Proton-Coupled Electron Transfer in the *Escherichia coli* Ribonucleotide Reductase

by

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Michelle C.Y. Chang

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Abstract

Ribonucleotide reductases (RNRs) comprise a biologically indispensable enzyme class which converts the four common nucleotides to their corresponding deoxynucleotides via a thyl radical hydrogen abstraction mechanism. As this is the only cellular pathway for production of the monomeric precursors required for DNA synthesis, the regulation of RNR is crucial to controlling the cell cycle by the availability of substrate pools for DNA replication. The class I RNR from *E. coli* is composed of two subunits; the R1 subunit contains the active site for nucleotide reduction, as well as the allosteric effector binding sites, and the R2 subunit contains the stable Y122*-diiron cofactor. Based on the conserved mode of action of the class II and III RNRs, Y122* is proposed to oxidize C439 in R1 to the active thyl radical species through a long-range radical transfer pathway involving several conserved aromatic residues (in R2: Y122, W48, Y356; in R1: Y731, Y730), which is unprecedented for its length (35 Å). Although this model is supported by *in vitro* and *in vivo* mutagenesis studies as well as sequence conservation, direct evidence for radical intermediates along this pathway has been masked by conformational gating. We have initiated R1 nucleotide reduction in the absence of R2, using a photoactive derivative of the R2 C-terminal peptide, containing the essential Y356. This peptide is capable of generating a tyrosyl radical upon photolysis and provides a specific entry point into the radical initiation pathway of R1. We now report that catalytic turnover in R1 may be triggered in the absence of R2 by the photogeneration of a conserved aromatic amino acid radical located within a synthetic 21-mer peptide derived from the R2 C-terminus. Mutation of conserved tyrosines on the pathway, corresponding to Y356 and Y731, to phenylalanine effectively deactivates radical initiation. These results provide the first direct evidence of the radical transfer pathway of the class I RNRs and underscore the importance of aromatic amino acid radical intermediates for proton-coupled electron transfer in physiologically-relevant processes.

Thesis Advisor: Professor Daniel G. Nocera
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<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
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<td>Az</td>
<td>azurin</td>
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<td>2'-deoxycytidine-5'-diphosphate</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethyl</td>
</tr>
<tr>
<td>DIC</td>
<td>diisopropyl(carbodiimide)</td>
</tr>
<tr>
<td>dGTP</td>
<td>2'-deoxyguanosine-5'-triphosphate</td>
</tr>
<tr>
<td>DIEA</td>
<td>diisopropylamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMPO</td>
<td>5,5-dimethyl-1-pyrrolone-N-oxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxynucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>1,4-dithiothreitol</td>
</tr>
<tr>
<td>dU</td>
<td>2'-deoxyuridine</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELDOR</td>
<td>electron-electron double resonance</td>
</tr>
<tr>
<td>ENDOR</td>
<td>electron-nuclear double resonance</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>ET</td>
<td>electron transfer</td>
</tr>
<tr>
<td>FAB</td>
<td>fast atom bombardment</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Fl</td>
<td>flavin</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast-protein liquid chromatography</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
</tr>
<tr>
<td>HATU</td>
<td>N - [(dimethylamine) - 1H - 1, 2, 3 - triazolo [4,5-b] pyridine - 1-ylmethylene] - N - methylmethanammunium hexafluorophosphate N-oxide</td>
</tr>
<tr>
<td>HBTU</td>
<td>2 - (1H-benzotriazole-1-yl) -1,1,3,3- tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HOBi</td>
<td>N-hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HRFAB</td>
<td>high-resolution fast atom bombardment</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MADH</td>
<td>methylamine dehydrogenase</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption ionization time-of-flight</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry or mass spectrum</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>NADPH</td>
<td>β-nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NHE</td>
<td>normal hydrogen electrode</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-pyrrolidine</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OEC</td>
<td>oxygen-evolving complex</td>
</tr>
<tr>
<td>OPO</td>
<td>optical parametric oscillator</td>
</tr>
<tr>
<td>PCET</td>
<td>proton-coupled electron transfer</td>
</tr>
<tr>
<td>phen</td>
<td>1,10-phenanthroline</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>PyBOP</td>
<td>benzotriazole-1-yl-oxy-tris-pyrrlicino-phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>Q</td>
<td>quinone</td>
</tr>
<tr>
<td>RC</td>
<td>reaction center</td>
</tr>
<tr>
<td>r.m.s.d.</td>
<td>root mean squared deviation</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>TAPS</td>
<td>N-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>TIS</td>
<td>triisopropylsilane</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer liquid chromatography</td>
</tr>
<tr>
<td>TLCK</td>
<td>N-tosyl-L-lysine chloromethyl ketone</td>
</tr>
<tr>
<td>t.o.</td>
<td>turnovers</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-tosyl-L-phenylalanine chloromethyl ketone</td>
</tr>
<tr>
<td>TR</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TRR</td>
<td>thioredoxin reductase</td>
</tr>
<tr>
<td>TTP</td>
<td>2'-deoxythymidine-5'-triphosphate</td>
</tr>
<tr>
<td>TTQ</td>
<td>tryptophan tryptophylquinone</td>
</tr>
<tr>
<td>t&lt;sub&gt;r&lt;/sub&gt;</td>
<td>retention time</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>k&lt;sub&gt;cat&lt;/sub&gt;[enzyme]</td>
</tr>
<tr>
<td>V/K</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Chapter 1

Proton-Coupled Electron Transfer in Protein Systems
1.1 Ribonucleotide Reductases (RNR)

1.1.1 Introduction

RNRs comprise a biologically indispensable enzyme family that converts the four common ribonucleotides to their corresponding deoxynucleotides through a thyl radical hydrogen abstraction mechanism [1]. As this is the only cellular pathway for production of the monomeric precursors required for DNA synthesis, the regulation of RNR is crucial to controlling the cell cycle by the availability of substrate pools for DNA replication. The catalytic subunit that carries out the nucleotide reduction is conserved throughout prokaryotes and eukaryotes [2]; however, the hole generation required for thyl radical production is performed by a wide variety of metallo-cofactors and radicals, including a diiron/tyrosine cluster in class I enzymes [3], an S-adenosylcobalamin in class II enzymes [4,5], and an iron-sulfur/S-adenosylmethionine/glycine system in the class II RNRs [6] (Figure 1.1). In spite of this evolutionary divergence in form and structure, all classes are proposed to serve in the identical capacity of radical chain initiation, propagating the radical through the complex to the active site cysteine until it is finally transferred to the substrate during the catalytic cycle. Thus the activity of RNRs relies entirely on the ability of the cofactor to assemble and produce the hole for transfer into the active site for nucleotide reduction.

![Figure 1.1](image-url)
1.1.2 *Mechanism of Nucleotide Reduction*

Although the activation domains must diverge structurally, the chemistry of nucleotide reduction is thought to be conserved in a common catalytic active site. Structural studies of the class I [7], II [2], and III [8] enzymes show that the active sites are remarkably superimposable (Cα r.m.s.d. of 1.5 Å in the barrel core, Figure 1.2) despite the very low sequence homology between families. All three classes share the ten-stranded α,β barrel with a protruding finger loop containing the catalytic cysteine as well as very similar connectivities between secondary structure elements. The major functional and structural difference exists between the class I and II enzymes and the class III RNR, as the latter uses formate as the source of reducing equivalents rather than the formation of a disulfide pair [9]; hence, the class II RNR contains only two essential cysteines in its active site whereas the class I and II enzymes contain three.

Once the transient thiol radical (C439, *Escherichia coli* numbering) [10] is generated by any of the diverse metallo-cofactors, it can abstract the 3'-H from the ribose ring forming the initial substrate radical (Scheme 1.1) [11]. Upon deprotonation of the 3'-OH group by an active site carboxylate group, water is eliminated irreversibly to generate a 3'-keto-2'-deoxynucleotide radical, driving the C-H hydrogen abstraction by the thiol radical.

![Class I, Class II, Class III](image-url)
Scheme 1.1 Mechanistic model for nucleotide reduction by class I and II RNRs (E. coli numbering).

Hydrogen atom transfer or proton-coupled electron transfer (PCET) from a pair of active site cysteines (C225/C462, E. coli numbering) produces the 3'-keto-2'-deoxynucleotide and a protein-based disulfide radical anion, which has been observed by high-field EPR and stopped-flow UV-visible spectroscopy in a mutant RNR [12]. A second PCET step results in the 3'-deoxynucleotide radical and protein disulfide (except for the class III enzymes, which produces carbon dioxide). In the final step, the hydrogen atom is transferred back to the 3' position to yield one equivalent of product and regenerate the thyl radical. The terminal reducing equivalents are provided either by NADPH through a thioredoxin/thioredoxin reductase system after disulfide exchange (class I and II) or formate (as previously mentioned).

1.1.3 The Class I RNR from Escherichia coli

The class I diiron-containing enzymes, including the E. coli enzyme, although perhaps
the most complex, make up the most studied of the RNR classes. They consist of two subunits, R1 and R2, which carry out the nucleotide reduction and stable tyrosyl radical generation, respectively. The initial assembly of R2 involves Fe$^{2+}$ chelation to the apo enzyme followed by oxygen binding with its concomitant four-electron reduction,

$$2 \text{Fe}^{2+} + \text{Y122} + 1 \text{e}^- + \text{O}_2 + 2 \text{H}^+ \rightarrow \text{Fe}^{3+}-\text{O}-\text{Fe}^{3+} + \cdot\text{Y122} + \text{H}_2\text{O}$$

leading to production of the oxidized $\cdot$Y122 and a diferric iron center, along with extensive ligand rearrangements and large fluctuations in the Fe–Fe distance. This process is complicated by the fact that the in vivo source of the fourth electron, imperative for the diiron/tyrosyl radical cofactor formation, has yet to be identified; to carry out iron center/tyrosyl radical reconstitution in vitro, an external reductant such as Fe$^{2+}$ or ascorbate is provided in the buffer [13]. In addition, the oligomeric state of the R1–R2 complex remains ambiguous. As R1 and R2 are both isolated as dimers, RNR is typically modeled as a dimer of dimers (Figure 1.3). However, recent studies have showed the existence of higher-order structures in the mammalian enzyme [14,15], which may or may not be important in catalysis. Crystal structures of both subunits have been solved in various states, but the structure of the complex remains elusive [7,16-18]. In the meantime, the two subunits have been docked according to the dimer of dimers model using shape complementarity and electrostatics, to result in a 35 Å hole transfer pathway between the

![Nucleotides Reduction](image)

**Figure 1.3** Dimer of dimers model for the E. coli RNR. Each subunit is isolated and crystallized as a dimer. R1 and R2 are then docked by shape complementarity and electrostatics, resulting in a 35 Å separation between $\cdot$Y122 and C439.
tyrosyl radical, •Y122, on the radical-generating R2 subunit to the catalytic C439 in the R1 active site [7]. Recent pulsed electron-electron double resonance (ELDOR) measure a large distance (33 Å) between the two •Y122s of R2 consistent with the crystal structure [19] and will hopefully provide information on the cross distance between •Y122 and an active site radical on R1 generated by a mechanism-based inhibitor.

One prediction of this model is that •Y122 must be reduced upon oxidation of C439 and that it remains reduced throughout the nucleotide reduction process [20]. However, this process is masked by a rate-limiting conformational changes and stopped-flow UV-visible spectroscopy shows no change in the steady-state concentration of •Y122 upon mixing of R1 and R2 in the presence of various substrates and effectors, including 3'-deuterated substrate [21]. However, pre-steady state and steady-state analysis show that the rate-limiting step occurs before PCET and nucleotide reduction [21], consistent with the absence of a V_{max} isotope effect and existence of a V/K isotope effect on 3'-hydrogen bond cleavage [11]. The larger $k_{cat}$ for R2 (with excess R1) versus R1 (with excess R2) also implies that forward and reverse PCET occurs on each turnover, similar to the Class II enzyme [21,22]. In addition, dissociation of R2 is likely required for re-reduction of the disulfides formed in R1 concomitant with nucleotide reduction. Although activation of C439 is kinetically masked, reduction of •Y122 results in complete functional loss of nucleotide reduction activity. In addition, a direct chemical link between these residues is drawn in studies with mechanism-based inhibitors (Figure 1.4) as well as R1 mutant

![Figure 1.4 Mechanism based inhibitors of R1 that cause concomitant •Y122 reduction.](image-url)
C225S with CDP. In reactions of this mutant and wild-type R1 with nucleotide analogs, the initial step of 3'-hydrogen atom abstraction by •C439 takes place and a 3'-ketyl-deoxynucleotide radical is generated (downstream products are observed by EPR for 2'-azido-2'-deoxyuridine [23] and 2'-methylenefluoro-2'-deoxycytidine [20,24]). If reduction of the inhibitor takes place from the bottom face, the thiyl radical is not regenerated, leading to •Y122 loss. Reduction from the top face reforms the thiyl radical, but in either circumstance, the resulting 3'-keto-deoxynucleotide product can dissociate from R1 and decompose to form a furanone that stoichiometrically inactivates R1 by nonspecific alkylation. The number of equivalents required for complete tyrosyl radical reduction is related to the partitioning between these reactions and differs between analogs. In R1C225S, the normal substrate acts as a mechanism-based inhibitor itself, as hydrogen atom abstraction becomes decoupled from nucleotide reduction; in this case, there is both an isotope effect on tyrosyl radical loss [25] and a stoichiometric R1 cleavage at S225 [26]. Additional EPR studies with an R1 active site mutant, E441Q, show the decay of •Y122 coupled to the rise of a disulfide radical anion [12]. All these studies serve to show that chemistry and substrate radical generation at the active site of R1 are directly coupled to •Y122 reduction, however, the actual mechanism of radical initiation at C439 still remains unclear.

Pure electron tunneling over the 35 Å spanning •Y122 and C349 is not feasible as it would lead to electron transfer being the rate-determining step (10^−4 to 10^−9 s⁻¹, calculated from \( k_{ET} = k_{ET}(0) \exp(-\beta r) \) where \( \beta = 1.1 \text{ Å}^{-1} \) or 1.4 Å⁻¹ and \( k_{ET}(0) \) is approximated to be 10^13 s⁻¹ for an activationless process [27,28]) in nucleotide reduction, which is known to occur at a steady state rate of 2 to 10 s⁻¹ [27] in the Class I RNRs. Thus, either a large conformational change to bring two sites nearer to each other or a different mechanism of hole transfer must be invoked in order to support the oxidation of C439 by •Y122. These
Figure 1.5 Proposed PCET pathway between Y122 on the R2 subunit and C439 on the R1 subunit based on a docking model generated from crystal structures of the individual subunits using shape complementarity and electrostatics.
considerations have led to proposals of a radical hopping mechanism to account for the observed chemistry carried out by RNR [7]. The hypothetical radical transfer pathway (Figure 1.5) has been mapped out by mutagenesis [29-32] and sequence conservation [7], but has not yet been directly tested. Interestingly, the reduction potential (0.9 V) of the tyrosyl radical (•Y122) produced at the dinuclear iron center in the E. coli R2 subunit is insufficient to generate the thyl radical (C439) essential for catalysis from the cysteine thiol (1.3 V). Since the driving force of tyrosine reduction is adequate to oxidize a deprotonated cysteine thiolate (0.8 V), it is postulated that the electron transfer reaction must also include proton transfer steps, based on the absence of any obvious pKₐ perturbation in the active site [13]. Even though the radical transfer may or may not be an overall uphill reaction or involve uphill steps, the equilibrium can be driven to the right by coupling to the rapid, irreversible loss of water during nucleotide reduction. Theoretical studies have supported a hydrogen atom transfer mechanism, in the limit of proton and electron synchronization, with pure ET being highly energetically unfavorable [33]. In a PCET framework, the location of protons along the ET route would govern the directionality of ET as protonation and deprotonation events can tune the redox potentials of the amino acids involved (W, Y, and C). This provides a means for catalytic reactivity to be regulated by protein conformation and allosteric binding events, and the presence of the hole at either Y122 or C439 to be controlled by the enzyme.

When examining the putative radical transfer pathway in more depth, it becomes clear that assigning roles to conserved residues in R2 is difficult as there must be essential residues involved as both ligands to the diiron center and in cofactor assembly, as well as PCET. In the PCET pathway between Y122 and C439, presumably only the aromatic residues (W48, Y356, Y730, and Y731) are directly oxidized with acid/base groups (D237) as potential proton shuttles:
From studies on CcP, it is known the presence of an H-D-W triad, reminiscent of the pathway leading from \( ^*Y_{122} \) to W48 in R2 \textit{via} H118 and D237, guides the formation of a compound I intermediate in which the extra oxidizing equivalent is located on an adjacent tryptophan cation radical rather than the porphyrin macrocycle \cite{7}. In this case as well, there is an essential link between pK\(_a\) and redox potential that is important to the activity of CcP, as the pK\(_a\) of the radical is perturbed (from \( \sim 4 \)), resulting in a more oxidizing species. Indeed, studies of iron cluster-tyrosyl radical assembly under Fe\(^{2+}\)-limiting conditions show that a transient \( ^*W_{48}H^+ \) is generated and can provide the fourth reducing equivalent used to oxidize Y122 \cite{34,35}. Thus, communication between \( ^*Y_{122} \) and W48 can be established. In fact, as discussed later in this chapter, it may be possible that W48 plays a role of the gate-keeper for reduction and re-oxidation of Y122, as its reduction potential is easily modulated by protonation or deprotonation by the neighboring D237 \cite{36}. Mutational analysis of the pathway show that all these residues are important to nucleotide reduction activity, but the lower limit of detection of activity (10\(^{-2}\) of wt) is relatively high due to contaminating wt protein. \textit{In vivo} experiments, however, show that the residues in the putative PCET pathway are mostly essential. Complementation studies with chromosomally-encoded temperature (R1) and hydroxyurea (R2) sensitive mutants show no growth (detection \( \leq 10^{-7} \)) of any of the plasmid-encoded R1 and R2 mutants of the pathway except for D237E, which is a relatively conservative change \cite{37}. R2 semisynthesis using intein-mediated protein ligation has focused on the role of Y356 in the PCET pathway. Replacement of Y356 with the unnatural amino acid 2,3-difluorotyrosine (pK\(_a\) 7.6) allows analysis of the proton requirement at position 356 \cite{38}. The pH-rate profile from pH 6.6 to 9.0 shows a remarkable similarity to wt R2, although the overall rate of nucleotide reduction is
reduced. Even at pH 6.6, where 90% of the 2,3-difluorotyrosine would be deprotonated if the pKₐ is unperturbed, only a two to three-fold drop in activity is observed (compared to the maximal activity at pH 8.0), implying that a proton can be lost from the pathway with little effect on enzymatic activity. Thus, it appears as if the proton is loosely coupled at Y356 and that hydrogen atom transfer is not required at this position. Further studies with 3-nitrotyrosine show that an increase in the reduction potential by 200 mV, while maintaining hydrogen bonding, is sufficient to act as a redox block of nucleotide reduction [39].

1.1.4 Structural Basis for Thiyl Radical Generation

The structural conservation of the active sites for nucleotide reduction between the different classes of RNRs provide surprising chemical insight into the mechanism of thiyl radical generation in the class I enzyme. The α/β barrels are remarkably identical, an observation that is consistent with the highly conserved chemistry supported by biochemical experiments. However, the structural comparison also reveals a surprising detail concerning radical initiation; the three-dimensional arrangement of the AdoCbl and the •G580 (A580 in the T4 phage structure) with respect to the catalytic cysteine, is identical to the two conserved tyrosines Y730 and Y731 in the E. coli enzyme (Figure

![Class I, Class II, Class III](image)

**Figure 1.6** Structural alignment of Y731 (Class I), •CH₂Ad (Class II), and •G580 (Class III) with respect to the catalytic cysteine. Adapted from ref. 40.
1.6) [2,40]. The class II (AdoCbl) is known to generate the thiyl radical by direct hydrogen atom abstraction coupled to carbon-cobalt bond homolysis [41] and presumably the glycyl radical of the class III enzyme functions by H• abstraction as well. Although the mechanism of radical initiation in class I enzyme remains ambiguous, the overlay of the crystal structures points to an essential role for Y730 and Y731 in the PCET pathway and suggests the importance of hydrogen atom abstraction in S• formation in the class I RNRs.

1.1.5 Conclusions

The long-range radical initiation process of the class I RNRs is unique in Nature. A formal H• equivalent is transferred reversibly over 35 Å between two individual subunits, perhaps on each enzymatic turnover [21]. Thus the location of the radical, whether at the stable •Y122 or the transient •C439, must be regulated by the enzyme and governed by substrate and/or effector binding. If this reaction were not highly regulated, either cofactor destruction or uncontrolled radical chemistry could result. The directionality of radical transfer between the aromatic amino acids along the proposed pathway (Y122, W48, Y356, Y731, and Y730), whether in the forward or reverse direction, needs to be directed by conformational changes in R1 and R2, which would transiently alter their environment and consequently their redox potentials and/or pKs. If fluctuating inequivalencies between these residues are not created during the catalytic cycle of RNR, directional transport would be exceedingly difficult to accomplish. In the following sections, we present examples from the literature that aid in development of a model for long-range radical initiation in RNR. First, a historical overview of ET in protein systems is discussed. Next, we move forward to systems in which ET is observed to be gated by either proton-coupling, protein conformational changes, or transient protein–protein interactions. We then demonstrate examples in which the proton inventory determines the directionality of ET, as well as the location of radical equivalents, between seemingly
identical cofactors. Lastly, a strategy for studying radical initiation in the class I RNR from *E. coli* and the specific aims of this thesis are put forth.

1.2 **A Framework for Electron Transfer in Protein Systems**

1.2.1 **Introduction**

All current treatments of electron transfer (ET) are based on Fermi's golden rule, which allows separation of the timescales for electron and nuclear movement, thereby greatly simplifying the problem. Although the resulting theories for ET have been incredibly successful at describing behavior in biological and chemical systems in which ET takes place with little nuclear motion [27,42,43], it does not fully account for systems such as RNR in which proton and electron are thought to move on similar timescales [44].

1.2.2 **Evolution of Theoretical Models for ET**

The first picture for fixed-distance ET was developed by Marcus from activated complex theory in the 1950s. In this formulation, the potential energy of the system is the sum of the reactants and products as well as the surrounding medium (i.e. solvent) and is a function all their nuclear configurations [45]. One such surface demonstrating the intersection of the harmonic potential energy well of reactant and product states is shown in Figure 1.7. The intersection of the wells, represented by the dotted lines, corresponds to the electronic coupling ($H_{DA}$) between the initial and final states. The electron transfer event is described as a Franck-Condon transition, meaning that the nuclear coordinate is fixed during movement of the electron. Therefore, the

![Figure 1.7 Potential energy surface for an ET reaction as a function of the nuclear coordinates of the reactants, products, and surrounding medium. Q₀, Q⁺, and Qₚ represent the nuclear configuration for the initial state, transition state, and product state, respectively. $H_{DA}$ is defined as electronic coupling, $\Delta G^*$ is the activation barrier, $\Delta G^0$ is the driving force for the reaction, and $\lambda$ represents the reorganization energy.](image)
electron is only transferred at the transition state where the potential energy of the reactants equals that of the products (including the solvent coordinate), consequently, electron tunneling between redox sites in proteins would be governed by the fluctuations of the protein continuum ($\lambda$) as mediated by the intervening medium. From this model, Marcus developed an expression for the activation barrier of,

$$\Delta G^* = \frac{\left(\Delta G^0 + \lambda\right)^2}{4\lambda}$$

predicting the existence of an inverted region in which the rate of ET decreases when $-\Delta G^0 > \lambda$ [42]. This classical description for ET developed by Marcus was later merged with quantum mechanical perturbation theory. From the latter, the first-order rates of ET ($k_{ET}$) reactions with weak electronic coupling, are described by the electronic coupling ($H_{DA}$) and the Franck-Condon weighted density of states, $FC$ [46-48],

$$k_{ET} = \frac{2\pi}{h} H_{DA}^2 \cdot FC$$

Incorporating the nuclear Franck-Condon factor for a vertical transition given by Marcus' classical transition-state theory [42] yields the semiclassical Marcus-Levich expression,

$$k_{ET} = \frac{4\pi^3}{\sqrt{h^2 k_B T}} H_{DA}^2 \exp \left[ -\frac{\left(\Delta G^0 + \lambda\right)^2}{4\lambda k_B T} \right]$$

where $\Delta G^0$ is the reaction free energy, $\lambda$ is the nuclear reorganization energy, $T$ is the absolute temperature and $k_B$ is the Boltzmann constant. Thus, the electron tunneling rate is influenced by the electronic ($H_{DA}$) and structural properties ($\lambda$, $H_{DA}$) of the medium separating donor and acceptor. Although predictions resulting from this model were confirmed soon after for chemical model systems [49], the development of techniques for the specific modification of proteins with redox- and photo-active metals (e.g. Ru, Os, Re, Co), and the advent of the “flash-quench” method, only opened the field of protein ET for evaluation of Marcus theory some 30 years later [50-52].
The distance-dependence of ET was derived from early treatments of $H_{DA}$ in the 1970s by modeling the bridging medium as a one-dimensional square barrier, yielding the expression [53,54],

$$H_{DA} = H_{DA}^0 \exp\left[-\beta R_{DA}\right]$$

indicating an exponential modulation of electronic coupling (and hence $k_{ET}$) by the physical separation between donor and acceptor, $R_{DA}$, and a constant, $\beta$. Thus the corresponding rate of electron transfer is given by,

$$k_{ET} = k_{ET}(0) \exp(-\beta R_{DA})$$

which is maximized at zero separation between donor and acceptor, $k_{ET}(0)$, and decays exponentially with distance. A general protein decay factor of $\beta=1.4$ Å⁻¹ was estimated for biological ET, suppressing the contribution of protein structure and amino acid sequence [54]. Later formulations, which compute ET pathways through a given protein by balancing the competition between more highly coupled through-bond (including hydrogen bonds) and shorter through-space mechanisms, resolve differences between $\alpha$-helix ($\beta$=1.4 Å⁻¹) and $\beta$-sheet ($\beta$=1.0 Å⁻¹) secondary structures (Figure 1.8) [55,56]. Although, the question of whether electronic coupling within proteins depends on the structure of the intervening medium [52] or simply the distance between donor and acceptor [27,43] remains an arena of longstanding debate, the two viewpoints have converged as both descriptions must take into account the existence of multiple pathways [57] or an equivalent packing density of the protein medium [58].
1.2.3 Pathways for ET and PCET

Although both these approaches take into consideration the difference in electronic coupling between through-bond, through-space, and through-hydrogen bond paths by either a weighted average of different pathways [57] or by packing density [58], the question of whether Nature has tuned ET pathways by the precise positioning of amino acids along the trajectory of the electron remains unresolved. The evolutionary conservation of hydrogen-bonded networks in proteins suggests an important role for protons in even simple ET by increasing the electronic coupling between donor and acceptor. At the same time, however, it has also been observed that ET proceeds at similar rates in engineered proteins as naturally occurring ET proteins, implying that an evolutionarily optimized path guiding the flow of electrons is unnecessary [27]. Neither of these models of ET truly treats long-range PCET reactions, such as radical initiation in the class I RNR, which requires the localization of a proton equivalent along the pathway of the electron due to its short tunneling distance [59,60].

As shown in studies of ET through DNA base stacks, GG and GGG sites act as traps in which the oxidizing equivalent can localize [61,62]. This hopping mechanism allows for long distance hole transfer with increased rates compared to those predicted by exponential distance dependence for pure superexchange between donor and acceptor. When treated as a series of sequential charge transfer reactions, the rate is found to follow a power law related to the number of steps involved [63]:

\[
\begin{align*}
\text{direct tunneling} \\
k_{ET} \propto e^{-A(R)} \\
\Rightarrow \\
\text{multistep hopping} \\
k_{CT} \propto k_{\text{hop}} n^{-\eta}
\end{align*}
\]

where \( n \) is the number of intervening base pairs between donor and acceptor and \( R \) equated to the distance between base pairs, \( \eta = 1-2 \) and \( k_{\text{hop}} \) is the rate for inter-trap charge transfer. Thus, the rate of ET becomes highly sequence-dependent as it is
dominated by the location and number of GG and GGG sites. Indeed, the rate of ET has been found experimentally to be limited by the length of the longest hop [64]. This type of radical transport by a hopping mechanism has been observed in protein model systems [65-67], but has yet to be shown for physiologically-relevant reactions.

1.3 Gated Electron and Proton Transfer in Proteins

1.3.1 Introduction

In addition to the multiplicity of pathways available in a static three-dimensional protein fold, theorists have more recently addressed the problem of the fluctuating nature of the protein bridge [68-72]. Protein dynamics have long been believed to play an important role in their function and specificity; both large-scale movements, such as loop or domain "breathing" [73], and small-scale fluctuations of proteins about their average structure, such as vibrational coupling between protein and substrate [74], have been implicated in protein activity. Indeed, single-molecule spectroscopy of enzymes has shown that variations in state from molecule to molecule can be correlated to the activity of individual entities [75]. Depending on the time regime of motion, internal protein dynamics can influence ET rates by perturbing either $\lambda$, in a conformationally gated ET [76,77], or $H_{DA}$ [68-72], when the fluctuations are on the timescale of ET. The following examples describe systems where ET is shown to be gated by transient protein–protein interactions, substrate binding, and proton transfer, all elements which are believed to be involved in the radical initiation process of the class I RNRs.

1.3.2 Cytochrome c/Cytochrome c Peroxidase

One of the most well-studied interprotein ET reactions is the oxidation of cytochrome (cyt) c by cyt c peroxidase (CcP). Hydrogen peroxide reacts with CcP to generate the Fe(IV)=O/$^*$W191H$^+$ species, which can oxidize two equivalents of cyt c via the compound I and II states (Scheme 1.2) [78]. A docking model between these two proteins was proposed based on surface electrostatic interactions between four cyt c lysines
Scheme 1.2 Oxidation of cyt c by compound I and II of CcP, respectively.

located near the heme edge and complimentary CcP aspartates, placing the heme cofactors 17 to 24 Å apart (Fe–Fe distance) [79]. The crystal structures of two different cyt c/CcP complexes are consistent with the docking model, yielding Fe–Fe distances of 27 and 29 Å respectively [80].

Upon closer examination, it was discovered that a rigid docking model did not fully describe the binding between the protein partners, and that cyt c was able to transiently occupy different sites on CcP in the reduced and oxidized forms [79,81]. Molecular dynamic simulations further implicated different binding modes of the reactant and product states, which pointed towards the importance of interfacial motion in formation of the transition state for ET [82]. Upon cooling to 220 K, ET is shut down as it becomes limited by formation of the active complex [83]. A large reorganization energy of 1.5 eV has been calculated based on both temperature and driving force dependence studies [84], further emphasizing the existence of large scale redox-induced motion within the complex upon oxidation of cyt c.

These types of large reorganization energies have been observed in a variety of other systems that involve ET between physiological protein partners. In these cases, the overall reaction rate is governed by $\lambda$ rather than such quantities as $\Delta G^\circ$ or $H_{DA}$. This allows conformational control of reaction rates between protein partners, to be mediated by specific events such as substrate and/or effector binding as well as formation of transient protein complexes, an ability that is crucial to regulating interactions between
reacting species in the cell. R1 and R2 also exhibit a weak association \( (K_D \sim 0.1-0.2 \mu M) \) [85] that may indicate transient associations; indeed, subunit dissociation is likely required for enzyme turnover [27]. If a conformational change is required to establish radical transfer between Y122 and C439, either resulting from R1–R2 binding or binding of substrate and effector, radical initiation can then be regulated with specificity, as not to lose the oxidizing equivalent in an off-pathway reaction.

1.3.3 Methylamine Dehydrogenase/Amicyanin/Cytochrome c-551i

Methylamine dehydrogenase (MADH) uses a tryptophan tryptophylquinone (TTQ) cofactor to catalyze the oxidative deamination of primary amines to the corresponding aldehyde, allowing the host organism to utilize methylamine as a carbon source [86]. The resulting N-quinol form of the enzyme is re-oxidized one electron at a time by amicyanin,

\[
\begin{align*}
\text{CH}_3\text{NH}_2^+ + \text{MADH(O-quinone)} + \text{H}_2\text{O} & \rightarrow \text{CH}_2\text{O} + \text{MADH(N-quinol)} + \text{H}^+ \quad 1 \\
\text{MADH(N-quinol)} + \text{Ami(Cu}^{2+}) + \text{H}_2\text{O} & \rightarrow \text{MADH(N-semiquinone)} + \text{Ami(Cu}^+) + \text{H}^+ \quad 2 \\
\text{Ami(Cu}^+) + \text{cyt c-551i(Fe}^{3+}) & \rightarrow \text{Ami(Cu}^{2+}) + \text{cyt c-551i(Fe}^{2+}) \quad 3 \\
\text{MADH(N-semiquinone)} + \text{Ami(Cu}^{2+}) + \text{H}_2\text{O} & \rightarrow \text{MADH(O-quinone)} + \text{Ami(Cu}^+) + \text{NH}_3 \quad 4 \\
\text{Ami(Cu}^+) + \text{cyt c-551i(Fe}^{3+}) & \rightarrow \text{Ami(Cu}^{2+}) + \text{cyt c-551i(Fe}^{2+}) \quad 5
\end{align*}
\]

**Scheme 1.3 Oxidation of methylamine by the MADH, amicyanin (Ami), cyt c-551i ternary complex.** (1) Reduction of the substrate by the TTQ O-quinone of MADH leads to product formation and the two-electron reduced N-quinol. (2) ET from MADH to amicyanin generates the TTQ N-semiquinone and reduced amicyanin. (3) The electron is then passed on to cyt c-551i in the ternary complex. (4) A second ET from the TTQ N-semiquinone to amicyanin leads to formation of the fully-oxidized MADH O-quinone with release of ammonia. (5) Amicyanin again reduces cyt c-551i.

a Type I blue copper protein, which then passes the substrate-derived electrons to cyt c-551i (Scheme 1.3). Note that most of these studies have examined individual steps in a single-turnover fashion, so that re-oxidation of cyt c-551i is not required. In physiological reactions, the electrons received by cyt c-551i are eventually passed to cyt c oxidase (COX). MADH is unable to reduce cyt c-551i directly, although the reaction is energetically feasible, implying that amicyanin is an obligatory mediator [87]. Amicyanin
The rates of ET between MADH and amicyanin can be measured by stopped flow UV-visible spectroscopy from four different forms of MADH – the substrate-derived N-quinol and N-semiquinone states (Scheme 1.4) as well as dithionite-derived O-quinol and O-semiquinone states. Several variables, $\lambda$, $\Delta G^\circ$, and $H_{DA}$, can be extracted by fitting the temperature dependence of $k_{ET}$. Rates of ET from the N-semiquinone [92], O-quinol [93], and O-semiquinone forms show reasonable values for these quantities, indicative of an adiabatic (unmasked) ET event. In addition, an accurate distance between the TTQ and reduction of the cytochrome (190 mV) is also dependent on ternary complex formation as its reduction potential shifts from 294 mV to 221 mV vs. NHE in the presence of MADH [88, 89]. This system provides an excellent arena for the study of interprotein ET as the high-resolution crystal structure of the ternary complex [90] is available and is active in the crystalline form [91]. Thus, the geometric organization of the redox centers with respect to each other in an active complex is known (Figure 1.9), an essential piece of information that is unavailable in many enzyme systems.
Cu$^{2+}$ sites can be calculated using a general protein $\beta$ of 1.0 Å$^{-1}$. Interestingly, using Marcus theory, the ET from the $N$-quinol to amicyanin shows calculated quantities calculated for $\lambda$ (3.3 eV) and $H_{DA}$, which are extremely large, as well as a negative $r_{DA}$, all hallmarks of a gated ET [94]. Thus, Marcus theory can not be applied to this reaction as the rate of measured for amicyanin oxidation is limited by a physical change and not the rate of the actual ET, meaning that the $\lambda$, $H_{DA}$, and $r_{DA}$ extrapolated from this rate are unrelated to ET. The non-physiological $O$-quinol generated by dithionite reduction does not show the same gating as the substrate reduced $N$-quinol. From studies of model compounds, the reduction potential of the $N$-quinol is 41 mV more positive than the $O$-quinol/$O$-semiquinone couple, making ET from $N$-quinol MADH uphill by $-10$ mV [95]. There is a strong pH and monoclonal dependence on the reaction resulting from the requirement for binding of a cation and deprotonation of the $N$-quinol prior to ET [96]. The observed primary deuterium solvent isotope effect of this process is also consistent with the interpretation that ET is proton-gated [94]. The $O$-quinol does not show the same cation dependence as it does not need to be deprotonated in order to reduce amicyanin, however, it is interesting to note that the rate of ET is at least an order of magnitude slower than the ET from the $N$-quinol. Although ions such as $K^+$ and $Na^+$ are able to bind at the cation site and react with the $N$-quinol, it may be that the regulatory site is intended for the methylammonium ion, allowing ET to be activated by substrate binding [97]. Thus the first step in the ET from the substrate-reduced MADH by amicyanin, is proton-gated ET, possibly by substrate binding, which allows for regulation of activity and specificity in the ternary complex. By enforcing the deprotonation of the $N$-quinol prior to ET, the enzyme is able to both increase the overall rate of ET ($k_{ET} = 12$ s$^{-1}$ in the $O$-quinol versus the lower limit of $k_{PT} = 144$ s$^{-1}$ in the $N$-quinol) and retain chemical control over the reaction timing [97]. This is interesting with regard to RNR, as we believe that radical initiation is also likely gated by substrate and effector binding, in order synchronize nucleotide reduction with radical transport out of the protected hydrophobic
pocket of *Y122 [98]. In this way, the radical equivalent is not as susceptible to loss by off-pathway quenching at W48 [99].

Although ungated, the long-distance ET reaction (24 Å) between amicyanin and cyt c-551i has also yielded interesting observations. Mutation of P94 to phenylalanine in the loop containing the copper ligands of amicyanin increases its reduction potential by 195 mV to 415 mV vs. NHE by allowing the copper(II) site to relax to the preferred copper(I) geometry [100]. Thus, the reduction of cyt c-551i by the P94F amicyanin would be uphill by 260 mV [101]. The measured $k_{ET}$ (0.46 s$^{-1}$) for this process is two orders of magnitude slower than that of the native amicyanin, following the Marcus prediction, and becomes the rate-limiting step in turnover [101]. Despite the positive $\Delta G^\circ$ for the forward ET reaction, back ET is slower, presumably because the complex dissociates quickly or a conformational change occurs. Thus, forward ET can be driven in thermodynamically uphill processes, even when separated by a large distance as long as it is driven to the right by a physical or chemical event. This is analogous to the uphill radical initiations catalyzed by RNR, first in the radical transfer from *Y122 to C439 and second in the 3'-hydrogen atom abstraction carried out by *C439, both of which can be driven by the irreversible loss of water during nucleotide reduction (Scheme 1.1).

1.3.4 Ferredoxin I

Ferredoxins are small iron-sulfur (Fe-S) cluster containing proteins that can carry out redox reactions with other proteins or enzymes. The ferredoxin I (Fd I) from *Azotobacter venelandii contains one [3Fe-4S] and one [4Fe-4S] cluster. The one electron reduction of the buried [3Fe-4S]$^+$ cluster results in the corresponding uptake of one solvent-derived proton, a process which has been elucidated in detail by Armstrong and co-workers [102]. Using fast-scan protein-film voltammetry, the authors are able to deconvolute the PCET kinetics of this cluster in both native and mutant forms (Scheme 1.5). In wt-Fd I,
Scheme 1.5 Proton shuttling in the [3Fe-4S] cluster of ferredoxin I. If D15 is protonated, then proton transfer to the
cluster occurs rapidly at a unimolecular rate of \( k_{\text{on}} = 1,294 \, \text{s}^{-1} \). If D15 remains unprotonated, then a pH-sensitive slow
bimolecular rate of cluster protonation is observed (\( pK_a = 7.2 \)). In both cases, if D15 is re-protonated after transfer to the
cluster (\( pK_a = 5.9 \)) then back ET is inhibited. For both the D15N and D15E mutants, only the slow bimolecular cluster
protonation is observed, with loss of pH sensitivity in this step.

rapid forward ET to the cluster leads to a slower proton uptake (\( k_{\text{on}} = 1294 \, \text{s}^{-1} \), \( pK_a = 7.8 \)),
while the back ET is gated by a slow deprotonation (\( k_{\text{off}} = 332 \, \text{s}^{-1} \)) that must occur before
cluster oxidation. The proposed protonation site, a bridging sulfide group, is adjacent to
an aspartate, D15, which is postulated to act as a proton relay group. Consistent with this
interpretation, back ET is inhibited at low pH, due to protonation of D15 (\( pK_a \)). If D15 is
unprotonated at the time of ET, then the protonation of the cluster takes place in a slow
bimolecular step which is sensitive to the \( pK_a \) of D15 (\( pK_a \)). Several mutants were
examined, of which D15N and D15E were the most instructive. Changing both the \( pK_a \)
and geometry of D15 leads to uncoupling of the protonation event from ET as both D15N
and D15E show slow pH-independent bimolecular proton transfer kinetics. The fact that
elimination of the carboxylate has the same effect as addition of a methylene group
emphasizes the exacting requirements for the proton shuttle group in this system. In RNR
on the other hand, the proton shuttle, D237, is equally susceptible to changes in \( pK_a \),
although less geometrically sensitive [30,37]. Interestingly, high-resolution crystal
structures of the oxidized and reduced Fd I at high and low pH are available and show
conformational heterogeneity of the geometry of D15 [103,104]. Furthermore, molecular
dynamics simulations show that D15 is quite fluxional and capable of the rapid motion
that is required to catalyze the rapid protonation of the cluster. In R2 as well, we expect
that D237 is fluxional during enzymatic turnover in order to carry out protonation and deprotonation at W48.

1.3.5 Conclusions

The conformational changes associated with complex formation or substrate and effector binding are often rate limiting in enzyme systems and can mask chemical steps such as ET. Hence, for many physiological interprotein ET reactions, where specificity is essential, conformational gating allows for regulation of activity by precluding ET until the proper trigger is received. As shown in both the CcP/cyt c as well as the MADH/amicyanin/cyt c-551i complexes, intraprotein ET is regularly governed by large reorganization energies. Transient interactions may also play a role in MADH, as in CcP, since complex dissociation could be involved in multiple turnover cycles. Studies of PCET in Fd I demonstrate the close relationship between redox potential and pK_a, which provides a mechanism for controlling the timing and directionality of electron as well as proton transfer. Notably, in both Fd I and MADH, the proton-coupling to ET is indirect, as long-range ETs lead to short-range proton hopping or vice versa in these systems.

1.4 Proton-Coupled Electron Transfer in Protein Systems

1.4.1 Introduction

In primary metabolism, namely photosynthesis and respiration, controlled charge transport is the central mechanism in the conversion of various forms of energy to biological work. Likewise, redox transformations form one of the major classes of reactions in the intricate network of intermediary metabolism. Electron transfer (ET) in these protein systems is often linked to protonation or deprotonation events that serve to preserve charge neutrality in the low dielectric protein environment or conversely, protonation or deprotonation can aid electron or hole transfer. These effects are manifested in the pH dependence of reduction potentials as well as the converse dependence of pK_a on oxidation state. The delicate interplay between acid/base and redox
chemistry combined with ability of proteins to alter these properties allows enzymes to fine tune their control over the location of electron and proton equivalents. For example, hydrogen bonding and proton gating can often serve as a means to control ET to and from substrates to couple active site chemistry to physical events such as binding of a protein partner or allosteric effector [76,105]. By definition, PCET is excluded from a Marcus description, unless the timescales for electron versus proton transfer are clearly separated. Although these types of reactions may at times be examined using Marcus theory, cases in which proton and electron are transported in a concerted manner, namely hydrogen atom or radical transfer, continue to escape direct analysis, as it requires stepping beyond the Born-Oppenheimer regime. Current descriptions in the literature involve modifications of Marcus theory to take into account the four different states describing both the electron and the proton in their initial and final states, leading to effects of PCET in the terms $\lambda$, $\Delta G^*$, and $H_{DA}$ [106,107]. This describes situations where proton-gating occurs or vice versa, but does not describe true hydrogen atom transfer. We will first examine several systems involved in primary metabolism which have provided the major groundwork for PCET studies in biological systems. From this point, we will present examples of radical transfer in peroxidases, followed by a short discussion of the instances of radical hopping in long-range PCET processes in model proteins.

1.4.2 PCET in respiration and photosynthesis

Both respiration and photosynthesis consist of electron transport chains that are coupled to vectorial transport of protons (Figure 1.10). COX effects both the pumping of protons across the membrane to generate a chemiosmotic potential as well as the multiple electron and proton reduction of oxygen to water. In the reverse reaction, photosystem II (PS II) harvests light energy to evolve oxygen from water at a tetranuclear manganese cluster, in a tyrosyl radical-mediated process. PS II is a multi- subunit 500,000 Da protein
Figure 1.10 Schematic representations of COX and PS II. In COX, the four electron-four proton reduction of oxygen to water is effected with vectorial transport of protons. In PS II, stepwise excitation of the P680 chromophore leads to the storage of four oxidizing at the OEC, which leads to formation of oxygen from water with concomitant release of four protons.

complex located in the thylakoid membrane containing a pseudo-C2 axis of symmetry [108]. At the heart of PS II is the D1/D2 heterodimer containing the reaction center, with the cofactors of the electron transport chain located symmetrically with respect to the pseudo-C2 axis (Figure 1.11a). Two tyrosyl radicals [109], •YZ on the D1 subunit and •YD on the D2 subunit, are also symmetrically arranged about this axis (Figure 1.11b); however, •YZ becomes transiently oxidized during the catalytic cycle of PS II [110,111], while •YD exists as a stable radical [112,113]. Absorption of light by the specialized chlorophyll complex shared between D1 and D2, the P680 chromophore, drives ET to QA, generating a charge-separated state (•P680+QA−) that is stabilized by re-reduction of •P680+ by the neighboring YZ, with the electron eventually passed down an electron transport chain to effect proton pumping (Figure 1.10). The oxidizing equivalent of •YZ is then transferred to the adjacent tetra-manganese oxygen-evolving complex (OEC) to carry out the water-splitting reaction (Figure 1.10). As water oxidation at the OEC
Figure 1.11 Structure of an active PS II from Synechococcus elongatus and arrangement of cofactors from ref. 108. (Panel A) Transmembrane α-helices in the D1/D2 dimer with cofactors bound. The D1/D2 core is shown in yellow and orange. (Panel B) Arrangement of cofactors with respect to the pseudo C2-axix indicated by the dotted line. Center-to-center distances between cofactors are indicated with lines. The putative Qb binding site is indicated with an asterisk. Yz and Yd are indicated in red.

requires the release of four electrons and four protons, this process is repeated an additional three times before evolution of oxygen takes place. This phenomenon is summarized using the S-state notation (Sn), in which n represents the number of oxidizing equivalents stored within the OEC [114]. One of the particularly fascinating aspects of PS II is the functional asymmetry of the two seemingly identical D1 and D2 subunits. The D1/D2 core has somehow evolved to pass the oxidizing equivalent to the D1 side where chemistry occurs, while transporting the reducing equivalent to Qb of D2 to carry out proton-pumping. Upon closer examination, the environments of YD and YZ differ in ways that are consistent with their observed reactivities. YD exhibits high stability and is well-shielded from solvent, as shown by D2O exchange and tests of reductant accessibility [115]. Consistent with these observations, YD is located in a hydrophobic pocket and participates in a stable hydrogen bond with D2-H189 [115,116]. YD is also not essential to the water splitting reaction, as oxygen evolution is still observed when this residue is mutated to phenylalanine [117]. However, it is believed that the oxidation and protonation states of YD may tune the potentials of both P680 and the OEC, affecting the
quantum yield of charge separation and oxygen evolution as well as the kinetics of S state transitions [113,118]. On the other hand, the essential •\(Y_Z\) is readily accessible from solvent [119,120] and located in an area with a large number of hydrophilic residues [115]. Instead of being involved in a stable hydrogen bond, the corresponding histidine of D1 (H190) is believed to play a crucial role in deprotonation of the phenolic proton of \(Y_Z\) during oxidation as well as reprotoation upon its reduction. Interestingly, there is a difference between the intact and manganese-depleted PS IIIs. In the intact preparations of PS II, \(Y_Z\) oxidation by •\(P680^+\) (which are 12 Å apart) is believed to take place on a nanosecond timescale and limited by electron rather than proton transfer in an isotopically insensitive step [115]. Once released, the proton thought to be either rapidly delocalized or transferred to the lumen via D1-H190 [115,121]. In the Mn-depleted PS IIIs, the rate of \(Y_Z\) oxidation is slowed by two to three orders of magnitude and becomes gated by deprotonation [122]. This effect has been attributed to either a distortion or elongation of the \(Y_Z\)-histidine hydrogen bond in the absence of the OEC, which can increase the barrier for proton transfer by 10 kcal/mol or more [123,124]. More recently, Babcock proposed a more intimate role in water oxidation for •\(Y_Z\), in addition to its historical capacity as a redox shuttle [125-127]. In his model, he suggests that PS II functions as a metalloradical enzyme in much the same way as ribonucleotide reductase [128] or galactose oxidase [129,130]. In PS II, however, the hydrogen atom “current” is reversed, with the proton and electron equivalents transferred from the metalloradical active site to P680, as •\(Y_Z\) carries out four consecutive formal hydrogen atom abstraction reactions from the bound water molecules to evolve oxygen. Although the net reaction is exothermic, the individual hydrogen atom transfers to •\(Y_Z\) are either disfavored or, at most, approaching thermoneutral [126]. However, this process can still be driven to the right by the charge-separation process, which causes the reaction to be effectively irreversible.
From these studies of PS II, it is clear that the protein environment surrounding $Y_Z$ and $Y_D$ dramatically perturbs their chemical reactivity and that there exists quantitative differences between stable and transient tyrosyl radicals. It is the transient tyrosyl radical, •$Y_Z$, which is proposed to carry out the chemistry and which participates accordingly in fluxional rather than stable hydrogen bonds with D1-H190 that control its deprotonation and protonation, and consequently, its oxidation and reduction. Like the foregoing studies of Fd I, the geometry of this hydrogen is astonishingly sensitive to change, as it may be tuned by two- and three-dimensional effects such as bond length and bond angle. Both transient and stable tyrosyl radicals are proposed for the radical initiation catalyzed by the class I RNRs, and the wealth of knowledge assembled on $Y_Z$ and $Y_D$ should eventually aid development of a detailed chemical mechanism for radical transfer. In addition, half-site reactivity, as seen in PS II, has also been proposed for R2 as the wt enzyme is only isolated or reconstituted with ~1.2 •$Y_122$s per dimer [131,132]. As the docked complex of R1 and R2 contains a true two-fold symmetry, it seems unlikely that there would exist differences between the •$Y_122$ sites on each individual monomer that could lead to differences in reactivity for one monomer versus the other. However, the active form of R2 from *Saccharomyces cerevisiae* is a 1:1 heterodimer of Y2 and Y4 subunit, which contain a functional diiron-tyrosyl cofactor and an inactive center, respectively [133]. This enforced half-site reactivity may have interesting ramifications on the characteristics of the corresponding •$Y_122$ in the heterodimer or may simply provide method of regulating of RNR activity *in vivo*.

The bacterial reaction center (RC) has long-served as a model for PS II, due to the functional and structural similarities between the two. However, the bacterial RC does not carry out the water-splitting reaction and thus functions only to achieve the vectorial proton transport. Due to its smaller size, it has been much more amenable to study and
Figure 1.12 A schematic of the RC and the cyt bc1 complex. Photoinduced ET leads to the stepwise two-electron reduction of Qb, driving the uptake of two protons. QbH2 is then passed to the cyt bc1 complex and oxidized at the Q site. The two electrons derived from quinone oxidation are then transferred in a bifurcated reaction to cyt c2 and cyt b, respectively. After passing through two cycles, the oxidized quinone is returned to the RC. The net effect is the transfer of directional transfer of protons across the membrane into the periplasmic space.

has provided a mechanism for directional charge transport between two identical cofactors as directed by proton inventory. In a similar reaction scheme to PS II, ET is light-initiated from the bacteriochlorophyll special pair; the forward reaction is driven by charge separation as the electrons are passed through several cofactors, bacteriopheophytin and ubiquinone (QA), to the terminal electron acceptor, ubiquinone bacteriochlorophyll, Qb (Figure 1.12). Upon dissociation from the RC, the two-electron reduced Qb (QbH2) is oxidized by the cytochrome bc1 (cyt bc1) complex [134] with concomitant proton translocation across the membrane, via a modified Q cycle, before re-uptake by the RC. One of the electrons generated by the Q cycle is passed to cyt c2 (cyt c2), which then re-reduces the oxidized special pair during charge separation.
The first series of steps from the primary donor (bacteriochlorophyll special pair) to QA are pure ET reactions and do not directly entail proton coupling until the reduction of QB by QA. Ubiquinone reduction to the corresponding quinol requires two electrons and two protons. In solution, the intermediate semiquinone has a relatively low pKa of ~5 [135] while the doubly-reduced state has pKa's of 14 and 12 for the first and second protonation, respectively [136]. Conversely, the first reduction of ubiquinone to the semiquinone is ~400 mV less favorable than the second reduction, which is assisted by the uptake of two protons to form the quinol. Thus, ET and proton uptake are closely intertwined in this system as one process can drive or aid the other. Since partitioning between the different quinone states can be determined by protonation, interquinone ET in the RC can be controlled by the protein environment surrounding either quinone. In fact, QA resides in a hydrophobic pocket and cycles only between the quinone and anionic semiquinone states. This one-electron chemistry is most likely enforced by the inaccessibility of QA to protons that would be needed to undergo a second reduction to the quinol. QB, on the other hand, is situated in a rather polar environment with several hydrogen bond donors, which leads to stabilization of the QB•− state and confers a possible mechanism for conformational gating by hydrogen bonding.

The QB reduction cycle begins with the excitation of the primary donor (D) to generate the first charge-separated state D+QA•−QB, followed by re-reduction of D+ by cyt c2 (Scheme 1.6). ET from QA•− to QB spans 15 Å and produces the stable anionic semiquinone, QB•−. The rate of ET (kET1) from QA•− to QB is both temperature-dependent and invariable with driving force, suggesting that ET is not rate-limiting and that a conformational change may be required before ET. The proposed gating motion involves the twisting of QB from the inactive distal state with only one hydrogen bond to the active proximal conformation with four hydrogen bonds [137]. In addition to gating by QB, the
pH-rate profile shows that a residue with a $pK_a$ of 8.5 is involved and must uptake a proton prior to ET. From structural and spectroscopic studies of multiple forms of the RC, E212 was proposed as the critical residue, although this would require the $pK_a$ to be substantially perturbed by four orders of magnitude [138]. The $Q_B$ semiquinone is then protonated reversibly during the second light-initiated ET from $Q_A$ ($k_{h+}$). The second slow ET from $Q_A^{•−}$ coupled to this protonation event occurs in the rate-determining step, followed by a rapid proton transfer from E212 in the $Q_B$ binding site [139-141]. Pathways for both protons into the $Q_B$ site have been put forth, but remain incompletely defined. Although several residues have been implicated, the divergence of the pathways for first and second protons as well as the possibility of multiple proton channels continue to be areas of study. The amenability of the RC to mutational analysis, quinone replacement, and crystallography has permitted careful studies directed at disentangling the complicated cycle of proton and electron transfers [142]. In the RC, PCET allows for conformational gating and directionality of ET between two identical redox cofactors by tuning proton uptake in the protein microenvironments surrounding each quinone. As
discussed later, this provides an important mechanism for distinguishing the tyrosines of
the radical transfer chain of RNR, which further involves the added complication of
forward and reverse PCET.

Upon release of the reduced Q_{B}H_{2}, the quinol is bound at the Q_{o} active site of the
cytochrome bc1 complex and oxidized back to the quinone form (Figure 1.12). The two
electrons generated in this overall process are bifurcated with one electron being
transferred to the high potential chain comprising a Rieske-type [Fe-S] cluster, cyt c1 and
cyt c2, and the other being passed to the low potential chain consisting of two cyt b
hemes. The electron transferred to cyt c2 is returned to the RC to re-reduce the special
pair, while the second electron is passed through the cyt b chain to the Q_{i} site and used to
reduce the quinone formed at the Q_{o} site. Therefore, two Q cycles are required before the
quinone is fully reduced at the Q_{i} site and before an oxidized Q_{B} can be passed back to
the RC. The net result of this modified Q cycle is the vectorial transport of protons into
the periplasmic space to generate a proton gradient. The first electron transfer from Q_{B}H_{2}
to the high potential chain at the Q_{o} site takes place at a surprisingly slow rate (k_{ET}\sim 2 \times 10^{3} \text{ s}^{-1}) and shows a high activation barrier (\sim 65 \text{ kJ/mol}), as well as a large
reorganization energy (\sim 2.0-2.5 \text{ eV}), that are unexpected from Marcus theory given the
separation between the quinol and the [Fe-S] cluster [143,144]. As this step is rate-
limiting, the one electron-reduced semiquinone intermediate has not been directly
observed. In addition, although the activation barrier drops upon increase of pH, the rate
of quinol oxidation was found to increase at lower pH and decrease at alkaline pH [145].
This pH dependence was attributed to two groups with pK_{s}s of 6.5 and 8.5 respectively
[145,146], which were proposed to involve dissociation of a histidine ligand (H161) from
the Rieske-type [Fe-S] cluster in order to bind the quinol reactant [147,148]. Mutagenesis
of the [Fe-S] cluster-containing subunit can alter the pK_{s} of the cluster and indeed, the
pH-rate profile for quinol reduction shifts accordingly [149,150]. The anomalies in this

52
first ET can be explained in terms of a proton-gated ET in which both a proton and electron are transferred to the [Fe-S] cluster during quinol oxidation [151]. This would account for both the slow rate of ET and the large activation barrier and reorganization energy and also provides for a mechanism for proton translocation. The second electron transfer to form the fully reduced quinol is proposed to take place after rotation of QB in the Qo pocket, which both brings the semiquinone closer to the cyt b subunit face as well as deterring charge recombination with the reduced [Fe-S] cluster. Consistent with this model, the Qo site occupies a large volume in the available crystal structures of these enzymes [152,153].

1.4.3 Location of Radical Sites: Heme Peroxidases

As discussed in Section 1.1.3, cytochrome c peroxidase (CcP) contains a His-Asp-Trp triad at the proximal site to guide formation of a compound I intermediate with the oxidizing equivalent located on W191H+, rather than on the porphyrin macrocycle [154,155] (Figure 1.13). Although there is another tryptophan in the immediate vicinity of the heme, the oxidizing equivalent resides specifically with W191 [156]. This triad is reminiscent of the H118-D237-W48 triad in R2 [16], which is thought to control the radical transfer between Y122 and W48 (Figure 1.13). Mutational studies of the

![Figure 1.13 Structural comparison of the H-D-W triads from CcP and RNR. Note that the distances measured for CcP are between the heterocatoms rather than the hydrogens.](image-url)
aspartate bridge show that the D235A and D235N mutants are inactive, while the D235E mutant retains \(~40\%\) activity [157]. In addition, the D235A and D235N are found to exist in a low-spin state, while the wt and D235E enzymes are found in a high-spin state, implying the hydrogen bond to the axial H175 is intact in the D235E mutant. Disruption of the hydrogen bond leads to shifts in the Fe(III)/Fe(II) redox couple, with values of \(-182\) mV, \(-113\) mV, \(-79\) mV, and \(-78\) mV vs. NHE for wt, D235E, D235N, and D235A respectively. The midpoint potential for iron reduction indicates the strength of the hydrogen bond formed with H175, as wt CcP is thought to exist effectively with an imidazolate ligand to the heme [155]. X-ray structural studies confirm that the hydrogen bond is maintained the D235E, with the position of W191 unchanged [157]. Although the length of the hydrogen bond is the same as wt there exists a change in the geometry with respect to the H175 ligand that attenuates the strength of the interaction, involving a \(~15^\circ\) twist of the carboxylate oxygens as well as a \(~20^\circ\) decrease in the angle of the \(N_{\text{His}}-\text{H}-O_{\text{Glu}}\) hydrogen bond [157]. This is in contrast with D235N [158] and D235A [157] mutants, in which W191 was re-oriented with the indole NH hydrogen-bonded with a main chain carbonyl. All mutants exhibit an EPR spectrum indicative of an organic radical, however, only D235E exhibits the unique properties known to be associated with \(\cdot W191H^+\) [159,160]. In spite of the structural similarity between the active sites of the wt and D235E enzymes, the EPR spectrum of the \(\cdot W191H^+\) in the D235E mutant shows that one of the exchange coupling modes of the radical with the heme is lost, perhaps because of loss of the hydrogen-bonding mode where H175 is fully deprotonated with the proton located on D235. Although RNR possesses this same Fe-H-D-W motif, the crystal structure of R2 shows that the intervening D237 forms a one-point to W48 rather than a two-point hydrogen bond with both the analogous histidine and tryptophan [16]. In R2 as well, the D237E mutant retains activity while the D237N mutant does not, pointing to an important role of hydrogen bonding in radical initiation [30,37]. It may be that shifts in
D237 between one-point and two-point binding modes can gate the communication between W48 and Y122 via the iron center and H118.

CcP is unique compared to most heme peroxidases, as the oxidizing equivalent resides on the neighboring tryptophan rather than the porphyrin itself. In fact, most peroxidases contain a phenylalanine at the location corresponding to W191 [161-163]. More recently, the crystal structure of ascorbate peroxidase (APX) has been solved, which contains a nearly identical H-D-W catalytic at the distal heme pocket [164]. Mutation of the analogous W179 to phenylalanine has no effect on the catalytic reactivity of APX [165], although this same mutation eliminates activity in CcP [166]. Consistent with the mutational analysis, APX forms a porphyrin-based cation radical rather than the analogous tryptophan cation radical (•PorH⁺), despite its structural homology with CcP [167]. The origin of the partitioning between •PorH⁺ and •WH⁺ in APX versus CcP may derive from stabilization of •WH⁺ in CcP, destabilization of •WH⁺ in APX, or a combination of the two. The midpoint potential of W191 in CcP has been measured to be 740 mV vs NHE, which is unusually low compared to free tryptophan [168,169]. This has been attributed to a cation binding site motif surrounding W191, which is calculated to stabilize •W191H⁺ by 7.8 kcal/mol or 355 mV [170]. If W191 is mutated to glycine or glutamine, the monovalent cation site is unmasked and cations such as K⁺, NH₃⁺, and Tris are found in the resulting cavity. A comparison between the crystal structures of CcP and APX reveals a cation site that is 9 Å removed from W179 in APX, which is thought to destabilize formation of a tryptophan cation radical [164]. Indeed, when this cation site is engineering into the homologous loop of CcP, the stability of the •W191H⁺ species is dramatically decreased and titratable with K⁺ [171,172]. Calculations show that the formation of •W179H⁺ would be destabilized by 330 mV with respect to •W191H⁺ in CcP, although the effects are not entirely attributed to the existence of the cation site [173]. Comparison of CcP with APX shows that the surrounding protein environment is
essential for controlling the location of radical equivalents and can cause critical inequivalencies in structurally identical units. Although the peroxidases involve formation of a stable •WH+ rather than the transient •W48H+ proposed for RNR, these studies suggest that distant conformational changes could be sufficient to stabilize and destabilize this species during the catalytic cycle as required by our model.

1.4.4 Radical Transfer: DNA Photolyase

DNA photolyases provide the first observed example of radical hopping between aromatic amino acids in a protein system. These enzymes use a light-mediated radical reaction to repair the cyclobutane rings between adjacent pyrimidines generated by UV damage [174]. DNA photolyases typically consist of a single polypeptide chain of 50 to 65 kDa containing a noncovalently bound flavin adenine dinucleotide (FAD), the essential cofactor that serves to reduce the pyrimidine dimers, along with a secondary antenna cofactor, usually tetrahydrofolate or deazaflavin. The enzyme is isolated in the flavin semiquinone (•FADH) form as an artifact of aerobic purification [175], although it is only active in the fully reduced FADH2 form. It can, however, be activated by illumination with visible light. Although the photoactivation process seems to be irrelevant in vivo, it has been studied extensively as an informative model system for radical transfer as the reducing hydrogen atom equivalent is found to come from a protein-based tryptophan residue [176].

In the E. coli photolyase, the excited •FADH (•FADH*) oxidizes a solvent-accessible tryptophan, W306, 13.4 Å away that is then quenched by an external reductant to stabilize the reduced flavin state [176]. Using transient spectroscopy, Aubert et al propose that the oxidation of W306 occurs stepwise through a chain of two additional tryptophans, W382 and W359 [66] (Figure 1.14), a pathway supported by more recent theoretical calculations [177,178]. The characteristic signal in the near-IR region for •FADH* decays 30 ps after excitation, presumably by fast ET from the neighboring
Figure 1.14 Pathway between FAD and W306 with the intervening tryptophan residues, W382 and W359, in the E. coli photolyase. Adapted from ref. 66.

W382 (4.2 Å), which is reduced in turn by W359 (5.2 Å). The tryptophan cation radical spectrum in the visible region is observed within the pulse width (7 ns) of laser and is attributed to the formation of $\cdot W306 H^+$ (3.9 Å from W359). The radical cation spectrum slowly converts to the neutral radical in 300 ns by loss the proton to solvent, a reaction that shows pH, isotope, buffer, and viscosity dependence. The quenching of $\cdot W306$ competes with charge recombination, which can occur upon reprotonation of the neutral radical ($pK_a \sim 4$), as shown by pH studies. Mutation of W382 to phenylalanine reduces the amount of photoproduct by over two orders of magnitude as the intrinsic 80 ps decay of the excited $\cdot FADH$ outcompetes ET to W359 [67].

In DNA photolyase from A. nidulans, the photoactivation occurs in a slightly different fashion. [65,179] The external reducing equivalent is thought to be funneled into the flavin active site through a transient tyrosyl radical ($pK_a \sim -2$) via a neutral tryptophan radical, slowing the back reaction due to uphill re-oxidation of tryptophan. In similar experiments using laser flash photolysis, the radical equivalent was found to reside 40% on the tyrosine while 60% decays by an isotope-sensitive charge recombination with FADH. In addition, the reduction in the A. nidulans photolyase could involve an actual hydrogen atom transfer rather than the fast ET followed by slow proton dissociation seen in E. coli. Interestingly, both the tryptophan triad and the neighboring tyrosine that putatively becomes the observed tyrosyl radical are conserved in both the enzymes from A. nidulans (W390–W367–W314, Y468) and E. coli (W382–W359–W306, Y464). Although the tyrosine is further away from the terminal tryptophan in the A. nidulans
Figure 1.15 The arrangement of the FAD cofactor, tryptophan triad, and tyrosine in the photolyases from A. nidulans and E. coli. Y468 in the A. nidulans enzyme is on the surface of the protein while the corresponding Y464 is shielded from solvent in the E. coli enzyme.

photolyase (8.6 Å vs 3.6 Å in E. coli), it resides on the surface and is therefore solvent accessible, whereas the corresponding tyrosine is buried in the E. coli enzyme (Figure 1.15). If the buried tyrosine (E. coli Y464) were unable to undergo deprotonation due to a low protein dielectric, the ET from •W306H+ to form •Y464H+ is computed to be uphill by 750 mV. Calculations based on this value show that the forward ET rate in this case would be only 2 s⁻¹, which could be outcompeted by charge recombination between W306 and FAD (τ = 17 ms). Moreover, the calculated lifetime of •Y464H⁺ (10⁻¹³ s) is also limited by the back reaction with W306 [178]. Thus, even in virtually identical systems, the mechanism of radical transfer or PCET can differ depending on the specifics of pKₐ and redox potential as dictated by the protein environment. These studies may have implications with regard to radical transfer in RNR as they show that formation of Y* from •WH⁺ can be prevented by the surrounding environment of both residues. Thus, forward or reverse transfer from •W48H⁺ to either Y356 or Y122 respectively can be achieved by appropriate conformational changes in R2.

1.4.5 Engineered Systems: Rhenium-modified Azurin

Radical transfer is also beginning to be studied in engineered systems using flash-quench methodology, a technique that revolutionized the field of fixed-distance biological ET [180]. This allows for the physical parameters of PCET to be elucidated without being
bound by evolution or the particulars of enzyme function. Azurin (Az) is a simple blue copper protein, consisting mainly of β-sheets. Attachment of either a ruthenium(II) bis(bipyridyl) imidazole [181] or rhenium(I) tricarbonyl phenanthroline [182] complex through a protein-based histidine allows the photoinduced reduction or oxidation of the copper center to take place. Flash-quench oxidation of Re(CO)$_3$(phen)(H107)]$^+$AzCu$^+$ with an intervening tyrosine, Y108, is over two orders of magnitude faster than the rate of copper oxidation without the adjacent tyrosine (Figure 1.16) [183]. •Y108 can be generated in frozen samples in the H107-labelled Y72F mutant, which is consistent with the model that the rate increase is attributable to hopping through this residue [182]. Although the photogenerated •Y108 has not yet been shown to be kinetically competent, the observed rate increase is intriguing with respect to radical chain hopping in proteins. Interestingly, the attachment of the rhenium complex at H83 leads to the generation of •W48, which does not equilibrate to •Y108 (Figure 1.16) [182]. This may mean that the redox potential of tryptophan (1.05 to 0.50 V between pH 0-14) and tyrosine (1.25 to 0.65 V between pH 0-14) [36] within the protein are perturbed from the free amino acids (1.0 V and 0.9 V respectively) in neutral aqueous solution. These studies show that radical transfer in proteins by a hopping mechanism may potentially increase the
transport rates of a redox equivalent as it does in DNA. Thus, the large separation between redox sites (Y122 and C439) can be reconciled with the rate of nucleotide reduction if this mechanism is truly in effect for the class I RNRs.

1.4.6 Conclusions

PCET provides a tuning mechanism for both proton and electron transfer in protein environments. The interplay between redox and acid/base chemistry allows for protein control of timing and directionality of both events even when the redox cofactors, whether quinones or aromatic amino acids, have very similar properties. Mutational analysis of these protein systems, such as in CcP or PS II, indicate that the proper geometry and strength of a single hydrogen bond can be key with respect to catalytic activity. The kinetic parameters for long-range PCET have been best described for the RC, as the ability to photoinitiate the reaction has permitted the resolution between proton and electron by careful analysis of wild-type and mutant RCs. In addition, the option of replacing the non-covalently bound quinones has allowed methodical driving force and pKₐ studies. Although amino acid radicals have been proposed as PCET intermediates, their study has been limited by the inability to replace these redox cofactors systematically as in the RC. Although amino acid radical intermediates have not yet been observed in physiologically relevant systems, the preceding model proteins have contributed significantly to our understanding of radical transfer as the respective PCET reactions may be initiated synchronously for spectroscopic studies.

1.6 Specific Aims and Organization of the Thesis

While studies of radical transfer in model proteins have contributed considerably to our understanding of biological PCET, the 35 Å intrasubunit PCET of RNR provides a unique opportunity to dissect a long-range physiological PCET reaction that is required for each enzymatic turnover. However, conformational changes gated by substrate and effector binding have masked analysis of PCET and consequently, no direct evidence for
any radical intermediates exists. In addition, the long-range radical transfer between R2 and R1 is a complicated problem that involves tyrosyl radical formation at the dinuclear iron core and may involve ligands to the diiron center. For example, in the mammalian class I RNR, CDP is turned over at a rate that is 0.5% of wt activity with no stable tyrosyl radical in the Y122F mutant (Y177, mouse numbering) and may derive non-specific radical generation upon formation of the diiron cluster assembly, which occurs approximately every 100 turnovers in the mouse R1 [184]. The Y122F mutation in *E. coli* [185-187], as well as other mutations [188], lead to generation of radical species which are believed to be off-pathway. Therefore, we require the development of methodology to trigger the RNR PCET reaction synchronously using light-initiated amino acid radical generation. Our experimental strategy exploits the fact that Y356, the last member of the PCET pathway in R2, is present on the unstructured C-terminal tail of R2. This 20-mer tail (Y356 to L375, abbreviated R2C20) competes with the full subunit for binding to R1 ($k_D \sim 20 \mu M$) [85]. Generation of a tyrosyl radical at position 356 on a synthetic C-terminal peptide in complex with R1, in the presence of substrate and effector, could provide a

![Diagram](image.png)

*Figure 1.17* Strategy used in this thesis for the study of the PCET pathway of RNR. Photoinduced decaging of a tyrosyl radical at position Y356 on the R2 C-terminal tail generates a PCET intermediate, which should be able to propagate the radical forward to C439 in the R1 active site to generate thyl radical and deoxynucleotide.
specific entryway into the radical initiation pathway of R1 (Figure 1.17). The competence of the peptide to initiate enzymatic turnover by radical initiation could then be monitored both biochemically by nucleotide reduction activity and spectroscopically by thyl radical generation. By eliminating the requirement for R2, the inter-subunit PCET can be isolated to the pathway between Y356 on the R2 C-terminal peptide and C439 in the R1 active site. Thus, we hope that reduction of the complexity of the PCET pathway of Figure 1.10 will allow us to further elucidate the radical initiation process.

This thesis is structured as follows: Chapter 2 presents the synthesis of the R2 C-terminal peptide, R2C20, while Chapter 3 explores the different methodologies undertaken to trigger tyrosyl radical generation by light-mediated methods. Chapter 4 explores the development of assays for light-mediated nucleotide reduction catalyzed by modified peptides. Chapter 5 presents ongoing work and future directions of this project.
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Chapter 2

Synthesis of the R2 C-Terminal Tail
2.1 Introduction

Insight into the model for radical initiation in the class I RNRs relies on the docking model generated from the individual crystal structures of R1 [1,2] and R2 [3]. In addition to the shape complementarity between the two subunits, the docking model is also based on the observation that the binding interactions between R1 and R2 are mediated by the highly acidic C-terminal tail of R2; removal of this tail from R2 effectively eliminates interactions between the two subunits without affecting the catalytic efficiency of the active site of either subunit [4,5]. ²H-NMR studies of both the *E. coli* [6] and mammalian R2s [7] demonstrate that their C-termini are highly flexible in absence of R1, but order upon the formation of the R1-R2 complex. In fact, addition of R2C20 aids the crystallization of R1, presumably to help order R1 homogenously [1]. Unsurprisingly, the R2 C-terminus of *E. coli* is also disordered in the crystal structure and is invisible until residue 340 [3], while the 20-mer peptide, present in the crystallization of R1, is only visible from residue 360 on (to residue 375). In the docking model (Figure 1.1), R1 and R2 are oriented such that the termini of the disordered region of R2 may be spanned by

![Figure 2.1 A docking model of individual crystal structures of R1 and R2 generated by shape complementarity showing the residues involved in the PCET pathway. R1 is shown in green and blue and R2 is shown in yellow and red. The R2 peptide (R2C20) present in the crystal structure of R1 is indicated in magenta. Panel A shows the termini of R2C20 (380-375) as visualized in the crystal structure of R1 as well as the last visible amino acid of R2 (340). Panel B shows a 90° rotation with the residues involved in PCET shown in light blue as well as substrate (GDP) and specificity effector (TTP) in CPK.](image-url)
the twenty amino acids (residue 340 to 360) missing from the R2 structure. Unfortunately, Y356 is contained within this disordered segment and its geometric position within the PCET pathway is ambiguous.

Mutational analysis of both the *E. coli* and mammalian RNRs shows that in the R2Y356A [5], R2Y356F [8], and corresponding mammalian R2Y356F (Y370, mouse numbering) [9] mutants enzymatic activity is lost by a factor of $5 \times 10^{-3}$ without affecting cofactor or complex formation; moreover, the Y356W mutation is lethal *in vivo* [10]. More recent studies with a His$_6$–R2 show that Y356F mutation reduces activity to $5 \times 10^{-5}$ of the corresponding wt enzyme [11]. Since Y356 is quite distant from either the R1 or R2 active site, it must be required as either a structural residue or as a radical mediator between subunits. As Y356 is absolutely conserved as well as essential for nucleotide reduction, a role in radical initiation is the more attractive hypothesis, as mutation of tyrosine to phenylalanine is structurally conservative while abolishing physiological redox activity. Consistent with this interpretation, replacement of Y356 with 3-nitrotyrosine using intein ligation methods shows that nucleotide reduction activity is reduced by a factor of $10^{-3}$, within the lower limit of detection of wt R2 contamination from heterodimer formation with the semi-synthetic R2s [11]. As hydrogen-bonding capability is maintained in this R2 derivative, it is likely that again the change in reduction potential is responsible for the loss of activity. In addition, Y356 is thought to mediate redox chemistry related to Y122 oxidation during diiron center assembly [8] as well as cofactor regeneration from met-R2 [12]. Thus, we hope that our experimental strategy will allow us to break down the PCET pathway and isolate the radical initiation process from Y356 to C439 by modeling the *Y356 PCET intermediate with light triggered decaging of the corresponding Y• on a synthetic R2 peptide.
2.2 Peptide Syntheses

2.2.1 Introduction

The C-terminal tails of various class I R2s have been studied for use as potential inhibitors of nucleotide reduction activity in vivo. As DNA synthesis is a strong target for rapidly dividing tumor and virus cells, the selective inhibition of endogenous and exogenous RNRs is a potential line of inquiry for anti-cancer and anti-viral drugs. These competitive inhibitors act by binding to R1 in place of R2, thereby blocking nucleotide reduction. As many of the R2 C-terminal sequences show little homology, it is possible that these inhibitors will specifically inhibit only the RNRs from which they are derived. Both the mammalian and the herpes simplex virus C-termini (7 and 9 amino acids long respectively) have been studied in depth, leading to the development of highly potent peptide and peptidomimetic inhibitors, which are indeed not cross reactive. [13]

Less is known about the E. coli R2 peptide, but it has also been demonstrated to competitively inhibit nucleotide reduction activity resulting from formation of the R1 and R2 complex. Kinetic analysis further shows that two peptides are capable of binding to each R1 dimer with equal affinity [14]. The 37-mer (Table 2.1, 1) and 30-mer (Table 2.1,

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K/\mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LVSDNVQVAPQEVEVSSYLVGQIDSEVTDDDLNSFQL</td>
<td>18.3</td>
</tr>
<tr>
<td>2</td>
<td>VAPQEVEVSSYLVGQIDSEVTDDDLNSFQL</td>
<td>21.5</td>
</tr>
<tr>
<td>3</td>
<td>YLVGQIDSEVTDDDLNSFQL</td>
<td>20.0</td>
</tr>
<tr>
<td>4</td>
<td>LVGQIDSEVTDDDLNSFQL</td>
<td>40.0</td>
</tr>
<tr>
<td>5</td>
<td>Ac-DDLSNFQL</td>
<td>370.0</td>
</tr>
<tr>
<td>6</td>
<td>Ac-YLVGQIDSE</td>
<td>4000.0</td>
</tr>
</tbody>
</table>

Table 2.1 R2 C-terminal peptides from E. coli and inhibition constants from ref. 14.
2) C-terminal peptides have similar inhibitory abilities as the 20-mer peptide (Table 2.1, 3), implying that the N-terminus of the peptide may be modified without any detrimental effects in recognition. Complete removal of Y356 itself from the peptide actually only results in a two-fold decrease in the inhibitory ability of the peptide (Table 2.1, 4). Indeed, most of the binding energy can be attributed to the eight C-terminal amino acids (Table 2.1, 5) with the nine N-terminal amino acids contributing minimal binding energy (Table 2.1, 6). Interestingly, parallel studies with R2 heterodimers composed of one full-length monomer and one truncated monomer missing the C-terminus show that these heterodimers bind with a similar dissociation constant as a single peptide, consistent with the interpretation that the majority of binding energy for formation of the R1–R2 complex derives from the C-terminus [5].

Retention of the binding mode of the derivatized peptide is one of the major concerns in this strategy, as we will require the addition of photoactive groups to R2C20. Fortunately there is some freedom in the N-terminal binding mode adjacent to Y356, as it is disordered and conformationally flexible in the crystal structure of the R1-R2C20 complex. For caged designs, the introduced modifications will be removed irreversibly by photolysis, leaving a bare tyrosyl radical for single turnover experiments that can be probed by a sensitive radioactively assay for nucleotide reduction. For optical studies, however, it is preferable to incorporate a reversible flash-quench system, such as ruthenium [15] and rhenium [16] based metal complexes, in which the spectra of transient species may be time-averaged to improve the signal-to-noise ratios, especially in consideration of the relatively small extinction coefficients of the amino acid radicals of interest. If binding remains an issue with these systems, flash-flow experiments with the caged tyrosines or bimolecular flash-quench experiments are a possibility.

2.2.2 Results and Discussion

At the outset, the primary structure of the R2 C-terminal tail did not project any obvious
areas of difficulty for the synthesis of the 19-mer peptide, (R2C19 = LVQIDSEVDTDDLSNFQL). Thus, we initially started with a single-coupling DIC/HOBt activation scheme on a highly-substituted Wang resin with seven eq of amino acid (Figure 2.2). The peptide was cleaved from the resin in a TFA cocktail and analyzed by reverse-phase HPLC and MALDI-TOF mass spectrometry. As the 19-mer peptide was suspected to precipitate in the standard reverse-phase HPLC gradient in 0.05% TFA used for peptides, the HPLC was run using both the standard acidic gradient (Figure 2.3) and a modified gradient in basic conditions (Figure 2.4). Note that the 19-mer peptide is present in a smaller percentage in Figure 2.3 due to low solubility below pH 6-7. The peaks in Figures 2.3 and 2.4 corresponding to the 19-mer peptide were identified by MALDI-TOF.

![HPLC trace](image_url)
Figure 2.4 HPLC trace of R2C19 synthesis using a highly substituted Wang resin and DIC/HOBt coupling chemistry in a linear gradient of 10%-75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1 mL/min) over 45 min. Product peak is indicated with a red asterisk (*) and identified by MALDI-TOF MS.

MS and are indicated with a red asterisk. Unfortunately, this initial synthesis of the 19-mer peptide showed multiple products and only a small amount of desired product as evidenced by reverse-phase HPLC in combination with MALDI-TOF MS. Since the mass spectrum of the crude peptide material showed that majority of the side-products were due to early chain-termination at ΔL and ΔLV, the synthetic scheme was modified by using stronger coupling reagents as well as double-coupling steps.

Double-coupling steps were included at β-branched amino acids and the residues immediately following, as these sterically bulky residues are known to reduce the coupling efficiency. A stronger activation scheme of PyBOP/HOBt/DIEA [17] was also used. These synthetic conditions led to a greatly increased amount of 19-mer peptide, however, the yields were found to vary from 60-80% (Figure 2.5). Decreasing the resin substitution (from 0.4 to 0.2 mmol/g) and increasing the linker length with a PEG–PAC–
PS resin (Figure 2.6) also aided in improving the yield of 19-mer peptide by reducing the amount of chain terminated products to the 18-mer peptide alone (Figure 2.7). Unexpectedly, the variance in product yield was not caused by early chain termination, as the amount of 18-mer peptide remained consistent (~15%), but was due to several higher molecular weight products with a mass difference of 67 Da (Figure 2.7). One possible explanation for the observed mass difference is that the peptide could chelate a metal ion through one of the six carboxylates. Thus, the peptide was treated by passing through a

Figure 2.5 HPLC trace of R2C19 synthesis using a highly substituted Wang resin and PyBOP/HOBt/DIEA activation scheme in a linear gradient of 10%-75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1 mL/min) over 45 min. Product peak is indicated with a red asterisk (*) and identified by MALDI-TOF MS. Chain-terminated peptides are those migrating earlier than R2C19.

Figure 2.6 Synthesis of R2C19 on a PEG-PAC-PS resin with double-coupling PyBOP/HOBt chemistry.
Chelex resin and re-chromatographed in the presence of 1 mM EDTA, however, the elution profile was unchanged from the untreated peptide, and retained the several peaks deriving from +67 Da species (data not shown). As metal ion chelation was not believed to be the source of the higher molecular weight products, the additional mass most likely results from an organic modification of the peptide during synthesis. At this time, we were unable to determine the source of the multiple +67 Da species and turned our attention to reducing the amount of 18-mer peptide in the synthesis.

As the 18-mer peptide was always present at considerable levels in the peptide products (Figure 2.7), it was clear that there was either difficulty deprotecting or coupling at that position, which would not be surprising as the preceding amino acid is a valine. Interestingly, the primary structure of the 19-mer peptide from the R2 C-terminus does not show any high propensity for aggregation or secondary structure formation until L19.
Figure 2.8 Aggregation potential of R2C19 calculated by Peptide Companion. Residues with score values above 1.1 (indicated in red) are defined as positions of difficult coupling due to aggregation or secondary structure formation.

(Figure 2.8). Therefore, the 18-mer peptide was then synthesized using a continuous-flow Millipore 9050 system to further explore the problems with addition of the N-terminal leucine of R2C19. The synthesis of the Fmoc-protected 18-mer peptide proceeded smoothly at 90% yield (Figure 2.9a), however, removal of the N-terminal Fmoc was a considerable problem. After one round of deprotection with 20% piperidine for 20 min, 15-20% of Fmoc-R2C18 still remained (Figure 2.9b). As shown by the difficulty in deprotection at V18, the 18-mer peptide was clearly forming significant secondary structure. We then decided to carry out a manual synthesis on large-scale while monitoring the release of the Fmoc-fulvene during deprotection with piperidine. R2C19 was synthesized in an end-capped fritted column using an elliptical shaker. Amino acids were added by double coupling for one h with five equivalents of Fmoc amino acid per resin site and followed with capping reactions with acetic anhydride. The extent of Fmoc deprotection was monitored by the decrease in fulvene absorption at 301 nm in the piperdine flow through (ε = 5315 M⁻¹cm⁻¹). The total amount of Fmoc removed was approximately 90% at each cycle; typically six or seven rounds of deprotection were required before the A₃₀₁ nm was less than 0.1 OD. The synthesis was analyzed by reverse-phase HPLC at the 7-mer, 10-mer, 12-mer, 17-mer, and 19-mer stages. Figure 2.10 shows representative HPLC traces using a C-18 column. At the 7-mer stage (DLSNFQL), the synthesis is proceeding smoothly with few peptide side-products (Figure 2.10a). Upon addition of three more amino acids (DTDDLSNFQL), the +67 Da species begins to
Figure 2.9 HPLC traces of Fmoc-R2C18 continuous-flow synthesis using a low substitution PEG-PAC-PS resin and HBTU/HOBt coupling chemistry in linear gradient of 10%-75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel A) Before and (Panel B) after deprotection in 20% piperidine. Fmoc-R2C18 peak is indicated with a blue asterisk (*) and R2C18 peak is indicated with a red asterisk (*). These peaks were identified by MALDI-TOF MS.
Figure 2.10 Manual synthesis of R2C19 with Fmoc deprotection monitoring. (Panel A) Reverse-phase HPLC trace of the 7-mer peptide using a linear gradient of 10-55% acetonitrile versus 0.05% TFA (1.0 mL/min) over 30 min. (Panel B) Reverse-phase HPLC trace of the 10-mer peptide using a linear gradient of 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel C) Reverse-phase HPLC trace of the 19-mer peptide using the gradient described for Panel B. Product peaks indicated with a red asterisk (*) and +67 Da peaks indicated with a blue asterisk (†).
Scheme 2.1. Aspartimide formation from aspartate and ring-opening by piperidine during peptide synthesis.

appear (Figure 2.10b). In the HPLC traces of R2C12 (EVDTDDLNSFQL) and R2C17 (QIDSEVDTDDLNSFQL), the amount +67 Da species has increased to ~80% of the parent peaks with few chain-terminated peptides (data not shown). The trace showing the final product, R2C19, shows a similar profile to the 12-mer and 17-mer peptides, with an extremely large percentage of +67 Da peptides with one predominate form (Figure 2.10c). The set of small peaks immediately preceding the 19-mer result from capped and uncapped R2C17 and R2C18. Although this particular synthesis was not of preparative utility, it allowed us to isolate the onset of the +67 Da modification to the stretch of carboxylate residues added between the 7-mer and 12-mer peptides. As the major change in the synthesis was the increased amount piperidine deprotection of the Fmoc groups, we realized that the modification was arising from β-piperidide adducts [18,19] formed by the attack of piperidine upon cyclized aspartate residues [20,21]. Although other pathways for aspartimide decomposition are possible, the relevant pathway for generation of the β-piperidide of aspartate (+67 Da) is shown in Scheme 2.1. Formation of the β-piperidide can be suppressed by use of N,O-bis-Fmoc-amino acid 2-hydroxy-4-methoxybenzyl (Hmb) derivatives immediately preceding aspartates to protect the amide backbone [22] or by simple addition of HOBT or 2,4-dinitrophenol to the piperidine solution during deprotection [23].

A solution to the two-fold problem of difficult deprotection at position 18 and β-piperidide formation was to both incorporate a pseudoproline dipeptide into the synthesis.
Scheme 2.2 Generation of an Asp-Ser dipeptide from the Asp(OBu)-Ser(YMc,Mc-pro)-OH pseudoproline derivative at position 13-14 of R2C19 upon treatment with TFA.

of R2C19 and to add hydroxybenzotriazole during removal of the Fmoc protecting groups [23]. The pseudoproline dipeptide derivatives are designed to break up helical structure during synthesis by adding a kink, as a proline would. Upon cleavage with TFA, the pseudoproline structure opens to a dipeptide containing either serine or threonine at the terminal position; the Asp(OBu)-Ser(YMc,Mc-pro)-OH derivative was used at position

Figure 2.11 HPLC trace of R2C19 synthesis using a pseudoproline dipeptide and 0.1 M HOBr in the piperidine deprotection solution on a continuous flow Pioneer synthesizer with a low substitution PEG-PAC-PS resin and HATU coupling chemistry in a linear gradient of 10%-75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. Product peak is indicated with a red asterisk (*) and identified by MALDI-TOF MS.
6-7 of R2C19 (Scheme 2.2). Upon addition of the pseudoproline amino acid, the synthesis of R2C19 proceeds smoothly at almost quantitative yield using HATU chemistry (Figure 2.11). In addition, the simple inclusion of 0.1 M HOBr to the piperidine deprotection solution satisfactorily inhibits β-piperidide C- formation, which is a much simpler resolution to the problem of aspartate cyclization than backbone protection with Hmb amino acid derivatives. The use of the pseudoproline also prevents piperidide formation at that particular aspartate.

The purified R2C19 was also characterized by MALDI-TOF mass spectrometry. The lack of basic residues in the sequence difficulties in data collection by MALDI-

![MALDI-TOF MS](image)

**Figure 2.12** MALDI-TOF MS of purified R2C19. 256 scans were collected in both negative and positive ion mode. The measured molecular weights fall within the accepted error of the MS measurement (1-2 Da) of the calculated molecular weights.
TOF and electrospray methods, which are typically run in positive ion modes. A characteristic MALDI-TOF MS in both negative and positive ion mode is shown in Figure 2.12, calibrated externally with a mixture of three peptides bracketing the m/z range of our peptide. Each spectrum is a composite of the parent ion as well as various sodium and potassium salts. We find that positive ion mass spectra are unusually difficult to obtain with the C-terminal peptide unless the sample concentration is orders of magnitude higher than that required for typical peptides. Note that the total counts for the positive ion collection in Figure 2.12 is two-fold less, although the laser power for ion desorption was increased, and that the positive ion spectrum is dominated by the Na⁺ form of the peptide. Thus, MS of the R2 peptide must instead be collected in negative ion mode. This leads to decreased sensitivity and problems using established peptide calibrants developed for positive ion detection.

2.3 Conclusions

Inspection of R2C19 sequence does not present any obvious synthetic challenges at the outset, however, preliminary attempts at synthesis showed several areas of difficulty. Firstly, the high acidity of the peptide (no basic residues and six carboxylates including the C-terminus) required the development of purification methods other than the standard 0.05-0.10% TFA (pH 2.0) gradient due to insolubility at low pH conditions. The purification gradient using 0.1 M ammonium bicarbonate also leads to increased salt content, consequently, peptides needed to be lyophilized completely before mass spectrometry to remove the buffer ions. Other HPLC gradients were assayed (i.e. 0.1 M triethylammonium acetate, pH 7.0), however, ammonium bicarbonate still yielded the best separations.

Although the large number of aspartates in the sequence of R2C19 initially lead to formation of piperidides during deprotection, the simple addition of HOBt to the deblock solution virtually eliminated this problem. With the inclusion of the pseudoproline
dipeptide at position D₆S₇ as well as the use of a low substitution resin, the issues related
to peptide aggregation and secondary structure formation were resolved. With the kink
placed early in the growing peptide chain, removal of the Fmoc groups was no longer
problematic. The use of the pseudoproline also removed the need for double-coupling
steps and all couplings were reduced to a single extended coupling to yield a clean 19-
mer starting material for further synthesis (Figure 2.10).

2.4 Experimental Methods

**Reagent Information.** N,N-Dimethylformamide (DMF) and N-methylpyrrolidine
(NMP) were treated with molecular trap from Chemassist Corporation (Brookline, MA)
for a minimum of 2 days before usage to remove water and dimethylamine. Piperidine
was either purchased from Sigma-Aldrich (St. Louis, MA) or as a 20% (v/v) solution in
DMF from Applied Biosystems (Foster City, CA). Wang resins were obtained from
Novabiochem (San Diego, CA) at ~0.4 mmol/g substitution and PEG-PAC-PS resins
were obtained from Applied Biosystems at ~0.2 mmol/g substitution with the C-terminal
Fmoc amino acid attached. Fmoc amino acids were purchased from either Novabiochem
or Applied Biosystems with trityl or tert-butyl protecting groups. Pseudoproline
dipeptide Fmoc-Asp(O'Bu)-Ser(ψⁿΜe,Μe₃pro)-OH was purchased from Novabiochem.
Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), 2-
(1H-Benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) and N-
hydroxybenzotriazole (HOBt) were procured from Novabiochem while N-
[(dimethylamine)- 1H - 1, 2, 3 - triazolo [4,5-b] pyridine-1-ylmethylene] - N -
methylmethanammonium hexafluorophosphate N-oxide (HATU) was purchased from
Applied Biosystems. Peptide synthesis grade trifluoroacetic acid (TFA),
triisopropylsilane (TIS), diisopropylethylamine (DIEA), and ammonium bicarbonate
were bought from Sigma-Aldrich (St. Louis, MA). C-8 Vydac columns were purchased
from Vydac Corporation (Hesperia, CA). C-18 Econosil column was purchased from
Alltech (Deerfield, IL). XTerra MS 5 μm C-8 and XTerra MS 5 μm C-18 columns obtained from Waters Corporation (Milford, MA). Peptides for MALDI-TOF calibration were obtained from two sources. Angiotensin I and ACTH 18-39 were purchased from Bachem Chemicals (King of Prussia, PA), while oxidized bovine insulin obtained from Sigma-Aldrich.

**Synthesis of Ac-LVGQIDSEVDSTDLSNFQL with dicyclohexylcarbodiimide activation.** The synthesis was carried out on a Wang resin on a 0.05 μmol/well scale using Fmoc chemistry on an Advanced Chem Tech 32 with a 1 DIC:1 HOBT activation scheme with 7 equivalents of Fmoc amino acid. DIC and HOBT were dissolved separately to 0.5 M in DMF and Fmoc amino acids were dissolved separately to 0.4 M in NMP before mixing. Each cycle consisted of single-coupling of the amino acid for 60 min, capping with 1 acetic anhydride:1 DIEA for 5 min, and followed by two cycles of deprotection in 25% (v/v) piperidine in DMF for 5 min; these steps were interspaced with 1 min washes in DMF. Acetic anhydride and DIEA were diluted to 0.1 M in DMF.

**Synthesis of LVGQIDSEVDSTDLSNFQL with PYBOP activation.** The synthesis was carried out both on the Wang and PEG–PAC–PS resins on a 50 or 100 μmol/well scale using Fmoc chemistry on an ACT 32 with 0.8 PYBOP:1 HOBT:2 DIEA activation scheme with 4 equivalents of Fmoc amino acid. PyBOP, HOBT. DIEA were dissolved separately to 0.4 M, 0.5 M, and 1.0 M respectively in DMF, Fmoc amino acids were dissolved to 0.4 M in NMP before mixing. Double-coupling was programmed at residues L19, V18, Q16, I15, E12, V11, D10, and T9. Each cycle consisted of either single-coupling of the amino acid for 60 min or double-coupling for two 30 or 60 min periods, capping with 1 acetic anhydride: 1 DIEA for 5 min, and followed by two rounds of deprotection in 25% (v/v) piperidine in DMF for 5 min and 10 min each; these steps were interspaced with 1 min washes in DMF. Acetic anhydride and DIEA were diluted to 0.1 M in DMF.
Synthesis of Fmoc-VGQIDSEVDTDDLSNFQL with HBTU chemistry. The synthesis was carried out on a PEG–PAC–PS resin on a 100 µmol scale using Fmoc chemistry on a continuous-flow Millipore 9050 with a 1 HBTU:1 HOBT:2 DIEA activation scheme with 4 equivalents of Fmoc amino acid. Amino acids and HBTU were weighed into each vial and dissolved each cycle in 0.5 M HOBT before activation with DIEA. Each cycle consisted of either a single-coupling of the amino acid for 60 min or double-coupling for two 60 min periods, capping with 1 acetic anhydride: 1 HOBT (10.2 g HOBT (0.26 M), 7.1 mL acetic anhydride (0.24 M), 25 mL CH₂Cl₂, 225 mL DMF) for 10 min, and followed by deprotection in 20% (v/v) piperidine in DMF.

Manual synthesis of LVQIDSEVDTDDLSNFQL with HBTU chemistry. The manual synthesis was carried out on a PEG–PAC–PS resin on a 1.2 mmol scale in a fritted column with a screw top and teflon seal tied down to an elliptical shaker. Fmoc chemistry was used with a 1 HBTU:1 HOBT:2 DIEA activation scheme with 5 equivalents of Fmoc amino acid. Each cycle was performed as follows. Amino acid (5.75 mmol) and HBTU (5.12 mmol) were dissolved together with sonication in 11.5 mL of DMF; this solution was then mixed with 0.5 M HOBT (11.5 mL) and activated 1.0 M DIEA (11.5 mL) prior to addition to the resin and shaking (2 × 1 h). After washing with DMF (5 × 50 mL), a ninhydrin test was conducted as a macroscopic check for coupling efficiency. The resin was then capped with 1 acetic anhydride:1 HOBT in DMF/CH₂Cl₂ (50 mL) for 20 min and washed with DMF (4 × 50 mL). The resin was deprotected with 20% (v/v) piperidine in DMF (50 mL) until the A₃₀₁ was 0.1 OD or less, typically 6 or 7 times. Before starting the next coupling cycle, the resin was washed again with DMF (5 × 50 mL).

Synthesis of LVGQIDSEVDTDDLSNFQL with HATU/pseudoproline chemistry. The synthesis was carried out on a PEG–PAC–PS resin using Fmoc chemistry on a continuous flow Pioneer Synthesis System from Applied Biosystems with a 1 HATU:2 DIEA activation scheme with 4 equivalents of Fmoc amino acid. Amino acids D₈S₇ were
replaced with the pseudoproline dipeptide, Fmoc–Asp(OtBu)–Ser(YMe,Mepro)–OH from Novabiochem. Deprotection was carried out in 20% (v/v) piperidine with 0.1 M HOBT. Each cycle consisted of a 1 h coupling, followed by 5 min capping reaction with acetic anhydride for 5 min, and finally a 5 min deprotection as described above.

**Ninhydrin test for free amines** 95 parts of solution A (0.2% (w/v) ninhydrin in n-butanol) with 5 parts solution B (10% (v/v) acetic acid in water) was added to 5 to 10 beads of resin in a test tube. This was heated above 100 °C for less than five min. A dark blue or purple color indicates the presence of a free amine.

**Cleavage from the resin and isolation of peptide material** Cleavage of the peptide from the resin was accomplished by vortexing for 2-3 hs in a Reagent K cocktail (87.5% trifluoroacetic acid with 5% (w/v) phenol, 5% thioanisole, 2.5% 1,2-ethanedithiol, and 2.5 % water) in a disposable 20 mL fritted Bio-Rad column. Later, the cleavage mixture was changed to 95% (v/v) TFA, 2.5% (v/v) TIS, and 2.5% (v/v) water as the peptide did not contain any leaving groups that required the stronger scavengers. After this time, the eluent was collected and concentrated with a stream of N₂ in either a 14 mL or 50 mL polypropylene Falcon tube, depending on the amount of peptide. After concentration to under 1 mL or 3 mL respectively, diethyl ether was added in a > 1:7 ratio to visibly precipitate the white peptide material. This suspension was incubated at -10 °C for 1 h to overnight and collected by centrifugation. The solid was then washed twice with diethyl ether before air-drying the pellet. This pellet was resuspended in 0.1 M ammonium bicarbonate, pH 8.0 and lyophilized.

**Peptide purification by reverse-phase HPLC** R2C19 was isolated by reverse-phase HPLC on a pH-stable C-8 Vydac column (analytical, 4.6 × 250 mm; semi-preparative, 9 mm × 250 mm) or C-8 XTerra MS 5 μm column (analytical, 4.6 × 100 mm; preparative, 30 × 100 mm). A C-18 XTerra MS 5 μm column (analytical, 4.6 × 100 mm) was also
used where indicated. A linear gradient of 10% to 65% acetonitrile versus 100 mM ammonium bicarbonate, pH 8.0 over 45 min was used to separate the peptide products. The flow rates were as follows: analytical, 1.0 mL/min; semi-preparative, 4.0 mL/min; preparative, 22.5 mL/min. The fractions were collected and lyophilized either for mass spectral analysis or sample preparation. When a standard TFA gradient is indicated, analytical reverse-phase HPLC was also carried out a C-18 Econosil column (4.6 × 250 mm) using a linear gradient of 2% to 60% acetonitrile versus 0.05% TFA over 60 min.

**Peptide characterization by MALDI-TOF MS** The mass of peptides were determined using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry on a Voyager workstation (PE Biosystems). The matrix consisted of a >10 mg/mL suspension of α-cyano-4-hydroxycinnamic acid in a solution of 50% acetonitrile (v/v), 49% (v/v) water, and 1% (v/v) TFA. If needed, the α-cyano-4-hydroxycinnamic acid may be recrystallized from hot ethanol and water. The suspension was sonicated and centrifuged before spotting 1 μL of the supernatant with 1 μL of a salt-free sample dissolved in water onto the 100-well MALDI target. Mass spectra were collected in negative ion and linear mode using a 600-1000 MW cutoff. A linear calibration was conducted using angiotensin I (M_{ave} = 1295.5 Da), ACTH 18-39 (M_{ave} = 2464.7 Da), and oxidized bovine insulin (M_{ave} = 3495.9).

**Chelex treatment of R2C16** Chelex resin (0.5 mL) was placed in a Bio-Rad spin column and equilibrated with water. R2C17 was incubated with shaking for 10 min before eluting by centrifugation.
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Chapter 3

Methods for Light-Initiated Tyrosyl Radical Generation
3.1 Introduction

Interest in the role of protein radicals in enzymatic catalysis has steadily escalated as stable and transient radicals have been increasingly identified in Nature [7]. Oxidized tyrosines and their derivatives, in particular, are ubiquitous in a variety of biological systems besides the class I ribonucleotide reductases, including PS II [2,3], galactose oxidase [4,5], and prostaglandin H synthase [6]. Although radical-based catalysis is now being widely recognized as a paradigm for enzyme reactivity, there are few time-resolved methods available to date for the investigation of reaction mechanisms enabled by the generation of amino acid radicals, especially on fast timescales. In this regard, laser flash-

\[
\text{R} \quad \text{hv} \rightarrow \quad \text{R}^* + \text{H}
\]

*Scheme 3.1 Generation of amino acid radical in a light-mediated reaction. \( R \) = amino acid side-chain of cysteine, glycine, tyrosine, and tryptophan.*

photolysis techniques have led to significant advances in studies of biochemical reactions that require information at time-scales shorter than the millisecond resolution provided by stopped-flow methods and limited by mixing. Thus, the development of techniques to “turn on” amino acid radicals quickly by light would contribute an important new methodology for the study of radical chemistry in biology (Scheme 3.1).

Compared to other approaches of initiating chemical reactions, such as pH and temperature jumps, optical triggers are far more desirable because they allow a relatively instantaneous release of a reactive species from an inert molecule with minimal damage to the protein if the photochemistry occurs outside the protein absorption envelope. Flash-photolysis tactics have led to significant advances in detailed studies of other systems that require information at timescales shorter than the millisecond resolution.
provided by stopped flow approaches. For example, time-resolved Laue diffraction [7-9] has been most successful when using either photo-deprotection of shielded groups [10,11] or photochemical liberation of caged substrates [12,13]. Another example where photochemical triggers has uncovered new insights is the field of protein folding where electron injection into metalloproteins by flash photolysis [14,15] or photocleavage of unnatural amino acids in constrained peptides [16] allows fast and synchronized initiation of folding. In addition, protein chemical [17,18] and semi-synthesis [19,20] based on intein splicing [21] are becoming more widely used as methods of incorporation of labeled or unnatural amino acids into full-length proteins in vitro and in vivo [22], permitting site-specific modification of an intact protein or model peptide with an entire spectrum of amino acid derivatives without being limited by the genetic code. On a similar front, the recent realization of in vivo methods for stop codon suppression in E. coli [23,24] and yeast [25] have added to the available methodology for site-specific incorporation of unnatural amino acids [26-29] into proteins.

We have taken several approaches to the problem of tyrosyl radical generation, consisting of organic caging of amino acids together with covalent attachment of inorganic and organic photooxidants. Among the issues to consider in molecular design are: (1) stability of the radical precursors to peptide synthesis conditions, (2) their solubility in aqueous environments, (3) the steric bulk introduced by functionalization, (4) the reactivity of additional photoproducts, (5) the kinetics of tyrosyl radical generation, (6) the wavelength of light required for photolysis, and (7) the quantum yield of radical formation.
3.2 Diaryl Oxalate Esters

3.2.1 Introduction

One approach for fast tyrosyl radical generation is the linkage of tyrosine to transition-metal complexes that can undergo photoinduced electron transfer with the pendant amino acid. Systems based on covalently tethered ruthenium(II) [30] or rhenium(I) [31] and polypyridine complexes can generate tyrosyl radicals within 20 µs for the former and is believed to occur on the nanosecond timescale for the latter, using a bimolecular flash-quench technique. These derivatives may be cumbersome, in some instances, for biochemical studies because of their steric bulk and the requirement for high concentrations of external quencher. Thus, we sought a simple photochemical method that could cleanly deliver the tyrosyl radical on a sub-microsecond timescale with chemically benign byproducts and no added chemical quencher. We became interested in the advances made by Lahti and co-workers, who were able to generate aryloxyl radicals

![Scheme 3.2 Photochemistry of diaryl oxalates. Irradiation of the nπ* transition of diaryl oxalates leads to the elimination of two molecules of carbon monoxide with the concomitant formation of an aryloxyl radical pair.]

in solid state [32] and solution [33] environments by photolysis of diaryl oxalate esters with a quantum yield of ~0.1 [33] (Scheme 3.2). Inspired by this work, we have utilized this strategy for the preparation and study of oxalate-based unnatural amino acid derivatives for fast tyrosyl radical generation upon UV irradiation. We have developed tyrosine-based derivatives (where ArO = Tyr) that may be linked through their carboxy-terminus and describe their attempted incorporation into synthetic peptides using standard solid-phase synthetic techniques. In addition, we present the synthesis and
characterization of napthol and nitrophenol derivatives, which were designed to shift the wavelength of photolysis towards the visible range.

3.2.2 Results and Discussion

Bis(2,4,6-tri-tert-butylphenyl) oxalate ester 1 was prepared by modifications of literature methods [33]. Steady-state photolyses of 1 in our hands under anaerobic conditions show that the 2,4,6-tri-tert-butylphenoxyl radical is indeed stable due to the steric buttressing provided by the bulky substituents at the ortho position (Figure 3.1a). Steady-state EPR spectroscopy further confirms that a stable radical product is formed (Figure 3.1b), whose spectrum is characteristic of the 2,4,6-tri-tert-butylphenoxyl radical [34]. The ortho position of phenoxy radicals is susceptible to dimerization by radical recombination through their keto resonance structures followed by rapid re-aromatization. Thus, this type of tert-butyl protected radical does not undergo this reaction and provides an ideal caging group for tyrosyl radical generation from the oxalate ester family of compounds. Phenoxy radical generation from 1 was then probed by time-resolved absorption spectroscopy; direct laser excitation of 1 ($\lambda_{\text{exc}} = 270$ nm) produces a species that has a transient difference spectrum exhibiting positive absorption maxima at 382 and 402 nm (Figure 3.1c); the spectrum obtained shows the phenoxy radical absorption signature (Figure 3.1b) and develops within the microsecond timescale of the integration time of the CCD.

As the preliminary results with 1 established the light-mediated release of the phenoxy radical in our hands, both symmetric and asymmetric caged tyrosine radicals were prepared in gram-scale quantities by adaptation of literature procedures as outlined in Scheme 3.3. Symmetric bis(N-acetyl tyrosyl methyl ester)oxalate ester 2 is prepared in good yield (65%) by addition of N-acetyltirosyl methyl ester and triethylamine to oxalyl
Figure 3.1 Generation of the 2,4,6-tri-tert-butylphenoxyl radical from 1 under photolytic conditions. (Panel A) Steady-state photolysis of 1 over 45 min in THF at λ > 285 nm using a 90% neutral density filter shows the growth of the absorption spectrum characteristic of the 2,4,6-tri-tert-butylphenoxyl radical, which is stable in the absence of oxygen for days. (Panel B) 9 GHz EPR spectrum of the radical product generated from photolysis of 1 at λ = 308 nm. (Panel C) Transient absorption spectrum of 1 upon nanosecond laser excitation at λ = 270 nm with a 1.0 μs integration time (blue line) overlaid upon the spectrum of the 2,4,6-tri-tert-butylphenoxyl radical generated in the steady state (black line).
Scheme 3.3 Synthesis of symmetric and asymmetric tyrosine oxalate ester derivatives. (a) \( \text{NEt}_3, \text{THF}, N_2, -78 \degree \text{C} \); (b) \( \text{NEt}_3, \text{THF}, N_2 \); (c) 10\% Pd/C, THF, H\(_2\).}

chloride at \(-78 \degree \text{C}\) in THF followed by gradual warming to room temperature. The product was collected by filtration, followed by removal of triethylammonium chloride by extraction. Similarly, asymmetric diaryl oxalate esters 3 and 4 are obtained in 42\% and 41\% yield, respectively, by reaction of the corresponding aryl oxalyl acid chloride with a protected tyrosine derivative and triethylamine. Deprotection of 4 by hydrogenation over 10\% Pd/C gives carboxylic acid 5 in high yield (92\%), without loss of the oxalate ester moiety. All compounds were fully characterized by \(^1\text{H} \text{NMR spectroscopy and high-resolution mass spectrometry.}

Tyrosyl radical generation from symmetric tyrosine derivative 2 was then investigated by time-resolved absorption spectroscopy on the nanosecond timescale. As with compound 1, 2 undergoes an analogous photoinduced decaging to deliver a tyrosyl radical on the nanosecond timescale. Laser excitation of 2 (\( \lambda_{\text{exc}} = 270 \text{ nm} \)) results in a transient absorption spectrum characteristic of a tyrosyl radical, with maxima at 394 and 407 nm.
Figure 3.2 Photochemistry of 2. (Panel A) Transient absorption spectrum of the tyrosyl radical formed from laser flash-photolysis at $\lambda = 270$ nm of 2 using a 50 ns integration time and delays of 0 ns (black line), 200 ns (red line), 900 ns (blue line). (Panel B) Steady-state $^1$H-NMR showing the release of free tyrosine (blue) upon photolysis of 2 at $\lambda > 285$ nm.
(Figure 3.2a). The signal intensity maximizes within the 50 ns integration time of the gated CCD detector and the tyrosyl radical subsequently decays after 200 ns. Spin-trapping by 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) confirms the formation of a radical species from 2 by excimer laser photolysis at 308 nm. In addition, the production of free tyrosine from the steady-state photolysis of 2 is also observed by $^1$H-NMR (Figure 3.2b). Parallel studies of 3, the asymmetric derivative of 2 (Scheme 3.2), demonstrate formation of phenoxy radicals on a sub-microsecond timescale. In this case, laser excitation ($\lambda_{\text{exc}} = 270$ nm) of 3 produces a transient spectrum that appears to be a superposition of both the tyrosyl and 2,4,6-tri-tert-butylphenoxy radicals, as it lacks a clear valley between the two signature peaks (data not shown). Steady-state photolysis shows no production of the stable 2,4,6-tri-tert-butylphenoxy radical by UV-Vis spectroscopy, as with 1, in either aprotic (dichloromethane), protic (methanol), or hydrogen atom-donating (tetrahydrofuran) solvents, although both free tyrosine and 2,4,6-tri-tert-butylphenol are observed by $^1$H-NMR. This may indicate that the 2,4,6-tri-tert-butylphenoxy radical is able exchange with the tyrosine generated under these conditions (mM concentrations) and thereby quench the radical equivalent in a bimolecular reaction. N-Acetyl methyl ester protected derivative 3 was then incubated in neat TFA and Reagent L (88% TFA, 2% triisopropylsilane, 10% water 5% (w/v) DTT) for 3 h to test the stability of the oxalate ester linkage to solid-phase peptide synthesis conditions. As less than 5% release of free N-acetyltyrosine methyl ester was observed in both cases by $^1$H-NMR, the oxalyl aryl ester modification was judged stable to the cleavage conditions. In addition, 3 was also tested in the peptide bond formation conditions (PyBOP, DIEA, and HOBr in DMF) and found to be stable. Thus the synthesis of asymmetric tyrosine 4 was carried out, with the intention of incorporating tyrosine with a 2,4,6-tri-tert-butylphenyl oxalate ester caging group into peptides. The synthesis of asymmetric tyrosine derivative 4 proceeds similarly to 3. The hydrogenation of the benzyl ester moiety of 4 to yield free carboxylic acid 5, provides a functional group that
can be used to couple this amino acid derivative through the C-terminus to form amide or ester linkages. A wide variety of other N-terminal protecting groups, such as Fmoc or acetyl, are compatible with the deprotection step and can be facilely integrated into this class of compounds.

Although this class of compounds exhibits rapid generation of tyrosyl radicals upon photolysis, the wavelength required for the photoreaction occurs within the protein absorption envelope. First, the high extinction coefficients of proteins in the UV may cause them to act as an inner filter. Second, nonspecific excitation of tryptophan and tyrosines within proteins could lead to their denaturation. Thus, we have attempted to shift the wavelength of irradiation towards the visible region by modification of the tyrosyl radical caging group. Towards this end, synthetic targets 6-8 were designed (Figure 3.3). Targets 6 and 7 would address phenyl ring substitution with electron withdrawing (6) and donating (7) groups with the possibility of stable phenoxyl radical observation with ortho tert-buty! protection. 2-Naphthol derivative 8 would allow us to quickly assess the effect of larger aromatic ring systems without an involved synthesis of the corresponding tert-buty! protected alcohol. We would also have the potential of studying the decaging reaction by steady-state UV-visible absorption spectroscopy if the 2,4,6-tri-tert-butylphenoxyl radical is stable in this system.

Synthesis of symmetric derivative 6 proved to be exceedingly difficult. Nitration of 2,6-di-tert-butylphenol to generate the 2,6-di-tert-butyl-4-nitrophenol starting material was carried out by literature procedures [35]. However, the coupling reaction with oxalyl
chloride was problematic and the majority of the phenol starting material was isolated as a deep purple side product, while only trace amounts of the desired compound (0.6%) was isolable as a pale yellow solid. The reaction seemed to proceed in a straightforward manner at -78 °C, but warming to room temperature invariably caused development of the deep red color resulting from the unidentified side reaction. Fortunately, 6 was finally obtained and characterized by 1H NMR and high-resolution MS in sufficient amounts to study the photochemical reactivity. Steady-state photolyses at $\lambda_{\text{exc}} > 285$ nm in tetrahydrofuran showed no changes in the UV-visible absorption spectrum indicative of formation of a stable phenoxy radical species, instead, only a decrease of absorption at 425 nm was observed as the bright yellow solution turned to a pale yellow (Figure 3.4). The photolyzed sample was then examined by 1H-NMR; one unidentified photoproduct predominated, with only a trace amount of free phenol. We then undertook time-resolved studies of 6 in dichloromethane using nanosecond transient absorption spectroscopy. Upon laser excitation of 6 ($\lambda_{\text{exc}} = 320$ nm), no changes in the difference spectrum were observed were observed up to 3 μs. As the photochemistry of the nitro-substituted diary oxalate ester 6 seemed to be different from 3, perhaps due to changes in the electronic structure of the phenol moiety, we then undertook the synthesis of derivative 7, which is substituted with an electron-donating group.

Synthesis of bis(2,6-di-tert-butyl-4-N,N-dimethylaminophenyl) oxalate ester target 7 was attempted as outlined in Scheme 3.4. 2,6-di-tert-butyl-4-aminophenol 7a was prepared by palladium-catalyzed hydrogenation of the nitropheno1 starting material from the synthesis
of 6 in tetrahydrofuran over 4 days with a catalytic amount of triethylamine. Preparation of the 2,6-di-tert-butyl-4-N,N-dimethylaminophenol 7b starting material proceeded smoothly from the reductive methylation of 2,6-di-tert-butyl-4-aminophenol [36]. The literature procedure was modified slightly, due to the facility with which 7a and 7b were oxidized in air. The symmetric diaryl oxalate ester derivative of 7b was attempted at −78 °C using the same methods for the preparation of 2 and 6. Although some evidence of product formation was observed in the initial crude mixture, quantitative regeneration of the phenol starting material 7b was observed after work-up. It is also possible that 7 also might be synthesized from 6 using a similar reaction sequence as shown above in Scheme 3.4. Due the fragility of target 7 as well as the low availability of 6, we then proceeded to the synthesis of naphthol-based derivative 8.

The synthesis of naphthol-based target 8 (Figure 3.3) proceeds smoothly upon reaction of 2,4,6-tri-tert-butylphenyl oxalyl chloride with recrystallized 2-napthol. The product was characterized by both 1H NMR and high-resolution mass spectrometry. Steady-state photolyses at λ_{exc} > 285 nm showed no changes in the UV-visible absorption

![Diagram of reactions](image)

**Scheme 3.4 Proposed synthetic route to 7.** (a) HNO₃, 60 °C, hexanes; (b) 10% Pd/C, NEt₃, H₂, THF; (c) H₂SO₄, NaBH₄, CH₃O, N₂, THF; (d) oxalyl chloride, 2,6-lutidine, N₂, −78 °C THF.

![UV-visible absorption spectrum](image)

**Figure 3.5 Changes in the UV-visible absorption spectrum of 8 upon steady-state photolysis over 3h.**
spectrum characteristic of the 2,4,6-tri-tert-butylphenoxyl radical, instead, only a decrease in a broad band at 400 nm was observed (Figure 3.5). Although, the UV-visible spectra collected showed no evidence of radical formation, significant levels of free 2,4,6-tri-tert-butylphenol and 2-naphthol were observed by 'H-NMR at the end of the photoreaction. As the desired photochemistry (Scheme 3.1) seems to be preserved in 8, it may demonstrate tyrosyl radical decaging at longer wavelengths and could thus provide a general methodology for shifting the wavelength of photochemistry towards the visible range by substitution of the caging group with larger aromatic ring systems.

Before undertaking any large synthetic effort towards the preparation of appropriately blocked tyrosine derivatives caged with tert-butyl-protected multi-ring aromatic oxalate esters, we decided to attempt the synthesis of the R2 C-terminal peptide modified with the 2,4,6-tri-tert-butylphenyl oxalyl ester from the coupling of R2C19 and free carboxylic acid 5. Unfortunately, reverse-phase HPLC (Figure 3.6a) of the resulting peptide (9) shows that the aryl oxalate ester modification is lost by hydrolysis under purification in basic conditions, as 9 elutes at the expected retention time for Y-R2C19. In addition, the UV-Vis spectrum (λ_max = 276 nm) of 9 is consistent with a free tyrosine.

![Figure 3.6 Characterization of 9. (Panel A) Analytical reverse-phase HPLC trace of 9 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel B) MALDI-TOF MS of 9 in negative ion mode. The calculated m/z(−H) for Y-R2C19 is 2270 Da, while the expected mass for 9 is 2587 Da.](image-url)
rather than a diaryl oxalate ester. MALDI-TOF MS (Figure 3.6b) further confirms that the mass of 9 is identical to Y-R2C19. Hydrolysis of the oxalate ester linkage was then tested by lyophilizing 3 in ammonium bicarbonate; $^1$H-NMR shows that the compound has been mostly degraded to the free phenol and N-acetyltyrosine methyl ester components. At this time, we decided to synthesize a model peptide to incorporate 5 without need for purification in basic conditions.

A 5-mer peptide, LVGGQ, deriving from the N-terminus of R2C19 (10) with the carboxylate side-chains removed was synthesized so that standard acidic HPLC conditions could be used to purify the peptide product formed upon coupling with 5. An analytical amount of peptide 10 was cleaved from the resin, in order to characterize the peptide starting material by analytical reverse-phase HPLC. Figure 3.7 shows an HPLC trace of the crude peptide on a C-18 column in 0.05% TFA, which indicates that the peptide starting material is relatively clean. As the molecular weight of 10 is too low to analyze by MALDI-TOF MS (due to matrix background), its identity was confirmed by ESI MS. With the peptide 10 in hand, the extension reaction upon coupling with 5 was

![Figure 3.7 Analytical reverse-phase HPLC trace of starting material LVGGQ (10) in a linear gradient of 10%-75% acetonitrile versus 0.05% TFA (1.0 mL/min) over 45 min.](image-url)
attempted to produce peptide 11, which we hoped to isolate as the oxalate ester-modified species. A similar loss of the oxalate aryl ester moiety as with peptide 9 was observed and a peptide corresponding to YLVGQI was generated instead. Reverse-phase HPLC of 11 on a C-18 column of the crude peptide material (Figure 3.8a) shows both the starting material 7 ($t_R = 10.9$ min) and the product ($t_R = 14.8$ min). 11 was not analyzed by MALDI-TOF MS as laser excitation would possibly lead to decaging of the oxalate aryl ester group, however, the ESI MS measures a molecular mass identical to that calculated for YLVGQI. In addition, the absorption spectrum of 11 (Figure 3.8b) is identical to that of free tyrosine with a sharp peak at 276 nm rather than the broad spectrum of the corresponding diaryl oxalate ester. To ensure that this interpretation was correct, the sixmer peptide YLVGQI (12) was synthesized independently from the coupling of tyrosine to 10 in order to compare retention times and UV-visible absorption spectra (data not shown for 12). 12 was characterized by both MALDI-TOF MS and ESI MS to confirm its identity. Indeed, 12 elutes at the same retention time ($t_R = 15.1$ min) as peptide 11 ($t_R = 14.8$ min, Figure 3.8a) and the UV-visible absorption spectrum is also identical to 11. Because the oxalate linkage does not persist under either acidic or basic conditions, the
modified tyrosine is best introduced in solution phase synthesis at neutral pH and may be
effected enzymatically [37].

We have outlined a novel method for the fast photochemical generation of tyrosyl radicals by direct photocleavage of organic tyrosine derivatives based on diaryl oxalate esters. The timescale for generation of radical product (< 50 ns) from these unnatural amino acids is over 500 times faster than the ruthenium(II) polypyridine system [30] without the need for an external chemical quencher. Moreover, the preparation of 5 contributes to methods aimed at generating tyrosyl radicals on peptides. Although initial attempts at incorporating 5 into synthetic peptides using traditional solid-phase synthetic techniques were unsuccessful, these types of compounds may be able to be coupled to a purified peptide by solution-phase synthesis. Preliminary studies with caging group substitution (6-8) have afforded some indications of the path that should be taken towards shifting the wavelength of photochemistry outside the protein absorption envelope. Addition of the electron-withdrawing nitro group in 6 does shift the nπ* absorption, but preliminary photochemical studies seem to indicate that the reactivity outlined in Scheme 3.2 has been altered. Initial studies with napthol derivative 8, seem promising with regard to phenoxy radical generation. Although the 2,4,6-tri-tert-butylphenoxy radical was not observed by the steady-state photolysis, analysis of the photoproducts indicate formation of both the uncaged phenol and napthol. This is similar to what was observed for asymmetric tyrosine derivative 3, which indeed showed light-initiated release of both the tyrosyl and phenoxy radicals. Thus it may be constructive to pursue time-resolved photolysis experiments to determine whether tyrosyl radical may be formed by photolysis of 8 outside the protein absorption envelope.

3.3 Ruthenium(II) Polypyridyl Complexes

3.3.1 Introduction

Ruthenation of proteins is a common method used in the investigation of biological
electron transfer studies by laser flash-photolysis [38]. The photochemistry of ruthenium(II) polypyridyl complexes is well-defined and has been reviewed extensively [39]. The production of a ruthenium(III) oxidizing or a ruthenium(I) reducing equivalent by flash-quenching electronically excited ruthenium(II) complexes permits electron transfer to be established promptly upon laser excitation [40-42]. The flash-quench method has been used to generate a tyrosyl radical covalently appended to a polypyridine ligand of a ruthenium(II) tris(bipyridyl) (Rubpy_3) complex [30] (Scheme 3.5).

Scheme 3.5 Bimolecular flash-quench scheme for generation of ruthenium (III) from ruthenium (II). Quenching of the excited state of ruthenium (II) with an oxidative quencher, either MV^2+ or cobalt (III) pentaamine chloride, generates the ruthenium (III) center and reduced quencher. The ruthenium (III) is then capable of oxidized the covalently-linked tyrosine to a tyrosyl radical.

Excitation of the metal-to-ligand charge transfer (MLCT) of ruthenium(II) followed by oxidative quenching of the ligand with methylviologen (MV^2+) or cobalt(III) pentaamine chloride produces ruthenium(III). The oxidized metal center (ε^o = 1.26 V vs. NHE [43]) is competent then to oxidize the adjacent tyrosine (ε^o = 0.93 V vs. NHE [44]) to a tyrosyl radical. Both the reversible quencher, MV^2+, and a irreversible quencher, cobalt(III) pentaamine chloride, produced the tyrosyl radical with a slow intramolecular rate constant of 5 × 10^4 s^{-1}. This rate constant for tyrosyl radical production may be too sluggish for our planned RNR studies, due to the low driving force for tyrosine oxidation. If hole propagation through R1 is faster than 20 μs, which may well be the case, then flash-quench generation of •Y356 will be rate determining. However, we may still be able to carry out biochemical studies with the corresponding ruthenium-modified 20-mer
peptide in complex with R1, even if kinetic analysis of the PCET pathway in R1 is not possible using this system.

As the measurement of tyrosyl radical formation by transient absorption in the Rubpy3-Y complex (14) is quite complex, a short description of the methodology presented in the literature is summarized below [30]. Irradiation into the MLCT band at 455 nm creates the excited state of 14, whose emission ($\lambda_{\text{max}} = 640$ ns) has a lifetime of 370 ns. Upon addition of quencher, the emission lifetime should be shortened due to depletion of the excited state upon bimolecular electron transfer with the external quencher to form the oxidized ruthenium(III) metal center. Indeed, when 14 is incubated with 10-20 mM quencher (MV$^{2+}$ or cobalt(III) pentaamine chloride), the emission lifetime is reduced to 100-150 ns [30]. If MV$^{2+}$ is used as the sacrificial oxidant, both products from the quenching reaction, ruthenium(III) and •MV$^+$ (Scheme 3.5), can be observed spectroscopically by the negative change in absorption of the MLCT band at 450 nm or positive absorption at 396 nm and 600 nm, respectively. The tyrosyl radical absorption maximum (410 nm, $\varepsilon \sim 3000$ M$^{-1}$cm$^{-1}$) overlaps with the bleach of the MLCT band ($\sim$350-550 nm, $\varepsilon \sim 14,000$ M$^{-1}$cm$^{-1}$); consequently, the transient absorption at 410 nm is dominated by the metal center and the tyrosyl radical signature is obscured [30]. Instead, the kinetics of tyrosyl radical production were inferred from ruthenium(II) reappearance at 450 nm ($\tau_0 = 15$ µs), which was more rapid than the decay of •MV$^+$ at 600 nm ($\tau_0 = 250$ µs). Thus, the authors conclude that the reappearance of ruthenium(II) is not caused by bimolecular recombination with •MV$^+$, but stems from ET from the adjacent tyrosine. In addition, the kinetic trace collected at 410 nm shows appearance of a positive absorption attributed to the sum of the rise of tyrosyl radical at 410 nm and the return of ruthenium(II) centered around 460 nm. This was contrasted with the redox-inert alanine congener of 14, which showed matched half-lives of 80 µs for ruthenium(II) appearance and •MV$^+$ decay, indicative of recombination between the oxidized metal center and
reduced quencher. When cobalt(III) pentaamine chloride was used with the alanine derivative as an irreversible quencher, there is no recovery at all of ruthenium(II) observed on the 200 μs timescale.

Based on this chemistry, we set out to synthesize and characterize the analogous R2 peptide modified with Rubpy₃ (15). Quite surprisingly, this peptide retains a similar affinity for R1 as the peptide without the N-terminal metal complex. Initial photochemical studies directed towards reproducing the results above indicate that ruthenium(III) is generated on 15 by flash-quench with an electron acceptor, however, formation of the tyrosyl radical was not observed due to precipitation of 15 upon metal center oxidation.

3.3.2 Results and Discussion

We sought to prepare 14 as well as the R2 peptide derivative 15 in order verify tyrosyl radical production for ourselves by these methods [30]. The carboxylic acid-modified ruthenium(II) tris(bipyridyl) complex (13) was synthesized according to literature procedures [45]. Rubpy₃-Y-OEt (14) was then prepared from the coupling of 13 and tyrosine ethyl ester as a model compound for flash-quench tyrosyl radical generation on the ruthenated R2 C-terminal peptide (15). The preparation of 14 was modified from the previously reported method [30] by substituting the coupling method for one employing
standard peptide synthesis reagents, with the goal of introducing 13 on the resin during solid phase peptide synthesis of 15. The identity of 14 was confirmed by $^1$H-NMR and the electronic absorption spectrum of 14 in acetonitrile (Figure 3.9a) is characteristic of ruthenium(II) tris(bipyridyl) complexes with peaks located at 245 nm, 286 nm, and 455 nm [46]. With 14 in our hands, verification of tyrosyl radical generation by the flash-quench method was then attempted. Addition of either 10 mM cobalt(III) pentaamine chloride or methyl viologen achieves the quenching of emission ($\lambda_{ex} = 475$ nm, $\lambda_{em} = 640$ nm) and reduces the emission lifetime to 171 ns (Figure 3.9b), which is similar to the value reported [30]. In addition, transient absorption experiments demonstrate changes in the absorption spectrum which indicate the formation of both $^\star$MV$^+$ and ruthenium(III). However, the kinetic traces collected at 410 nm and 600 nm do not clearly show evidence of tyrosyl radical as discussed above as the kinetics of ruthenium(III) and methyl viologen radical decay are quite similar (data not shown). There is also no positive absorption observed at 410 nm after the bleach recovery.

In preparation for the synthesis of the R2 peptide derivative of 14, metal complex 13 and
the metal-tyrosine complex 14 were tested for their stability to peptide cleavage conditions by incubation in Reagent K for 4 h (87.5% TFA, 5.0% water, 5.0% thioanisole, 2.5% 1,2-ethanediol, with 5% (w/v) phenol). Both compounds remained intact by $^1$H-NMR, thus allowing a viable route to the preparation of peptide 15 by standard solid-phase peptide synthesis methods. Synthesis of 15 proceeded smoothly from the coupling of 13 and R2C19 on resin. The metal complex remains intact through cleavage in TFA, thus affording the ruthenium-modified peptide by standard peptide synthesis conditions. A reverse-phase C-8 HPLC trace of 15 is shown in Figure 3.10a, with the electronic absorption spectrum (Figure 3.10b). The binding of the peptide was assayed by competitive inhibition with R2 in
a standard spectrophotometric assay for nucleotide reduction and compared to the curve generated with Ac-Y-R2C19 (16), which has a known $K_D$ of 20 $\mu$M [47] (Figure 3.11). The competition assay was not carried out to completion (to full inhibition), due to the overlapping absorption of 15 with NADPH at 340 nm. However, it does show that binding to R1 is retained by 15, even with the large N-terminal modification of a +2 charged metal complex. Other methods to measure the dissociation constant between R1 and 15 employing equilibrium dialysis and gel filtration spin columns were attempted, however, equilibration was not reached in either case. In the former case, the peptide did not dialyze freely and tended to stick to the dialysis membrane while the spin column did not allow appropriate equilibration upon elution.

Assured of the identity of 15, as well as its ability to bind R1, we next undertook studies to investigate the production of *Y356 by the flash-quench technique. The methyl viologen dichloride quencher was not soluble under the conditions used, consequently, cobalt(III) pentaamine chloride was used as the oxidant and the ruthenium(II) bleach kinetics could not be compared to the decay of the oxidized quencher. The emission quenching of Rubpy3-Y-R2C19 by 15 mM cobalt(III) pentaamine chloride was characterized in 10 mM HEPES, pH 7.6 ($\lambda_{ex} = 485$ nm) and demonstrates formation of ruthenium(III). Steady-state emission spectra at pH 7.6 show an intensity reduction of 82% upon addition of quencher (Figure 3.12a) and time-resolved studies demonstrate that the emission lifetime reduced from 448 ns to 270 ns in the absence and presence of the quencher, respectively (Figure 3.12b and Table 3.1). Upon raising the pH to 10.1 (at the p$K_a$ of tyrosine), the emission lifetime of 15 is similar to that measured at pH 7.6 with $\tau_0 = 448$ ns (Table 3.1). Addition of cobalt(III) pentaamine chloride at pH 10.1 reduces the intensity the steady-state emission and shortens its lifetime substantially, again confirming generation of ruthenium(III). The majority of the amplitude resides with a rapid 68 ns phase and a much smaller amplitude (0.2%) residing with a longer 266 ns
Figure 3.12 Emission of Rubpy$_2$-Y-R2C19 (15). (Panel A) Steady-state fluorescence spectra of 15 alone (blue) and in the presence of 10 mM cobalt(III) pentaamine chloride (red). (Panel B) Normalized emission lifetimes of 15 alone (blue) and in the presence of 10 mM cobalt(III) pentaamine chloride (red).
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$^a$from ref. 30

Table 3.1 Excited-state lifetimes of Rubpy$_3$Y-R2C19 compared to literature values for Rubpy$_3$Y.

phase (Table 3.1). The longer phase is similar to the emission lifetime measured upon quenching at pH 7.6 while the shorter phase may be due to faster reaction with cobalt(III) with the negatively-charged tyrosinate, present at pH 10.1.

After verifying production of ruthenium(III) by flash-quench, time-resolved absorption studies of Rubpy$_3$Y-R2C19 (15) monitoring kinetics of ruthenium(II) reappearance were carried out to address tyrosyl radical generation. Figure 3.13a shows the transient spectrum generated upon laser-excitation ($\lambda_{exc} = 475$ nm) with a positive absorption at

![Figure 3.13](image-url)
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²from ref. 30

Table 3.2 Kinetics of ruthenium(II) recovery in Rubpy₃⁻Y-R2C19 (15) after flash-quench compared to Rubpy₃ and literature values for Rubpy₃⁻Y and Rubpy₃⁻A.

375 nm arising from the ruthenium(II) excited state and negative absorption changes at 458 nm due to bleaching of the MLCT state of ruthenium(II) (black line). The time evolution of this spectrum over 5.0 µs shows a decay of the excited state and corresponding recovery of the MLCT bleach at 430 nm. The single-wavelength kinetics at 430 nm measure the half-life of bleach recovery to be 413 ns (Figure 3.13b, Table 3.2). In the presence of cobalt(III) quencher, reappearance of ruthenium(II) at 430 nm is markedly slower, with a half-life of 200 ± 2.0 µs (Table 3.2). The lifetime of the ruthenium(II) bleach has indeed been extended by the addition of cobalt(III) due to ruthenium(III) formation, however, it is much slower than the reported decay of 15 µs observed when the tyrosyl radical is generated by intramolecular ET between the oxidized metal center and the adjacent tyrosine in the Rubpy₃⁻Y model compound 14 [30]. Nonetheless, the recovery of ruthenium(II) is still more rapid than that observed for the alanine derivative of 14 (Rubpy₃⁻A) or Rubpy₃ alone (Table 3.2) and may indicate a much slower formation of tyrosyl radical in peptide 15 than in model compound 14. Thus tyrosyl generation was tested by raising the pH from 7.6 to 10.1, as the more rapid tyrosyl
radical formation upon deprotonation of tyrosine ($pK_a = 10.1$) should lead to faster recovery of ruthenium(II) from the ruthenium(III) generated by flash-quench. At pH 10.1, the decay half-lives of ruthenium(III) at 430 nm are $250 \pm 20 \mu$s and $439.5 \pm 7.2$ ns, with and without quencher respectively (Table 3.2), which is quite similar to the time constants seen at pH 7.6. As the data is fairly noisy, it is possible, that a second, faster phase is present at pH 10.1. At this time, we also realized that there was significant peptide precipitation at the end of experiments with Rubpy$_3$-Y-R2C19 and quencher that did not occur with model compound 14 and quencher. This aggregation was not alleviated upon changing buffers from the anionic HEPES to the cationic Tris. In addition, precipitation did not occur with ruthenium(II) tris(bipyridyl) and quencher in HEPES, Tris, or potassium phosphate buffers. Since the precipitation was specific to the peptidyl portion of 15, we concluded that peptide precipitation may be occurring upon oxidation of the metal center to $+3$ state, causing aggregation of the highly negatively-charged portion of the R2 peptide around the ruthenium(III) site. If the precipitation outcompetes $^\ast$Y356 generation, then we would be unable to observe the tyrosyl radical spectroscopically.

Although tyrosyl radical formation was not verified in Rubpy$_3$-Y-R2C19 due to precipitation of the peptide upon oxidation to ruthenium(III), it is possible that the

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**Figure 3.14** Crystal structure of Ac-Y-R2C19 bound to R1 rendered in CPK [48]. R1 is shown in blue, while the peptide is shown in CPK coloring. The general location of Y356 is indicated, the last residue visible at the N-terminus is Q360.
aggregation would be inhibited upon binding to R1. The C-terminus of the peptide, where the carboxylates reside, is visible in the crystal structure of R1 and has relatively low thermal B-factors [48]. If these carboxylates are indeed held away from the oxidized +3 metal center in the presence of R1 (Figure 3.14), formation of •Y356 should established by flash-quench of Rubpy₃-Y-R2C19 (15). Although •Y356 generation was not directly observed, we can infer tyrosine oxidation by ruthenium(III), which was indeed produced with 15. Thus, R1 turnover by light-mediated generation could be tested as the radioactive assay for nucleotide reduction should be sensitive enough to detect less than 0.1% turnover over background.

3.4 Rhenium(I) Tricarbonyl Polypyridyl Complexes

3.4.1 Introduction

The slow production of tyrosyl radical in the Rubpy₃-Y (14) model system by intramolecular electron transfer is due to the relatively modest ruthenium II/III couple of 1.26 V vs NHE, and may not be rapid enough for measuring the PCET kinetics in R1 [30]. Replacement with the ruthenium(II) complex of 15 with the rhenium(I)(tricarbonyl) (polypyridyl)(X) photosensitizer [49-51] should help resolve hurdles facing the utility of 15: (i) the low driving force for tyrosyl radical generation and (ii) peptide precipitation upon metal center oxidation. The rhenium(I) complex in which X = imidazole has a rhenium(II/I) couple of 2.09 V vs. NHE [51], accelerating the intramolecular quenching rate constant to 10⁶-10⁹ s⁻¹, as estimated from a simple Marcus analysis. Indeed, flash-quench methodology analogous to that used for ruthenium has been established in protein systems by attaching the rhenium complex through histidine side-chains and found to generate both tyrosyl and tryptophan radicals [31]. In addition, tyrosyl radical generation may be possible directly from the excited state, which has a rhenium(I*)/0) couple of 1.44 V vs NHE [51], to avoid the use of external quenchers that may interfere with the enzymatic activity of RNR. Secondly, the rhenium(I) metal complex has a lower charge
of either +1 or 0 (if X is an anionic ligand) and consequently derivatized R2 peptides will have less tendency to precipitate, as we know that the R2 peptide can support up to a +2 charge at the N-terminus from the solubility of 15.

In the following section, we begin with the preparation of peptides covalently linked to the rhenium(I) complex through the bpy ligand, since we believe that this will provide a more stable complex that histidine ligation (Figure 3.15). The peptide in which \(X\) = imidazole (Im) (20) was first synthesized because it is analogous to that used in the literature for tyrosyl radical generation [31] but was not isolable as a single species. Preparation of the chloride complex (21) showed the same pattern of ligand substitution with a peptide-derived carboxylate ligand.

The R2 peptide was then modified with the N-terminal ligands, bpy (22) and N-acetylhistidine (23), with the intention of carrying out the metallation reaction off the resin and thereby avoiding ligand exchange during cleavage in TFA.

However, peptide 22 did not withstand the synthetic conditions required to add the bpy ligand nor was histidine found to compete adequately with carboxylate for the ligand site, X. We then later sought to take advantage of the stable association of carboxylate with rhenium(I), but as presented below, the excited-state lifetimes of these types of complexes where \(X\) = carboxylate were found to be too short to implement flash-quench methodology. We end this section with the synthesis and preliminary characterization of peptides where \(X\) = CN (38), SCN (40) (Figure 3.15), which may

\[
\begin{align*}
X & = \text{Im} \quad 20 \\
& = \text{Cl} \quad 21 \\
& = \text{CN} \quad 38 \\
& = \text{SCN} \quad 40
\end{align*}
\]

Figure 3.15 Structures of R2 C-terminal peptides based on the rhenium(I){tricarbonyl(1polypyridyl)(X)} platform.
provide promising routes to successful generation of •Y356 by flash-quench methodologies.

3.4.2 Results and Discussion

The previously reported rhenium-based flash-quench systems for tyrosyl radical generation utilize a rhenium(I)(tricarbonyl)(phenanthroline)(X) complex, whose sixth ligand is a protein-derived histidine side chain [31]. These compounds where X is a π-acid, such as imidazole or pyridine, have relatively long excited-state lifetimes [50], allowing the implementation of the bimolecular ET reaction with the sacrificial electron acceptor. Indeed, it is difficult to quench reactions under typical biological concentrations in which the lifetime of the excited state is less than 100 ns. We then set out to prepare the analogous rhenium(I) (tricarbonyl) (4-methyl-4’-carboxylic acid-2,2’- bipyridine) (imidazole) complex, which maintains the imidazole ligand to yield excited-state lifetimes greater than 100 ns, yet allows a more stable attachment of the metal complex to the peptide (Scheme 3.6). Synthesis of metal complex 19 was accomplished in a

\[
\begin{align*}
\text{ReCl}_3(\text{CO})_3 & \xrightarrow{a} \text{ReCl}_3(\text{CO})_3(\text{imidazole})_6 \\
\text{ReCl}_3(\text{CO})_3(\text{imidazole})_6 & \xrightarrow{b} \text{ReCl}_3(\text{CO})_3(\text{imidazole})_6(\text{carboxylic acid})_2 \\
\text{ReCl}_3(\text{CO})_3(\text{imidazole})_6(\text{carboxylic acid})_2 & \xrightarrow{c, d} \text{ReCl}_3(\text{CO})_3(\text{imidazole})_6(\text{carboxylic acid})_2(\text{amino-acid})_2
\end{align*}
\]

Scheme 3.6 Synthesis rhenium(I)-modified peptide 20 from 19 and Y-R2C19. (a) Toluene, N\textsubscript{2}, 80 °C; (b) water, 60 °C (c) PyBOP/DIEA/HOBt, DMF/acetone; (d) 95% TFA/2.5% trisopropylsilane/2.5% water.
Figure 3.16 Characterization of Relm-Y-R2C19 (20). (Panel A) Analytical reverse-phase HPLC trace of a crude peptide mixture containing 20 in a linear gradient of 10%-75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. Peaks labeled b, c, and d were isolated and re-chromatographed using the same gradient. (Panel B) Analytical HPLC trace of peak b from Panel A. (Panel C) Analytical HPLC trace of peak c from Panel A. (Panel D) Analytical HPLC trace of peak d from Panel A. (Panel E) Representative MALDI-TOF spectrum of all peaks collected by HPLC in Panels A-D collected in negative ion mode. The calculated m/z(-H) for 20 is 2805 Da. The mass observed by MALDI-TOF corresponds to the mass of 20 minus the imidazole ligand (67 Da).
straightforward manner following literature procedures for similar metal complexes [39,51]. As the solubility of 19 was less than the typical Fmoc amino acid, additional DMF and acetone was added to the coupling reaction with Y-R2C19. Although the concentrations of reagents were less than optimal for solid-phase peptide synthesis reactions, the formation of the amide bond proceeded smoothly as evidenced by the negative ninhydrin reaction for primary amines after coupling and the large amount of bright yellow peptide precipitate observed during ether trituration. ReIm-Y-R2C19 (20) was then chromatographed on a C-8 column by HPLC in 0.1 M ammonium bicarbonate, pH 8.0 (Figure 3.16). The HPLC trace at 330 nm of the crude peptide mixture shows multiple peaks containing rhenium-linked peptides (Figure 3.16a). All the peaks collected from this HPLC run were found to have masses indicating the loss of the imidazole ligand (MW_{calc}(-imidazole) = 2738) from 20 by MALDI-TOF MS (Figure 3.16e). One possible explanation for this phenomenon is that the X ligand can be lost photochemically due to laser excitation during collection of the MALDI-TOF mass spectra. Three of the major peaks (peaks b-d, Figure 3.16a) were isolated and analyzed by reverse-phase HPLC in 0.1 M ammonium bicarbonate, pH 8.0. Figures 3.16b-d show the traces from the analytical re-injections of these peptides, which demonstrate re-speciation of the peptides into multiple forms. These peaks were also isolated and analyzed by MALDI-TOF MS and again found to have masses corresponding to that of 20 with imidazole removed (Figure 3.16e). In addition, the elution profile of ReIm-Y-R2C19 changed with time; samples dissolved in ammonium bicarbonate for over 24 h had different elution profile than those that were dissolved for under 6 h. In order to check the buffer dependence of this phenomenon, ReIm-Y-R2C19 was then chromatographed by reverse-phase HPLC in 0.1 M triethylammonium chloride buffer, pH 8.0, yielding similar results to those observed in ammonium bicarbonate. Again, multiple peaks were present in the HPLC trace at 330 nm and loss of imidazole in all MALDI-TOF mass spectra was
observed. A likely explanation for both the redistributing populations of the rhenium-modified peptides and the missing ligand in the mass spectra is that the coordinated imidazole was being replaced with one of six peptide-derived carboxylates. Multiple structures would be possible, including cross-linked aggregates, which could exchange easily. Consequently, we attempted the synthesis of the chloride derivative, peptide 21, from Y-R2C19 and intermediate metal complex 18 (Scheme 3.6) with the belief that an anionic ligand would be able to compete better with the carboxylates of the peptide. Unfortunately ReCl-Y-R2C19 behaved in the same manner as ReIm-Y-R2C19 and also showed multiple species of rhenium-containing peptides by HPLC with varying populations (Figure 3.17) as well as MALDI-TOF mass spectra corresponding to the mass of the rhenium-modified peptide missing the chloride ligand.

We sought alternate strategies to synthesize rhenium(I) (tricarbonyl)(polypyridyl)(X) derivatives of the R2 C-terminal tail. Rather than adding the metal complex to the peptide on the solid-phase, it could be possible to react a peptide, covalently-linked to a ligand, in
Scheme 3.7 Solution route to synthesis of rhenium(I)(tricarbonyl)(polypyridyl)(X) labeled peptides from peptide 22 and 23. Polypyridyl ligand NN = 2,2'-bipyridine or 1,10-phenanthroline.

solution with the appropriate rhenium(I) starting material (Scheme 3.7). This synthetic scheme would also allow easy substitution of the X ligand. Either reaction of a polypyridyl-modified peptide (22) or imidazole-modified peptide (23) provides simple paths to the target peptides. Peptide 22, bpy-Y-R2C19, was synthesized and isolated by reverse-phase HPLC. The purified peptide was characterized by analytical HPLC (Figure 3.18) and MALDI-TOF MS. As a preliminary test reaction, peptide 22 was heated to both 60 °C and 80 °C to monitor the behavior of the peptide under the conditions required to metallate the bipyridine ligand at the N-terminus. In both cases, bpy-Y-R2C19 was found to be destroyed upon analysis by reverse-phase HPLC, with almost all peptide material running at the void volume of the column. Another method for exchange of the carbonyls with the bipyridine ligand of 22 was attempted using trimethylamine N-oxide as a mediator, based on an approach for synthesis of Re(CO)₅(PPh)(CH₃CN) type complexes (where PPh = bisphosphine ligand) from Re(CO)₄(PPh) [52] (Scheme 3.8). As we require the
exchange of two carbonyls to coordinate the bipyridine ligand of 22, this reaction was first tested with 2,2'-bipyridine and rhenium(I) (pentacarbonyl)(chloride). The reaction of 2 trimethylamine N-oxide: 1 Re(CO)₅Cl: 1 2,2'-bipyridine was analyzed by ¹H NMR in CD₃CN and showed a 1:1 spectrum of free and coordinated ligand. This could either result from a 1:1 mixture of the desired Re(CO)₅(bpy)(Cl) with free ligand or a quantitative reaction to generate Re(CO)₄(bpy)(Cl) in which the bipyridine is coordinated in an η¹ fashion. The chemical shifts corresponding to the coordinated bipyridine were

\[
\begin{align*}
\text{OC-} & \quad \text{Re} & \quad \text{P} & \quad \text{OC} \\
\text{OC} & \quad \text{Re} & \quad \text{P} & \quad \text{OC} \\
\end{align*}
\]

**Scheme 3.8**: Exchange of carbonyls mediated by Me₃N-O. Exchange of a single carbonyl with acetonitrile can be catalyzed by addition of 1 eq of Me₃N-O (top). Exchange of two carbonyls with the bpy was attempted using 2 eq of Me₃N-O (bottom)

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consistently 0.1-0.2 ppm upfield from the chemical shifts for the independently prepared Re(CO)_3(bpy)(Cl) (24). Presumably, displacement of only one carbonyl ligand using this method was achieved. This interpretation was tested with asymmetric bipyridine ligand 17. Addition of one equivalent of trimethylamine N-oxide to Re(CO)_3Cl and 17 led to a quantitative reaction, while addition of a second equivalent of trimethylamine N-oxide re-equilibrated to multiple products. From the ^1H NMR, it appeared as if only one end of the ligand, either the carboxylate or pyridine moiety of 17, was coordinated. Therefore we believe that only the chemistry represented in the top portion of Scheme 3.8 occurs.

At this time, Ac-His-Y-R2C19 (23) was also synthesized and the purified peptide characterized by analytical HPLC (Figure 3.19) and MALDI-TOF MS. The intention was to label 23 with the same methodology as used to achieve protein labeling by reaction with a rhenium(I)(tricarbonyl)(1,10-phenanthroline)(aqua) complex. Because the side reaction with the peptide carboxylates remained a concern, we first tested the exchange reaction of rhenium(I)(tricarbonyl)(1,10-phenanthroline)(triflate) (27) with imidazole, sodium acetate or acetic acid/triethylamine, N-acetyl-histidine (Ac-His-OH), and N-

![Figure 3.19](image_url)

*Figure 3.19* Analytical reverse-phase HPLC trace of Ac-His-Y-R2C19 (23) in a linear gradient of 10%–75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min.
acetyl-histidine methyl ester (Ac-His-OMe) (Scheme 3.9) and monitored the extent of reaction by $^1$H NMR. Full exchange of the triflate ligand of 27 with imidazole (Figure 3.20) requires several days, while exchange with acetic acid in the presence of triethylamine (Figure 3.21) takes place rapidly in under 4 h. Acetic acid itself, without a base to deprotonate the carboxylic acid, does not exchange at all. Full exchange of sodium acetate (Figure 3.22) occurs somewhat more slowly in approximately 6-8 h, perhaps due to issues with solubility of CD$_3$OD. Complex 27 was then tested with Ac-His-OH (Figure 3.23) to see whether exchange with the carboxylate or imidazole moieties was preferred; exchange was rapid and completed within 1 day and thus kinetically more similar to the reaction of 27 with carboxylates. In addition, the upfield shift of H2 and H9 (at $\delta = 9.6$) of the 1,10-phenanthroline ligand upon triflate exchange is consistent with carboxylate ligation rather than imidazole coordination, in which H2 and H9 are shifted downfield. Next, a 1:1 mixture of Ac-His-OMe and acetic acid (Figure 3.24) was incubated with 27 to gauge the preference of rhenium(I) for imidazole versus carboxylate. Acetate ligation occurs immediately, with no visible coordination of Ac-His-OMe. However, full exchange takes longer than the reaction with the other carboxylates, perhaps because less of the acetic acid is available in acetate form due to lower $pK_a$ of
Figure 3.20 Exchange reaction of 27 with imidazole monitored by 'H NMR.
Figure 3.21 Exchange reaction of 27 with HOAc initiated by addition of NEt₃ monitored by ¹H NMR.
Figure 3.22 Exchange reaction of Z7 with NaOAc monitored by $^1H$ NMR.
Figure 3.23 Exchange reaction of 27 with Ac-His-OH monitored by $^1$H NMR.
Figure 3.24 Exchange reaction of 27 with Ac-His-OMe versus HOAc monitored by $^1$H NMR.
Figure 3.25 Exchange kinetics of (Panel A) acetic acid/triethylamine, (Panel B) sodium acetate, (Panel C) imidazole, (Panel D) and Ac-His-OH with Re(CO)₅(phen)(OTf) in CD₃OD as measured by ¹H NMR.
Ac-His-OMe as compared to triethylamine. The exchange kinetics as measured by $^1$H NMR from comparative integration of the 1,10-phenanthroline peaks are plotted in Figure 3.25. From these exchange reactions, as well as the phenomenological observations reported above in efforts to prepare ReI\textsubscript{m}-Y-R2C\textsubscript{19} (20) and ReCl-Y-R2C\textsubscript{19} (21), it is clear that the anionic carboxylates are greatly preferred to imidazole. Thus, labeling of peptide 23 by an exchange reaction does not seem possible due to the greater reactivity of the peptide-based carboxylates.

Because the carboxylates of the R2 C-terminal peptide seem to provide a preferred ligand for rhenium(I), we sought to prepare a tridentate polypyridyl ligand with a carboxylic acid side-arm. Modification of the 6 position of 2,2$'$-bipyridine with an ethanoic acid group would provide the appropriate diimine unit with a carboxylate donor. Modeling of the metal complex in both HyperChem and Chem3D using a simple molecular mechanics geometry optimization demonstrates formation of an

![Figure 3.26 Model of a rhenium(I)(tricarbonyl) complex with a tridentate bipyridine ligand generated by Chem3D. Atom colors: C=grey, N=blue, O=red, Re=fuschia.](image)

**Scheme 3.10** Synthesis of a tridentate ligand for rhenium(I) and the corresponding metal complex. (a) $-5$ °C, (b) Na\textsubscript{2}O\textsubscript{2}, 0 °C, (c) NaOH, 25 °C, (d) Bu\textsubscript{4}NBr/K\textsubscript{2}CO\textsubscript{3}/PrOH, DMF/H\textsubscript{2}O, 115 °C, (e) ether, $-78$ °C, (f) toluene, N\textsubscript{2}, 80 °C, (g) HATU/DIEA, DMF, (h) 95% TFA/2.5% trisopropylsilane/2.5% water.
Figure 3.27 Characterization of rhenium(II)(tricarbonyl)(1,10-phenanthroline)(acetate) (32). (Panel A) UV-visible absorption spectrum of 32. (Panel B) Steady-state emission spectrum of 32. (Panel C) Time-resolved emission of 32 ($\lambda_{exc} = 380$ nm) collected at 640 nm.
unstrained octahedral complex with a six-membered ring chelate (Figure 3.26). Thus, symmetric dicarboxylic acid, 6,6'-ethanoic acid-2,2'-bipyridine was designated as the synthetic target, as it can provide both a ligand for rhenium(I) as well as a carboxylate group for formation of an amide bond with Y-R2C19 (Scheme 3.10). 2-bromo-6-picoline (31) was prepared by standard procedures [53,54] from the commercially-available 2-amino-6-picoline starting material. Palladium cross-coupling afforded the 6,6'-dimethyl-2,2'-bipyridine ligand in 27%-35% yield. Although the ligand synthesis was proceeding smoothly, rhenium (I) (tricarbonyl) (1,10-phenanthroline) (acetate) complex (32) was synthesized in order to investigate the photochemical properties of carboxylate complexes of this type. The absorption and emission spectra of 32 shown in Figure 3.27a and Figure 3.27b is typical of complexes of this type. Unfortunately, the luminescence lifetime in methanol is quite short and comparable to compounds in which X = chloride (Figure 3.27c) [50]. 32 already contains the relatively rigid 1,10-phenanthroline ligand rather than the more flexible 2,2'-bipyridine ligand, which extends the lifetimes of complexes containing the former ligand 4-5 fold compared to those containing the latter ligand [50]. The lifetime of 32 could be lengthened using a more elaborate 1,10-phenanthroline ligand [55], however, it would be synthetically difficult to incorporate both the two required carboxylate arms as well as methyl and phenyl substituents onto the ligand. In addition, the radiative lifetimes of these rhenium(I) complexes decrease in water as compared to methanol [50]. Although carboxylates provide a stable ligand, the emission lifetimes of these complexes are most likely too short for establishment of flash-quench tyrosyl radical generation on the R2 C-terminal peptide.

Consequently, we were in search of a strong-field ligand with π-acceptor character to extend the lifetime of the excited state. Cyanide and thiocyanate ligands are possible candidates to fulfill these requirements as they are both anionic ligands with π-backbonding capabilities. Indeed, rhenium(I)(tricarbonyl)(polypyridyl)(cyanide)
complexes have been reported to have excited states with several microsecond lifetimes in a 4:1 methanol:water mixture at room temperature [56]. The cyanide complexes are easily obtainable by reaction of the triflate complex with an excess of potassium cyanide; both 33 and 35, where the polypyridyl ligand is 1,10-phenanthroline and 2,2'-bipyridine respectively, were made easily by this method. Removal of excess potassium cyanide from this reaction is then accomplished by extraction of the organic layer with water. Unfortunately, the synthesis of the cyanide complex with ligand 17 for the purpose of peptide modification was problematic due to solubility issues involving the carboxylic acid group on the ligand (Scheme 3.11). The rhenium complex 37 was no longer soluble.

Figure 3.26 Synthesis and characterization of ReCN-Y-R1C19 (38). (Panel A) Reverse-phase HPLC trace of 38 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel B) ESI MS of 38 in negative ion mode. The calculated m/z(−2H) is 1381.2 Da.
in water, and thus the excess potassium cyanide was not removable by extraction. Attempts at recrystallization were not completely successful. The emission lifetime of 37 synthesized using potassium cyanide (\(\tau_0\)(methanol) = 250 ns, \(\tau_0\)(water) = 90 ns) was much shorter than those reported for analogous compounds [56], perhaps due to quenching by the contaminating salts (data not shown). Changing the cyanide source to tetrabutylammonium cyanide allowed a more complete removal of salts. However, at this time we decided that further purification of 37 was not required for synthesis of peptide 38. Coupling of metal complex 37 with Y-R2C19 proceeded smoothly to afford ReCN-Y-R2C19. The peptide was isolated by reverse-phase HPLC in 0.1 M ammonium bicarbonate as a single species (Figure 3.28a). Characterization by MALDI-TOF MS shows dissociation of a ligand, but does not conclusively distinguish between loss of the cyanide ligand (\(-\Delta 26\) Da) and loss of a carbonyl ligand (\(-\Delta 28\) Da). Although the parent ion of 38 was outside the range of the ESI mass spectrometer, the \(-2\) charged ion confirms the identity of 38 (Figure 3.28b). Binding studies were not carried out because the extinction coefficient of metal complex 37 was not known because it was prepared in situ without purification. ReCN-Y-R2C19 was then exchanged in potassium phosphate buffer by re-chromatographing by reverse-phase HPLC in 4 mM potassium phosphate, pH 7.0. Buffer was exchanged in this manner rather than dialysis with the 1000 MWCO tubing as 38 was found to associate tightly with the cellulose membrane.

The rhenium(II)(tricarbonyl)(polypyridyl)(X) metal complex (39), where X = thiocyanate, was prepared by refluxing the corresponding chloride starting material (18) with an excess of potassium thiocyanate. As 39 was soluble in dichloromethane, excess salts were removed by extraction with water. Reaction of rhenium(II) complex 39 with Y-R2C19 provided the modified peptide, ReSCN-Y-R2C19 (40). This peptide was also isolated as a single species using our standard reverse-phase conditions in 0.1 M ammonium bicarbonate, pH 8.0. An analytical HPLC trace of the purified ReSCN-Y-R2C19 is
Figure 3.29 Characterization of ReSCN-Y-R2C19 (40). (Panel A) Analytical reverse-phase HPLC trace of 40 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel B) UV-visible absorption spectrum of 40 in water. (Panel C) MALDI-TOF MS of 40 in negative ion mode. The calculated m/z(-H) is 2795 Da. (Panel D) Competitive inhibition of nucleotide reduction by 40 (•) overlaid on Ac-Y-R2C19 (−) with a measured K_c of 6 μM.

shown in Figure 3.29a, along with the UV-visible absorption spectrum in water in Figure 3.29b. Although evidence of ligand loss during ionization is observed by MALDI-TOF MS, the parent ion of 40 constituted the major species (Figure 3.29c). The additional peaks correspond to photodissociation of carbon monoxide (−Δ28 Da), thiocyanate (−Δ58 Da), and the loss of both thiocyanate and carbon monoxide (−Δ86 Da). If the laser power for ion desorption is reduced, the relative intensity of these peaks compared to the parent ion decrease, but still remain visible. The concentration of 40 was determined using the extinction coefficient for rhenium complex 39 in water and the binding of ReSCN-Y-
R2C19 to R1 was then assessed by competitive inhibition of nucleotide reduction (Figure 3.29d). The relative activities are plotted against peptide concentration and overlaid on data from Ac-Y-R2C19 ($K_D \sim 20 \mu M$) [47]. The binding appears to be significantly tighter than Ac-Y-R2C19 ($K_D \sim 6 \mu M$) if the measured extinction coefficient is accurate to 100% (see Experimental Methods for fitting procedures). The rhenium(I) peptides of this family ($X = CN, SCN$) appear to be quite promising for photochemical studies with R1, as they both exist as a single species and retain binding affinity for R1.

Preliminary photochemical studies were carried out on ReSCN-Y-R2C19, since it was fully characterized at the time. Time-resolved emission studies demonstrate that the fluorescence lifetime of 40 is within the pulse-width of the laser pulse ($\tau_0 < 10$ ns) upon excitation into MLCT transition. This short lifetime could be attributable to either quenching by ET from the adjacent tyrosine by the rhenium(I) excited state (1.4 V) or an intrinsically short-lived excited state lifetime of the thiocyanate complex (39) alone. Initial attempts to observe the tyrosyl radical upon excitation were unsuccessful. Thus, if ET is indeed occurring between tyrosine and rhenium(I)$^*$, the tyrosyl radical population

![HPLC trace](image)

*Figure 3.30* Analytical reverse-phase HPLC trace of ReSCN-F-R2C19 (41) in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min.
is being rapidly depleted by the back ET from rhenium(II). The phenylalanine derivative of 40, Re(SCN)-F-R2C19 (41) was then synthesized and characterized by reverse-phase HPLC (Figure 3.30) and MALDI-TOF MS with the intention of further investigating this phenomenon. Because the putative tyrosyl radical is short-lived, we would need to turn to flash-quench methodology to generate a tyrosyl radical with a longer lifetime. However, luminescence lifetimes of a model complex of Re(SCN)-Y-R2C19 with the 19-mer peptide removed (ReSCN-Y) was also measured in methanol and found to be less than 100 ns [57]. In addition, we may expect that the anionic thiocyanate sigma donor may not support the longer lifetimes exhibited in complexes where X = pyridine, imidazole, phosphine, or cyanide [50,56]. Taken together, the data is more consistent with the idea that the metal complex alone has a naturally short emission lifetime. Consequently, it appears as if the excited-state lifetime of 40 may be too short to support flash-quench generation of the tyrosyl radical and that the lifetimes of both 38 and 40 may need to be extended by synthetic modifications.

As mentioned above, the excited-state lifetimes of the rhenium(I)(tricarbonyl) (polypyridyl)(X) complexes can be lengthened considerably by the substitution of 2,2'-bipyridine (bpy) for 1,10-phenanthroline (phen) as the rigid ligand prevents excited-state conformational deactivation. We therefore intended to replace bpy-derived ligand 17 with either phen-derived ligand 42 or 43 (Figure 3.31). Ligand 42 has the advantage that it could be synthesized in a one-pot reaction by lithiation of the 4-methyl-1,10-phenanthroline followed by addition of carbon dioxide based on previous work with derivatized bpy ligands [58]. The reaction did not go to completion and both starting material and product were

![Figure 3.31 Exchange of bpy-derived ligand 17 for more rigid phen-based ligands 42 and 43.](image)
detected in the insoluble powder. The synthesis of 4-carboxylic acid-1,10-phenanthroline (43) proceeds through a two-step reaction sequence analogous to 17. Synthesis of aldehyde intermediate, 43a, was carried out without difficulty using literature methods [59]. Further oxidation to the carboxylate-bearing ligand should easily yield ligand 43.

Our work with the rhenium(I)(tricarbonyl) (polypyridyld)(X) complexes has uncovered a surprising issue with ligand substitution at the third-row d⁶ metal center. The presence of an unusually large number of carboxylate side-chains on the R2 C-terminal peptide causes aggregation and cross-linking at the site of ligand X. The typical π-acid ligands used for implementation of flash-quench methodologies (X = pyridine or imidazole), owing to their relatively long excited-state lifetimes, are exchanged for carboxylate ligands in the presence of R2C19. Indeed, even the anionic chloride is replaced in favor of glutamate and aspartate. Attempts to incorporate X = carboxylate into our experimental design demonstrates that the excited-state lifetimes of these types of complexes are too short for quench bimolecularly with the sacrificial electron acceptor (Scheme 3.5). Further investigations into the chemistry of complexes where X = CN or SCN show that these ligands are able to compete effectively with the carboxylates and allow isolation of cleanly metallated peptides, ReCN-Y-R2C19 and Re-SCN-Y-R2C19. Preliminary photochemical studies of these systems show that some ligand modification may be required, but should easily by achieved. Overall, it is quite possible that ReCN-Y-R2C19 will eventually provide a route to generation of *Y356.

3.5 Lumiflavin-Modified Peptides

3.5.1 Introduction

Flavinoid compounds comprise a family of well-known photochemically-active redox agents. Excitation of flavin (Fl) to the triplet state (3Fl*) takes place with a high quantum yield (Φ~0.7) and creates a strong oxidant (1.7 V) capable of hydrogen atom abstraction from nucleophiles [60-62]. Tyrosine and tryptophan photooxidation by 3F* has been
well-established [63-65] and shown to generate the flavin semiquinone radical (•FlH) with approximate quantum yields of 0.5 and 0.4 respectively [64]. Hydrogen atom abstraction from tyrosine takes place with a bimolecular rate constant of $1.7 \times 10^9$ M$^{-1}$s$^{-1}$ [64,66], however the back reaction with •FlH can occur rapidly [67]. Thus, •Y356 could be produced on a R2 peptide covalently modified with flavin according to Scheme 3.12 if the forward reaction to Y731 is faster than the back reaction with •FlH. In anaerobic conditions, photobleaching of the flavin chromophore eventually occurs, as the

$$\text{Fl-Tyr} \xrightarrow{\text{hv}} \text{•FlH-Tyr} \xrightarrow{\text{Co}^{III}(\text{NH}_3)_5\text{Cl}} \text{•Fl}_{\text{ox}}-\text{Tyr} + \text{Co}^{II}(\text{NH}_3)_5\text{Cl} \rightarrow \text{Fl-Tyr}^* + \text{Co}^{II}(\text{H}_2\text{O})_6$$

*Scheme 3.12* Tyrosyl radical generation by triplet flavin or the oxidized flavin radical using an external electron acceptor.

semiquinone can either be quenched by another reaction component to produce the colorless fully reduced dihydroflavin (FlH$_2$) or disproportionate with a second molecule of •FlH to produce fully oxidized and reduced flavin [65,67]. Therefore, the FlH$_2$ would need to be re-oxidized to effect multiple turnovers in R1. In the presence of oxygen, Fl can be partially regenerated from •FlH [65], however, the aerobic photochemistry of flavin with tyrosine is quite complicated and involves singlet oxygen [68]. Although the upper arm of Scheme 3.12 does not provide for continual turnover, generation of the tyrosyl radical by direct reaction with $^3$F*$ offers the benefit of not requiring the external quenchers needed for ruthenium(II) or rhenium(I) systems. If multiple turnovers are necessary, a flavin redox cycle can also be constructed using external quenchers (Scheme 3.12). Oxidative quenching of $^3$Fl* by electron acceptors, such as cobalt(III) pentaamine chloride, produces the oxidized flavin cation radical (•FlH$^+$, pK$_a$ ~ 6.1, 2.3 V) which deprotonates to form the neutral radical (•Fl$_{\text{ox}}$), both strong oxidants which have similar hydrogen abstraction capabilities to $^3$Fl* [62,66,69-72]. Indeed, •Fl$_{\text{ox}}$ has been observed

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to oxidize tyrosine to the tyrosyl radical at a bimolecular rate of $1.9 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ [66] (Scheme 3.12). Thus in the presence of external quenchers, the tyrosyl radical produced should have a longer lifetime, and consequently, a greater probability of being able to carry out radical initiation at C439 in R1.

This section presents the synthesis and characterization of a R2 peptide derivative covalently modified with a lumiflavim amino acid [73] (44). Preliminary photochemical studies with 44 indicate the excited-state lifetime is quenched compared to free lumiflavim by the presence of tyrosine, however, the back electron transfer with \textsuperscript{*}FlH is rapid. Further experiments with an oxidative quencher, ruthenium(III) hexamine, provide promising evidence for tyrosyl radical generation.

3.5.2 Results and Discussion

The synthesis of flavin-Y-R2C19 (44) proceeded smoothly from the coupling of the Fmoc-flavin amino acid and Y-R2C19. The Fmoc group was removed, but the N-terminus was not acetylated. The characterization of flavin-Y-R2C19 by reverse-phase HPLC is shown in Figure 3.32a with the absorption spectrum (Figure 3.32b). The identity of 44 was further confirmed by MALDI-TOF MS (Figure 3.32c). A full binding curve for 44 was not generated, but addition of flavin-Y-R2C19 (10 \text{ \mu M}) inhibits nucleotide reduction by 70%. Photochemical studies of 44 were carried out in collaboration with Jeremiah Miller and Jay Winkler at the California Institute of Technology. Initial time-resolved luminescence measurements show that the lifetime is considerably shorter than
lumiflavin itself and decays within the pulse-width of the laser excitation ($\tau_0 < 10 \text{ ns}$), implying fluorescence quenching by the adjacent Y356. Nanosecond transient absorption studies of 44 also show that the lifetime of the triplet excited state is shortened compared to lumiflavin. Point-by-point data collection shows the characteristic changes in the absorption spectra corresponding to the flavin triplet state [64,66] with absorption between 300-400 nm and a bleach centered at 445 nm (Figure 3.33a). The single-wavelength trace collected at 300 nm corresponding to the decay of $^3$F1* in peptide 44 is shown in Figure 3.33b, and indicates that the lifetime of the triplet flavin on the R2 peptide in deaerated buffer potassium phosphate buffer is approximately 200 ns at neutral pH. Thus, the observed lifetime of the triplet flavin in 44 is substantially quenched.
Figure 3.33 Transient absorption of the triplet excited-state ($\lambda_{\text{exc}}=450$ nm) of flavin-Y-R2C19 in 50 mM potassium phosphate, pH 7.2. (Panel A) Spectrum generated point-by-point 150 ns after the laser pulse. (Panel B) Single-wavelength kinetics collected at 300 nm.

compared to the lifetime of free lumiflavin, which has been reported in the literature to be on the 10 to 100 $\mu$s timescale in aqueous solution [74]. This observation is consistent with the reported steady-state quenching of up to 90% of the lumiflavin amino acid in peptides which contain several tyrosines compared to those which do not [73]. Unfortunately, an electronic instrumental artifact from 500 nm to 600 nm obscures the spectral area corresponding to the flavin semiquinone radical absorption [64,66], which would confirm triplet quenching by hydrogen abstraction or PCET from the neighboring $\cdot$Y356. Control experiments with the lumiflavin model compound under the same conditions exhibits a triplet lifetime on the order of ~0.1 ms, as measured by the both the absorption at 330 nm (Figure 3.34a) and the bleach at 425 nm (Figure 3.34b), yielding an electron transfer rate of $5 \times 10^6$ s$^{-1}$. It appears as if the back electron transfer with the reduced flavin semiquinone radical is rapid, because no build-up of the tyrosyl radical was observed by transient absorption. Thus, we decided to pursue tyrosyl radical generation through the flash-quench method shown in Scheme 3.12. In this scheme, oxidative quenching of the triplet excited state by an electron acceptor generates the oxidized flavin radical, which can then oxidize the tyrosine to the tyrosyl radical. As ground-state flavin would be formed concomitant with the tyrosyl radical, $\cdot$Y356 should

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be generated stably and thus have a longer lifetime. Quenching of $^3\text{Fl}^*$ was first tested on the lumiflavin model before proceeding to peptide 44. Indeed upon addition of the oxidative quencher, ruthenium(III) hexaamine (20 mM), the lifetime of the lumiflavin triplet state was reduced to $\sim 3$ $\mu$s, as measured by monitoring both the triplet absorption and decay at 380 nm (Figure 3.34c) and 435 nm (Figure 3.34d), respectively. As oxidative quenching of $^3\text{Fl}^*$ was successfully achieved, we then proceeded to study the photochemistry of flavin-Y-R2C19 in the presence of ruthenium(III) hexaamine. Figure 3.35 shows the transient absorption spectrum generated point-by-point of 44 with 20 mM quencher both 300 ns and 5 $\mu$s after the laser pulse. At 300 ns, we see mainly features that correspond to either the excited triplet state [64,66] or the oxidized flavin radical [66,71]. In the trace collected at 400 nm, 405 nm, 408 nm, and 415 nm, the formation of a shelf is observed, which corresponds to the absorption of a much longer-lived species.
Long after decay of the flavin triplet and oxidized radical, we are left with a species exhibiting a spectral signature that is characteristic of the tyrosyl radical (Figure 3.35). Our contention that $\cdot{Y}356$ is generated upon flash photolysis of 44 is bolstered by the fact that the oxidized flavin radical is known to generate both tryptophan and tyrosyl radicals in bimolecular reactions [66]. From these studies, it appears as if $\cdot{Y}356$ generation can be achieved using both paths of Scheme 3.12 involving either direct oxidation by $^{3}Fl^{*}$ or by flash-quench of the triplet state to form oxidized flavin radical. It is our hope that these studies will aid development of R2-derived peptides for light-initiated PCET in R1.

### 3.6 Conclusions

In this chapter, we present the development of methods to initiate formation of $\cdot{Y}356$ on the R2 C-terminal peptide with the input of light. We began with the synthesis of the diaryl oxalate ester derivatives of tyrosine, which provide rapid release ($<50$ ns) of the tyrosyl radical upon UV excitation driven by loss of carbon monoxide. Although the
appropriate Boc-protected derivative with a free carboxylic acid could be prepared for incorporation into the 20-mer peptide, the oxalate ester linkage was found to be too fragile to withstand deprotection in TFA cleavage cocktails followed by reverse-phase HPLC purification in basic or acidic conditions. Further studies substituting a naphthyl ring for one of the phenyl moieties shows promise for shifting the wavelength of photochemical phenoxy radical release outside the protein absorption envelope. Although this type of compound cannot be used for our studies, they may be of great utility in model complexes or in peptides in which the aryl oxalate ester moiety may be added in a solution synthetic step.

Initial studies with ruthenium(II) polypyridyl-modified peptides show that binding to R1 is retained, in spite of the charge and large size of the covalently attached metal complex. Following previous studies that show tyrosine oxidation at an intramolecular rate of $5 \times 10^4$ s$^{-1}$, flash-quench studies were implemented with Rubpy$_3$-Y-R2C19. Although generation of the oxidized metal center was achieved, studies of tyrosyl radical generation remain inconclusive. In addition, the R2 peptide was found to catalyze the precipitation of the ruthenium(III) complex, most likely due to association of the negatively-charged carboxylates with the +3 metal ion. Since we expect that binding of R1 should prevent contact between the carboxylate side-chains of the peptide with the metal center, light-initiated turnover reactions with R1 will be pursued in Chapter 4.

Forays into the chemistry of rhenium(I)(tricarbonyl)(polypyridyl)(X) photooxidants reveal a surprising lability of the ligand X when covalently attached to the R2 C-terminal peptide. Again, the multiple carboxylate side-chains of the peptide are responsible, as carboxylate was found to be a more stable ligand for rhenium(I) than either imidazole or chloride. Attempts to metallate purified bpy-Y-R2C19 and Ac-His-Y-R2C19 were unsuccessful, as the peptide was denatured in conditions required for the reaction of the former and carboxylate was found to kinetically and thermodynamically outcompete
ligation of histidine for the latter. The observation that carboxylate provided a stable ligand for rhenium(I) led to studies in which a tridentate bpy ligand with an intramolecular carboxylate donor was targeted. The excited-state lifetime of the complex in which $X = \text{acetate}$ were observed to be short and consequently, we abandoned this line of approach. The long excited-state lifetimes ($\mu s$) reported in the literature for complexes in which $X = \text{CN}$, led to synthesis of peptide derivatives, ReCN-Y-R2C19 and ReSCN-Y-R2C19. These peptides were isolated as single species and found to retain their respective CN and SCN ligands by mass spectrometry. Although preliminary photochemical studies show that the thiocyanate complex may have an intrinsically short lifetime similar to the chloride complexes, their lifetimes may be extended by switching the bpy ligand for the more rigid phen ligand. Overall, we believe that the peptides in which $X = \text{CN}$, maybe provide a route for tyrosyl radical generation at position 356 and should be examined further.

The photochemistry of flavinoid compounds is quite rich and provides two separate paths to tyrosyl radical generation. Thus, a lumiflavin-modified peptide, flavin-Y-R2C19 was also synthesized and characterized. Preliminary studies of the first path via hydrogen atom abstraction by the excited-state triplet indicate that tyrosyl radical is most likely formed, but rapid reverse ET with semiquinone prevents observation of the tyrosyl radical species by transient absorption. Although eventual photobleaching of the flavin moiety is expected, this may provide a means for single turnover reactions with R1 in the absence of external quencher. If multiple turnovers are required, a redox cycle involving the oxidative quenching of the triplet to form oxidized flavin radical provides another route to generation of $\cdot \text{Y356}$ on flavin-Y-R2C19. Initial time-resolved studies using ruthenium(III) hexaamine as the electron acceptor are promising with regard to tyrosyl radical generation and will hopefully be pursued in the future.
3.7 Experimental Methods

**Materials.** All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Ruthenium(III) chloride was purchased from Alfa Aesar (Ward Hill, MA). Silica gel 60 (70-230 and 230-400 mesh, Merck) was used for column chromatography. Analytical thin layer chromatography was performed using Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick). Solvents for synthesis were of reagent grade or better, and were dried according to standard methods. Triethylamine was distilled over calcium hydride and stored at 4 °C under nitrogen. The material was further purified by dissolution in dichloromethane and filtration to remove any remaining LiCl. Oxalyl chloride was purchased in 5g ampules from Sigma-Aldrich. Phenol starting materials were dried *in vacuo* for 24 h before use. Spectroscopic experiments employed tetrahydrofuran (spectroscopic grade, Burdick & Jackson), which was stored over sodium/benzophenone under vacuum. Deuterated solvents were purchased from Cambridge Isotope Labs (Andover, MA). N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) was purchased from EM Science (Gibbstown, NJ). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Roche (Indianapolis, IN).

(5)-2-Amino-N\(^{\alpha}\)-Fmoc-6-(7',8'-dimethylisoalloxazin-10'-yl)hexanoic acid (Fmoc-flavin-OH) was obtained as a gift from Professor T. Carell at the University of Marbourg, Germany. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), and N-hydroxybenzotriazole (HOBr) were procured from Novabiochem (San Diego, CA). Fmoc amino acids with standard tert-butyl and trityl protecting groups, N-[(dimethylamine)- 1H - 1, 2, 3 - triazolo [4,5-b] pyridine-1-ylmethylene] - N - methylmethanammonium hexafluorophosphate N-oxide (HATU), and 20% (v/v) piperidine in DMF were purchased from Applied Biosystems (Foster City, CA). Disposable columns were purchased from Bio-Rad Corporation (Hercules, CA). C-8 Vydac columns were purchased from Vydac Corporation (Hesperia, CA). C-8 and C-18
XTerra MS 5 μM columns purchased from Waters Corporation (Milford, MA). Dialysis tubing was purchased from Spectrum Labs (Rancho Dominguez, CA).

**General method for manual addition of Fmoc-amino acids to Fmoc-R2C19 using PyBOP (method A).** Fmoc-R2C19 (100 mg, 0.025 mmol) was deprotected by 3 × 7 min treatments with 20% (v/v) piperidine containing 0.1 M HOBt in DMF in a 20 mL fritted column. The reaction was mixed by a Vortex mixer using a foam adapter for 50 mL Falcon tubes, in which the column was placed. After washing 5 × DMF and 3 × acetone, Fmoc amino acids were added using a 0.9 PyBOP:1 HOBt:2 DIEA activation scheme. Briefly, Fmoc amino acid, PyBOP, and HOBt were dissolved in 150 μL DMF to 0.5 M (0.075 mmol), 0.45 M (0.068 mmol), 0.5M (0.075 mmol) respectively. All three components were combined and then activated by the addition of DIEA (26 μL, 0.15 mmol) and added to the resin. The coupling was allowed to proceed for 1 hr before washing 5 × DMF. Removal of the N-terminal Fmoc was then carried out as described for Fmoc-R2C19 above.

**General method for manual addition of Fmoc-amino acids to Fmoc-R2C19 using HATU (method B).** Fmoc-R2C19 (100 mg, 0.025 mmol) was deprotected by 3 × 7 min treatments with 20% (v/v) piperidine containing 0.1 M HOBt in DMF in a 20 mL fritted column. The reaction was mixed by a Vortex mixer using a foam adapter for 50 mL Falcon tubes, in which the column was placed. After washing 5 × DMF and 3 × acetone, Fmoc amino acids were added using a 0.9 HATU:2 DIEA activation scheme. Briefly, Fmoc amino acid and HATU were dissolved in 250 μL DMF to 0.5 M (0.125 mmol) and 0.45 M (0.113 mmol) respectively. All three components were combined and then activated by the addition of DIEA (44 μL, 0.25 mmol). The coupling was allowed to proceed for 1 hr before washing 5 × DMF. Removal of the N-terminal Fmoc was then carried out as described for Fmoc-R2C19 above.
Isolation of crude peptide material. Peptides were cleaved from the resin by vortexing for 3 h in a solution composed of 95% (v/v) TFA, 2.5% (v/v) TIS, and 2.5% (v/v) water. The peptide solution was collected by filtration and concentrated under a stream of N₂. The peptide was then precipitated by addition of diethyl ether. The suspension was incubated at -10 °C and pelleted by centrifugation. The peptide pellet was washed twice with ether and air-dried. The semi-dried pellet was solubilized in 0.1 M ammonium bicarbonate, pH 8.0.

Semi-preparative reverse-phase HPLC (Gradient A). Peptides were isolated by semi-preparative HPLC on a pH-stable C-8 Vydac column (9 mm × 250 mm) using a linear gradient (4.0 mL/min) of 10% to 75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 45 min. Fractions were collected by hand and lyophilized.

Analytical reverse-phase HPLC (Gradient B). Peptides were analyzed by HPLC on a pH-stable C-8 Vydac column (4.6 mm × 250 mm) using a linear gradient (1.0 mL/min) of 10% to 75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 45 min. Fractions were collected by hand and lyophilized.

Preparative reverse-phase HPLC (Gradient C). Peptides were isolated by preparative HPLC was on an Waters C-8 X Terra MS 5 μM column (30 mm × 150 mm) using a linear gradient (22.5 mL/min) of 10% to 65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 45 min. Fractions were collected by hand and lyophilized.

Analytical reverse-phase HPLC (Gradient D). Peptides were analyzed by HPLC on a C-8 X Terra MS 5 μM column (4.6 mm × 100 mm) using a linear gradient (1.0 mL/min) of 10% to 65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 45 min. Fractions were collected by hand and lyophilized.
Buffer exchange by dialysis. Purified peptides were resuspended after lyophilization in a minimal volume of 0.1 M ammonium bicarbonate, pH 8.0. Buffers were then exchanged using Spectra/Por 1000 MWCO dialysis tubing to a final of 1:1,000,000 by two successive 1:1,000 dilutions. Each dialysis was allowed to proceed for a minimum of 4 h.

Buffer exchange using HPLC. Purified peptides were resuspended after lyophilization in a minimal volume of 0.1 M ammonium bicarbonate, pH 8.0 and rechromatographed by reverse-phase HPLC on either an analytical or preparative scale on a C-8 XTerra MS 5 μM column using a linear gradient of 10% to 75% acetonitrile versus 4 mM potassium phosphate over 45 min. Column sizes and flow rates are as follows. Analytical: 4.6 mm × 100 mm, 1.0 mL/min. Preparative: 30 × 150 mm, 22.5 mL/min. Lyophilized peptides were then resuspended in water.

Bis(2,4,6-tri-tert-butylyphenyl) oxalate ester (1). Compound 1 was prepared from the condensation of two equivalents of 2,4,6-tri-tert-butylyphenol with oxalyl chloride by a modified literature procedure [33]. 2,4,6-tri-tert-butylyphenol (5 g, 19.1 mmol) was dried in vacuo overnight in a 100 mL round-bottom Schlenk flask. The flask was then filled with N₂ and cooled in an isopropanol/ice bath. THF (40 mL) was collected in a syringe from the solvent stills and injected into the reaction. n-Butyllithium (1.6 M, 11.9 mL, 19.1 mmol) was then injected into the flask. A white precipitate formed shortly after addition was allowed to stir for an 5 additional min. The oxalyl chloride was transferred into a round-bottom flask and capped with a septum in the dry box and removed from the glove box to inject (0.84 mL, 9.6 mmol) into the reaction. The white solid cleared and the solution turned green. The solution was stirred for an additional h in the cold bath before removing and stirring overnight at room temperature. THF was removed by rotary evaporation and the yellow solid was suspended in diethyl ether and filtered. The filtrate was washed twice with 10% sulfuric acid, followed by sodium carbonate. Upon washing with a solution of 10% sodium carbonate, a white precipitate formed that was removed
by filtration. The organic phase was dried over sodium sulfate, filtered, and evaporated to dryness. The product was then recrystallized as a white solid from dichloromethane by the addition of methanol. $^1$H NMR (500 MHz, CDCl$_3$, 25 °C): $\delta = 7.39$ (s, 4H, ArH), 1.42 (s, 36H, $^3$Bu), 1.35 (s, 18H, $^3$Bu). HRFAB MS (M): calcd for C$_{38}$H$_{58}$O$_4$, m/z 578.43, found 578.43.

Bis(N-Acetyltyrosyl methyl ester) oxalate ester (2). In a 100-mL Schlenk flask, N-Acetyltyrosyl methyl ester (4.0 g, 15.6 mmol) was dissolved in dry THF (30 mL). The solution was stirred overnight over activated 4 Å molecular sieves after which triethylamine (2.17 mL, 15.6 mmol) was added. A solution of oxalyl chloride (0.64 mL, 7.3 mmol) in THF (10 mL) was prepared in a separate 250-mL Schlenk flask. Both flasks were cooled to –78 °C and the solution of N-acetyltyrosyl methyl ester was transferred dropwise via cannula to the solution of oxalyl chloride. The solution was stirred at –78 °C for 1 h, and then allowed to warm to room temperature and stirred for an additional h. The resulting white precipitate was collected by filtration in air and redissolved in dichloromethane containing a small amount of methanol. The organic layer was washed with HCl (3 × 25 mL, 3.7% v/v) and dried over Na$_2$SO$_4$. The solution was evaporated to dryness to yield pure 1 as a white powder (2.5 g, 65% yield). $^1$H NMR (500 MHz, CDCl$_3$, 25 °C): $\delta = 7.20$ (m, 8H, ArH), 5.96 (d, $J = 8$ Hz, 2H, NH), 4.92 (dd, $J_1 = 9.5$ Hz, $J_2 = 6$ Hz, 2H, CH), 3.75 (s, 6H, methyl ester), 3.17 (ddd, $J_1 = 29.5$ Hz, $J_2 = 14$ Hz, $J_3 = 6$ Hz, 2H, CH$_2$), 2.02 (s, 3H, acetyl). HRFAB MS (MH$^+$): calcd for C$_{26}$H$_{29}$N$_2$O$_{10}$, m/z 529.182, found 529.181.

(N-Acetyltyrosyl methyl ester)(2,4,6-tri-tert-butylphenyl) oxalate ester (3). 2,4,6-Tri-tert-butylphenyl oxalyl chloride was prepared according to literature procedures [33]. N-Acetyl tyrosyl methyl ester (1.5 g, 5.9 mmol) was dissolved in THF (30 mL) containing pyridine (0.475 mL, 5.9 mmol). The solution was transferred via cannula to a solution of 2,4,6-tri-tert-butylphenyl oxalyl chloride (2.8 g, 8.0 mmol) in THF (20 mL) and stirred
overnight at room temperature. Pyridine hydrochloride was removed by filtration and the filtrate was dried to a yellow oil that was triturated with hexanes. The resulting white precipitate was collected and redissolved in dichloromethane. Ether was added to precipitate unreacted tyrosine. The remaining solution was collected, washed with saturated aq. Na₂CO₃ (1 × 50 mL), and dried over Na₂SO₄. The solvent was removed to deliver pure 2 as a white solid (1.37 g, 42% yield). ¹H NMR (500 MHz, CDCl₃, 25 °C): δ = 7.38 (s, 2H, ArH), 7.21 (dd, J₁ = 23 Hz, J₂ = 9 Hz, 4H, ArH), 5.96 (d, J = 7.5 Hz, 1H, NH), 4.92 (dd, J₁ = 13.5 Hz, J₂ = 6 Hz, 1H, CH), 3.76 (s, 3H, methyl ester), 3.18 (ddd, J₁ = 30 Hz, J₂ = 14 Hz, J₃ = 5.5 Hz, 2H, CH₂), 2.03 (s, 3H, Acetyl), 1.41 (s, 18H, 'Bu), 1.35 (s, 9H, 'Bu). HRFAB MS (MH⁺): calcd for C₃₂H₄₄NO₇, m/z 554.312, found 554.312.

(N-α-Boc-tyrosyl benzyl ester)(2,4,6-tri-tert-butylphenyl) oxalate ester (4). 2,4,6-Tri-tert-butylphenyl oxalyl chloride was prepared according to literature procedures [33]. N-α-Boc-tyrosyl benzyl ester (2.0 g, 7.4 mmol) was dissolved in THF (15 mL) containing triethylamine (1.4 mL, 10 mmol). The solution was transferred dropwise via cannula into a solution of 2,4,6-tri-tert-butylphenyl oxalyl chloride (2.8 g, 8.0 mmol) in THF (20 mL) and resulting solution was stirred overnight at room temperature. The solvent was removed in vacuo and purification by flash column chromatography (silica gel, 98/2 dichloromethane/methanol) afforded pure 3 as a white powder (2.1 g, 41% yield). ¹H NMR (300 MHz, CDCl₃, 25 °C): δ = 7.39 (s, 2H, ArH), 7.36 (m, 5H, benzyl-ArH), 7.12 (dd, J₁ = 14.1 Hz, J₂ = 8.4 Hz, 4H, ArH), 5.16 (dd, J₁ = 27.3 Hz, J₂ = 12 Hz, 2H, benzyl-CH₂), 5.03 (d, J = 8.7 Hz, 1H, NH), 4.65 (dd, J₁ = 14.7 Hz, J₂ = 7.2 Hz, 1H, CH), 3.13 (m, 2H, CH₂), 1.45 (s, 9H, 'Boc), 1.42 (s, 18H, 'Bu), 1.36 (s, 9H, 'Bu). HRFAB MS (MNa⁺): calcd for NaC₄₁H₅₅NO₈, m/z 710.367, found 710.368.

(N-α-Boc-tyrosyl)(2,4,6-tri-tert-butylphenyl) oxalate ester (5). Ester 3 (1.0 g, 1.45 mmol) was dissolved in THF (50 mL) and stirred over 10% Pd/C (250 mg) for 6 h under a hydrogen atmosphere. The Pd/C was removed by filtration and the solvent was
evaporated to give pure 4 as a pale manila solid (0.8 g, 92% yield). \(^1\)H NMR (300 MHz, CDCl\(_3\), 25 °C): \(\delta = 7.38\) (s, 2H, ArH), 7.27 (dd, \(J_1 = 15.9\) Hz, \(J_2 = 9\) Hz, 4H, ArH), 4.99 (d, \(J = 10.2\) Hz, 1H, NH), 4.63 (m, 1H, CH), 3.20 (dd, \(J_1 = 45\) Hz, \(J_2 = 6.9\) Hz, 2H, CH\(_2\)), 1.45 (s, 9H, 'Boc), 1.41 (s, 18H, 'Bu), 1.36 (s, 9H, 'Bu). HRFABMS (MNa\(^+\)): calcd for NaC\(_{34}\)H\(_{72}\)NO\(_8\), \(m/z\) 620.320, found 620.319.

**Bis(2,6-di-tert-butyl-4-nitro-phenyl) oxalate ester (6).** 2,6-di-tert-butyl-4-nitrophenol was synthesized from 2,6-di-tert-butylphenol in 40% yield by heating in hexanes with nitric acid following literature methods (\(\lambda_{\text{max}}\) (CHCl\(_3\)) = 320 nm) [35]. The phenol (4.3 g, 17.1 mmol) was dissolved in dichloromethane (30 mL) and dried over activated 4 Å overnight. The liquid was then transferred into a 250 mL Schlenk flask and dried in vacuo. The phenol was redissolved in THF (30 mL) before adding pyridine (1.38 mL, 17.1 mmol). The reaction was cooled in an ice/acetone bath. Oxalyl chloride (0.75 mL, 8.55 mmol) was transferred in slowly by syringe. The yellow reaction was allowed to warm up to room temperature and was stirred for an additional 3 h. The reaction was filtered to remove pyridinium hydrochloride before evaporating to a red solid. The solid was dissolved in a mixture of dichloromethane and methanol. Upon removal of dichloromethane, a fine yellow powder was collected by filtration. Additional product was collected from the filtrate by addition of hexanes and evaporation of dichloromethane (60 mg, 0.6 %). \(^1\)H NMR (300 MHz, CDCl\(_3\), 25 °C): \(\delta = 8.31\) (s, 4H, ArH), 1.46 (s, 36H, 'Bu). HRFAB MS (MH\(^+\)): calcd for C\(_{30}\)H\(_{41}\)N\(_2\)O\(_8\), \(m/z\) 557.286, found 557.286.

**Bis(2,6-di-tert-butyl-4-N,N-dimethylamino-phenyl) oxalate ester (7).** 2,6-Di-tert-butyl-4-aminophenol (7a) was synthesized by palladium-catalyzed hydrogenation of the corresponding nitrophenol starting material [35]. 2,6-di-tert-butyl-4-nitrophenol (10.0 g, 39.8 mmol) was dissolved in THF (75 mL) in a 250 mL round-bottom flask. 10% Pd/C (100 mg) and triethylamine (0.1 mL) were then added. The reaction was purged three
times with hydrogen before filling the reaction with hydrogen using a balloon reservoir. The reaction was stirred for 4 days until the reaction changed color from yellow to clear. The flask was evacuated before bringing into the glove box and filtering through a Celite pad in a fritted glass. The solvent was then removed in vacuo (4.2 g, 48%) and stored under nitrogen. \(^1\)H NMR (300 MHz, CDCl\(_3\), 25 °C): \(\delta = 6.58\) (s, 2H, ArH), 4.62 (s, 1H, OH), 3.36 (s, 2H, NH\(_2\)), 1.42 (s, 18H, \(^t\)Bu).

2,6-Di-\(t\)-butyl-4-N,N-dimethylaminophenol (7b) was synthesized by reductive methylation following literature procedures [36]. 7a (1.0 g, 4.5 mmol) was dissolved in THF (20 mL) in a 50 mL Schlenk flask in the glove box. The phenol was then transferred by cannula into a solution of 3 M sulfuric acid (3.65 mL, 1.1 mmol) and formaldehyde (2.2 mL, 27 mmol) in a 100 mL Schlenk flask. Sodium borohydride (1.2 g, 31.7 mmol) was added as a solid while bubbling through the solution with nitrogen. The resulting clear solution was stirred overnight at room temperature. The unreacted sodium borohydride was removed by filtration. The filtrate was then partitioned in H\(_2\)O:dichloromethane. The organic layer was collected and washed 3 × H\(_2\)O, 3 × saturated sodium bicarbonate, and 1 × H\(_2\)O. The organic layer was dried over sodium sulfate and evaporated to dryness in vacuo to a white solid and stored in the glove box (0.975 g, 87%). \(^1\)H NMR (300 MHz, CDCl\(_3\), 25 °C): \(\delta = 6.73\) (s, 2H, ArH), 4.69 (s, 1H, OH), 2.89 (s, 6H, CH\(_3\)), 1.48 (s, 18H, \(^t\)Bu).

Synthesis of 7 was attempted from 7b based on the synthesis of 2. 7b (0.975 g, 3.91 mmol) was transferred into a round-bottom flask containing 2,6-lutidine (0.58 mL, 4.98 mmol) in tetrahydrofuran (25 mL) in the dry box. At the same time, oxalyl chloride (0.19 mL, 2.18 mmol) was transferred into a second Schlenk flask containing 5 mL of tetrahydrofuran. Both flasks were cooled in acetone/dry ice baths under nitrogen before transferring the phenol dropwise into the reaction flask containing oxalyl chloride by cannula. The brown reaction was stirred first at −78 °C and then allowed to warm up to
room temperature and stirred for an additional 4 h. First, a white precipitate was removed from the reaction by filtration and found to consist mainly of 2,6-lutidinium chloride and a small amount of possible product (7c) by $^1$H-NMR. After dissolving the solid in dichloromethane and extracting with 10% hydrochloric acid, the lutidinium chloride was removed, however, the product was quantitatively converted to another compound (7d). Both compounds demonstrated a similar downfield shift of the aromatic protons ($\delta_{7b} = 6.72$ ppm, $\delta_{7c} = 7.51$ ppm, $\delta_{7d} = 7.01$ ppm) observed in other diaryl oxalate ester compounds. As the majority of the reaction mass still remained in the filtrate, this was evaporated to dryness and redissolved in dichloromethane. $^1$H-NMR showed that both 7d and phenol 7b were present, along with a small amount of 2,6-lutidinium chloride. Attempts to 2,6-lutidinium chloride and 7b by extraction with 10% hydrochloric acid and sodium bicarbonate unfortunately lead to the quantitative conversion to phenol 7b.

(2-Naphthyl)(2,4,6-tri-tert-butylphenyl) oxalate ester (8). 2,4,6-tri-tert-butylphenol (5.0 g, 19.0 mmol) was dissolved in diethyl ether (30 mL) in a Schlenk flask and stirred in an ice/acetone bath for 5 min. n-Butyllithium (12 mL, 1.6 M) was added, precipitating a white solid. The suspension was allowed to stir for another 5 min before dumping into cooled 100 mL Schlenk flask with oxalyl chloride (3.3 mL, 39.4 mL). The reaction was stirred for another 30 min before removing the solvent in vacuo. The pale green solid was redissolved in THF (15 mL). Recrystallized 2-naphthol (0.6 g, 4.1 mmol) was dissolved in a 50 mL Schlenk flask with THF (30 mL) and pyridine (0.5 mL, 4.1 mmol). The solution containing the 2-naphthol was then transferred by cannula into the flask with 2,4,6-tri-tert-butylphenyl oxalyl chloride and stirred for 30 min. Pyridinium hydrochloride was removed by filtration and the filtrate was evaporated to a yellow oil. Methanol was added to the oil, precipitating a white solid that was collected in a finely fritted glass. The solid was collected and dissolved in dichloromethane and extracted with $4 \times$ sodium bicarbonate (1.0 M), $2 \times$ water, and $2 \times$ brine. The organic layer was dried
over sodium sulfate and evaporated to a manila powder (0.84 g, 53%). \(^1\)H NMR (300 MHz, CDCl\(_3\), 25 °C): \(\delta = 7.45\) (s, 2H, Ar\(_{phe}\)H), 1.45 (s, 18H, \('\)Bu), 1.36 (s, 9H, \('\)Bu). HRFABMS (M\(^+\)): calcd for C\(_{30}\)H\(_{36}\)O\(_4\), m/z 460.261, found 460.261.

Synthesis and characterization of [(Tyrosyl)(2,4,6-tri-tert-butylphenyl) oxalate ester]-R2C19 (9). The addition of 5 to R2C19 was carried out by manual solid-phase peptide synthesis with 0.8 PYBOP:1 HOBr:2 DIEA activation scheme with 4 eq of 5 by method A. The semi-dried pellet was solubilized in 0.1 M ammonium bicarbonate, pH 8.0. The product was then isolated using gradient A. The purified product was then analyzed by gradient B and MALDI-TOF MS. Analytical HPLC: \(t_R = 13.9\) min. MALDI-TOF MS: MW\(_{calc}\) = 2588 Da, MW\(_{obs}\) = 2271 Da. UV-Vis: \(\lambda_{max} = 276\) nm.

Synthesis and characterization of LVGQI (10). LVGQI was synthesized by manual solid-phase peptide synthesis on a PAC-PEG-PS resin functionalized with the C-terminal Fmoc-Ile. Successive addition of Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, and Fmoc-Leu-OH was carried out using coupling method A. Capping with 0.3 M acetic anhydride: 0.3 M DIEA: 0.1 M HOBr in DMF was also carried out for 30 min between coupling with the Fmoc amino acid and deprotection, followed by washing with DMF and dichloromethane. The peptide pellet was resuspended in water and characterized by analytical HPLC in a gradient of 10-75% acetonitrile in 0.05% TFA over 45 min (1.0 mL/min) on a C-18 X Terra MS 5 \(\mu\)m column (4.6 × 100 mm). HPLC: \(t_R = 10.5\) min. ESI MS (MH\(^+\)): m/z\(_{calc}\) = 529.33, m/z\(_{obs}\) = 529.33.

Synthesis and characterization of [(Tyrosyl)(2,4,6-tri-tert-butylphenyl) oxalate ester]-LVGQI (11). The addition of 5 to 10 and isolation of the ether-precipitated peptide was accomplished as described for 9. The peptide was resuspended in water and characterized by analytical HPLC in a gradient described for 10. HPLC: \(t_R = 14.8\) min. ESI MS (MH\(^+\)): m/z\(_{calc}\) = 1009.6, m/z\(_{obs}\) = 692.41. UV-Vis: \(\lambda_{max} = 276\) nm.
Synthesis and characterization of YLVGQI (12). The addition of Fmoc-Tyr(Bu)-OH to 10 was carried out by method A. The peptide was resuspended in water and analyzed by HPLC using the gradient described for 10. HPLC: t_R = 15.1 min. ESI MS (MH^+): m/z_{calcd} = 692.40, m/z_{obs} = 692.380. MALDI-TOF MS: MW_{calcd} = 692 Da, MW_{obs} = 694 Da. UV-Vis: λ_{max} = 276 nm.

Ruthenium(II) (bis (2,2'-bipyridine)) (4-methyl-4'-carboxylic acid-2,2'-bipyridine) (bis (hexafluorophosphate) (13). The metal complex was synthesized by literature methods [45]. ^1H NMR (300 MHz, CD_3CN, 25 °C): δ = 8.88 (s, 1H, ArH), 8.54 (s, 1H, ArH), 8.51 (d, J = 8.1 Hz, 4H, ArH), 8.06 (m, 4H, ArH), 7.90 (d, J = 6.0 Hz, 1H, ArH), 7.76 (dd, J_1 = 6.0 Hz, J_2 = 1.5 Hz, 1H, ArH), 7.72 (m, 4H, ArH), 7.55 (d, J = 5.7 Hz, 1H, ArH), 7.40 (td, J_1 = 7.2 Hz, J_2 = 1.2 Hz, 4H, ArH), 7.27 (d, J = 4.2 Hz, 1H, ArH), 2.55 (s, 3H, CH_3). UV-Vis: λ_{max} = 245 nm, 288 nm, 453 nm. IR (KBr): ν(cm⁻¹) = 1725, 1618, 1466, 1447, 1425, 1314, 1236, 842, 766. Analytical: calcd for C_{32}H_{26}F_{12}N_6O_2P_2Ru, C 41.88, H 2.86, N 9.16, found C 41.76 H 2.98, N 9.11.

Ruthenium(II)bis(2,2'-bipyridine))(4-methyl-4'-CONH-tyrosine ethyl ester-2,2'-bipyridine)(bis(hexafluorophosphate) (14) (=Rubpy_3-Y). 13 (100 mg, 0.11 mmol) was dissolved in DMF (10 mL) in a 50 mL round-bottom flask. HOBT (25 mg, 0.16 mmol) and DIEA (43 μL, 0.25 mmol) were then added. The solution was allowed to stir under N_2 in an ice/acetone bath for 5 min before PyBOP (85 mg, 0.16 mmol) was added. After stirring for an additional 5 min, tyrosine ethyl ester hydrochloride (40 mg, 0.16 mmol) was added. The reaction was followed by TLC in 90 dichloromethane: 10 methanol on neutral alumina and stopped after 5.5 h. The solvent was removed by rotary evaporation. The dark red oil was dissolved in dichloromethane. After removing a white precipitate by filtration, the solution was extracted with 4 × 25 mL ammonium hexafluorophosphate (10 mM). The aqueous phase was then extracted with 2 × 20 mL dichloromethane due to the orange color seen in the aqueous layer. The combined organic phases were dried over
sodium sulfate and concentrated to 3 mL by rotary evaporation. The solution was added dropwise to isopropanol (30 mL), forming a fine red-orange precipitate. The dichloromethane was removed by rotary evaporation and the solution was then stored overnight at -20 °C. The product was collected as a bright orange solid and redissolved to dichloromethane and extracted with 5% hydrochloric acid. Again, the aqueous phase was back-extracted with dichloromethane until it was no longer red and the combined organic phases were dried over sodium sulfate before removing the dichloromethane by rotary evaporation. The dark red solid was then dried in vacuo. \(^1\)H NMR (300 MHz, CD\(_3\)CN, 25 °C): \(\delta = 8.85\) (s, 1H, ArH), 8.61 (s, 1H, ArH), 8.49 (d, \(J = 8.1\) Hz, 4H, ArH), 8.06 (td, \(J_1 = 7.8\) Hz, \(J_2 = 1.5\) Hz, 4H, ArH), 7.89 (d, \(J = 6.6\) Hz, 1H, ArH), 7.84 (dd, \(J_1 = 6.3\) Hz, \(J_2 = 1.5\) Hz, 1H, ArH), 7.70 (m, 4H, ArH), 7.60 (m, 1H, ArH), 7.54 (d, \(J = 5.7\) Hz, 1H, ArH), 7.40 (m, 4H, ArH), 7.27 (d, \(J = 5.4\) Hz, 1H, amide), 7.10 (d, \(J = 8.1\) Hz, 1H, Ar\(_{\text{Tyr}}\)H), 6.71 (d, \(J = 8.1\) Hz, 1H, Ar\(_{\text{Tyr}}\)H), 4.78 (m, 1H, CH\(_{\text{Tyr}}\)H), 4.14 (q, \(J = 6.9\) Hz, 2H, ethyl ester), 3.14 (ddd, \(J_1 = 23.4\) Hz, \(J_2 = 13.2\) Hz, \(J_3 = 5.4\) Hz, 2H, CH\(_{2\text{Tyr}}\)H), 2.56 (s, 3H, CH\(_3\)), 1.20 (t, \(J = 7.2\) Hz, 3H, ethyl ester). UV-Vis: \(\lambda_{\text{max}}(\text{CH}_3\text{CN}) = 245\) nm, 286 nm, 455 nm.

**Ruthenium(II)(bis(2,2'-bipyridine) (4-methyl-4'-CONH-tyrosine-LVGQIDSEVDT DDLSNQL-2,2'-bipyridine) (15) (=Rubpy3-Y-R2C19).** 15 was synthesized from R2C19 by successive additions of Fmoc-Tyr(tBu)-OH and 13 using an 0.9 PyBOP:1.0 HOBt:2.0 DIEA activation scheme as described in Method A. The peptide was isolated by HPLC using gradient A. The purified 15 was then analyzed by gradient B. 15 was dialyzed against either water or R1 assay buffer (50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA, pH 7.6). Some loss of 15 was observed due to binding to the dialysis tubing, yielding only ~70% return after dialysis. HPLC: \(t_R = 19.7\) min. MALDI-TOF MS: MW\(_{\text{calc}} = 2881\) Da, MW\(_{\text{obs}} = 2881\) Da. UV-Vis: \(\lambda_{\text{max}} : 246\) nm, 288 nm, 458 nm (14,300 M\(^{-1}\)cm\(^{-1}\)).
Ac-Y-VGQIDSEVDTDDLNSNFQL (16) (=Ac-Y-R2C19). The 20-mer peptide was synthesized by addition of Fmoc-Tyr(‘Bu)-OH to R2C19 by method B. Upon removal of the N-terminal Fmoc group, the peptide was acetylated by mixing in a solution of 0.5 M acetic anhydride; 0.5 M DIEA in DMF (1 mL) for 30 min. 16 was then purified by HPLC with gradient A and re-analyzed in gradient B. HPLC: \( t_r = 18.2 \) min MALDI-TOF MS: \( MW_{\text{calc}} = 2313 \) Da, \( MW_{\text{obs}} = 2315 \) Da. UV-Vis: \( \lambda_{\text{max}} : 276 \) nm (1,420 M\(^{-1}\)cm\(^{-1}\)).

4-methyl-4′-carboxylic acid-2,2′-bipyridine (17). The ligand was synthesized by literature procedures [45]. \(^1\)H NMR (300 MHz, CD\(_2\)OD, 25 °C): \( \delta = 8.82 \) (s, 1H, ArH), 8.80 (s, 1H, ArH), 8.53 (d, \( J_1 = 4.8 \) Hz, 1H, ArH), 8.25 (s, 1H, ArH), 7.93 (dd, \( J_1 = 5.1 \) Hz, \( J_2 = 1.5 \) Hz, 1H, ArH), 7.34 (d, \( J_1 = 4.8 \) Hz, 1H, ArH), 2.49 (s, 3H, CH\(_3\)). ESI MS (MH\(^+\)): calcd for C\(_{12}\)H\(_{10}\)N\(_2\)O\(_2\), \( m/z \) 215.082, found 215.082.

Rhenium(I)(tricarbonyl)(4-methyl-4′-carboxylic acid-2,2′-bipyridine)(chloride) (18). The preparation for 18 was based on standard procedures [39]. Rhenium(I)(pentacarbonyl)(chloride) (500 mg, 1.38 mmol) was dried in vacuo in a 200 mL 3-neck round-bottom flask with a condenser and 2 septa. The flask was filled with nitrogen and purged. Distilled toluene (100 mL) was added by syringe. 17 (300 mg, 1.4 mmol) was added to the stirred suspension at 60 °C. After 1.5 h at 60 °C, the orange solution was then heated to 80 °C overnight. The reaction was then cooled and filtered. The bright orange filter cake was washed with diethyl ether and dried (690 mg, 96% yield). \(^1\)H NMR (300 MHz, CD\(_2\)OD, 25 °C): \( \delta = 9.18 \) (d, \( J_1 = 5.7 \) Hz, 1H, ArH), 8.98 (s, 1H, ArH), 8.87 (d, \( J_1 = 5.7 \) Hz, 1H, ArH), 8.56 (s, 1H, ArH), 8.12 (dd, \( J_1 = 5.7 \) Hz, \( J_2 = 1.8 \) Hz, 1H, ArH), 7.55 (d, \( J_1 = 5.7 \) Hz, 1H, ArH), 2.62 (s, 3H, CH\(_3\)). \( \lambda_{\text{max}} = 387 \) nm (520 M\(^{-1}\)cm\(^{-1}\)).

Rhenium(I) (tricarbonyl) (4-methyl-4′-carboxylic acid-2,2′-bipyridine) (imidazole) (19). The chloride ligand was exchanged for imidazole using previously reported
procedures [5]. Aqueous imidazole (3.4 g, 90 mmol) was brought to pH 7.0 with concentrated sulfuric acid. To this solution, 18 (300 mg, 0.58 mmol) was added and sonicated. The opaque mixture was placed in a pre-heated oil bath at 60 °C for 1 h before filtering while the solution was still hot. The product was precipitated from the clear filtrate with the addition of ammonium sulfate (~15 g) followed by concentration by rotary evaporation. The yellow solid was collected by filtration and dried in vacuo (250 mg, 78% yield). 1H NMR (300 MHz, CD3Cl, 25 °C): δ = 9.02 (d, J1 = 5.4 Hz, 1H, ArH), 8.90 (d, J1 = 5.4 Hz, 1H, ArH), 8.78 (s, 1H, ArH), 8.20 (s, 1H, ArH), 8.16 (dd, J1 = 5.7 Hz, J2 = 1.5 Hz, 1H, ArH), 7.41 (d, J1 = 6.5 Hz, 1H, ArH), 7.01 (s, 1H, ArmH), 6.90 (s, 1H, ArmH), 6.71 (s, 1H, ArmH), 2.61 (s, 3H, CH3). Analysis: C 39.20, H 2.38, N 10.16, calculated. C 37.15, H. 2.51 N 10.45, found.

Rhenium(I)(tricarbonyl)(imidazole)(4-methyl-4'-CONH-tyrosine-LVGQIDSEVDTDDLSNFQL-2,2'-bipyridine) (=ReIm-Y-R2C19) (20). 20 was synthesized from R2C19 using 0.8 PyBOP: 1 HOBr: 2.0 DIEA activation chemistry by successive additions of Fmoc-Tyr(Bu)-OH and 19 by method A. However, metal complex 19 was suspended in a mixture of DMF (300 μL) and acetone (300 μL) to help solubilize the metal complex before adding to the coupling reaction with Y-R2C19. 20 was then analyzed by HPLC using gradient B. MALDI-TOF MS: MWcalc = 2805 Da, MWobs = 2740 Da.

Rhenium(I)(tricarbonyl)(chloride) (4-methyl-4'-CONH-tyrosine-LVGQIDSEVDTD DLSNFQL-2,2'-bipyridine) (=ReCl-Y-R2C19) (21). 21 was synthesized from R2C19 using 0.8 PyBOP: 1 HOBr: 2.0 DIEA activation chemistry by successive additions of Fmoc-Tyr(Bu)-OH and 18 as described for 20. 21 was then analyzed by HPLC using gradient B. MALDI-TOF MS: MWcalc = 2773 Da, MWobs = 2739 Da.
4-methyl-4'-CONH-tyrosine-LVGQIDSEVDTDDLSNFQL-2,2'-bipyridine (=bpy-Y-R2C19) (22). 22 was synthesized from R2C19 by successive additions of Fmoc-Tyr(Bu)-OH and 17 using 0.8 PyBOP: 1 HOBt: 2.0 DIEA activation chemistry as described in method A. 17 was solubilized in 2 volumes of DMF (300 μL) and one additional volume acetone (150 μL) before adding to the coupling reaction. The peptide was then isolated by HPLC using gradient B and re-analyzed in gradient B. HPLC: \( t_R = 15.9 \) min. MALDI-TOF MS: \( MW_{\text{calc}} = 2467 \text{ Da}, MW_{\text{obs}}=2467 \text{ Da} \).

Ac-HY-LVGQIDSEVDTDDLSNFQL (=Ac-His-Y-R2C19) (23). 23 was synthesized from R2C19 by successive additions of Fmoc-Tyr(Bu)-OH and Fmoc-His-OH, using 0.8 PyBOP: 1 HOBt: 2.0 DIEA activation chemistry by method A. The N-terminus was acetylated by incubation in 0.5 M acetic anhydride: 0.5 M DIEA in DMF for 30 min. 23 was then isolated by gradient A. Purified 23 was analyzed by gradient B and dialyzed against water. HPLC: \( t_R = 11.2 \) min. MALDI-TOF MS: \( MW_{\text{calc}} = 2409 \text{ Da}, MW_{\text{obs}}=2409 \text{ Da} \).

Rhenium(I)(tricarbonyl)(chloride)(2,2'-bipyridine) (24). 24 was prepared by literature methods [39]. \(^1\)H NMR (300 MHz, CD\(_3\)OD, 25 °C): \( \delta = 9.12 \) (ddd, \( J_1 = 4.5 \text{ Hz}, J_2 = 1.5 \text{ Hz}, J_3 = 0.9 \text{ Hz}, 2H, ArH), 8.71 \) (dt, \( J_1 = 7.2 \text{ Hz}, J_2 = 0.9 \text{ Hz}, 2H, ArH), 8.35 \) (td, \( J_1 = 8.1 \text{ Hz}, J_2 = 1.5 \text{ Hz}, 2H, ArH), 8.05 \) (ddd, \( J_1 = 7.5 \text{ Hz}, J_2 = 5.4 \text{ Hz}, J_3 = 0.9 \text{ Hz}, 2H, ArH).

Rhenium(I)(1,10-phenanthroline)(tricarbonyl)(chloride) (25). 25 was synthesized by literature procedures in benzene [39]. \(^1\)H NMR (300 MHz, CD\(_3\)Cl, 25 °C): \( \delta = 9.43 \) (dd, \( J_1 = 5.1 \text{ Hz}, J_2 = 1.2 \text{ Hz}, 2H, ArH), 8.58 \) (dd, \( J_1 = 8.1 \text{ Hz}, J_2 = 1.5 \text{ Hz}, 2H, ArH), 8.05 \) (s, \( 2H, ArH), 7.91 \) (dd, \( J_1 = 8.1 \text{ Hz}, J_2 = 4.8 \text{ Hz}, 2H, ArH).

Rhenium(I)(pentacarbonyl)(triflate) (26). 26 was prepared as described in the literature [75]. Rhenium(I)(pentacarbonyl)(chloride) (2.0 g, 5.5 mmol) was suspended in dichloromethane (140 mL) under nitrogen in a 3-neck round-bottom flask. Silver triflate
(1.8 g, 7.0 mmol) was added slowly through one opening with a positive nitrogen flow. The white reaction was stirred in the dark for 1.5 h before filtering through celite. The filtrate was then evaporated and recrystallized from dichloromethane to give a white crystalline solid (2.2 g, 83% yield)

**Rhenium(I)(1,10-phenanthroline)(tricarbonyl)(triflate)** (27). 27 was prepared by two separate methods from either 25 or 26. In the first method [76], 25 (50 mg, 0.103 mmol) was dissolved in dichloromethane (50 mL) in a 125 mL Erlenmeyer flask. Triflic acid (2.0 mL, 22.6 mmol) was added dropwise using a Pasteur pipet while stirring. After stirring for 1 h, diethyl ether was added to collect a bright yellow precipitate in low yield (~25%). \(^1\)H NMR (300 MHz, CD\(_3\)CN, 25 °C): \(\delta = 9.39 \text{ (dd, } J_1 = 5.1 \text{ Hz, } J_2 = 1.5 \text{ Hz, 2H, ArH)}, \ 8.86 \text{ (dd, } J_1 = 8.1 \text{ Hz, } J_2 = 1.5 \text{ Hz, 2H, ArH)}, \ 8.23 \text{ (s, 2H, ArH)}, \ 8.04 \text{ (dd, } J_1 = 8.4 \text{ Hz, } J_2 = 5.4 \text{ Hz, 2H, ArH}).

In the second method, distilled toluene (100 mL) was added by syringe to 26 (200 mg, 0.42 mmol) in a 3-neck round-bottom flask equipped with a reflux condenser under nitrogen. The clear solution was heated to 80 °C before adding 1,10-phenanthroline (76 mg, 0.42 mmol) under a positive nitrogen pressure. The bright yellow reaction was stirred in the dark for 2 days at 80 °C. Upon cooling to room temperature, a canary yellow solid was collected by filtration and dried (220 mg, 87% yield). \(^1\)H NMR (300 MHz, CD\(_3\)CN, 25 °C): \(\delta = 9.39 \text{ (dd, } J_1 = 5.1 \text{ Hz, } J_2 = 1.5 \text{ Hz, 2H, ArH)}, \ 8.86 \text{ (dd, } J_1 = 8.4 \text{ Hz, } J_2 = 1.2 \text{ Hz, 2H, ArH)}, \ 8.23 \text{ (2, 2H, ArH)}, \ 8.05 \text{ (dd, } J_1 = 8.4 \text{ Hz, } J_2 = 5.4 \text{ Hz, 2H, ArH}).

**Rhenium (I) (tricarbonyl) (1,10-phenanthroline) (imidazole)** (28). One eq of imidazole was added to a solution of 26 was dissolved in methanol. \(^1\)H NMR (300 MHz, CD\(_3\)OD, 25 °C): \(\delta = 9.52 \text{ (dd, } J_1 = 5.4 \text{ Hz, } J_2 = 1.2 \text{ Hz, 2H, ArH)}, \ 8.82 \text{ (dd, } J_1 = 8.4 \text{ Hz, } J_2 = 1.2 \text{ Hz, 2H, ArH)}, \ 8.17 \text{ (s, 2H, ArH)}, \ 8.04 \text{ (dd, } J_1 = 8.1 \text{ Hz, } J_2 = 2.1 \text{ Hz, 2H, ArH)}, \ 7.63 \text{ (s, 1H, Ar\textsubscript{im}H)}, \ 6.87 \text{ (t, 1H, } J = 1.5 \text{ Hz, Ar\textsubscript{im}H}), \ 6.54 \text{ (t, } J = 1.5 \text{ Hz, 1H, Ar\textsubscript{im}H}).
Rhenium (I) (tricarbonyl) (1,10-phenanthroline) (N-acetyl-histidine) (29). One eq of N-acetyl-histidine was added to a solution of 26 was dissolved in methanol. (500 MHz, CD$_3$OD, 25 °C): $\delta = 9.43$ (ddd, $J_1 = 5.4$ Hz, $J_2 = 6.0$ Hz, $J_3 = 1.0$ Hz, 2H, ArH), 8.84 (ddd, $J_1 = 8.0$ Hz, $J_2 = 6.0$ Hz, $J_3 = 1.5$ Hz, 2H, ArH), 8.52 (d, $J = 1.5$ Hz, 1H, ArHisH), 8.21 (s, 2H, ArH), 8.02 (dd, $J_1 = 14.0$ Hz, $J_2 = 8.5$ Hz, $J_3 = 5.0$ Hz, 2H, ArH), 6.89 (s, 1H, ArHisH), 3.94 (dd, $J_1 = 8.5$ Hz, $J_2 = 4.5$, 1H, C$_\alpha$H), 2.65 (dd, $J_1 = 15.0$, $J_3 = 5.5$ Hz, 2H, C$_{\beta_1}$H), 2.45 (dd, $J_1 = 15.5$, $J_3 = 6.5$ Hz, 2H, C$_{\beta_1}$H), 1.4 (s, 3H, CH$_3$).

2-Bromo-6-picoline (30). 2-Amino-6-picoline (50 g, 0.5 mol) was added as a solid to hydrobromic acid (48%, 247 mL, 4.5 mol) in a 2 L 3-neck flask with a mechanical stirrer in an ice/acetone bath. Bromine (75 mL, 1.46 mol) was added dropwise with a 125 mL addition funnel over 2.5 h. Aqueous sodium nitrite (85 g, 130 ml, 1.23 mol) was then added dropwise over 1.5 h, turning the orange suspension to a dark brown color. The cold bath was removed and the reaction was allowed to stir for an additional h. The reaction was re-immersed in an ice/acetone bath before adding aqueous sodium hydroxide (190 g, 200 mL, 4.75 mol) dropwise over 40 min, keeping the temperature under 25 °C. The biphasic reaction was extracted with ether (4 x 150 mL) in a 1 L separatory funnel. The organic layer was extracted with brine (150 mL) and water (150 mL) and dried over sodium sulfate. The ether was removed by rotary evaporation and the crude liquid was distilled under vacuum using a dry ice/acetone bath. The clear, oily liquid was collected from 40 °C to 55 °C (30 g, 35%). $^1$H NMR (500 MHz, CDCl$_3$, 25 °C): $\delta = 7.45$ (t, $J = 8.0$ Hz, 1H, ArH), 7.30 (d, $J_1 = 8.0$ Hz, 1H, ArH), 7.11 (d, $J_1 = 8.0$ Hz, 1H, ArH), 2.55 (s, 3H, CH$_3$).

6,6'-Dimethyl-2,2'-bipyridine (31). 2-Bromo-6-picoline (5.0 g, 29.1 mmol), palladium(II) acetate (0.33 g, 1.45 mmol), tetrabutylammonium bromide (4.67 g, 14.5 mmol), and potassium carbonate (4.02 g, 29.1 mmol) were combined in DMF (3.3 mL) and water (1.3 mL) in a 50 mL 3-neck flask. The red reaction was heated to 115 °C with a
reflux condenser while purging with nitrogen through with a glass pipet. Isopropanol (2.2 mL, 29.1 mmol) was added. After 48 h, the reaction was cooled partitioned between water and diethyl ether. The ethereal layer was collected and washed with 3 × water and dried over sodium sulfate. The brown solution was evaporated to dryness and redissolved in dichloromethane and chromatographed on silica gel with 95 dichloromethane:5 methanol to obtain an eggshell powder (1.5 g, 28%). 1H NMR (500 MHz, CDCl3, 25 °C): δ = 8.18 (d, J = 8.0 Hz, 2H, ArH), 7.69 (t, J1 = 8.0 Hz, 2H, ArH), 7.16 (d, J1 = 7.5 Hz, 2H, ArH), 2.64 (s, 6H, CH3). GC-MS (M+): calcd for C12H12N2, m/z 184.2 found 184.

Rhenium(I)(tricarbonyl)(1,10-phenanthroline)(acetate) (32). 25 (250 mg, 0.42 mmol) was dissolved in methanol (100 mL) with a 1:1 mixture of glacial acetic acid (35.8 μL, 0.63 mmol)/triethylamine (87.2 μL, 0.63 mmol). The orange reaction was stirred under nitrogen overnight and then concentrated by rotary evaporation to 10 mL. The resulting yellow crystalline solid was collected by filtration. Additional product was collected by recrystallization from dichloromethane (192 mg, 90%). 1H NMR (500 MHz, CD3OD, 25 °C): δ = 9.47 (dd, J1 = 5.0 Hz, J2 = 1.5 Hz, 2H, ArH), 8.84 (dd, J1 = 8.5 Hz, J2 = 1.5 Hz, 2H, ArH), 8.21 (s, 2H, ArH), 8.048 (dd, J1 = 8.0 Hz, J2 = 5.0 Hz, 1H, ArH), 1.42 (s, 3H, CH3). HRFAB MS (MH+) : calcd for C17H12N2O3Re, m/z 511.304, found 511.304. λmax = 365 nm (4000 M−1 cm−1). Emission: λmax = 637 nm, τ0 = 46.6 ± 0.3 ns.

Rhenium(I)(tricarbonyl)(cyanide)(1,10-phenanthroline) (33). 27 (25 mg, 0.146 mmol) and potassium cyanide (850 mg, 13.0 mmol) were dissolved in a 1:1 ethanol/water mixture (25 mL) and refluxed under nitrogen overnight. The ethanol was removed by rotary evaporation before extracting the aqueous phase with dichloromethane. The organic layer was collected and dried over Na2SO4. The solution was evaporated to give a yellow solid (40 mg, 60% yield). 1H NMR (300 MHz, CD3OD, 25 °C): δ = 9.08 (d, J1 = 4.8 Hz, 2H, ArH), 8.62 (d, J1 = 8.4 Hz, 2H, ArH), 8.26 (t, J1 = 7.5 Hz, 2H, ArH), 7.70 (t, J1 = 6.6 Hz, 1H, ArH).
Rhenium(I)(tricarbonyl)(triflate)(2,2′-bipyridine) (34). 34 was prepared as described for the analogous 27. ¹H NMR (300 MHz, CD₃OD, 25 °C): δ = 9.12 (ddd, J₁ = 5.4 Hz, J₂ = 1.5 Hz, J₃ = 0.9 Hz, 2H, ArH), 8.68 (dt, J₁ = 8.4 Hz, J₂ = 0.9 Hz, 2H, ArH), 8.23 (td, J₁ = 8.4 Hz, J₂ = 1.5 Hz, 2H, ArH), 8.05 (ddd, J₁ = 7.5 Hz, J₂ = 5.1 Hz, J₃ = 1.2 Hz, 2H, ArH).

Rhenium (I)(tricarbonyl)(cyanide)(2,2′-bipyridine) (35). 34 (75 mg, 0.146 mmol) was refluxed overnight under nitrogen in a 1:3 ethanol: 1 water mixture (25 mL). The ethanol was removed by rotary evaporation and then partitioned with dichloromethane. The organic layer was washed with water and dried over sodium sulfate before evaporating to dryness (40 mg, 60% yield). HRFAB MS (MH⁺): calcd for C₁₄H₈N₃O₃Re, m/z 454.020, 454.0177 found.

Rhenium(I) (tricarbonyl)(triflate) (4-methyl-4′-carboxylic acid-2,2′-bipyridine) (36). 26 (400 mg, 0.91 mmol) and ligand 17 (200 mg, 0.93 mmol) were heated under nitrogen at 80 °C overnight in freshly-distilled toluene (30 mL). The yellow solid was collected by filtration and washed with ether before drying in vacuo (330 mg, 61% yield). ¹H NMR (300 MHz, CD₃OD, 25 °C): δ = 9.17 (d, J₁ = 5.7 Hz, 1H, ArH), 8.97 (s, 1H, ArH), 8.85 (d, J₁ = 5.4 Hz, 1H, ArH), 8.56 (s, 1H, ArH), 8.12 (d, J₁ = 5.1 Hz, 1H, ArH), 7.54 (d, J₁ = 5.4 Hz, 1H, ArH), 2.62 (s, 3H, CH₃). UV-Vis (ethanol): λ_max = 384 nm.

Rhenium(I)(tricarbonyl)(cyanide)(4-methyl-4′-carboxylic acid-2,2′-bipyridine) (37). 36 (150 mg, 0.25 mmol) was added to a solution of tributylammonium cyanide (125 mg, 0.47 mmol) in water (30 mL) and was stirred overnight at room temperature. The bright yellow solution was then evaporated to dryness by rotary evaporation. The resulting brown solid was redissolved in acetone. A fine white water-soluble precipitate was formed and removed by filtration. This brown solution was evaporated to dryness and used in its entirety for the synthesis of peptide 38.
Rhenium(I) (tricarbonyl)(cyanide) (4-methyl-4'-CONH-YLVGQIDSEVDTDLLSFNQL-2,2'-bipyridine) (=ReCN-Y-R2C19) (38). 38 was synthesized from R2C19 by successive additions of Fmoc-Tyr('Bu)-OH and 37 using 0.9 HATU:2.0 DIEA activation chemistry by method B. 38 was then isolated by reverse-phase HPLC by gradient C. Purified 38 was exchanged into potassium phosphate buffer by HPLC. HPLC: \( t_R = 22.7 \) min. MALDI-TOF MS: \( MW_{calc} = 2764 \) Da, \( MW_{obs} = 2740 \) Da. ESI MS: \( MW_{calc} = 2764, MW_{obs} = 2765 \) Da.

Rhenium(I) (tricarbonyl)(thiocyanate) (4-methyl-4'-carboxylic acid-2,2'-bipyridine) (39). 18 (40 mg, 0.08 mmol) was dissolved in a 1:1 ethanol:water mixture (40 mL) with potassium thiocyanate (200 mg, 2.06 mmol). The solution was refluxed overnight under \( N_2 \). The ethanol was removed by rotary evaporation and the product isolated by extraction with dichloromethane. The organic layer was dried over magnesium sulfate, filtered, and evaporated to dryness. \(^1\)H NMR (300 MHz, CD\(_2\)OD, 25 °C): \( \delta = 9.17 \) (dd, \( J_1 = 5.4 \) Hz, \( J_2 = 0.6 \) Hz, 1H, ArH), 8.99 (s, 1H, ArH), 8.86 (d, \( J_1 = 5.7 \) Hz, 1H, ArH), 8.59 (s, 1H, ArH), 8.15 (dd, \( J_1 = 5.7 \) Hz, \( J_2 = 1.8 \) Hz, 1H, ArH), 7.55 (dd, \( J_1 = 5.7 \) Hz, \( J_2 = 0.9 \) Hz, 1H, ArH), 2.64 (s, 3H, CH\(_3\)). ESI MS (M–H\(^–\)): calcd for \( C_{16}H_{15}N_3O_5ReS \), \( m/z \) 541.98, found 541.98. \( \lambda_{max} \) (methanol) = 390 nm (3,600 M\(^{-1}\)cm\(^{-1}\)), \( \lambda_{max} \) (H\(_2\)O) = 365 nm (3,900 M\(^{-1}\)cm\(^{-1}\)).

Rhenium(I)(tricarbonyl)(thiocyanate)(4-methyl-4'-CONH-YLVGQIDSEVDTDLLSFNLQ-2,2'-bipyridine) (=ReSCN-Y-R2C19) (40). 40 was synthesized from R2C19 using 0.9 HATU: 2.0 DIEA activation chemistry by successive additions of Fmoc-Tyr('Bu)-OH and metal complex 39 by method B. 40 was isolated by reverse-phase HPLC using gradient C. Purified 40 was then analyzed by reverse-phase HPLC by gradient D and exchanged into potassium phosphate buffer by HPLC. HPLC: \( t_R = 24.5 \) min. MALDI-TOF MS: \( MW_{calc} = 2796 \) Da, \( MW_{obs} = 2796 \) Da. UV-Vis: \( \lambda_{max} = 247 \) nm, 296 nm, 376 nm.
Rhenium(I) (tricarbonyl)(thiocyanate) (4-methyl-4'-CONH-FLVGQIDSEVDTDDL SFNQL-2,2'-bipyridine) (=ReSCN-F-R2C19) (41). 41 was synthesized from R2C19 using 0.9 HATU: 2.0 DIEA activation chemistry by successive additions of Fmoc-Phe-OH by method B. 41 was then isolated by HPLC using gradient C. Purified 41 was characterized by HPLC in gradient D. HPLC: t<sub>r</sub> = 30.2 min. MALDI-TOF MS: M<sub>W</sub><sub>calc</sub> = 2780 Da, M<sub>W</sub><sub>obs</sub> = 2780 Da. UV-Vis: λ<sub>max</sub> = 376 nm.

4-Ethanoic acid-1,10-phenanthroline (42). The synthesis of 42 was attempted from 4-methyl-1,10-phenanthroline using literature methods [58]. Dry THF (5 mL) and DIEA (0.34 mL, 2.4 mmol) were injected into a 100 mL 3-neck flask under N<sub>2</sub>. 4-methyl-1,10-phenanthroline (0.5 g, 2.6 mmol). After cooling in a dry ice/acetone bath, n-butyllithium (1.6 M, 1.5 mL, 2.4 mmol) was also injected into this flask. 4-methyl-1,10-phenanthroline (0.5 g, 2.6 mmol) was then dissolved in dry THF (50 mL) and transferred to the reaction flask by cannula. The resulting brown solution was stirred for an additional 2 h before dumping into a slurry of dry ice (100 g) and diethyl ether (180 mL) in a 1L Erlenmeyer flask and stirring overnight. The orange solution was filtered to obtain a brown powder, which was to contain both product and starting material by <sup>1</sup>H-NMR.

4-Carboxaldehyde-1,10-phenanthroline (43a). 43a was prepared by literature methods [59] from 4-methyl-1,10-phenanthroline using selenium dioxide as an oxidant. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>Cl, 25 °C): δ = 10.61 (s, 1H, CHO), 9.50 (d, J = 4.5 Hz, 1H, ArH), 9.29 (dd, J<sub>1</sub> = 4.5 Hz, J<sub>2</sub> = 1.5 Hz 1H, ArH), 9.06 (d, J = 9.0 Hz, 1H, ArH), 8.36 (dd, J<sub>1</sub> = 6.6 Hz, J<sub>2</sub> = 1.5 Hz 1H, ArH), 8.03 (d, J = 15.3 Hz, 1H, ArH), 8.04 (d, J = 1.2 Hz, 1H, ArH), 7.75 (dd, J<sub>1</sub> = 8.1 Hz, J<sub>2</sub> = 4.2 Hz 1H, ArH).

Flavin-Y-LVGQIDSEVDTDDLNSFQNL (=flavin-Y-R2C19) (44). The flavin-modified peptide (44) was synthesized from R2C19 by the successive additions of Fmoc-Tyr(tBu)-
OH and Fmoc-flavin-OH using 0.9 HATU: 2 DIEA activation chemistry by method B. 44 was isolated by HPLC in gradient C. The purified peptide was then characterized by HPLC using gradient D and exchanged into potassium phosphate buffer by HPLC. HPLC: $t_R = 14.0$ min. UV-Vis: $\lambda_{\text{max}} = 268$ nm, $370$ nm (6,000 $\text{M}^{-1}\text{cm}^{-1}$), $445$ nm (8,000 $\text{M}^{-1}\text{cm}^{-1}$). MALDI-TOF MS: $M_{\text{calcd}} = 2623$ Da, $M_{\text{obs}} = 2624$ Da.

**Peptide characterization by MALDI-TOF MS.** The mass of peptides were determined using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry on a Voyager workstation (PE Biosystems). The matrix consisted of a >10 mg/mL suspension of $\alpha$-cyano-4-hydroxycinnamic acid in a solution of 50% acetonitrile (v/v), 49% (v/v) water, and 1% (v/v) TFA. The suspension was sonicated and centrifuged before spotting 1 $\mu$L of the supernatant with 1 $\mu$L of a salt-free sample dissolved in water onto the 100-well MALDI target. Mass spectra were collected in negative ion and linear mode using a 600-1000 MW cutoff. An external linear calibration was carried out before every use with angiotensin I ($M_{\text{ave}} = 1295.5$ Da), ACTH 18-39 ($M_{\text{ave}} = 2464.7$ Da), and oxidized bovine insulin ($M_{\text{ave}} = 3495.9$).

**Characterization of Peptide Binding to R1.** The binding of peptides to R1 was assessed by competitive inhibition of nucleotide reduction. R1 (0.1 $\mu$M), R2 (0.2 $\mu$M), thioredoxin (50 $\mu$M), thioredoxin reductase (1.0 $\mu$M), CDP (1.0 mM), ATP (1.6 mM), NADPH (0.2 mM) were incubated with varying concentrations of peptide. Activity was measured by the decrease in $A_{340}$ nm.

**Determination of R1-peptide Dissociation Constants from Inhibition Curves.** The assumption is made that one peptide binding to R1 is sufficient to inhibit nucleotide reduction. The relative activity is used as a scaling factor for the amount of R1–R2 complex formed in the presence of peptide ($([R1–R2]_{p=n} = \text{relative activity} \times [R1–R2]_{p=0})$ as compared to the amount of R1–R2 complex formed with no peptide ($[R1–R2]_{p=0}$).
Under the conditions described, with $[R1] = 0.1 \, \mu M$ and $[R2] = 0.2 \, \mu M$, $[R1-R2]_{p=0}$ is equal to 0.05 $\mu M$ using a $K_D$ of 0.15 $\mu M$ for R1 and R2. The difference in active complex at $[P]_{=n}$, $[R1-R2]_{p=0} - [R1-P]_{p=0}$, is then set equal to the quantity $[R1-P]_{p=0}$. From this value, $[P]_{p=0}$ can be calculated from $[P]_{Total} - [R1-P]_{p=0}$. The plot of $[R1-P]_{p=0}$ versus $[R1-P]_{p=0}/[P]_{p=0}$ is then fit linearly to the equation:

$$[R1-P]_{p=0} = [R1]_{Total} - K_i \frac{[R1-P]_{p=0}}{[P]_{p=0}}$$

where $K_i = K_D$ for a competitive inhibitor. This fitting routine was tested with binding curves generated using Ac-Y-R2C19 and Ac-R2C19, which have published inhibition constants of 20.0 $\mu M$ and 40.0 $\mu M$, respectively [47]. Using these approximations, both fits fall within error of the literature values for the dissociation constants, which were determined using a multi-dimensional fit (Figure 3.30a-b).

**Physical Measurements.** $^1$H NMR spectra were collected at the MIT Department of Chemistry Instrumentation Facility (DCIF) using either Inova 500 or Unity 300 spectrometers from Varian (Palto Alto, CA) at 25 $^\circ$C. All chemical shifts are reported using the standard $\delta$ notation in parts-per-million; positive chemical shifts are to higher frequency from the given reference. FAB or ESI mass spectral analyses were performed.
by the University of Illinois, Urbana-Champaign School of Chemical Science Mass Spectrometry Service.

**Diaryl Oxalate Esters (Section 3.2):** Steady-state photolyses were carried out at 25 °C using a 1000 W high-pressure HgXe lamp from Oriel Instruments (Stratford, CT) with either a 240 nm or a 285 nm longpass filter. Samples were prepared by multiple freeze-pump-thaw cycles to \(10^{-5}\) torr in exact pathlength 1 cm quartz cells equipped with a solvent reservoir from Starna Cells, Inc. (Atascadero, CA). The two chambers were isolated from each other by a high-vacuum Teflon valve and from the environment with a second high-vacuum Teflon valve. Absorption spectra were obtained using an OLIS-modified Cary-17 absorption spectrophotometer. Spin-trapping experiments were carried out using an X-band (9 GHz) CW EPR from Varian and excitation at 308 nm (2 W) from a Lambda Physik HCl/Xe excimer laser running at 50 Hz.

Nanosecond transient absorption measurements were performed using a Coherent Infinity-XPO Nd:YAG laser. The third harmonic of the 1064 nm fundamental is used to pump an optical parametric oscillator (OPO) that is tunable throughout the visible region (420-700 nm). The OPO produced laser pulses at a repetition rate of 20 Hz with a pulse width of 5-7 ns. The samples were excited at 270 nm after frequency doubling from 540 nm or at 320 nm after frequency doubling from 640 nm in a beta barium borate (BBO) crystal. A 50-W Xe arc lamp (PTI 1000, unpulsed) provided the probe light. A Vincent Associates Uniblitz shutter was used to block the probe light. The signal was dispersed with a SPEX Triax 320 spectrometer and detected using an Andor DH520-25F-01 gated intensified CCD camera (ICCD) calibrated using a mercury lamp. The timing of the ICCD, probe light shutter, and laser were controlled using two Stanford DG535 delay generators. Series of four spectra were taken: \(I\) (pump on/probe on), \(I_p\) (pump on/probe off), \(I_0\) (pump off/probe on), \(I_0p\) (pump off/probe off). Transient spectra corrected for fluorescence and laser signals were calculated from these spectra: \(\Delta OD = -\log(I_0 - \ldots\)
\( I_{OF} / (I - I_F) \). Spectra reported are the average of 250 of the 4-spectra sequences. Instrument control and data analysis were performed using software written in LabView from National Instruments (Austin, TX). Samples for photochemical measurements were contained within a cell equipped with a solvent reservoir and a 2 mm clear fused-quartz cell. The two chambers were isolated from each other by a high-vacuum Teflon valve and from the environment with a second high-vacuum Teflon valve.

*Ruthenium(III) polypyridyl complexes (Section 3.3):* 14 and methyl viologen dichloride hydrate were dissolved in MilliQ water at 60 \( \mu \text{M} \) and 10 mM concentrations respectively. The sample was transferred to a 2 mm exact-path length quartz cell (Starna Cells, Inc.) and sealed with a rubber septum. This solution was then degassed with argon for 20 min. A stock solution of peptide 15 was made at 300 \( \mu \text{M} \) in MilliQ water. Quencher Co(NH$_3$)$_3$Cl$_3$ was prepared as a stock solution at 20 mM in MilliQ water. Peptide (200 \( \mu \text{L} \)) was added to a cell equipped with a solvent reservoir and a 2-mm clear fused-quartz cell (Starna Cells, Inc.). The two chambers were isolated from each other by a high-vacuum Teflon valve and from the environment with a second high-vacuum Teflon valve. The peptide solution was evaporated to dryness in the cell. Quencher (800 \( \mu \text{L} \)) was then added to the solvent reservoir and also evaporated to dryness. Buffer (1.2 mL of either 10 mM HEPES (pH 7.6), 10 mM HEPES (pH 10.1), or 10 mM Tris (pH 7.0)) was added to the reservoir and subjected to at least three freeze-pump-thaw cycles (10$^{-3}$ torr) before mixing. Final concentrations were approximately 40 to 80 \( \mu \text{M} \) and 15 mM in peptide and quencher respectively.

Luminescence lifetime measurements were carried out using a Hamamatsu C4334-0 Streak scope streak camera, which was controlled with the high-performance digital temporal analyzer (HPDTA) software provided by Hamamatsu Photonics (Bridgewater, NJ). Excitation pulses (95 \( \mu \text{J} \), 485 nm) were generated from a commercial laser system consisting of a Coherent/BMI Comet-400S two-stage optical parametric amplifier (OPA).
The OPA generates laser pulses at a repetition rate of 1 kHz and offers wavelength tunability from 475 to 710 nm. The OPA is pumped by a Coherent/BMI Alpha-1000 chirped-pulse regenerative amplifier, which, in turn, is pumped by a 10-W, 1-kHz Nd:YLF laser and seeded by a Coherent Mira femtosecond Ti:sapphire oscillator. The oscillator is pumped by a 5-W CW Coherent Verdi solid-state, frequency-doubled Nd:YVO4 laser.

Nanosecond transient absorption measurements were performed using a Coherent Infinity Nd:YAG laser with OPO running at 20 Hz to excite the samples at 475 nm; detection was performed as previously described in Section 3.2.2. Luminescence lifetimes were also collected using a Hamamatsu R928 photomultiplier tube (PMT) attached to the lateral exit of the SPEX Triax 320 spectrometer. The output from the PMT was channeled into a Lecroy 9384 CM digital oscilloscope.

Rhenium(I) tricarbonyl polypyridyl complexes (Section 3.4): Steady-state emission spectra were collected on a modular instrument consisting of a SPEX monochromator attached to a CDD cooled to 77K. Luminescence lifetime measurements were collected as described above for Section 3.3 with 380 nm excitation.

Lumiflavin-modified peptides (Section 3.5): These experiments were carried out in collaboration with Jeremiah Miller and Jay Winkler at the California Institute of Technology. Samples of flavin peptide 44 were prepared at 30-40 μM concentration in 50 mM potassium phosphate buffer, pH 7.2 and degassed by purging with argon. Lumiflavin was prepared at 230 μM in the same buffer and also degassed by purging with argon. When added, the final concentration of ruthenium(III) hexaamine was 20 mM. Nanosecond transient absorption and emission studies were carried out with λexc = 450 nm at 10 Hz at a power of ~1 mJ/pulse. The probe light was generated using a Xe arc
lamb equipped with a 300 nm longpass filter. Single-wavelength data was collected using a PMT attached to an oscilloscope. Typically 100 shots were collected per trace.
References


Chapter 4

Triggering Turnover in R1 with Photoactive Peptides
4.1 Introduction

Ribonucleotide reductases (RNRs) catalyze the conversion of nucleotides to deoxynucleotides in all organisms, supplying a balanced pool of deoxynucleoside triphosphates required for DNA replication and repair. The central role of RNRs in nucleic acid metabolism, their exquisite control of free radical chemistry, as well as the proposed part they play in the conversion of an RNA to a DNA world, have fascinated scientists since their discovery. As discussed in Chapter 1, RNRs have been divided into three classes based on their different methods of radical initiation. The class I RNRs use a diiron-tyrosyl radical (\(*Y122, E. coli\) numbering) cofactor, the class II RNRs use the coupled homolysis of the carbon-cobalt bond of a B_{12} cofactor, while the class III RNRs utilize a glycyl radical generated by an S-adenosylmethionine/[4Fe4S] activase [7]. In each case, the radical initiator generates a thyl radical (S^*) [2] in a structurally homologous active site where the nucleotide is reduced to the corresponding deoxynucleotide concomitant with the oxidation of either two cysteines to a disulfide (class I and II) or formate to CO_2 (class III) (Eq. 1):

![Equation 1]

While much has been learned about the control over protein and nucleotide radical intermediates exerted by RNRs, a major unresolved problem is the mechanism of radical initiation by the class I enzyme [3].

The class I RNRs are thought to be composed of a 1:1: complex of two homodimeric
subunits, R1 and R2. In the class I RNR from *E. coli*, the stable $\gamma$Y122 is located on R2 whereas the cysteine (C439) that becomes the transient S$^\bullet$ is contained in R1. In addition, R1 also contains the allosteric effector binding sites that determine both the specificity (h-site: TTP, dGTP, dATP, and ATP) and rate (l-site: dATP and ATP) of nucleotide reduction [4,5]. As the formation of the thiyl radical is thought to occur on every enzymatic turnover [6], the radical transport between the two subunits process is key to the mechanism of the class I RNRs. The Y122 and C439 residues are proposed to be separated by 35 Å, based on a docking model generated from the individual crystal structures of R1 and R2 using shape complementarity and electrostatics [7]. The observed rate constants of 2-10 s$^{-1}$ [6,8] for enzyme turnover is much faster than a single electron transfer step over this distance based on Marcus theory ($10^{-4}$–$10^{-9}$ s$^{-1}$ for $\beta = 1.1$–1.4 Å$^{-1}$). If this model is correct, the long distance for radical initiation therefore requires the existence of intermediates, namely aromatic amino acid radicals, to accommodate the observed turnover numbers of these enzymes. The hypothetical pathway [7] has been constructed by a docking model of R1 and R2 (Figure 4.1) and has been mapped by

![Figure 4.1 PCET pathway and distances generated from the docking model of the R1 and R2 subunits of the class I RNR from *E. coli*. The last 35 to 40 amino acids of the C-terminal tail of R2, in which Y356 resides, are thermally labile and undetectable in available crystal structures. Thus, the distance between W48 on R2 and Y731 in R1 is based only on the docking model of a 1:1 complex of R1 and R2.](image-url)
sequence alignment (absolutely conserved across ~140 sequences) as well as in vitro [9-13] and in vivo [14] mutagenesis studies. Whereas these studies have shown these residues to be important for enzyme activity, they have not shown the existence of amino acid radical intermediates along the proposed pathway or provided any mechanistic details of the radical initiation process.

The essential nature of the three tyrosine residues [14], Y356 in R2 and Y731 and Y730 in R1, is striking as they have no known role in either nucleotide reduction or biosynthesis of the diiron-Y• cofactor. Although structural roles may be proposed for these residues, the crystal structure of the Y730F mutant shows no meaningful structural perturbations from wild-type R1 [5]. Accordingly, biochemical studies demonstrate further that neither the Y730F nor the Y731F mutant exhibit significantly altered substrate or R2 binding [11]. Recent studies in which Y356 has been replaced with 3-nitrotyrosine, preserving hydrogen-bonding networks, show that the activity observed is 5 × 10⁻³ lower than wild-type R2. Although 1-2 turnovers are observed, this specific activity is within the lower limit of detection due to heterodimer formation of the semisynthetic R2 with contaminating wild-type enzyme [15]. Taken together, these results imply that the Y356F mutation, and perhaps the 3-nitrotyrosine substitution, abolish nucleotide reduction activity by acting as a redox block and it is the ability to carry out redox chemistry that makes it essential. Although less is known about Y731 and Y730, the structural superposition of the 5'-adenosyl (class II) and glycylyl radicals (class III) with Y730 and Y731 in R1 points to an evolutionarily conserved role for these residues in radical initiation at •C439. Remarkably, the three-dimensional arrangement of the chemically diverse cofactors with respect to the catalytic cysteine residue has been preserved, providing the most compelling evidence for the conserved tyrosines in the radical initiation pathway [16]. The redox potentials of the amino acids along the pathway (tyrosine, tryptophan, and cysteine) require changes in protonation state to
become transiently oxidized and control the directionality of radical propagation, implicating proton-coupled electron transfer (PCET) [17]. Whereas the role of aromatic amino acid radical intermediates in long-range charge transport has recently been recognized in model proteins [18,19], the radical initiation in class I RNRs is unique because of its exceptionally long PCET distance. In addition, the existence of aromatic amino acid radicals in biological charge transport has not yet been shown to be physiologically relevant, and the class I RNRs could provide the first example of such protein-based intermediates in Nature.

Although the arguments for a conserved PCET pathway are persuasive, no direct evidence for this pathway or the proposed radical intermediates exists because the radical initiation step is kinetically masked by rate-limiting conformational changes preceding radical initiation [6]. The developments of Chapter 3 provide a methodology to trigger the RNR PCET reaction synchronously using light-initiated amino acid radical

![Diagram](image)

**Figure 4.2** Strategy for the photochemical triggering of the Y731→Y730→C439 PCET pathway in the E. coli R1. NDP = nucleoside diphosphate substrate, dNDP = deoxynucleoside diphosphate product. This strategy exploits the weak interaction between R1 and R2 (0.2 μM) and the ability of peptides to the C-terminus of R2 to completely inhibit nucleotide reduction in a competitive fashion.
generation. Our experimental strategy exploits the fact that the essential Y356, the last member of the PCET pathway in R2, is present on the unstructured C-terminal tail of R2. This 20-mer tail (Y356 to L375) competes with the full subunit for binding to R1 (K_D ~ 20 μM) [20] and predominately accounts for the majority of interaction required for subunit association [9, 21], as well as aiding the crystallization of R1 [5, 7]. Generation of a tyrosyl radical at position 356 on a synthetic C-terminal peptide in complex with R1, in the presence of substrate and effector, would allow the Y356→Y731→Y730→C439 pathway of the R1–R2 complex to be isolated (Figure 4.2). Thus, a specific entryway into the radical initiation pathway of R1 can be achieved, while bypassing the complexity of the R2 pathway through the metallo-cofactor [22] and W48 [23, 24]. The competence of the photogenerated PCET intermediate may be tested both biochemically by nucleotide reduction activity and spectroscopically by thyl radical detection.

This chapter will first address the development of the assay used to assess turnover in R1–peptide complexes in Section 4.2 using the Ru(bpy)_3-Y-R2C19 and flavin-Y-R2C19 peptides presented in Chapter 3. Section 4.3 reports the observation of dCDP formation in a R1–peptide complex, in which a tryptophan is used as the phototrigger for •Y356 generation, and consequently the first evidence for the participation of aromatic amino acid radical intermediates in the radical initiation process catalyzed by class I RNRs.

4.2 Development of Assays for Light-Mediated R1–Peptide Turnover with Ru(bpy)_3-Y-R2C19 and Flavin-Y-R2C19

4.2.1 Introduction

The first test of the participation of Y356 in the proposed PCET pathway is to assess the ability of the R1–peptide complex to catalyze nucleotide reduction upon photolysis. Generation of •Y356 by light enables us to intercept a putative PCET intermediate in which chemical competence in RNR activity should be observed. The radioactive assay for deoxynucleotide formation using [^{14}C]-CDP as substrate is an extremely sensitive
probe for enzyme activity and should allow the measurement of >0.1% turnover. As both activity and specificity binding sites are located on R1 [5], this strategy should also allow us to gauge the role of these effectors in modulating the activity of RNR. However, our eventual goal is to move beyond the steady-state regime to time-resolved studies of radical initiation in the class I RNRs and to address the kinetic competence of the R1–peptide by correlating tyrosyl radical decay with thiol radical (•C439) appearance and examine the substrate and effector dependence of the PCET reaction.

We begin with the developments presented in Chapter 3 concerning the photoactive peptides, Rubpy3-Y-R2C19 and flavin-Y-R2C19. Although tyrosyl radical generation was not firmly established in either case, the coupling of •Y356 to R1 activity can amplify a small amount of tyrosyl radical formation with multiple turnovers to produce a measurable amount of dCDP. In the case of Rubpy3-Y-R2C19, a flash-quench scheme utilizing the irreversible oxidative quencher, cobalt (III) pentaamine chloride, leads to the generation of the oxidized ruthenium center. The ruthenium (III) complex can then oxidize the adjacent Y356 with an intramolecular rate constant of \(5 \times 10^4 \text{ s}^{-1}\), as shown in model systems [25] (Eq 4.2):

\[
\text{Ru}^{3+}(\text{bpy})_3\text{-Tyr} + \text{Co}^{II}(\text{NH}_3)_5\text{Cl} \xrightarrow{hv} \text{Ru}^{3+}(\text{bpy})_3\text{-Tyr} + \text{Co}^{II}(\text{NH}_3)_5\text{Cl} \xrightarrow{ET} \text{Ru}^{3+}(\text{bpy})_3\text{-Tyr} + \text{Co}^{II}(\text{H}_2\text{O})_6
\]

*Equation 4.2* Flash-quench generation of •Y356 from Rubpy3-Y-R2C19.

Our hope is that the precipitation observed in the ruthenium-modified peptide will be prevented upon binding of R1, as the negatively charged carboxylates should be held away from the oxidized metal center. If peptide precipitation remains a problem, we can also turn to flavin-Y-R2C19 as flavin (F1) also has a well-known photochemistry with regard to tyrosine photo-oxidation [26-29]. Either the triplet flavin (3F1*) or the oxidized flavin radical (•F1ox) is competent to generate the tyrosyl radical (Eq 4.3) [30,31]:

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Although tyrosyl radical was not detected with flavin-Y-R2C19, possibly due to relatively fast back reaction with the flavin semiquinone (•FlH), it may be possible to drive nucleotide reduction if forward PCET to Y731 occurs at a competitive rate.

4.2.2 Results and Discussion

Development of single-turnover assays. We began by assessing the effect of the oxidative quenchers, cobalt(III) pentaamine chloride and ruthenium(III) hexaamine, on RNR activity by using a standard coupled assay. The coupled assay for RNR is based on the generation of an active site disulfide concomitant with nucleotide reduction (Eq. 1) [8]. The disulfide formed by C225 and C462 is exchanged with the C-terminal C754–C759 pair, which is in turn reduced by thioredoxin through a second disulfide exchange reaction [32]. Nucleotide reduction is then coupled to NADPH oxidation through thioredoxin reductase, which contains a flavin and redox-active thiols [33] (Figure 4.3). Unfortunately, both quenchers uncouple nucleotide reduction from NADPH oxidation by reacting with TRR. NADPH is turned over rapidly when TRR is incubated alone with quencher; the most probable explanation is that the quenchers are able to either oxidize the flavin cofactor or the redox-active thiols, as they are both chemically competent to do so, leading to a futile cycle with overall NADPH consumption. The rate of NADPH turnover by TRR was dependent on cobalt(III) pentaamine chloride concentration, with a specific activity of 280 nmol min⁻¹mg⁻¹ and 490 nmol min⁻¹mg⁻¹ at concentrations of 10 mM and 20 mM respectively. Addition of ruthenium(III) hexaamine (20 mM) to TRR caused NADPH oxidation at a rate of 950 nmol min⁻¹mg⁻¹. We then turned to chemical rather than enzymatic methods for reduction of the R1 C754–C759 disulfide (Figure 4.3).
R1 and R2 were incubated with substrate, effector, and cobalt(III) pentaamine chloride (20 mM) in the presence of dithiothreitol (20 mM) to turn over R1. Upon addition of DTT, a thick brown precipitate was formed, possibly due to generation of exchange-labile cobalt(II) in a redox reaction with DTT followed by polymer formation. When the concentrations of quencher (5 mM) and DTT (10 mM) were reduced, the precipitate was also reduced but still very significant. At this time, neither enzymatic nor chemical methods for achieving multiple turnovers in the presence of oxidative quenchers seemed promising; consequently, attempts to carry out multiple-turnover assays were halted in favor of developing single-turnover assays with pre-reduced R1. Although both quenchers were assayed at the outset, further studies were pursued with cobalt(III) pentaamine chloride because it irreversibly aquates upon reduction to form the cobalt(II) hexaaqua species, preventing back electron transfer with the oxidized ruthenium(III) center of the peptide.

The two cysteine pairs of R1 (C225/C462 and C754/C759) can also be pre-reduced by treatment with DTT. Upon removal of DTT, ~3 of the potential 4 dCDPs are observed on average upon addition of R2 and substrate, with the first two equivalents produced in a
rapid kinetic phase and the third equivalent formed in a second slower kinetic phase [6].

Thus, the chemical competence of R1 to turnover in the presence of peptide and light may still be measured in the absence of a disulfide-reducing system. For these assays, R1 was prepared by standard procedures [6] by incubating in 20 mM DTT (final concentration) for 20 min at room temperature. Following this incubation, hydroxyurea was added to final concentration of 20 mM to this solution and incubated for an additional 5 min at room temperature to reduce the *Y122 in the small amount of contaminating R2 that co-purifies with R1. The protein solution was then loaded onto a Sephadex G-25 column to remove the small molecules and to exchange the R1 into assay buffer (50 mM HEPES, 15 mM MgSO₄, 1 mM EDTA, pH 7.6). The protein-containing fractions were concentrated by Amicon using a PM30 membrane and stored on ice before assaying. Single-turnover assays were carried out at 25 °C for 8 min with the pre-reduced R1, R2, [¹⁴C]-cytidine-5′-diphosphate (CDP), and allosteric effector adenosine-5′-triphosphate (ATP) in the presence of cobalt(III) pentaamine chloride at varying concentrations. The samples were quenched by placing in a boiling water bath, which resulted in the formation of a small amount of brown precipitate that increased upon longer quenching times. After boiling the reactions, the samples were centrifuged and the supernatant dephosphorylated enzymatically. dC carrier is added to the sample.

<table>
<thead>
<tr>
<th>No.</th>
<th>[R1]/μM</th>
<th>[R2]/μM</th>
<th>[Co]/mM</th>
<th>t.o. per R1</th>
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<tr>
<td>1</td>
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</tr>
<tr>
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<td>-</td>
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<tr>
<td></td>
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<td>5</td>
<td>20</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*Table 4.1 Two trials of single-turnover assays with R1 and R2 with varying concentrations of cobalt (III) pentaamine chloride. Samples were quenched by boiling. The results are reported in turnovers (t.o.) per R1.*

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before passing it through a Dowex-1 borate column that removes the geminate diol substrate. A fraction of the eluent from the borate column is then counted to determine the equivalents of dCDP produced per R1. The results from two separate trials are tabulated in Table 4.1. When R1 and R2 are mixed, only about half of the expected dCDP is measured, perhaps due to poor pre-reduction or oxidation following pre-reduction. In addition, there was a high background in the presence of the quencher, which is observed in the absence of R1 or R2 (Table 4.1, No. 2). Lowering the cobalt(III) pentaamine chloride concentration almost twenty-fold from 13 mM to 0.7 mM did not lower this background significantly (Table 4.1, No. 1). When the control samples containing cobalt(III) pentaamine chloride without R1 are chromatographed by reverse-phase HPLC, the radioactivity migrates early with contaminants rather than with the dC carrier. Although it was possible to distinguish the background radioactivity from that of the dC product, we decided to try single-turnover assays using a different quenching method than boiling to reduce the high backgrounds. RNR can also be quenched by the addition of 0.5 volumes of 2 % perchloric acid, which precipitates the proteins present. If the solution is neutralized quickly to pH 7.0 after protein precipitation with addition of 0.5 N potassium hydroxide, background due to base elimination usually remains low. This was confirmed by repeating the assays of Table 4.1 using perchloric acid to quench the reaction. In addition, we found that the background in samples containing cobalt(III) pentaamine chloride was only increased 2-5% from those without quencher added.

**Light reactions with Rubpy3-Y-R2C19.** With the mechanics of the assay under control, we proceeded onto photolysis reactions with R1 and Rubpy3-Y-R2C20. Although the ruthenium(II)-modified peptide would precipitate upon oxidation to ruthenium(III) when alone, it is possible that it would remain in solution when bound by R1. In the ideal circumstance, the experiment would be designed such that the peptide would be fully bound by excess R1, however, the assay is limited by the low solubility of R1 (<100 µM
and <70 μM if it will be purged with argon for anaerobic assays), the low solubility of the cobalt(III) pentaamine chloride quencher (~25 mM), and the high $K_D$ of the R1–peptide complex (~20 μM). Thus, the experiment was typically carried out using a final peptide concentration of 20 μM, with R1 (25-30 μM) and cobalt(III) pentaamine chloride quencher (10-13 mM) making up ~90% of the reaction volume, due to their low solubility. Under these conditions, 65-70% of the peptide will be bound, if indeed each R1 monomer acts independently with respect to peptide binding as previously reported [20]. The incubation time for photolysis of the reaction was based on the stability of R1 to the photolysis conditions; upon light irradiation at 20 °C using a 1000 W Hg-Xe lamp with 90% neutral density and 455 nm longpass filters, 70-80% R1 activity was retained after 15 min and 60% activity was retained after 45 min. The first set of reactions was carried out anaerobically with 25 μM R1, 20 μM Rubpy3-Y-R2C19, 1.6 mM ATP, 1.0 mM CDP (7,700 cpm/nmol), and 10 mM cobalt (III) pentaamine chloride in the final sample (400 μL). A control was run under these same conditions except that peptide was excluded. The stock materials were degassed and purged with argon before transfer into the glove box and loaded into a quartz cuvette modified with a J. Young teflon top. The samples were then photolyzed in series at 20 °C for 2 h at which time they were quenched with perchloric acid. After removal of the phosphates from the nucleotides with alkaline phosphatase, the samples were passed through a 6.0 mL Dowex-1 borate column and eluted with 45 mL of deionized water. The eluent was lyophilized in two 50 mL pear-shaped flasks treated with Sigma-Cote. The white solid was re-dissolved in 2.0 mL of deionized water per flask, which was then combined and lyophilized in a 10 mL pear-shaped flask. These samples were brought up in 1.0 mL deionized water and counted with 9.0 mL of scintillation fluid. Although peptide precipitate was formed during the course of the reaction, the initial results were promising, with the experiment with R1 and peptide yielding 3.9% dCDP formation per R1, a 22% increase over the control with R1 in the absence of peptide (3.0%). If we account for the 3 possible turnovers per R1 for the
amount of R1-peptide complex formed (13 µM), then 5.8% and 4.5% dCDP is produced with and without peptide respectively. As the stoichiometry of the photoreaction could be different than what is observed with R2 and we cannot be certain if we can indeed approximate peptide binding to independent monomers of R1, we prefer to report the amount of dCDP measured per R1 added because these assumptions are not needed for this value.

Although the preliminary studies showed potential formation of dCDP above the control of R1 without peptide, the experimental background was high due to incomplete reduction of contaminating R2 in the R1 sample in conjunction with the long reaction time of the assay. Thus, the assay time was reduced to 1 h, which also facilitated the inclusion of several other controls, and the hydroxyurea treatment of R1 was extended to 30 min. The filter was changed to a 435 nm long pass to help compensate for the shorter reaction time.

In addition, the eluent from the Dowex-1 borate columns was chromatographed by reverse-phase HPLC to separate the dC from other radioactive contaminants. The results from these experiments are reported in Table 4.2 and are consistent with a light-initiated reaction between Rubpy3-Y-R2C19 and R1. The light reaction in both trials (Table 4.2, line 1) is higher than either the dark reaction (Table 4.2, line 2) or R1 alone (Table 4.2, line 3). Further controls with peptide (Table 4.2, line 4) and CDP alone (Table 4.2, line 5) with quencher produces no deoxynucleotide and are thus consistent with the

<table>
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<tr>
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<td>3.5%</td>
</tr>
<tr>
<td>R1 + Rubpy3-Y-R2C19</td>
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</tr>
<tr>
<td>R1</td>
<td>hν</td>
<td>2.9%</td>
</tr>
<tr>
<td>Rubpy3-Y-R2C19</td>
<td>hν</td>
<td>n.d.*</td>
</tr>
<tr>
<td>CDP alone</td>
<td>hν</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*none detected.

Table 4.2 dCDP formation in light-mediated reaction of R1 and Rubpy3-Y-R2C20. R1 was treated with hydroxyurea (20 mM) and DTT (20 mM) for 30 min at room temperature. All reactions contain 10 mM cobalt (III) pentaamine chloride quencher and 1.8 mM ATP. Samples (300 µL) were irradiated using 90% neutral density and 435 nm long pass filters at 25 °C for 1 h. Specific activity of the substrate used was 5,300 cpm/nmol. dCDP formation per R1 is calculated from the amount of radioactive dC isolated by HPLC and normalized for carrier recovery.
interpretation that the observed background derives from small amounts of contaminating R2 since there is no reaction that generates dCDP with light and quencher alone or with peptide, light, and quencher. Correspondingly, the background of the dark reaction control (Table 4.2, line 2) is likely lower than the control with R1 alone (Table 4.2, line 3) because the peptide is inhibiting R1 interaction with the contaminating R2 in the preceding case. Thus, it is essential that the light reaction of peptide and R1 be greater than that of R1 alone, because peptide precipitation can lead to a false positive due to loss of competitive inhibition with any contaminating R2. Additional assays with TTP as effector in place of ATP as well as with peptide Rubpy3-F-R2C19, in which tyrosine is replaced with phenylalanine, were formulated to aid in clarifying the reactivity of the ruthenated 20-mer with R1.

The control peptide, Rubpy3-F-R2C19 (1), was synthesized and isolated according to standard procedures. An analytical HPLC trace of the purified peptide is shown (Figure 4.4a) with the absorption spectrum (Figure 4.4b). Binding to R1 was then characterized by competitive inhibition of nucleotide reduction monitored by NADPH oxidation [20]. The inhibition profile is overlaid upon that of Rubpy3-Y-R2C19, which has a similar affinity as the 20-mer peptide Ac-Y-R2C19 \((K_d \approx 20 \, \mu M)\) [20], and is unchanged within experimental error (Figure 4.4c). With this peptide in hand, a full set of photolysis assays were carried out with the additional controls. The results from trials 3, 4, and 5 are shown in Table 4.3. Trial 3 does show increased dCDP production (10.0\%; Table 4.3, line 1) over the dark reaction (5.1\%; Table 4.3, line 5) as expected, however, the amount is less than the control with R1 alone (10.7\%; Table 4.3, line 6). As discussed above, this leads to a fundamental ambiguity in the analysis of the results due to peptide precipitation. Again, no dCDP is observed with peptide (Table 4.3, 7) or substrate only (Table 4.3, 8). The high background of dCDP production was attributed to poor hydroxyurea-reduction.
Figure 4.4 Synthesis and characterization of Rubpy-F-R2C19 (1). (Panel A) Analytical reverse-phase HPLC trace of 1 in a linear gradient of 0%-55% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min on a C-8 column. (Panel B) UV-Visible absorption spectrum of 1. (Panel C) Competitive inhibition of nucleotide reduction by 1 (■) overlaid onto that of Rubpy-Y-R2C19 (○).
<table>
<thead>
<tr>
<th>Reaction</th>
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<th>ATP</th>
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<th>4</th>
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<td>R(^1) + Rubpy(_2)-Y-R2C19</td>
<td></td>
<td></td>
<td>10.0%</td>
<td>6.4%</td>
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<td>R(^1) + Rubpy(_3)-Y-R2C19</td>
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<td>4.3%</td>
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</tr>
<tr>
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<td>dark</td>
<td>ATP</td>
<td>–</td>
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</table>

\(^{a}[R1] = 30 \mu M; \(^{b}[R1] = 40 \mu M; \)none detected.

Table 4.3 dCDP formation in light-mediated reaction of R1 and Rubpy\(_3\)-Y-R2C20. All reactions contain 8-10 mM cobalt (III) pentaamine chloride quencher. Samples (300 \mu L) were irradiated using 90% neutral density and 435 nm long pass filters at 25 °C for 1 h. For trial 3 and 4, R1 was treated with hydroxyurea and DTT for 30 min and specific activity of the substrate used was 5,700 cpm/nmol. For trial 5, R1 was pre-reduced for 60 min and specific activity of the substrate used was 9,100 cpm/nmol. dCDP formation per R1 is calculated from the amount of radioactive dC isolated by HPLC and normalized for carrier recovery. For trials 4 and 5, the isolated dC was converted into dU quantitatively.

of the contaminating R2 in the R1 sample and consistent with the especially large amounts of dCDP formation that were two-fold larger than previously observed in trials 1 and 2. Therefore, a new R1 sample was prepared before trial 4. Trial 4 reproduces the results of trial 1 and 2 with light-mediated R1–peptide catalyzed dCDP formation (6.4%; Table 4.3, line 1) above both the dark reaction (6.1%; Table 4.3, line 5) and R1 alone (5.5%; Table 4.3, line 6), although not convincingly so. Increasing the amount of R1 correspondingly raised the amount of dCDP observed, but may derive from increased background activity from the additional R1 (7.7%; Table 4.3, line 2). In both trial 3 and 4, the samples in which activity effector, ATP, was replaced with specificity effector, TTP, (Figure 4.2) produced less dCDP (Table 4.3, line 3), which is consistent with the activity profiles in the steady state. Also in both trials, the control peptide Rubpy\(_3\)-F-R2C19 generated more dCDP than the peptide containing tyrosine (Table 4.3, line 4), significantly so in trial 4. One explanation for this phenomenon would be that direct oxidation of Y731 in R1 (Figure 4.1) can be more productive with regard to •C439 generation than an indirect pathway through •Y356. At this time, we also wanted to
confirm the identity of the product as dC, rather than any co-migrating side-products (although this is unlikely because of the late retention time of dC compared to other cytosine-containing compounds). Samples were converted dU enzymatically using cytidine deaminase and re-chromatographed by reverse-phase HPLC using a modified two-step gradient. After quenching the deamination reaction by boiling, dC carrier was added so that both the dC and dU peaks could be collected and quantitated. Indeed, the radioactivity quantitatively migrated with the dU peak with none remaining in the dC peak. A fifth trial was carried out in hope of reproducing the results from trials 1, 2, and 4 and to determine whether trial 3 could be considered an anomaly (Table 4.3). For trial 5, the incubation time for treatment of R1 with hydroxyurea and DTT was extended to 60 min in hopes of lowering the dCDP background formed with R1 alone. Again, the light reaction with R1 and Rubpy3-Y-R2C19 (3.4%; Table 4.3, line 1) produces significant amounts of dCDP above the dark reaction (1.0%; Table 4.3, line 5), but remains below the experiment with R1 in the absence of peptide (4.3%; Table 4.3, line 6). Thus, trial 5 was consistent trial 3 and not trials 1, 2, and 4. In addition, the high dCDP formation with Rubpy3-F-R2C19 did not reproduce in trial 5 (2.2%; Table 4.3, line 4) and was lower than the turnover observed with Rubpy3-Y-R2C19. Overall, the foregoing results showed great promise, but the relatively high backgrounds due to the small amounts of copurifying R2 present in the R1 sample made it difficult to unconditionally establish light-mediated turnover in the R1–peptide complex. This problem is intrinsic to photochemistry of the ruthenium-modified peptide itself as the precipitation from solution upon oxidation fundamentally prevents the direct comparison between the dark reaction and light reactions.

Light reactions with flavin-Y-R2C19. We then began photolysis assays with the flavin-modified peptide using similar conditions, with the intention of exploiting the anaerobic flavin photochemistry in which tyrosyl radical is formed from hydrogen atom abstraction
by the triplet excited state to form the reduced semiquinone radical [28,29] (Eq 2). Since we can only carry out single-turnover assays in the presence of external quencher due to constraints with R1, as shown in the beginning of this section, we did not feel that inability to re-generate the oxidized flavin for multiple rounds of tyrosyl radical generation would offer any additional disadvantage. Also, the flavin can be continually re-excited until forward propagation of \( \cdot Y356 \) to C439 occurs. As discussed in Chapter 3, the photochemistry of flavin-mediated photochemistry is complicated by the many pathways for redox chemistry available. For instance, in the presence of EDTA (a component of the RNR assay buffer), reductive photochemistry from the flavin semiquinone state becomes the predominant pathway as EDTA acts as a sacrificial reductant of the excited triplet flavin [29,34]. In addition, it was unclear whether the HEPES buffer could also react with the flavin excited state, as amines are capable of similar reactivity [35]. Thus, EDTA was excluded from the reaction and the assays with flavin-Y-R2C20 were carried out using potassium phosphate buffer. This lead to an additional problem, as magnesium phosphate has a \( K_{sp} \) of \( 10^{-24} \) [36]. Although magnesium is proposed as a requirement for R1–R2 association [37] and not for magnesium-nucleotide binding, it would be difficult to ascertain whether the 20-mer peptide retains binding under these conditions by the standard inhibition assay which requires R1–R2 interaction. The magnesium concentration was varied from normal assay conditions (15 mM) and the activity was found to be reduced correspondingly (10 mM, 82% activity; 5 mM, 55% activity) and is undetectable by the spectrophotometric assay at 1 mM (<5-10% activity). Another concern was that phosphate ions have been observed to be inhibitory at high concentration [38]. We thus chose a buffer of 20 mM potassium phosphate, 10 mM magnesium chloride, pH 7.6. After preparation of the protein, including the Sephadex G-25 column and concentration (to 55 \( \mu \)M) in this buffer, the final R1 was measured to have an activity of \(~\)60% of normal activity in the phosphate buffer. An added complication was that flavin-Y-R2C19 had already been dialyzed into
the standard assay buffer. Unfortunately, the EDTA was unable to be removed by dialysis and consequently the peptide was re-chromatographed by reverse-phase HPLC in 5 mM potassium phosphate, pH 7.0 to obtain an EDTA-free sample. The preliminary photolysis assays were carried out anaerobically with R1 (25 µM), flavin-Y-R2C19 (20 µM), [14C]-CDP (0.75 mM), and ATP (1.6 mM) at 25 °C by irradiating with a 1000 W Hg-Xe lamp with 90% neutral density and 360 nm longpass filters. Samples containing flavin-Y-R2C19 were visibly yellow and fluorescent at the outset but had photobleached by the end of the 1 h incubation, as expected under anaerobic conditions. When all the samples were completed, the R1 sample used was assayed in phosphate buffer to test for time inactivation of R1 throughout the course of the assays and found to have lost ~50% activity. As the sample volumes had been reduced to 200 µL (from the initial 400 µL), Dowex-1 borate columns were run using two standard columns prepared in Pasteur pipets rather in a single large column. The crude dC sample was then counted and the amounts deriving from this trial is presented in Table 4.4. Note that equivalents of dC calculated before HPLC removal of radioactive contaminants are typically much higher, as observed in experiments with the ruthenated peptide. The samples are listed in order in which they were carried out due to the gradual loss of R1 activity during the photolysis assays. In addition, the dark reaction (Table 4.4, line 3) is always run simultaneously with the light reaction (Table 4.4, line 2). Chromatographic isolation of dC was not carried out because the preliminary results were unpromising. The R1 has incredibly high background (83.0%; Table 4.4, line 4). It also

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<tbody>
<tr>
<td>Flavin-Y-R2C19</td>
</tr>
<tr>
<td>R1 + Flavin-Y-R2C19</td>
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<td>R1</td>
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<tr>
<td>CDP alone</td>
</tr>
<tr>
<td>R1 + R2</td>
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Table 4.4 dCDP formation in light-mediated reaction of R1 and flavin-Y-R2C20. R1 was treated with hydroxyurea (20 mM) and DTT (20 mM) for 50 min at room temperature. Samples (200 µL) were irradiated using 90% neutral density and 360 nm long pass filters at 25 °C for 1 h. Specific activity of the substrate used was 7,800 cpm/nmol. dCDP formation per R1 reported in column 1 is calculated from the amount of crude dC recovery from the Dowex-1 borate column.
appears as if flavin-mediated photochemistry is somehow destroying the R1 as the photoreaction with flavin-Y-R2C19 and R1 has much lower dCDP formation (15.2%; Table 4.4, line 2) than either the dark reaction (57.8%; Table 4.4, line 3) or the reaction with R1 alone (83.0%; Table 4.4, line 4). This may result from the incomplete removal of oxygen from the sample; R1 in incompatible with vigorous degasing and there is mostly likely a minimal concentration of oxygen that is equimolar with peptide that can mediate singlet oxygen chemistry. Although only a preliminary foray has been made into the photolysis of the R1–peptide complex with triplet flavin, the results are rather unpromising and should not be repeated under the same conditions. It may be possible to remove oxygen using an enzymatic oxygen-scrubbing system such as glucose oxidase [39,40], however, it will most likely be more fruitful to pursue assays with flavin-Y-R2C19 using external quencher to generate •Y356 through reaction with the oxidized flavin radical [30,31]. This provides a closed redox cycle and may allow stable generation of •Y356, which can then lead to R1 turnover by photolysis in the visible range. However, at this time, we decided that the flavin photochemistry was too complex to carry out in the R1 assay without further spectroscopic study.

4.3 Initiating Turnover in R1–Peptide Complexes by Amino Acid Radical Generation

4.3.1 Introduction

The 20-mer peptide was synthesized with an N-terminal tryptophan adjacent to Y356 as the phototigger for radical formation at Y356. Irradiation of the tryptophan moiety generates its neutral and cation radical [41-43], which can in turn produce •Y356 on a microsecond timescale [44] (Eq 4.4):

Equation 4.4 Generation of •Y356 by radical transfer from the tryptophan or tryptophan cation radical.
Tyrosine itself can also undergo photoionization upon UV excitation to generate the corresponding radical [45-47]. Because tryptophan radical generation requires UV light to drive the photoionization reaction, the activity of R1 needs to be tested under UV irradiation.

The use of a tryptophan or tryptophan cation radical to generate •Y356 on the R2 C-terminal peptide would also allow us to take one step backward in the PCET pathway of RNR and also model the reaction between W48 and Y356 (Figure 4.1). We now report the first direct evidence for the participation of aromatic amino acid radical intermediates and consequently the long distance of the conserved PCET pathway in the radical initiation process catalyzed by class I RNRs.

4.4.3 Results and Discussion

Efforts to lower the R1 background resulting from contaminating R2. Although the results of Section 4.3 with Rubpy3-Y-R2C19 showed great promise, the relatively high backgrounds compared to dCDP formed by irradiation of the R1−peptide complex made their interpretation problematic. Much of this has to do with problems in the inconsistent preparations of R1. First, the incubation time with hydroxyurea was extended to 1 h, to help further reduce the •Y122 of the contaminating R2. Single-turnover assays were carried out for one h with 25 μM R1 and [14C]-CDP (specific activity = 18,000 cpmn/nmol) to make a direct comparison with the photolysis assay. When R1 was incubated alone, in the absence of R2, 0.2 equivalents of dCDP was generated, 8% of the 2.6 equivalents observed upon addition of R2 (5 μM). As this background is unacceptable for our studies, we then decided to attempt to remove the small amounts of contaminating R2 by anion exchange FPLC chromatography. A trial mixture of R1 and R2 was separated by a linear gradient of 0 mM to 800 mM sodium chloride using a Poros HQ/20
column (Figure 4.5). As R2 is highly anionic, it elutes at higher ionic strength than R1. R1 was then chromatographed with the same gradient; the protein peak was collected manually and concentrated by Amicon with a PM30 membrane to 20-30 μM. The purified R1 was pre-reduced with DTT and hydroxyurea for 45 min. The specific activity of R1 was then measured after pre-reduction by the spectrophotometric assay and found to be identical to the starting R1. When R1 (25 μM) was incubated alone with ATP and \(^{14}\text{C}\)-CDP (specific activity = 18,000 cpm/nmol), the amount of dCDP measured was less than the background of dCDP alone. Thus R1 was then chromatographed on semi-preparative scale, loading 12-14 mg of protein per run. The protein was concentrated to ~30 μM before flash-freezing with liquid nitrogen.

An SDS-PAGE gel of the R1 sample before (Figure 4.6, lane 2) and after (Figure 4.6, lane 3) FPLC is shown in Figure 4.6; 10 μg of protein is loaded in both lanes. There is faint band running
at the molecular weight of the R2 molecular weight (MW 47.5 kD) that we currently attribute to R2, however, this has not been confirmed by Western blot analysis. The intensity of the R2 band has decreased in the sample that has been further purified by anion-exchange FPLC. This R1 sample (Figure 4.6, lane 3) was treated with hydroxyurea (20 mM) for a total incubation time of 60 min and DTT (20 mM) for 20 min and concentrated to 90 µM using a YM30 Centriprep.

**Single turnover assays with Ac-WY-R2C19.** The photoactive peptide, Ac-WY-R2C19, was synthesized by standard methods from Fmoc-R2C19. The acetylation was carried out in a solution of 1 acetic anhydride: 1 DIEA in DMF. The crude peptide was purified using the standard reverse-phase HPLC gradient for the R2 peptide in 0.1 M ammonium bicarbonate, pH 8.0. The purified peptide was then dialyzed against 5 mM potassium phosphate, pH 7.0 and characterized by analytical HPLC (Figure 4.7a), MALDI-TOF (Figure 4.7b), and competitive inhibition of nucleotide reduction (Figure 4.7c). Binding to R1 is unchanged from Ac-Y-R2C19 ($K_D$=20 µM) within error [20]. One major concern was the possible denaturation of R1 under UV photolysis, but the activity was maintained over 30 min with irradiation from the 1000 W Hg-Xe lamp with 90% neutral density and 285 nm longpass filters. With the assay established for Ac-WY-R2C19, the competence of the R1-peptide complex to initiate PCET in the presence of light was assayed by measuring deoxynucleotide formation under single turnover conditions (Figure 4.8). Photolysis ($\lambda > 285$ nm) of pre-reduced R1 incubated with Ac-WY-R2C19 in the presence of substrate, CDP, and ATP effector, produced a significant amount of dCDP product (Table 4.5); 6.3% of dCDP is observed based on the amount of R1 added. If we account for the possible number of turnovers per R1 for the given amount of R1-peptide complex formed, then we calculate that 9.8% ± 2.6% of possible dCDP is produced. As with Rubpy$_3$-Y-R2C19 and flavin-Y-R2C19, we prefer to list in Table 4.5 the observed amount of dCDP measured per R1 added because no assumptions are needed in
Figure 4.7 Characterization of Ac-WY-R2C19 (2). (Panel A) Analytical reverse-phase HPLC trace of 2 in a linear gradient of 0%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 45 min using a C-18 column. (Panel B) MALDI-TOF MS of 2 in negative ion mode. (Panel C) Competitive inhibition of nucleotide reduction by 2 (●) overlaid on Ac-WY-R2C19 (■).
reporting this value. Also as before, the dCDP is isolated by reverse-phase HPLC as dC (Figure 4.9a) and then quantitatively converted into dU (Figure 4.9b). dC carrier is added to both samples to visualize the peak during HPLC analysis. When the peptide concentration was increased ten-fold, the amount of dCDP produced increased 3.1-fold to 19.8%, scaling with the amount of R1-peptide complex (3.8 fold increase). No additional dCDP was observed upon photolysis for another 60 min (90 min total), implying that the photoreaction may be completed in well less than the 30 min time course of the assay, possibly due to peptide denaturation. The observed product must be derived from •C439 generation in the R1 active site as there is no chemical precedence for direct nucleotide reduction in any system studied to date. No dCDP formation above background (0.32-0.48%; Table 4.5, controls 1, 5, and 6) was observed in dark reactions at either peptide concentration used indicating that this reaction is light dependent. Furthermore, no product was detected when substrate was irradiated solely in the presence of the peptide (Table 4.5, control 4). Other controls in which R1 was irradiated in the absence of peptide are consistent with low levels of contaminating R2 (Table 4.5, controls 2 and 3). Interestingly, an experiment in which the allosteric effector was varied showed a
<table>
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<tr>
<th>No.</th>
<th>Sample</th>
<th>Condition</th>
<th>Effector</th>
<th>dC/ %R1</th>
<th>σ/ %R1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Δ/ %R1&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
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<tr>
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<td>ATP</td>
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<td>0.23</td>
<td>0.61</td>
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<tr>
<td>4</td>
<td>R1 + Ac-WY-R2C19</td>
<td>hv</td>
<td>ATP</td>
<td>n.d.&lt;sup&gt;o&lt;/sup&gt;</td>
<td>–</td>
<td>n.a.</td>
</tr>
<tr>
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<td>ATP</td>
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<td>0.28</td>
<td>0.33</td>
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<td>6</td>
<td>R1 + Ac-WY-R2C19(×10)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>dark</td>
<td>ATP</td>
<td>0.32</td>
<td>0.23</td>
<td>0.61</td>
</tr>
</tbody>
</table>

<sup>a</sup>σ is the standard deviation measured for 2-4 experiments. <sup>b</sup>Δ is the error associated with the amount of dC measured by scintillation counting. <sup>c</sup>1.0 mm pathlength cell. <sup>d</sup>Due to protein precipitation, experiment was performed only once. <sup>o</sup>None detected.

**Table 4.5** Light-initiated single turnover experiments in R1-peptide complexes. Samples were irradiated using 90% neutral density and 285 nm long pass filters at 30 °C for 30 min. R1 was re-purified by anion-exchange FPLC using a Poros HQ column and treated with hydroxyurea and DTT for 60 min and 20 min respectively. The specific activity of the substrate was 18,000 cpmnmol<sup>−1</sup>. dCDP formation per R1 is calculated from the amount of radioactive dC isolated by HPLC and normalized for carrier recovery. The isolated dC was also converted into dU quantitatively.
clear difference between an activity/specificity effector (ATP, 6.3%), a specificity effector (TTP, 2.4%), and no effector (0.68%) (Table 4.5, effector dependence 1, 3 and 4, respectively). These results follow the trends observed by steady state kinetic studies of R1 in combination with R2, in which the turnover numbers for ATP, TTP, and no effector were measured under steady state conditions using the spectrophotometric assay to be 11.3 s\(^{-1}\), 7.5 s\(^{-1}\) and 2.1 s\(^{-1}\) respectively. As noted above, ATP can bind to activity and specificity sites, while TTP binds only to the specificity site within R1; thus the variance in activity most likely arises from distinct conformations of R1 in these three cases.

We next turned our attention to examine the role of individual amino acids in the radical initiation pathway of R1 (Figure 4.1). Three additional peptides, Ac-WF-R2C19, Ac-Y-R2C19, and Ac-F-R2C19, were synthesized to probe the specificity of \(\bullet\)Y356 as an entryway into the R1 pathway. The analytical reverse-phase HPLC traces are shown in Figure 4.10a-10c. Competitive inhibition studies with R2 show that, within error, these three peptides bind to R1 with the same \(K_D\) as Ac-WY-R2C20 (Figure 4.10d). Ac-R2C19 was also synthesized and the resulting inhibition curve falls outside of these peptides as its \(K_D\) is measured to be \(\sim 40 \mu\text{M}\) (data not shown) [20]. Not surprisingly, both Ac-WF-
Figure 4.16 Characterization of Ac-WF-R2C19 (3), Ac-Y-R2C19 (4), and Ac-F-R2C19 (5). Analytical reverse-phase HPLC traces were collected in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel A) Analytical HPLC trace of 3. (Panel B) Analytical HPLC trace of 4. (Panel C) Analytical HPLC trace of 5. (Panel D) Competitive inhibition of nucleotide reduction by peptides 3-5 overlaid on Ac-WY-R2C19.
R2C19 (2.7%) and Ac-Y-R2C19 (2.3%) were competent to turnover CDP to dCDP (Table 4.5, Y356 pathway dependence 1 and 2, respectively). In the crystal structure of R1 with the 20-mer peptide bound (Figure 4.11a and Figure 4.11b), only the last sixteen amino acids (360 to 375) are visible, suggesting that the N-terminus on which Y356 is located is fluxional and unstructured [5]. Thus, the observed dCDP can be attributed to the ability of Ac-WF-R2C19 to oxidize the solvent-exposed Y731 directly when the adjacent Y356 is deleted. As for Ac-Y-R2C19, it is known that direct irradiation of tyrosine can also generate Y* at lower quantum efficiencies [42], and accordingly can also directly promote radical initiation in R1. Using a relative quantum yield for Y*/W* formation of 0.45 calculated from [42], the theoretical value for dCDP formation from Ac-Y-R2C19 would be 2.0% ±0.5%, a range in which the experimental value (2.3%) falls. The results with these peptides also aid in setting a lower limit on time required to
enter the PCET pathway of R1, as it must occur before quenching of either W* or Y* (µs timescale). Being a redox inactive peptide, photolysis of Ac-F-R2C19 understandably yields (0.75%) no nucleotide reduction above background within experimental error (Table 4.5, Y356 pathway dependence 3 as compared to controls 1, 2, 4, 5, 6).

Although the peptide has a looser geometric requirement for radical initiation due to its intrinsic lack of structure in the vicinity of Y356, we would expect the stringency to be much higher within R1 itself. If the PCET reaction is indeed pathway specific, mutation of Y731 to a phenylalanine would block nucleotide reduction if no other pathway exists that could circumvent Y731. To test this hypothesis, we prepared the inactive R1Y731F mutant using the standard R1 procedure [48] omitting the DEAE column, as fractions are usually pooled by activity assays (Figure 4.12). The mutant was further purified by anion-exchange FPLC, however, only ~50% of the mutant protein was recovered due to precipitation after the R1 fraction was collected. As the band running at the molecular weight of R2 has not decreased after this step (Figure 4.12, lane 4 vs. lane 5), it can probably be omitted from future preparations. R1Y731F was then treated with hydroxyurea and DTT for 60 min and 20 min respectively. The specific activity of the mutant was measured by a standard discontinuous assay for dCDP formation to be 20 nmol min⁻¹ mg⁻¹, which is approximately 1.5-2.0% of the typical specific activity of wild-type R1 measured by this method. The Y731F mutation in the PCET pathway was found to reduce dCDP formation to almost background levels (Table 4.5, R1-Y731 pathway.
dependence 1 and 2 compared to 3-5). We note that a 10-fold increase in peptide
congcentration, in contrast to wild-type R1 (Table 4.5, effector dependence 1 and 2), does
not result in increased dCDP production. If the observed dCDP were related to activity
intrinsic to the R1Y731F-peptide complex, whether direct Y730 oxidation or tunneling
through Y731F, we would expect the dCDP formation to scale to 2.1% ± 0.27%. Thus,
the very small amount of dCDP observed is likely due to the contaminating wild-type R1
(2%) in the sample. As these results are consistent with the effective deactivation of the
Y356→Y731→Y730→C439 pathway, we favor a PCET mechanism over an electron
tunneling mechanism with protonation/deprotonation controlled by solvent. In bypassing
position Y731 in the radical transfer pathway, the hopping distance would increase by 3.4
Å (Figure 4.1). In this case, the electron tunneling rate would only decrease by 0.8-2.0%
(with β=1.1 Å⁻¹ and 1.4 Å⁻¹, respectively) while the proton tunneling required for PCET
would be shut down.

These studies may also implicate structural changes within R2, communicated by the
binding of substrate and effector in R1, as the major conformational gate of radical
initiation. A recent crystal structure of the class Iib R1 (R1e) from Salmonella
typhimurium was solved in the absence of its corresponding R2f C-terminal peptide [49].
The R1e structure exhibits a very similar overall conformation, including the helices
against which the 20-mer peptide docks in the E. coli R1. An overlay of the Cα traces of
R1 and R1e is shown in Figure 4.13. Note that there are two major differences between
the two structures, marked with red asterisks. The first derives from the missing N-
terminal ATP regulatory domain of R1 that has no counterpart in the R1e family [50].
The second difference is within the R2 peptide-binding region of R1, however, the R2f
peptide is four residues shorter at the C-terminus and not believed to make contacts in
this area. Consistent with this, the hydrophobic pocket on R1e for the conserved C-
terminal hydrophobic pocket is found at a location which corresponds to the shorter
length of the R1f peptide of *S. typhimurium* and in an area which is structurally conserved. Since the foregoing studies with photoactive peptides show that R1–peptide complexes are chemically competent, it seems plausible that R2 binding may not effect a large conformational change in R1. On the other hand, while the binding of substrates and effectors to R1 appears to be independent of R2 [51], the susceptibility of •Y122 in R2 to hydroxyurea scavenging is increased by an order of magnitude in the presence of R1 and ATP and CDP or dCDP [52].

Thus the conformational changes in R1 upon substrate and effector binding are somehow communicated to the •Y122 site in R2. Since •Y122 is protected in a hydrophobic pocket [53,54], it may be that a conformational change is required to initiate radical transfer to W48, and thus confer hydroxyurea sensitivity, and allow the •Y122 access to a proton or hydrogen-bond donor.

### 4.4 Conclusions

Our results demonstrate that a small peptide can replace R2 of RNR and effect nucleotide reduction by light-initiated amino acid radical generation. Radical initiation in the R1–peptide complex is dependent on both Y731 within R1 and an aromatic amino acid within the peptide. These results have important implications for the radical initiation process of RNR. They provide the first direct experimental support for the long-range, intrasubunit pathway shown in Figure 4.1, and also demonstrate the chemical competence of aromatic
amino acid radical intermediates in RNR catalysis. Although only the pathway between Y356 and C349 has been established, we believe that validation of the intermediacy of Y356 as a radical conduit between the R1 and R2 subunits does indeed confirm the radical transfer pathway as a whole. More generally, the ability of a small peptide to replace the entire R2 subunit reproduces the radical initiation process of monomeric class II RNRs, in which the small molecule adenosylcobalamin initiates thyl radical formation directly on the R1 equivalent. Thus, by controlling the radical initiation pathway, we have succeeded in converting class I RNR to its evolutionary class II predecessor. Taken together, these results underscore the importance of conserved pathways for biological radical transport and begin to disentangle the complicated process of radical initiation of an essential enzyme involved in DNA replication and repair.

4.5 Experimental Methods

Materials. [2-14C]-Cytidine-5’-diphosphate (MC480, 55 mCi/mmol, >99% pure) was purchased from Moravek Biochemicals (Brea, CA). Higher specific activity [2-14C]-cytidine-5’-diphosphate (18,000 cpmmol−1) was synthesized by Dr. Jie Ge from [2-14C]-cytidine (MC128) was purchased from Moravek Biochemicals (Brea, CA) [6]. Hydroxyurea, adenosine-5’-triphosphate (ATP), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), ampicillin, phenylmethanesulfonyl fluoride (PMSF), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N-tosyl-L-lysine chloromethyl ketone (TLCK), streptomycin sulfate, G-25, DEAE-sepharose, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES) was purchased from EM Science (Gibbstown, NJ). Tris(hydroxymethyl)aminomethane (Tris) was purchased from Roche (Indianapolis, IN). 1,4-Dithiothreitol (DTT) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). LB was purchased from Difco (Kansas City, MO). 2’-Deoxyadenosine-5’-triphosphate (dATP) sepharose resin
was synthesized by standard procedures by Dr. Hiroshi Abe. Calf intestine alkaline phosphatase was purchased from Roche (Indianapolis, IN). Cytidine deaminase (CDA) was obtained as a gift from Professor Richard Wolfenden at University of North Carolina-Chapel Hill and frozen in 100 μL fractions and stored at the original concentration (13.9 μM, ε_{280 nm} = 39,000 M^{-1}cm^{-1}). Dowex-1 resin was purchased from Bio-Rad (AG 1-X8 (50-100 mesh), chloride form) (Hercules, CA). Dialysis tubing was purchased from Spectrum Laboratories (Rancho Dominguez, CA). Emulsifier-Safe scintillation fluid was purchased from Packard Biosciences (Boston, MA). Poros HQ/20 column was purchased from PerSeptive Biosystems. PM30 and YM30 membranes as well as YM30 Centripreps were purchased from Millipore (Billerica, MA). C-18 Econosil 10 μm column was obtained from Alltech (Deerfield, IL). C-8 and C-18 XTerra MS 5 μm and XTerra MS 5 μm columns obtained from Waters Corporation (Milford, MA). C-18 Adsorbosphere Nucleotide/nucleotide column was obtained from Alltech (Deerfield, IL).

**Synthesis and Characterization of Peptides.** Fmoc-R2C19 starting material was synthesized by methods previously described in Chapter 2. Fmoc-R2C19 (100 mg, 0.025 mmol) was deprotected by 3 × 7 min treatments with 20% (v/v) piperidine containing 0.1 M HOBr in DMF in a 20 mL fritted column. The reaction was mixed by a Vortex mixer using a foam adapter for 50 mL Falcon tubes, in which the column was placed. After washing 5 × DMF and 3 × acetone, Fmoc amino acids were added using a 0.9 HATU:2 DIEA activation scheme. Briefly, Fmoc amino acid and HATU were dissolved in 250 μL DMF to 0.5 M (0.125 mmol) and 0.45 M (0.113 mmol) respectively. All three components were combined and then activated by the addition of DIEA (44 μL, 0.25 mmol). The coupling was allowed to proceed for 1 hr before washing 5 × DMF. Removal of the N-terminal Fmoc was then carried out as described for Fmoc-R2C19 above. N-
Acetylation was by incubation in 0.5 M acetic anhydride/0.5 M diisopropylethylamine in DMF for 45 min.

Peptides were cleaved from the resin by vortexing for 3 h in a solution composed of 95% (v/v) TFA, 2.5% (v/v) TIS, and 2.5% (v/v) water. The peptide solution was collected by filtration and concentrated under a stream of N₂. The peptide was then precipitated by addition of diethyl ether. The suspension was incubated at -10 °C and pelleted by centrifugation. The peptide pellet was washed twice with ether and air-dried. The semi-dried pellet was solubilized in 0.1 M ammonium bicarbonate, pH 8.0.

Peptides were purified by reverse-phase HPLC in a linear gradient of 10-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (10 mL/min) over 45 min using a semi-preparative C-18 column (XTerra MS 5 μM, 19 × 100 mm). Purified peptides were then dialyzed against 5 mM potassium phosphate, pH 7.0 using Spectra/Por CE 1,000 MWCO tubing at 4 °C. Purified peptides were analyzed by analytical HPLC (XTerra MS 5 μM, 4.6 × 100 mm) using a linear gradient of 10-65% acetonitrile over 45 min versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min).

The mass of peptides were determined using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry on a Voyager workstation (PE Biosystems). The matrix consisted of a >10 mg/mL suspension of α-cyano-4-hydroxycinnamic acid in a solution of 50% acetonitrile (v/v), 49% (v/v) water, and 1% (v/v) TFA. The suspension was sonicated and centrifuged before spotting 1 μL of the supernatant with 1 μL of a salt-free sample dissolved in water onto the 100-well MALDI target. Mass spectra were collected in negative ion and linear mode using a 600-1000 MW cutoff. An external linear calibration was carried out before every use with angiotensin I (M_	ext{ave} = 1295.5 Da), ACTH 18-39 (M_	ext{ave} = 2464.7 Da), and oxidized bovine insulin (M_	ext{ave} = 3495.9).
Peptide binding to R1 was characterized by competitive inhibition assays for nucleotide reduction [20]. R1 (0.1 μM), R2 (0.2 μM), thioredoxin (50 μM), thioredoxin reductase (1.0 μM), CDP (1.0 mM), ATP (1.6 mM), NADPH (0.2 mM) were incubated with varying concentrations of peptide (0-300 μM). Specific activity was measured from the decrease in A_{340 nm} using ε_{340 nm} = 6,220 M^{-1}cm^{-1} and defined as one nmol dCDP produced per min per mg R1.

**Rubpy3-F-R2C19** (1). Fmoc-Phe-OH and (bis(2,2'-bipyridyl)(4-methyl-4'-carboxylic acid-2,2'-bipyridyl) ruthenium (II) bis(hexafluorophosphate) were added in succession to the N-terminus of R2C19 using 0.9 HATU:2.0 DIEA activation chemistry. The concentration of the peptide was measured using ε_{458 nm} = 14,400 M^{-1}cm^{-1}. HPLC: t_R = 17.7 min. UV-Vis: λ_max = 246 nm, 288 nm, 458 nm. MALDI-TOF MS: MW_{calc} = 2865 Da, MW_{obs} = 2866 Da.

**Ac-WY-LVGQIDSEVDTDDLSFNL (=Ac-WY-R2C19)** (2). Fmoc-Tyr(O'Bu)-OH and Fmoc-Trp(Boc)-OH were added in succession to the N-terminus of R2C19 using 0.9 HATU:2.0 DIEA activation chemistry. The concentration of the peptide was measured using ε_{280 nm} = 7,110 M^{-1}cm^{-1}. HPLC: t_R = 18.9 min. MALDI-TOF MS: MW_{calc} = 2499 Da, MW_{obs} = 2498 Da.

**Ac-WF-LVGQIDSEVDTDDLSFNL (=Ac-WF-R2C19)** (3). Fmoc-Phe-OH and Fmoc-Trp(Boc)-OH were added in succession to the N-terminus of R2C19 using 0.9 HATU:2.0 DIEA activation chemistry. The concentration of the peptide was measured using ε_{280 nm} = 5,690 M^{-1}cm^{-1}. HPLC: t_R = 20.3 min. MALDI-TOF MS: MW_{calc} = 2484 Da, MW_{obs} = 2484 Da.

**Ac-Y-LVGQIDSEVDTDDLSFNL (=Ac-Y-R2C19)** (4). Fmoc-Tyr(O'Bu)-OH was added to the N-terminus of R2C19 using 0.9 HATU:2.0 DIEA activation chemistry. The
concentration of the peptide was measured using $\varepsilon_{280 \text{ nm}} = 1,500 \text{ M}^{-1}\text{cm}^{-1}$. HPLC: $t_R = 14.2$ min. MALDI-TOF MS: $MW_{\text{calc}} = 2313 \text{ Da}, MW_{\text{obs}} = 2315 \text{ Da}.$

**Ac-F-LVGQIDSEVDTDLDLSFNQL** (=Ac-F-R2C19) (5). Fmoc-Phe-OH was added to the N-terminus of R2C19 using 0.9 HATU:2.0 DIEA activation chemistry. The concentration of the peptide was measured using $\varepsilon_{258 \text{ nm}} = 390 \text{ M}^{-1}\text{cm}^{-1}$. HPLC: $t_R = 16.1$ min. MALDI-TOF MS: $MW_{\text{calc}} = 2297 \text{ Da}, MW_{\text{obs}} = 2297 \text{ Da}.$

**Isolation of R1** [48]. Freshly transformed BL21(DE3) cells containing pMJ1, the R1 overexpression plasmid were grown overnight in 100 mL LB with 100 $\mu$g/mL ampicillin. The seed culture (80 mL) was centrifuged at 5,000 rpm for 7 min and resuspended in 10 mL LB. The cell suspension was then used to inoculate the LB (20.0 L, pH 7.6) containing 100 $\mu$g/mL ampicillin, which was fermented at 37 $^\circ$C. Upon reaching $A_{600 \text{ nm}}$ of 1.0 OD, the cell culture was induced by the addition of IPTG (0.5 mM final concentration). The cells were grown for another 4 h before harvesting by continuous-flow centrifugation and flash-freezing with liquid nitrogen (71 g cell paste). Cells (71 g) were resuspended with homogenization in 370 mL R1 buffer (50 mM Tris, 15 mM magnesium acetate, 5% (w/v) glycerol, pH 7.6) with DTT (4mM) and PMSF (1.0 mM). Cell cracking was carried out in one pass at 14,000 psi. A cleared lysate (380 mL) was obtained after centrifugation for 10 min at 15K rpm. DNA was precipitated by addition of 15% (w/v) streptomycin sulfate solution (28 mL) dropwise over 10 min. After stirring for an additional 10 min, the sample was centrifuged for 30 min at 15K rpm. Ammonium sulfate was added to 60% saturation (148.2 g) to the cleared cell lysate (380 mL) over 20 min and stirred for an additional 20 min. The protein pellet was collected by centrifugation for 1 h at 10K rpm and resuspended in a minimal volume (66 mL). A Sephadex G-25 column (4.5 cm x 37 cm, 600 mL) was used to desalt the sample and the visibly brown protein fraction (200 mL) was collected in an Erlenmeyer flask. The protein was loaded onto a DEAE Sepharose column (7.0 cm x 7.0 cm, 300 mL) and the
column was washed (2.6 L) until the $A_{280\ nm}$ was less than 0.05 OD. R1 was eluted in 25 mL fractions with a 0 to 400 mM sodium chloride gradient (500 mL × 500 mL). Visibly yellow fractions (25-53) were assayed for R1 activity. Active fractions 29 to 49 (480 mL) were pooled and loaded onto two dATP affinity columns (2.4 cm × 4.0 cm, 20 mL, high-binding capacity; 5.0 × 6.0 cm, 120 mL, low-binding capacity). Both columns were washed until the $A_{280\ nm}$ was less than 0.05 OD before eluting with 10 mM ATP-2Na (pH 7.6) in R1 buffer with DTT (10 mM). Active fractions from both columns were collected and pooled (300 mL). R1 was concentrated by Amicon with a PM30 membrane (60 mL) and loaded onto a Sephadex G-25 column (6.0 cm × 18 cm, 500 mL) to remove the ATP. Protein-containing fractions were assayed for protein by Bradford assay and pooled and concentrated to 50 mL. This process was repeated again to obtain an $A_{\text{max}}$ of 278 nm. R1 concentration was measured using $\varepsilon_{280\ nm}=189,000$ M$^{-1}$cm$^{-1}$. Total yield of protein was 333 mg, which was stored at 10.1 mg/mL at −80 °C after flash-freezing in liquid nitrogen. R1 specific activity as measured by the spectrophotometric assay was found to be 2,700 nmol min$^{-1}$mg$^{-1}$.

**Isolation of R1-Y731F mutant.** The R1Y731F mutant was isolated by the same procedure as the wild-type R1 on a 20 g scale, omitting the anion exchange-DEAE chromatographic purification step. The mutant was the further purified by FPLC using a Poros HQ/20 column and pre-reduced according the procedure for the wild-type R1 purification. The activity of the mutant was measured by a standard discontinuous assay, using a boiling quench, measuring $[^{14}\text{C}]-dC$ formation [6] over 30 min with R1 (3.0 μM), R2 (3.0 μM), thioredoxin (50 μM), thioredoxin reductase (1.0 μM), $^{14}\text{C}-\text{CDP}$ (1.0 mM, 1,400 cpmnmol$^{-1}$), NADPH (1.0 mM), and ATP (1.6 mM) at 30 °C. R1 specific activity as measured by the radioactive assay was found to be 20 nmol min$^{-1}$mg$^{-1}$.

**Isolation of R2 [48].** Freshly transformed BL21(DE3) cells containing pTB2, the R2 overexpression plasmid were grown overnight in 100 mL LB with 100 μg/mL ampicillin.
The seed culture (80 mL) was centrifuged at 5,000 rpm for 7 min and resuspended in 10 mL LB. The cell suspension was then used to inoculate the LB (20.0 L) containing 100 μg/mL ampicillin, which was fermented at 37 °C. Upon reaching A_{600 nm} of 1.0 OD, the cell culture was induced by the addition of IPTG (0.5 mM final concentration). The cells were grown for another 4 h before harvesting by centrifugation and flash-freezing with liquid nitrogen (40 g). Cells were resuspended with homogenization in 3 mL/g R2 buffer (50 mM Tris, 10 % (w/v) glycerol, pH 7.6) with PMSF (1.0 mM). Cell cracking was carried out in one pass at 14,000 psi. A cleared lysate was obtained after centrifugation for 10 min at 15K rpm to which a purple mixture of ferrous ammonium sulfate and sodium ascorbate was added dropwise (0.4% w/w cell paste) followed by DNase (20 μg/g cell paste). Ammonium sulfate was added to 60% saturation (39 g/100 mL) to the cleared cell lysate over 20 min and stirred for an additional 20 min. The protein pellet was collected by centrifugation for 1 h at 10K rpm and resuspended in a minimal volume (50 mL). The visibly brown protein fraction was collected from a desalting Sephadex G-25 column (3.5 cm × 30 cm, 300 mL). The protein fraction was diluted two-fold to 600 mL and loaded onto a DEAE Sepharose column (7.0 cm × 7.0 cm, 300 mL). The column was washed until the A_{280 nm} was less than 0.1 OD. R2 was eluted in 20 mL fractions using a linear gradient of 0 to 400 mM sodium chloride (750 mL × 750 mL) in R2 buffer. R2 eluted from 200 mM to 250 mM sodium chloride and was pooled by monitoring A_{410 nm}. The pooled fractions were then loaded onto a Q-Sepharose column (5.0 cm × 11 cm, 230 mL) pre-equilibrated to 70 mM sodium chloride in R2 buffer and washed with the starting buffer. R2 was then eluted in 20 mL fractions using a linear gradient of 70-600 mM sodium chloride (750 mL × 750 mL). Active fractions 27 to 35 (180 mL) were pooled using the spectrophotometric assay and concentrated by Amicon using a YM30 membrane to 37 mL. Total yield of protein was 1200 mg, which was stored at 31.6 mg/mL at −80 °C after flash-freezing in liquid nitrogen. The radical content was measured using the formula, \[-(2A_{407 nm} + 3A_{416 nm})/5 + A_{410 nm}] × 73.1 ÷ A_{280 nm}, and
found to be 1.2 radicals per dimer. R1 specific activity as measured by the spectrophotometric assay was found to be 7,000 nmol min⁻¹mg⁻¹.

**Isolation of E. coli Thioredoxin (TR) [55]**. *E. coli* SK3981 were grown overnight in 100 mL LB with 100 µg/mL ampicillin. The seed culture was centrifuged at 5,000 rpm for 10 min and resuspended in 20 mL LB. The cell suspension was then used to inoculate 16.5 L TR media (1.0 L TR media: 5 g yeast extract, 10 g gas amino acids, 20 mL of a balanced salt solution containing 1 g magnesium sulfate heptahydrate, 10 g citric acid dihydrate, 50.5 g dibasic potassium phosphate, 17.6 g dibasic sodium ammonium phosphate tetrahydrate per 100 mL) with 100 µg/mL ampicillin, which was incubated at 37 °C at 200 rpm. Upon reaching A₆₀₀nm of 2.5 OD (15 h), the cells were harvested and flash-frozen with liquid nitrogen (67 g cell paste). Cells were resuspended in 355 mL TR buffer A (50 mM Tris·HCl, 3 mM EDTA, pH 7.4) with PMSF (17 mg/L), TLCK (37 mg/L) and TPCK (34 mg/L) and lysed at 14,000 psi with one pass through the French high-pressure cell. The cleared lysate was collected after centrifugation at 20K rpm for 20 min. Streptomycin sulfate was added to 0.8% (w/v) over 20 min and stirred for an additional 15 min before centrifugation at 20K rpm for 20 min. The supernatant was diluted to 1.2 L with TR buffer B (50 mM potassium phosphate, 3 mM EDTA, pH 7.3) and loaded onto a DE-52 column (6.0 cm × 30 cm, 850 mL) equilibrated in TR buffer B. Protein was eluted in 25 mL fractions with a linear gradient (3.0 L × 3.0 L) from 50 mM to 150 mM potassium phosphate. Active fractions 40 to 80 (1 L) were pooled and concentrated by Amicon with a YM3 membrane. This sample (13 mL) was then loaded onto a G-75 column (5 × 61 cm, 1.2 L) equilibrated with TR buffer B and eluted in 12 mL fractions at a flow rate of 0.4 mL/min. Active fractions 46 to 66 (250 mL) were pooled and exchanged into TR buffer A while concentrating by Amicon with a YM3 membrane to 31.5 mg/mL (ε₂₈₀nm = 11,400 M⁻¹cm⁻¹, 12 kDa, 760 µM). The isolated TR (158 mg) had a specific activity of 342 nmol min⁻¹mg⁻¹.
**Isolation of *E. coli* Thioredoxin Reductase (TRR)** [56]. Cell paste (29 g) was obtained from Dr. Chris Lawrence in the Stubbe Laboratory. Cells were resuspended in 145 mL TRR buffer A (50 mM Tris, 1 mM β-mercaptoethanol, pH 8.0) and lysed at 14,000 psi with two passes through the French high-pressure cell. The cleared lysate was collected after centrifugation at 20K rpm for 20 min. Streptomycin sulfate was added to 0.7% (w/v) over 20 min and stirred for an additional 15 min before centrifugation at 10K rpm for 30 min. The supernatant (175 mL) was loaded onto a DE-52 column (2.5 cm × 12 cm, 60 mL) equilibrated in TRR buffer B (100 mM potassium phosphate, 1 mM EDTA, pH 7.0). Protein was eluted in 25 mL fractions with a linear gradient (1.0 L × 1.0 L) from 100 mM to 250 mM potassium phosphate. Active fractions 17 to 37 (500 mL) were pooled and loaded onto a adenosine-2',5'-diphosphate agarose column (Sigma, 2.0 cm × 2.0 cm, 6 mL) equilibrated in TRR Buffer C (50 mM potassium phosphate, 3 mM EDTA, pH 7.6) at a flow rate of 1 to 2 mL/min. As there was insufficient resin to bind the total protein, the yellow flow-through from the column load (485 mL) was collected and saved for a second run of the ADP affinity column. The column was washed with 0.2 M sodium chloride (75 mL) until the A_{280} was less than 0.05 OD. TRR was eluted with 1 mM NADPH (15 mL) in 1 mL fractions, turning the column colorless. Upon re-oxidation, the yellow fractions 4 to 7 were pooled without further concentration (4.19 mg/mL, 20.5 mg, Lowry assay; 2 × 66 kDa, 350 μM; 860 nmol min⁻¹mg⁻¹). After washing the column with TRR buffer C to remove NADPH, the affinity column was loaded with the flow-through from the first load. The wash and elution were repeated and yellow fractions 8 to 11 were collected and concentrated by Amicon using a YM30 membrane (8.85 mg/mL, 4.4 mg, Lowry assay; 738 μM; 680 nmol min⁻¹mg⁻¹).

**Spectrophotometric assay for R1 and R2 activity.** The assay buffer consists of 50 mM HEPES, 15 mM magnesium sulfate, and 1 mM EDTA, pH 7.6. The specific activity of R1 and R2 subunits are measured using a 1:5 excess of the second subunit. A typical
assay contains R1 and R2 (either 0.1 μM or 0.5 μM), thioredoxin (50 μM), thioredoxin reductase (0.5-1.5 μM), CDP (1.0 mM), ATP (1.6 mM), and NADPH (0.2 mM). Specific activity is measured by the decrease in A340 nm using ε340 nm = 6,220 M⁻¹cm⁻¹ and defined as one nmol of dCDP produced per min per mg of enzyme. For effector dependent measurements of R1 turnover numbers, the following concentrations were used: R1 (0.1 μM), R2 (0.5 μM), thioredoxin (50 μM), thioredoxin reductase (1.0 μM), CDP (1.0 mM), NADPH (0.2 mM) were incubated with either ATP (1.6 mM), TTP (100 μM), or no effector.

**Spectrophotometric assay for TR and TRR activity.** The assay for TR/TRR activity consisted of approximately 2.5 μM TR and 0.1 μM TRR with 0.1 mM NADPH and 0.15 mM DTNB (10 mM stock solution in ethanol) in 100 mM Tris, 25 mM EDTA, pH 8.0. Upon mixing, the appearance of the thiolate was monitored at 412 nm (ε₄₁₂ nm = 13,600 M⁻¹cm⁻¹ at pH 8.0).

**Preparation of Dowex-1 borate columns.** The Dowex-1 borate resin was prepared by stirring AG 1-X8 resin (chloride form, 50-100 mesh) in a saturated potassium tetraborate solution overnight, filtering, and repeating twice. The columns (~1.7-2.0 mL) were poured in 9 inch Pasteur pipets with a glass wool plug and washed with 10 volumes of water.

**Isolation of dC by reverse-phase HPLC (gradient A).** dC was isolated after the Dowex-1 borate columns using a C-18 column (4.6 × 250 mm) in a buffer system of 4 mM potassium phosphate, pH 7.0 buffer versus methanol. The pump flow program consisted of two steps: an isocratic 0% methanol phase from 0 to 5 min followed by a linear gradient of 0% to 50% methanol from 5 to 35 min at a flow rate of 1.0 mL/min. dC was collected manually, typically eluting with a retention time of 15-17 min, and lyophilized in 1.5 mL Eppendorf tubes.
Separation of dU and dC by reverse-phase HPLC (gradient B). dC and dU were separated by reverse-phase HPLC on a C-18 column (4.6 x 250 mm) using 4 mM potassium phosphate, pH 7.0 buffer versus methanol. The pump flow program consisted of three steps: an isocratic 0% methanol phase from 0 to 5 min, a steep linear gradient of 0% to 10% methanol from 5 to 15 min, and a separating linear gradient of 10% to 20% methanol from 15 to 35 min. The flow rate was held constant at 1.0 mL/min. The dC and dU fractions were collected manually and lyophilized and typically eluted with retention times of 16-18 min and 20-22 min respectively.

Single turnover assays as carried out in Section 4.2:

Pre-reduction of R1. The R1 sample was treated with DTT and hydroxyurea to respectively reduce the R1 disulfides and the •Y122 in the small amounts of co-purifying R2. R1 was incubated with hydroxyurea (20 mM) and DTT (20 mM) for 60 min at room temperature. The reduced sample was then loaded on a Sephadex G-25 column (50-75 mL, or 1:10) equilibrated with either 50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA, pH 7.6 (for Ru(bpy)_3-Y-R2C19) or 20 mM potassium phosphate, 10 mM magnesium sulfate, pH 7.6 for (flavin-Y-R2C19). Protein fractions were pooled based on Bradford assays and concentrated to 60-70 μM by Amicon using a PM30 membrane. Samples were flash-frozen in 500 μL aliquots liquid nitrogen.

Anaerobic photolysis assay. Pre-reduced R1 (60-70 μM) was degassed by 5 cycles of gentle evacuation and purging with argon in a 10 mL pear-shaped flask on a Schlenk line. Substrate ([^14]C]-CDP), effectors (ATP and TTP), and peptides were also degassed by 5 cycles of evacuation and purging with argon in loosely capped screw-top microfuge tubes placed in a 100 mL 24/40 round-bottom flask. Assay buffer (either 50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA, pH 7.6 for Rubpy_3-Y-R2C19 or 20 mM potassium phosphate, 10 mM magnesium chloride, pH 7.6 for flavin-Y-R2C19), cobalt pentaamine trichloride (25 mM), and water were degassed by 5 cycles of evacuation and purging with
argon with stirring. All assay components were then brought into the anaerobic glove box. Substrate was first pre- aliquotted into individual 1.5 mL screw-top microfuge tube corresponding to each assay condition and left to degas in the refrigerator. For each reaction (250 μL), components were then added to final concentrations as follows: R1 (25-30 μM), peptide (20 μM), effector (ATP or TTP), [14C]-CDP (0.75 mM, 5,000-8,000 cpm/nmol). In assays with Rubpy3-Y-R2C19, cobalt (III) pentaamine chloride (8-10 mM) was also added. Upon mixing, the sample was spun down with a microcentrifuge and transferred to a 1.0 cm exact path length quartz semi-microcuvette adapted with graded seal and J. Young Teflon screw cap. Samples were irradiated at 25 °C for 1 h using a 1000-W high-pressure Oriel Hg-Xe lamp. The irradiation beam was passed through 435 nm long pass and 90% neutral density filters and a collimating lens prior to entering the sample chamber. A fixed sample volume (150-200 μL) was then removed from the cell and quenched with 0.5 volumes of 2.0% perchloric acid and neutralized to pH 7.0 with 0.5 N potassium hydroxide (volume of potassium hydroxide added is determined from titrations for each set of solutions, usually ranging from 0.9 to 1.2 volumes of the perchloric acid added).

**Quantitation of dCDP.** The supernatant was removed from the quenched sample into a 1.5 mL screw-top microfuge tube after centrifugation for 1 min at 14K rpm. The protein precipitate was washed twice with buffer (2 × 500 μL) and pooled with the supernatant. The dephosphorylation reactions (1.5 mL) were made to a final concentration of 100 mM Tris, 0.2 mM EDTA, pH 8.5 with dC carrier (10-20 nmol) and calf intestinal alkaline phosphatase (10 units) and incubated for 3 h at 37 °C. Four borate columns were used per sample. First, the sample was loaded onto each of the four columns. 500 μL was used to wash the tube and 125 μL of the wash was loaded per column. The columns were eluted with 8.5 mL water for a total volume of 36 mL for each sample. The samples were lyophilized in two 50 mL pear-shaped flasks treated with Sigma-Cote. The lyophilized
samples were resuspended in water (1.0 mL), and 50 μL was diluted to 1.0 mL with water and counted for 15 min with 9.0 mL of Emulsifier-Safe scintillation fluid using a Beckman LS-6500 scintillation counter (Fullerton, CA) to obtain the crude amount of dCDP produced.

Each sample (400 μL) was further characterized by reverse-phase HPLC on an Alltech Econosil 10 μm C-18 column using gradient A. The dC fraction was resuspended in water (1.0 mL). The sample (0.5 mL) was counted (water, 0.5 mL; scintillation fluid, 9.0 mL) for 30 min and normalized using carrier recovery ($\varepsilon_{271\text{ nm}} = 9,000 \text{ M}^{-1}\text{cm}^{-1}$).

**Conversion of deoxycytidine to deoxyuridine.** Cytidine deaminase (CDA) was added to the isolated dC (0.5 mL) with CDA buffer (50 mM Tris·HCl, pH 7.5; 0.5 mL) and incubated for 30 min at room temperature. The reaction was then quenched by placing in a boiling water bath for 1 to 2 min. dC standard (5 nmol) was added to the quenched sample before chromatographing. dU and dC were then isolated on an Alltech Econosil 10 μm C-18 column using gradient B. The solids were redisolved in water (1.0 mL) and quantitated against carrier recovery ($\varepsilon_{262\text{ nm}} = 10,100 \text{ M}^{-1}\text{cm}^{-1}$) and counted (sample 0.5 mL; water 0.5 mL; scintillation fluid, 9.0 mL) for 30 min.

*Single turnover assays as carried out in Section 4.3:*

**BioCAD Purification of R1.** The R1 sample was purified by FPLC by anion exchange chromatography with a Poros HQ/20 10 μm column (semi-preparative, 16.0 × 100 mm) using a Biocad Spring instrument (Applied Biosystems). The column was equilibrated in 50 mM Tris, pH 7.6 (at 25 °C). The gradient was begun with an isocratic step at 0 mM sodium chloride (1 min) before beginning the linear gradient from 0 to 800 mM sodium chloride (4.0 mL/min) over 30 min, which was used to separate R1 (300 mM sodium chloride) from R2 (400 mM sodium chloride). Each run consisted of 12-14 mg of R1.
The R1 fraction was collected and concentrated by Amicon using a PM30 membrane to ~30 μM before flash-freezing in liquid nitrogen.

**Pre-reduction of R1.** The R1 sample was treated with DTT and hydroxyurea to reduce R1 and R2 respectively. R1 was incubated with hydroxyurea for 40 min at a final concentration of 20 mM at room temperature. Following this incubation, DTT was added to a final concentration of 20 mM and incubated for an additional 20 min room temperature. The reduced sample was then loaded on a Sephadex G-25 column (1:10) equilibrated with assay buffer (50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA, pH 7.6). Protein fractions were pooled based on the Bradford assay and concentrated to 90 μM using a YM30 Centriprep concentrator. Samples were flash-frozen in 500 μL aliquots liquid nitrogen.

**Aerobic photolysis assays.** Pre-reduced R1 (30 μM), peptide (20 μM or 200 μM), ATP (1.6 mM) or TTP (100 μM), and [14C]-CDP (0.75 mM, 18,000 cpmnmol⁻¹) were incubated in either a 1.0 cm or 1.0 mm path length sealed quartz cell in 50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA, pH 7.6 (100-150 μL). Samples were irradiated at 30 °C for 30 min using a 1000-W high-pressure Oriel Hg-Xe lamp. The irradiation beam was passed through 285 nm long pass and 90% neutral density filters and a collimating lens prior to entering the sample chamber. Dark reactions (50 μL) were run simultaneously with the light reaction by incubating in a water bath at the same temperature. Samples of fixed volume, typically 75-100 μL, were withdrawn from the cells and were then quenched with 0.5 volumes of 2.0% perchloric acid and neutralized to pH 7.0 with 0.5 N potassium hydroxide (volume of potassium hydroxide added is determined from titrations for each set of solutions, usually ranging from 0.9 to 1.2 volumes of the perchloric acid added).
Quantitation of dCDP. The supernatant was removed from the quenched sample into a screw-top microfuge tube after centrifugation for 1 min at 14K rpm. The protein precipitate was washed twice with buffer and pooled with the supernatant. The dephosphorylation reactions (1.0 mL) were made to a final concentration of 100 mM Tris, 0.2 mM EDTA, pH 8.5 with dC (20 nmol) and calf intestinal alkaline phosphatase (10 units) and incubated for 3 h at 37 °C. One column was used per sample. Water (1.0 mL) was used to wash the reaction tube and was loaded on the column as well. The columns were eluted with 8.0 mL water for a total volume of 10 mL for each sample. The samples were lyophilized in one 25 mL pear-shaped flasks treated with Sigma-Cote. The lyophilized samples were resuspended in water (1.0 mL), and 50 µL was diluted to 1.0 mL with water and counted for 15 min with 9.0 mL of Emulsifier-Safe scintillation fluid.

These samples (500 µL) were separated by reverse-phase HPLC on an Adsorbosphere Nucleoside/nucleotide C-18 column using gradient A. The isolated dC was resuspended in water (1.0 mL). The sample (200 µL) was counted (water, 800 µL; scintillation fluid, 9.0 mL) for 30 min and normalized using carrier recovery ($\epsilon_{271 \text{ nm}} = 9,000 \text{ M}^{-1}\text{cm}^{-1}$).

Conversion of deoxycytidine to deoxyuridine. The isolated dC (400 µL) was incubated with CDA (0.025 units) for 30 min in 50 mM Tris·HCl, pH 7.0 at room temperature in a total reaction volume of 1.0 mL. The reaction was then quenched by placing in a boiling water bath for 1 to 2 min. dC standard (5 nmol) was added to the quenched sample before chromatographing. The sample was chromatographed by reverse-phase HPLC on an Alltech Adsorbosphere Nucleoside/nucleotide C-18 column using gradient B. Isolated dC and dU were resuspended in water (1.0 mL), quantitated against carrier recovery ($\epsilon_{262 \text{ nm}} = 10,100 \text{ M}^{-1}\text{cm}^{-1}$) and counted for 30 min (sample, 0.5 mL; water, 0.5 mL; scintillation fluid, 9.0 mL).
References


(36) http://www.ktf-split.hr/periodni/en/abc/kpt.html


Chapter 5

Towards Defining the Mechanism of Long-Range PCET in R1
5.1 Introduction

The existence of the PCET pathway of RNR has been demonstrated by the experiments presented in Chapter 4, however, the overall goal of understanding the mechanism and kinetics of long-range radical initiation has yet to be accomplished. This problem is difficult to study, as the kinetics are masked by rate determining conformational changes triggered by substrate and/or effector binding [7]. The discovery that peptides derived from the C-terminal tail of R2 are chemically competent to initiate turnover in R1 by light-mediated radical generation opens the path to examine the PCET pathway by time-

![Diagram](image)

*Figure 5.1 The PCET pathway between Y356 and C439 is isolated using photoactive derivatives of the R2 C-terminal tail to initiate turnover. As these peptides are chemically competent to generate the thyl radical, we can examine the mechanism and kinetics of radical initiation at C439 in the absence of R2, consequently eliminating rate-limiting conformational changes and issues related to cofactor assembly.*

resolved laser and EPR spectroscopy (Figure 5.1). With the ability to trigger transport along the PCET pathway with laser excitation, we gain the opportunity to optically detect radical intermediates and monitor PCET between Y356 and C439. Our eventual goal is to define the kinetics of the PCET pathway and the rate dependence of any perturbations that we may wish to introduce into the pathway using our strategy of light-initiated
radical generation and employing time-resolved transient absorption and EPR spectroscopy.

Although the experimental strategy has been validated, there are issues that need to addressed before this may be achieved. First, we need to develop second-generation R2 peptide derivatives that can selectively generate •Y356 by excitation outside the protein absorption envelope. Our steady-state experiments using the tryptophan phototrigger depended on the fact that deoxynucleotide is only produced from productive reactions related to formation of •Y356 or •Y731. However, the time-resolved studies will be greatly complicated by the formation of background radical species, especially in light of the low extinction coefficients of the tyrosyl (ε_{410 nm} ~ 3000 M^{-1} cm^{-1}) and thyl (ε_{300nm-330 nm} ~ 400-1,200 M^{-1} cm^{-1}) radicals involved [2]. Second, we would like to pinpoint the location of Y356 and measure its distance from Y731. This unknown distance is important (Figure 5.1) and will aid in dissecting the mechanism of PCET. More specifically, the distance-dependence of radical transfer can distinguish whether individual steps can be considered an effective hydrogen atom transfer or whether electron and proton transfer are separated. Lastly, we will also need a method to distinguish hole transport along the tyrosine chain Y356, Y731, and Y730 since they will be spectrally identical. The absorption spectrum of the tyrosyl radical may be shifted by fluorination on the ring and these tyrosines may thus be differentiated from each other. The tyrosines in R1 can be globally replaced in vivo, using standard methods, with fluorinated tyrosines so that Y356 can be distinguished from Y730 and Y731 or vice versa. In addition, fluorination changes both the phenolic pK_a and the redox potential and may allow us to uncover important mechanistic aspects of the PCET pathway [3].

5.2 Development of Second-Generation Photoactive Peptides

5.2.2 Introduction

Although the biochemical assays presented in Chapter 4 have provided a sensitive probe
of the chemical competence of photoactive R2 peptides to initiate turnover in R1 by PCET, the mechanistic details of the radical initiation remain ambiguous. In order to study the kinetic mechanism of radical propagation into the R1 active site by transient absorption spectroscopy, we require the development of photoactive R2 peptide variants that selectively generate $\cdot$Y356 upon excitation so that we can track the progress of the oxidizing equivalent. Peptides modified with rhenium(I)(tricarbonyl)(polypyridyl) (cyanide) complexes or flavin described in Chapter 3 may eventually provide a path to achieving this goal, but the requirement of external quencher limits us to single turnover conditions. Thus, the use of peptides that do not require such quenchers would be preferable. For example, nitration of the indole ring of tryptophan may provide a method for shifting the wavelength for W$^\bullet$ generation outside the protein absorption envelope (Scheme 5.1). The Fmoc-6-nitrotryptophan starting material has been reported in the literature and used in synthetic peptides as a photocrosslinking agent [4]. The mechanism of crosslinking by nitroaryl amino acids is thought to proceed through radical intermediates [5], so it is our belief that the 6-nitrotryptophan may be able to provide radical initiation at the adjacent Y356 by generation of the nitrotryptophan radical followed by radical transfer to Y356. In addition, the benzophenone amino acid
derivative, \( p \)-benzoylphenylalanine (Bpa), could also provide a method for tyrosyl radical generation (Scheme 5.1). Both the benzophenone triplet (\( \lambda_{\text{max}} \approx 535 \text{ nm} \)) and the ketyl radical product (\( \lambda_{\text{max}} \approx 545 \text{ nm} \)) have optical signatures can be monitored by transient absorption spectroscopy. Excitation to the \( n \pi^* \) triplet state of benzophenone at 350-360 nm (\( E_{0,0} = 3.0 \text{ eV} \) [6]) has been observed to oxidize tyrosine in aqueous solution at a bimolecular rate of \( 2.6 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) [6], measured from the rate of triplet decay at 520 nm. The ketyl radical that forms concomitantly with tyrosyl radical is quenched at a near diffusion-controlled rate of \( 2.3 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \) [6], assuming that it is scavenged by oxygen. Studies of an ether-linked benzophenone and phenol in acetonitrile have shown that the ketyl-phenoxyl biradical forms at a rate of \( 5.3 \times 10^7 \text{ s}^{-1} \), which has a lifetime of 285 ns at room temperature [7]. Although the quantum yield for phenoxyl radical decreases in the presence of oxygen, it should adequately inhibit the back reaction. The overall benefit of both of these unnatural amino acids are robust and can be incorporated into synthetic peptides and perhaps into R2 using semisynthetic methods.

5.2.2 Results and Discussion

Nitration of L-tryptophan affords the 6-nitrotryptophan starting material 1. Addition of the Fmoc protecting group using Fmoc-Cl proceeds smoothly in quantitative yield to generate Fmoc-6-nitrotryptophan 2. Addition of 2 to R2C19 is accomplished using standard procedures and without further protection of the indole side-chain. However, when N-terminal acetylation is attempted on this peptide, the indole nitrogen reacts as well, as evidenced by a blue-shifted absorption spectrum compared to the amino acid starting material 1 and MALDI-TOF MS, which shows addition of two acetyl groups to NO\(_2\)WY-R2C19. The reactivity of the indole nitrogen is due to the lowered pK\(_a\) upon nitration, as addition of DIEA during acetylation led color changes associated with deprotonation. Therefore, peptide 3 was synthesized without the N-terminal protecting group, which is added only to aid binding to R1 by masking the positively charge amino
terminus. If the acetyl group is indeed necessary for enhanced binding, N-acetyl-6-nitrotryptophan can most likely be synthesized by nitration of an N-acetyl-tryptophan starting material or Boc protection of the indole nitrogen of 2 can be carried out. The characterization of NO₂WY-R2C19 is presented in Figure 5.2. The analytical reverse-phase HPLC trace of purified 3 is shown in Figure 5.2a with the characteristic absorption spectrum (Figure 5.2b). The identity of 3 was further confirmed by MALDI-TOF MS (Figure 5.2c). The concentration of 3 was determined using the literature values for the extinction coefficient of amino acid 1 of \( \varepsilon_{335 \text{ nm}} \approx 7,000 \text{ M}^{-1}\text{cm}^{-1} \) [8]. The binding

**Figure 5.2** Characterization of NO₂WY-R2C19 (3). (Panel A) Analytical reverse-phase HPLC trace of purified 3 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel B) UV-visible absorption spectrum of 3. (Panel C) MALDI-TOF mass spectrum of 3 in negative ion mode The calculated m/z(−H) is 2500 Da. (Panel D) Characterization of binding of 3 (●) to R1 by competitive inhibition of nucleotide reduction overlaid on Ac-Y-R2C19 (□) with a measured \( K_D \) of 10 \( \mu \text{M} \).
The benzophenone-derivatized peptide 4 was synthesized by addition of the commercially available Fmoc-para-benzoylphenylalanine (Fmoc-Bpa-OH) amino acid to Y-R2C19. The analytical reverse-phase HPLC trace is shown in Figure 5.3a. The absorption spectrum of Ac-BpaY-R2C19 (Figure 5.3b) is similar to that of the Bpa amino acid generated by competitive inhibition studies is shown in Figure 5.2d. Not only is binding retained, the dissociation constant is substantially tighter than either the Ac-Y-R2C19 control or Ac-WY-R2C19 ($K_{D{s}} \sim 20 \mu M$) [9].

Figure 5.3 Characterization of Ac-BpaY-R2C19 (4). (Panel A) Analytical reverse-phase HPLC trace of purified 4 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel B) UV-visible absorption spectrum of 4. (Panel C) MALDI-TOF mass spectrum of 4 in negative ion mode. The calculated $m/z$ (–H) is 2562 Da. (Panel D) Characterization of binding of 4 (•) to R1 by competitive inhibition of nucleotide reduction overlaid on Ac-Y-R2C19 (○) with a measured $K_D$ of 13 µM.
acid. Unlike free benzophenone, the amino acid derivative has a low absorption cross-
section for the nπ* transition of only 200 M\(^{-1}\)cm\(^{-1}\) [10]. The peptide was also analyzed by
MALDI-TOF MS to ensure its identity (Figure 5.3c). Binding studies with Ac-BpaY-
R2C19 show that the affinity towards R1 is unchanged by addition of the bulky Bpa

Both these peptides were synthesized without problems from their respective amino acid
starting materials and can provide 3 and 4 in quantitative yield from the R2C19
precursor. In addition, they are also found to retain the binding mode to R1 similar to Ac-
Y-R2C19, as evidenced by their ability to compete with R2 and inhibit nucleotide
reduction. With these peptides in hand, we will study their photochemistry with regard to
•Y356 generation in hopes that they will provide an inroad to the kinetic resolution of
radical transfer in the R1 PCET pathway.

5.3 Photoaffinity Labeling of R1 using Modified R2 peptides

5.3.1 Introduction

Although the redox activity of Y356 and its participation in the PCET pathway of RNR
has been established, its location between W48 and Y731 and its contacts with R1 remain
ambiguous. If the N-terminus of the 20-mer peptide were fully extended toward R1,
Y356 could be as close as 3-5 Å to Y731, however if it is not fully extended toward R1, it
could be as distant as 12-14 Å. If the mechanism of radical propagation through R1 is to
be determined, the position of Y356 and its distance from Y731 and W48 needs to be
further defined. Photoaffinity crosslinking is a widely used methodology to examine the
interactions between biological macromolecules and their ligands and may provide a
method for fixing the position of Y356 with respect to Y731. In this approach, the natural
ligand is modified with a photochemically active group that can achieve selective
modification of the binding site upon light irradiation [11]. In protein systems, the region
of binding of a radiolabeled photocrosslinker can be defined by proteolytic digestion, followed by peptide mapping and identification of the labeled adduct by mass spectrometry or N-terminal sequencing. Photocrosslinking experiments have been carried out with both the Herpes Simplex Virus (HSV) [12] and the mouse C-terminal peptides [13]. In the case of the HSV peptide, the N-terminal tyrosine corresponding to Y356 in the sequence alignment (Figure 5.4) was replaced with a p-azidophenylalanine, and found to label R1 specifically in a crude cell lysate [12], however, the location of the crosslink was not identified. A similar approach was taken for the mouse C-terminal peptide, however, the FTLDADF heptamer does not contain the critical tyrosine (Figure 5.4). Two separate peptides were synthesized and derivatized with a 3-azido-4-hydroxyphenyl propionyl group at different locations. The peptide with the photocrosslinking group at the N-terminal phenylalanine (Q374, E. coli numbering) was found to label a peptide equivalent to 713-728 (E. coli numbering) [13], the helix against which the R2 peptide docks in the R1 structure from E. coli [14]. The linkage is believed to be made with a tryptophan residue corresponding to Y722 in
R1, which is immediately adjacent to Q375 on the peptide (Figure 5.5). If the crosslinking group is moved one residue downstream to threonine (Figure 5.4), the label is found attached at two sites. The first site is the same fragment identified above, and the second location is at the C-terminus of R1, which is missing in the crystal structure but in the vicinity of the peptide-binding site. If this methodology can be established in the *E. coli* RNR, we can address the position of Y356 in the R1-peptide complex. In addition, we also have the opportunity to examine the location in an R1–R2 complex using intein chemistry [3,15] to site-specifically incorporate a cross-linking group at Y356 in R2. This would allow us to gauge the position and changes in position of Y356 within the active RNR complex and in the presence and absence of different substrates and effectors.

Choice of the crosslinking group is essential, as it needs to be chemically stable towards synthesis and purification. In addition, the linkage formed in the photochemical reaction needs to persist during isolation and analysis of the crosslinked products. We have taken two approaches to the design of Y-R2C19-based photoaffinity probes (Scheme 5.2). In the first approach, Y356 would be modified by an azide group, which eliminates nitrogen under UV excitation to form an electrophilic nitrene intermediate [16]. For simple aryl azides (such as the 3-azidotyrosine of Scheme 5.2), the aryl nitrene itself is not reactive enough to undergo crosslinking; instead, the nitrene undergoes rapid ring expansion to a dehydroazepine species that can react with nucleophilic groups (Nu) to form stable C–Nu linkages with the side-chains of serine, threonine, tyrosine, cysteine, lysine, and histidine [16]. If the aryl ring is fluorinated [17], the electrophilicity of the nitrene species is increased dramatically and C–C linkages with aliphatic groups are made by the nitrene intermediate. Although the fluoroaryl azides are in more common usage because of their greater reactivity, the choice of the azidotyrosine allows us to incorporate the photocrosslinking group directly into the peptide with minimal structural perturbation. Therefore, we have the potential to assess the position of Y356 directly and map a more
Scheme 5.2 Photocrosslinking reactions of aryl azides (top) and benzophenone (bottom).

accurate location for the phenol side-chain. If we do indeed require the formation of C–C crosslinkages, we can turn to the benzophenone amino acid [10] of Section 5.2, which can also be directly integrated into the 20-mer peptide. Bpa has been incorporated into synthetic peptides and shown to have high reaction yields for photocrosslinking reactions (50-100%) [18]. Excitation into the nπ* triplet state generates a powerful hydrogen atom-abstracting biradical species, which forms C–C linkages as shown in Scheme 1 [19]. In the absence of a reaction, the triplet species relaxes back to ground state, allowing continuous excitation until hydrogen abstraction takes place and thus provides higher reaction yields than the aryl azide family of crosslinkers.

This section presents the synthesis and characterization of two different photoaffinity labels for R1, [3H]-Ac-N3Y-R2C19 and [3H]-Ac-Bpa-SY-R2C19. [3H]-Ac-N3Y-R2C19 was found to crosslink with R1, however, the R1 peptide adducts have yet to be identified. Attempts to prepare the 22-mer peptide with an N-terminal cysteine, CS-N3Y-R2C19, for the purposes of site-specifically incorporating the cross-linking group into R2
[3,15] have not yet succeeded and will require a different synthetic path than that used for the preparation of Ac-N3Y-R2C19.

5.3.2 Results and Discussion

Preparation of Ac*-N3Y-R2C19. The target peptide containing the 3-azidotyrosine crosslinking group, Ac*-N3Y-R2C19, was prepared as outlined in Scheme 5.3. Following addition of 3-nitrotyrosine to R2C19 using standard methods, a tritium label is added with the N-terminal acetyl group using 3H-acetic anhydride. Palladium-catalyzed hydrogenation of Ac*-NO2Y-R2C19 (5) provides the corresponding 3-aminotyrosine peptide (6), which can be converted to the final product (7) through a diazo intermediate.

Synthesis of the radiolabeled starting material, Ac*-NO2Y-R2C19, proceeded smoothly by standard procedures. Although unnecessary, peptide was purified first before proceeding to the next step. The characterization of 5 is shown in Figure 5.6a-c, and includes the analytical HPLC trace, absorption spectrum, and MALDI-TOF MS. 5 was converted quantitatively to Ac*-NH2Y-R2C19 by palladium-catalyzed hydrogenation in a 1:1 methanol:0.1 M ammonium bicarbonate (pH 8.0) solution. The reaction was shielded with aluminum foil to prevent light-induced decomposition of 6. After 48 h incubation at
Figure 5.6 Characterization of Ac⁺-NO₂-Y-R2C19 (5) and Ac⁺-NH₂-R2C19 (6). (Panel A) Analytical reverse-phase HPLC trace of purified 5 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min. (Panel B) UV-visible absorption spectrum of 5. (Panel C) MALDI-TOF mass spectrum of 5 in negative ion mode. The calculated m/z(–H) is 2558 Da. (Panel D) Analytical reverse-phase HPLC trace of purified 6 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min). (Panel E) UV-visible absorption spectrum of 6. (Panel F) MALDI-TOF mass spectrum of 6 in negative ion mode. The calculated m/z(–H) is 2528 Da.

an atmospheric pressure of hydrogen, the characteristic yellow color of 5 had completely disappeared. In this step, it is important to assure that the pH of the buffer is not above 8.0, because the gradual rise in pH of the 0.1 M ammonium bicarbonate buffer (to pH ~9) can lead to yellow decomposition products of 7. The methanol was removed in vacuo before filtering the clear aqueous solution through a 0.2 μM syringe filter. The peptide was then lyophilized before purifying by reverse-phase HPLC; again, it is essential that the peptide does not thaw during lyophilization as decomposition of 7 (perhaps oxidation) occurs at elevated pH. The reduction of the nitro group proceeded very cleanly based on the HPLC trace of the crude 6 (data not shown) and was isolated in quantitative yield. An analytical HPLC trace of purified 6 is shown in Figure 5.6d. The UV-visible absorption spectrum demonstrates a shift of the absorption band to 289 nm, which is characteristic of
3-aminotyrosine (Figure 5.6c) [20]. The identity of 6 is further confirmed by MALDI-TOF MS (Figure 5.6f).

Generation of Ac*-N3Y-R2C19 from 6 was accomplished using a slight adaptation of literature procedures [21]. The lyophilized starting material, Ac*-NH2Y-R2C19, was stirred as a fine suspension in 0.1 M hydrochloric acid at 0 °C, wrapped in aluminum foil. Sodium nitrite was then added to generate the diazo intermediate, producing a bright yellow color. This was stirred for an additional 20 min before adding sodium azide to generate the final product, peptide 7. The reaction was allowed to stir for an additional 4 h at room temperature. Buffer (0.1 M ammonium bicarbonate, pH 8.0) was added to dissolve the suspension and 7 was purified immediately by reverse-phase HPLC. Figure 5.7a shows the characterization of purified 7 by analytical reverse-phase HPLC. The retention time is characteristically later than that of the starting material 6. In addition, the absorption spectrum has shifted from 289 nm to 296 nm and a second peak at 248 nm becomes visible (Figure 5.7b). The concentration of 7 was calculated using the average specific activity of peptides 5 and 6 that is based on their respective extinction coefficients [15,20]. From this specific activity, the extinction coefficient at 296 nm of 7 was determined to be 3,500 M⁻¹ cm⁻¹. The MALDI-TOF spectrum shows a mass identical to that for 6, perhaps due to loss of nitrogen upon laser excitation followed by protonation in the acidic environment of the buffer used for mass spectrometry. Thus, the identity of the azidotyrosine peptide 7 was confirmed by ESI MS (Figure 5.7c). Only the –2 ion was detectable on the ESI MS instrument, as the spectrometer is only capable of measuring mass-to-charge (m/z) ratios of less than 2000. The spacing between the isotope peaks clearly indicates a doubly-charged species (the m/z ratios of those species are removed for clarity in Figure 5.7c). The binding to R1 of 7 was then assessed and found to be retained with affinity (KD ~ 24 μM) similar to Ac-Y-R2C19 (Figure 5.7d). With the peptide 7 in hand, we then proceeded to crosslinking studies with 7.
Figure 5.7 Characterization of Ac-N$_2$Y-R2C19 (7). (Panel A) Analytical reverse-phase HPLC trace of purified 7 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min). (Panel B) UV-visible absorption spectrum of 7. (Panel C) ESI MS of the -2 charged anion of 7 in negative ion mode. The calculated m/z(–2H) is 1176.2 Da. (Panel D) Characterization of binding of 4 (●) to R1 by competitive inhibition of nucleotide reduction overlaid on Ac-Y-R2C19 (○) with a measured $K_D$ of 24 μM.
Photoaffinity labeling studies of R1 using Ac*-N$_3$Y-R2C19. Photoaffinity labeling with Ac*-N$_3$Y-R2C19 was performed with saturating concentrations of R1, as we were concerned that non-specific crosslinking could be a problem if there was a significant amount of unbound peptide in solution. During the course of these studies, we also realized that R1 could be concentrated to much higher levels (160-180 μM) using a YM30 Centricon rather than an Amicon with a PM30 membrane (maximum 80-90 μM). Note that using this method of concentration, the R1 was entirely stable and no precipitation was observed even after storage at −80 °C. The crosslinking reactions were carried out by irradiating photoactive peptide 7 (20 μM) using a 1000 W Hg-Xe lamp with a 90% neutral density and 285 nm longpass filters for 25 min at room temperature in a quartz cuvette in the presence of R1 (100 μM), substrate (CDP), and effector (ATP or TTP). In these conditions, the peptide is 90% bound, assuming R1 behaves as independent monomers with respect to peptide binding [9]. After our labeling reaction, we attempted to remove unreacted peptide by a series of concentrations and dilutions using a YM30 Centriprep, however, only a small percentage of the radioactivity (~10%) was removed in this manner. Initial tryptic digests were based on previous studies of R1 [22]. Briefly, R1 was first reduced with DTT and the thiols protected by reaction with iodoacetamide in the presence of 6 M guanidinium. After dialysis against water, the protein precipitate was suspended and subjected to trypsin digestion for 24 h. Following the literature procedure, both samples (with ATP and TTP as effectors) were pre-reduced and treated with iodoacetamide in guanidinium before overnight dialysis against water and trypsin digestion for 24 h at 37 °C. At this time, ~40% of the radioactivity remained in the sample. Approximately one-third of each sample (~8,500 cpm) was injected directly onto the HPLC and separated in a linear gradient of 0% to 50% acetonitrile versus 0.1% TFA over 75 min as reported [22] (Figure 5.8a-b). The HPLC trace collected at 280 nm, monitoring the aromatic residues, is shown with the trace at 214 nm. Both
Figure 5.8 Tryptic digests of crosslinking reactions of Ac^-N6Y-R2C19 with R1. The reverse-phase HPLC traces were collected in a linear gradient of 0% to 50% acetonitrile versus 0.1% TFA (1.0 mL/min) over 75 min. The fractions in which the radiolabel elutes is shaded in grey. (Panel A) Photoaffinity labeling of R1 in the presence of CDP and ATP. (Panel B) Photoaffinity labeling of R1 in the presence of CDP and TTP.
samples have very similar elution profiles, which were visibly identical to a tryptic digest of R1 alone that was carried out simultaneously with the photoaffinity labeling experiment. With either ATP (Figure 5.8a) or TTP (Figure 5.8b) as effector, the radioactivity eluted fairly late, between 27% to 34% acetonitrile, in a single broad peak containing 5,500 cpm (or 65% of the tritium loaded). Fractions were collected through an extended wash period and counted; no additional radioactivity was detected during the wash. Therefore, the loss of counts could be due to TFA or acetonitrile-induced quenching of tritium. The fractions containing radiolabel were then lyophilized individually and each analyzed separately by MALDI-TOF MS in both positive and negative ion modes. Although many of the peptides found in these fractions were identified as R1 fragments, there were many peptides which had unique masses. Auspiciously, neither the parent peptide 7 nor the photolyzed peptide corresponding to the molecular weight of 6 were observed in these mass spectra. The mass spectra were compared directly against those collected in the same region of the R1 digest. Several of the unique peptides were also found in the R1 digest, and some were found to correspond to contaminating R2 tryptic fragments. At this time, it was clear that we would need to re-chromatograph the isolated fractions in order to characterize the crosslinked species. However, the amount of material and radiolabel remaining after analysis was insufficient to properly identify any crosslinked fragments. Preliminary attempts to isolate crosslinked peptides indicate that the radiolabel was not recoverable from lyophilized samples that were resuspended in water, but only from fractions in which the tube was washed with 0.1 M ammonium bicarbonate, pH 8.0. When these fractions using this basic wash were re-chromatographed in 0.1% TFA, the radiolabel was recovered quantitatively in the void volume of the column.

At this time, we found that there were still a few areas of concern related to the analysis of the data generated from the photoaffinity labeling experiments. First, the insolubility
of the R2 peptide in acidic solution could skew the elution profiles observed upon chromatography in 0.1% TFA. Second, it was possible that partial digestion by contaminating chymotrypsin was occurring during the 24 h incubation period with trypsin. Consequently, we could be looking for adducts of the wrong size since there are two potential chymotrypsin sites on Ac*-N3Y-R2C19, at the 3-azidotyrosine itself and at F373. Third, although the free peptide is probably removed during dialysis, we would like to use a method for removing the unreacted peptide that allows quantitation of the extent of labeling. To address the first matter, the digest with ATP as effector was reanalyzed using a linear gradient of 0% to 75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 60 min (Figure 5.9). The radiolabel elutes in a much more congested area of the gradient, increasing the difficulty of isolating the R1-linked fragments. However, 84% of the loaded radioactivity was recovered with this buffer.

![Figure 5.9](image)

*Figure 5.9* Tryptic digest of crosslinking reactions of Ac*-N3Y-R2C19 with R1 in the presence of CDP and ATP. The reverse-phase HPLC trace was collected in a linear gradient of 0% to 75% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 60 min. The fractions in which the radiolabel elutes is shaded in grey.

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Figure 5.10 Tryptic digest of NO$_2$Y-R2C19 over 30 hours monitored by reverse-phase HPLC at 430 nm in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min.
system, eluting from 27% to 33% acetonitrile as it did in the gradient using 0.1% TFA. These results may point to a solubility issue with the R2 peptide and its R1 adducts in 0.1% TFA. Although these fractions containing tritium were analyzed by MALDI-TOF MS, the results were again ambiguous. Next, proteolytic digestion of NO₂Y-R2C19 was carried out to assay for chymotrypsin activity with 10 μM peptide and 1.4 μM trypsin, a similar concentration range as that used for the digestions above. The presence of the 3-nitrotyrosine at the N-terminus of the peptide allows us to monitor at 430 nm the fate of the residue containing the radiolabel. A 30 h time-course for the tryptic digest is shown in Figure 5.10. Although there are no basic residues contained within the sequence of the R2 peptide, proteolysis begins as soon as 2 h (data not shown) and more than 50% bond scission occurs within 30 h. Since the proteolyzed peptide chromatographs near the full-length peptide, we suspect that the peptide is being clipped at F373, with loss of the C-terminal QL dipeptide. Thus, it appears as if the 3-nitrotyrosine is not a candidate for digestion, or it would elute near the void volume of the column. MALDI-TOF MS of the collected fractions confirms that F373 is the site of digestion for the clipped peptide

![Graph](image)

**Figure 5.11** Separation of R1 and peptide 6 using a S-100 column with a 1:50 dilution factor. R1 (α) was measured using the absorption at 600 nm by Bradford assay and 6 (ε) was measured by scintillation counting.
species growing in and consequently that the contaminating chymotrypsin is incompletely inhibited by TPCK. We next turned our attention to the separation of the R2 peptide from R1 using size-exclusion chromatography on a small scale (20 μL). R1 (100 μM) was mixed with Ac*-NH₂Y-R2C19 (20 μM) in the presence of substrate and effector. The reaction was diluted five-fold, leaving ~13% of peptide bound before loading onto various columns. No separation between peptide and protein was observed on either G-25 (1:50 dilution factor) or G-50 (1:50 dilution factor) columns as the R1 and peptide, measured respectively by Bradford assay and scintillation counting, were found to co-elute (data not shown). Therefore, we turned to an S-100 column (1:50 dilution factor), in which separation between the peptide and R1 was achieved (Figure 5.11). Approximately 45% of the radioactivity was recovered, of which 13% was found to elute with the R1 peak (fractions 8-12). As there two distinct radiolabeled peaks corresponding to the peptide, we believe that separation between the free peptide and R1 has been achieved and that the residual peptide eluting with R1 is most likely bound.

The crosslinking reaction was repeated under the same conditions with R1 (100 μM), Ac*-N₃Y-R2C19 (20 μM), CDP (1.0 mM), and ATP (1.6 mM). The reaction with TTP was omitted because the previous results with TTP were indistinguishable from those using ATP as the allosteric effector. This time, however, irradiation conditions using a hand-held UV lamp at room temperature for 10 min were based on the commercial protocol by Pierce Biotechnology. Following the photoreaction, the sample was diluted five-fold and passed through an S-100 column in the dark (1:75 dilution factor). Figure 5.12 shows the elution profile of R1 and the peptide. The free peptide was eluted in a very broad peak, possibly due to cross-linking of 7 on the column. Although the free peptide was not fully eluted, ~65% of the total radioactivity loaded was recovered from the column, again perhaps due to quenching of tritium. Due to the trailing of the peak
corresponding to free peptide, we base the following calculations on a conservative estimate that fractions 30 to 48 (Figure 5.12) contains 85% of the free peptide in the reaction. If so, the R1 peak contains 22% of the peptide (8,000 cpm), yielding approximately 10% crosslinking of the peptide if compared directly to the dark reaction with peptide 6. The extent of labeling may actually be higher than 10% because the dilution factor of the S-100 column from 1:50 to 1:75, thereby reducing the background of non-covalently bound peptide from that shown in Figure 5.11. Indeed, later studies with Bpa-modified peptide 11 show that a 1:75 dilution is sufficient to remove all non-covalently attached R2 peptide from R1. Fractions 20 to 27 were then pooled and concentrated using a YM50 Centriprep. Preparation of the protein for digestion was carried out in an identical fashion as presented above. Approximately 40% of the radioactivity recovered from the S-100 column was consumed in counting, Bradford assays, and transfers. Another large portion (~40%) was lost during overnight dialysis, perhaps due to instability of the linkage, leaving only ~20% (1,600 cpm) of the R1–peptide photocrosslinking reaction. The trypsin digest was only allowed to proceed
Figure 5.13  Tryptic digest of crosslinking reactions of Ac-N*Y-R2C19 with R1 in the presence of CDP and ATP. The free peptide 7 was removed using an S-100 column. The reverse-phase HPLC trace was collected in a linear gradient of 0% to 50% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 75 min. The fractions in which the radiolabel elutes is shaded in grey.

for 2.5 h, before HPLC analysis to prevent substantial proteolysis by the contaminating chymotrypsin. Figure 5.13 shows the reverse-phase HPLC trace of the tryptic digest in a linear gradient of 0% to 50% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 75 minutes. The elution profile is different from that shown for the 24 h tryptic digest in Figure 5.9, possibly due to the steeper gradient used to collect the HPLC trace of Figure 5.9 or the shorter digestion time for crosslinking reaction of Figure 5.13. A small amount of radioactivity was observed to elute from 21% to 27% acetonitrile, which is lower than previously observed. However, if one examines the three distinctive peaks in the HPLC trace at 280 nm (Figure 5.9: 20 min, 23 min, and 29 min; Figure 5.13: 33 min, 36 min, and 40 min), it appears as the radiolabel is eluting in the same region of fragments. At this time, there was insufficient amount of radiolabel to subject the sample any further analysis besides MALDI-TOF MS, which was again ambiguous.
Efforts to prepare a peptide directed at site-specific incorporation of 3-azidotyrosine at position Y356 in R2. The 22-mer peptide containing the 'Buthio-protected cysteine at its N-terminus and 3-nitrotyrosine at position 356 was synthesized for the purposes of incorporating the azidotyrosine site-specifically into R2 using protein semi-synthesis [15] (Scheme 5.4). The intention was to use the synthetic route of Scheme 3 on the 22-mer peptide in order to generate the C-terminal peptide containing 3-azidotyrosine at Y356 with the requisite cysteine at the N-terminus for ligation to an R2(1-353) thioester to produce an R2 with azidotyrosine at position 356 (Scheme 5.4). Thus, the starting material, Cys('Buthio)-SNO₂-Y-R2C19 (8) was synthesized using literature procedures and characterized by analytical HPLC and MALDI-TOF MS (Figure 5.14). Reduction of 3-nitrotyrosine to 3-aminotyrosine was attempted by hydrogenation with Pd/C, as it was achieved with the 20-mer peptide. Although the characteristic 3-nitrotyrosine absorption had disappeared, the product was not observed by reverse-phase HPLC, as the peaks

![Scheme 5.4 Intein-based method for generating R2 with 3-azidotyrosine incorporated at position 356. The R2(1-353) thioester is generated by standard methods from cleavage of a R2(1-353)-intein chimera with a small thiol (12). R2(1-353) is then ligated with the C-terminal (354-375) synthetic peptide containing the 3-azidotyrosine R2 with an N-terminal cysteine to produce R2 site-specifically modified at Y356.](image-url)
eluting had absorption maxima at 285 nm rather than at 289 nm. As there was concern that the cysteine deprotection was occurring upon hydrogenation, the peptide was chromatographed with and without 5 mM DTT in 5 mM potassium phosphate pH 7.0, however, no difference was seen in the elution profile. MALDI-TOF MS of the hydrogenated 8 showed some evidence of the desired product, with the cysteiny l 'Buthio protecting group removed, but the majority of the material existed as unidentified −30 and +25 Da modifications of the desired CS-NH₂Y-R2C19 peptide. Since the free 'Buthio group could be poisoning the palladium catalyst, the cysteine was first deprotected with DTT to remove half of the free thiols in the reaction and dialyzed before hydrogenation to generate CS-NO₂Y-R2C19 (9) [15]. Palladium-catalyzed hydrogenation was carried out on 9 in 50 mM Tris, pH 8.0 by preparing the reaction in the glove box and purging with hydrogen before stirring for 48 h under atmospheric hydrogen pressure. The reaction was then analyzed by reverse-phase HPLC in the presence of DTT, however, the CS-NH₂Y-R2C19 synthetic target was not observed. Again, the side-product exhibited an absorption maximum at 285 nm and was shown to be a +131 adduct by MALDI-TOF MS, which could be a combination of the original +25 Da modification and a coordinated

![Graph](image)

*Figure 5.14 Analytical reverse-phase HPLC trace at 430 nm of purified 8 in a linear gradient of 10%-65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 (1.0 mL/min) over 45 min.*
palladium (+106 Da). Anaerobic reduction by dithionite [20] was also assayed in both 0.1 M ammonium bicarbonate, pH 8.0 as well as in 50 mM Tris, pH 8.0 buffers. The appropriate color change was seen immediately upon addition of ~20 eq of dithionite. The desired product was observed by MALDI-TOF MS, along with a second species with a +50 Da adduct. The peptides elute at the void volume of the column in the presence of dithionite, even after salt removal using a C-18 Sep-Pak. The collective peptide material exhibit absorption maximums at 289 nm (3-aminotyrosine) and 310 nm. Thus it appears as though the N-terminal cysteine is causing complications during reduction. Other palladium catalysts, which are less susceptible to thiols, could be assayed. In addition, a protected Fmoc-3-aminotyrosine derivative could be synthesized and directly incorporated into the 22-mer peptide.

**Photoaffinity labeling studies with Ac*-Bpa-SY-R2C19.** Another possibility is to use the benzophenone derivative, Bpa, rather than 3-azidotyrosine to carry out crosslinking. This amino acid is more robust and synthetically accessible than either the 3-aminotyrosine or the 3-azidotyrosine and would be simpler to incorporate into our studies, especially those concerning site-specific modification of R2. In addition, it is capable of forming stable C-C linkages. As a preliminary foray into the Bpa photochemistry, tritium-labeled 21-mer peptide Ac*-Bpa-SY-R2C19 (10) was synthesized. The serine spacer was added due to optimistic concerns that •Y356 might be generated if it were immediately adjacent to the Bpa residue. The characterization of the purified peptide 10, is shown in Figure 5.15a-c and includes an analytical HPLC trace, UV-absorption spectrum, and MALDI-TOF mass spectrum. Binding studies were not carried out because the untritiated version of 10 was not synthesized as it was for Ac-N3Y-R2C19. In addition, we assume that its affinity for R1 should be similar to Ac-Bpa-R2C19 (Figure 15.3). Initial studies in HEPES and Tris buffers were carried out using
peptide 10 under the same conditions as described for peptide 7 with ATP as effector. Samples were chromatographed on a S-100 column (1:75 dilution factor) after irradiation using a hand-held UV lamp. As previously mentioned above, almost no co-elution of the peptide was observed in the dark reaction, meaning that a 1:75 S-100 column is sufficient to separate non-covalently bound peptide from R1 (Figure 5.16). However, light reactions in HEPES and Tris also showed no co-elution of the photoaffinity probe, and thus no crosslinking, with R1 (data not shown). Possible explanations for this phenomenon are that buffer molecules, especially Tris, may be reacting with Bpa, addition of the serine

Figure 5.16 Separation of R1 and peptide 10 using a S-100 column with a 1:75 dilution factor. R1 (●) was measured using the absorption at 600 nm with a Bradford assay and 6 (●) was measured by scintillation counting.
spacer may have placed the Bpa group too far from R1 to make contacts, or perhaps the light source or reaction time may have been inadequate for acceptable extent of labeling.

Overall, it appears as if R1 is being labeled by the Ac*-N₃-Y-R2C19 photoaffinity probe for Y356, meaning that a residue at position 356 is able to make contacts with R1 within the lifetime of the dehydroazepine intermediate. The radiolabel elutes consistently in the same region, whether the HPLC analysis is carried out in acidic or basic conditions. We believe that a greater extent of labeling occurred when the 1000 W lamp was used rather than the hand-held UV lamp, and that the experiments incorporating the S-100 column to remove unbound peptide and the shorter tryptic digest need to repeated under those conditions. We may also want to assay photocrosslinking conditions in which the peptide, rather than R1, is saturating. Even with a 10% to 20% yield for the crosslinking reaction, this only corresponds to a concentration of 2 to 4 μM R1–peptide adduct; as R1 is dimeric, only 1-2% of the tryptic digest corresponds to photoaffinity-labeled material. Additional studies are required with the Ac*-Bpa-SY-R2C19 probe to determine whether the conditions or the location of the probe needs to be altered. One possibility is that Bpa should be placed at position 356; this would also allow for better resolution of the geometric position of Y356. Due to the size of R1, the tryptic profile is quite complex and it may be therefore necessary to add an additional handle to the photoaffinity probe in order to aid in identification of the cross-linked adducts. This could either be an affinity tag such as biotin, or an optical label, such as a dye. Another possibility is to generate antibodies to the R2 C-terminal tail and pass the tryptic digest over an antibody column, as was done in the previous crosslinking studies with the mammalian RNR [13].

5.4 Fluorotyrosine Incorporation into the R1 Subunit

5.4.1 Introduction

Mechanistic enzymology has been limited by the 20 natural amino acids represented by the genetic code. Site-directed mutagenesis, although powerful, is often insufficient to
answer the detailed chemical questions about mechanism since conservative changes are difficult to make. Of late, attention has been focused on the use of unnatural amino acids designed to answer specific mechanistic questions [23]. For example, fluorinated tyrosine analogues have been used as probes because of the large range of pKₐs accessible to the phenolic proton upon fluorination with minimal structural perturbation. A full range of fluorotyrosines have been synthesized enzymatically that shift pKₐ of the phenolic proton from 10 in tyrosine to 5.2 in the tetrafluoro-substituted derivative [24]. Of course, as the pKₐ is lowered, there is a corresponding increase in the oxidation potential. Indeed, recent studies on R2 using intein-splicing methods have successfully incorporated the 2,3-difluorotyrosine as a site-specific probe of hydrogen atom transfer through Y356 [3]. Since the 3-fluorotyrosine and 2,3-difluorotyrosine derivatives are relatively similar to the natural amino acid (Figure 5.17), in vivo global replacement of tyrosine in proteins by its fluorinated equivalent has been possible [25-28]. In addition, as seen in 3FY-R2,

![Diagram of various fluorinated tyrosines and their pKₐs](image)

*Figure 5.17 Various fluorinated tyrosines and their pKₐs from ref. 24.*

fluorination of the tyrosine ring shifts the absorption of the tyrosyl radical, which may be used as an optical signature. For our purposes, replacement of tyrosine with a fluorinated derivative in the R1 subunit would be instructive both as a spectroscopic marker to differentiate the tyrosyl radicals belonging to one subunit from the other, as well as a probe of redox and pKₐ effects for PCET.
In this section, we describe the overproduction and purification of 3FY-R1 using in vivo methods for the global replacement of tyrosine with 3-fluorotyrosine. We further attempt the isolation of 2,3FY-R1, however, R1 is not induced in cells grown in the presence of 2,3-difluorotyrosine even if the pH of the media is lowered. The purification of 3FY-R1C225S is also presented with the intention of examining the kinetics of tyrosyl radical reduction using CDP as an inhibitor. Although the mutant was isolable, the instability of the protein prevents further studies using stopped-flow UV-visible spectroscopy.

5.4.2 Results and Discussion

Global replacement of a particular amino acid is typically achieved by growing auxotrophic cells in a minimal media containing all factors needed for growth, such as a carbon source and inorganic salts, in the absence of the particular amino acid. Just prior to induction, the unnatural amino acid is added to the growth media and is thus incorporated into the over-expressed protein. We based the rich media (Table 5.1) used for growth of E. coli BL21(DE3) cells containing the R1 overexpression plasmid on previous methods developed in the Stubbe group to incorporate 3-fluorotyrosine into R2 for the purposes of ENDOR studies. The glycerol/salt solution was problematic as magnesium phosphate was precipitated upon preparation and further precipitated upon raising the pH to 7.0 (K_{sp} = 10^{-24}) [29]. When M-9 [30] was used as the media base for the carbon and salt source, no growth of the cells were seen within 6 h. Thus the glycerol/salt solution was made to the specifications of Table 5.1 and the supernatant poured off for use in media preparation. Sterilized amino acid stock solution was prepared easily at a 20× concentration and stored at 4 °C for up to one month. Pyrimidine bases were also added, while purine bases were added as nucleosides due to solubility issues. This solution was stored at 40 °C overnight after autoclaving to prevent precipitation upon cooling. Vogel-Bonner salt solution was stored indefinitely after
autoclaving. Vitamins were added as solids just prior to inoculation. Inoculation and growth of *E. coli* was carried out using standard procedures. Doubling times (40-60 min) were not drastically different than those seen for growth in LB medium. 3-Fluorotyrosine was added as a solid 10-15 min before induction when the $A_{600\text{ nm}}$ reached 0.7–0.8 OD. IPTG (0.5 mM final concentration) was then added ($A_{600\text{ nm}} \sim 1.0$ OD) to induce R1 production. R1 was induced slightly later than usual, because it was observed that the cell mass yield was increased, with no detrimental effects on the level of induction of R1 as visualized by SDS-PAGE. Induction was allowed to proceed for 4-5h before harvesting and flash-freezing. Purification of the 3FY-R1 was carried using the standard procedure for R1 [31], except that the anion-exchange purification step was omitted. The protein is less stable than wt-R1, and tends to precipitate when stored above a 40 μM concentration. Figure 5.18a shows the SDS-PAGE gel of 3FY-R1 after each purification step. 3FY-R1 was found to bind normally to the dATP affinity column as no 3FY-R1 was observed in the flow-through or wash (data not shown). The specific activity for 3FY-R1 was measured to be 340 nmol min$^{-1}$mg$^{-1}$ using the spectrophotometric assay. 3FY-R1 was flash-frozen and stored at $-80$ °C. A fresh aliquot was used every time the enzyme was assayed, as precipitation was observed in samples that had been re-frozen. The final
protein was characterized by MALDI-TOF and ESI mass spectrometry to confirm incorporation of 3-fluorotyrosine. The expected mass of 3FY-R1 is 86,293 Da if 3-fluorotyrosine is fully substituted in place tyrosine, which is a difference of +738 Da from wt-R1. The spectrum shows the parent ion at an \( m/z \) of 86,424 Da, as well as the doubly-charged ion and the dimer species (Figure 5.18b). The observed molecular mass is much greater than expected for MALDI-TOF and lead us to suspect that methionine was being left on the N-terminus of 3FY-R1, which was confirmed by protein sequencing. Thus, the mass measured by MALDI-TOF is different from the expected molecular mass of 3FY-R1+Met (\( m/z_{\text{calc}} = 86,477 \) Da) by -53 Da or 0.06%, which falls within the error range of MALDI-TOF MS. This mass difference could either be due to instrument error or 96% incorporation of 3-fluorotyrosine. 3FY-R1 was also compared by ESI MS to wt-R1 because of the lower error for measurement of protein molecular masses (0.01%). The ESI MS was only obtainable when 3FY-R1 was submitted at 20 \( \mu \)m concentration. Data was collected by direct infusion at 5 \( \mu \)L/min in a 1:10 dilution with a solution composed of 50% acetonitrile/0.2% acetic acid. As the observed mass (86,411 Da) was less by 0.08% than expected for 100% incorporation, therefore it seems likely that slightly less than 100% incorporation of 3-fluorotyrosine is achieved. Note that the error in the molecular mass for wt-R1 was 0.02%-0.04%, which although greater than the theoretical error, is still less than the mass difference observed between the isolated 3FY-R1 and the expected mass for a protein with 100% incorporation of 3-fluorotyrosine.

A pH-rate dependence study was undertaken and compared to wt-R1. Although interpretation of the results would be complicated, we wanted to see if maximal activity of 3FY-R1 was shifted to lower pH due to the lower \( pK_a \) of the unnatural amino acid (\( pK_a = 9.0 \)). Both wt-R1 and 3FY-R1 were assayed at pH 7.0, 7.6, 8.0, and 9.0 using a standard discontinuous assay for dCDP formation [I]. The specific activity measurements
for wt-R1 and 3FY-R1 are shown in Figure 5.19a and 5.19b respectively. The pH-rate profile of both R1s are overlaid upon each other in Figure 5.19c for a direct comparison. The pH for maximal activity of 3FY-R1 is indeed shifted to lower pH compared to wt-R1. More dramatically, however, the relative activity has dropped reproducibly to less than 10% of the maximal activity for 3FY-R1 at the pKₐ of 3-fluorotyrosine, whereas the activity is maintained at about 65% at pH 9.0 for wt-R1. In addition, the specific activity measured for 3FY-R1 at pH 7.6 is higher than that measured using the spectrophotometric assay. Although all tyrosines in the protein are substituted with 3-fluorotyrosine and many factors could be involved, including structural perturbation [32], no other tyrosine residues in R1 are currently known to participate in the nucleotide reduction process besides the Y730 and Y731 tyrosine pair. Therefore, we decided to
attempt incorporation of 2,3-difluorotyrosine into R1 to further examine the effect of altering the phenolic $pK_a$.

Although many reports of global replacement of 3-fluorotyrosine are found in the literature, very few can claim in vivo substitution of 2,3-difluorotyrosine. High efficiencies of incorporation were seen in $\Delta^5$-3-ketosteroid isomerase, however, substitution of only one tyrosine was attempted [28]. R1, on the other hand, contains 41 tyrosines per monomer. 2,3-difluorotyrosine (11) was synthesized enzymatically using tyrosine phenol lyase and isolated as previously reported [24]. All attempts at R1 induction using 2,3-difluorotyrosine in the growth medium showed no evidence of expression. The pH of the media was varied from 6.3 to 7.2, but still no change in the induction profile was seen by eye. As it was possible that R1 was being induced in low yield and not visible by SDS-PAGE, we took one such growth of cells through purification since the affinity column should allow isolation of small amounts of enzyme. No R1 was eluted from the dATP column and it therefore appears that 2,3-difluorotyrosine is not easily substituted into R1.

A way to link changes observed in the pH-rate profile of 3FY-R1 to PCET would be to compare the R1C225S mutant with the 3-difluorotyrosine analogue. The R1C225S mutant is inactivated by the normal substrate, CDP, resulting in $\bullet$Y122 reduction, protein alkylation, and cleavage of R1 at S225 [33,34]. Interestingly, this mutant also shows a $V_{max}$ isotope effect of 2.0 on $\bullet$Y122 reduction with [3'-2H]-CDP [33]. It would be possible to examine the pH dependence of tyrosyl radical loss, and if necessary, the isotope effect on $\bullet$Y122 reduction could be studied as well. If an altered pH dependence on this reaction exists between R1C225S and 3FY-C225S, our model for radical transfer between the R1 active site and Y122 would necessarily invoke involvement of Y730 and Y731. Preparation of the R1C225 proceeded smoothly, however, there were problems
isolating the 3FY-R1C225 mutant. All steps proceeded normally until the final G-25 column for removal of ATP and DTT from the protein solution (after elution from the dATP affinity column using 10 mM ATP). At this time, ~75% of the protein precipitated on the column. Continued elution of the column allowed isolation a small amount of protein (~5 mg). The 3FY-R2C225S was concentrated to only 10-15 μM before flash-freezing. SDS-PAGE gels of both proteins are shown in Figure 5.20. The stability of the 3FY-R2C225S mutant remains a concern, as R1 precipitate is observed upon thawing. Thus, it appears as if the stopped-flow studies required to monitor tyrosyl radical reduction in R2 in the presence of CDP would be extremely difficult to do, as it requires very high concentrations of protein. Although 3FY-R1C225S can probably be concentrated to higher levels in the presence of R2, it is unlikely that these types of studies would be viable with this construct.

Global incorporation of 3-fluorotyrosine in R1 is carried out without difficulty with in vivo methods used for R2. This provides a spectral signature for tyrosyl radicals within R1, as fluorination shifts the absorption from unsubstituted tyrosine. Indeed, the 3FY-R2 protein exhibits a blue-shifted tyrosyl radical absorption from wt-R2. In addition, there may be an altered pH dependence on PCET that can be further examined using our methods for initiating turnover with the derivatized R2 C-terminal tail using light. The
mutant 3FY-R1C225S was not isolable in high yields. Although the yield may be increased by modifications to the protein preparation method, it is unlikely that it could ever stably concentrated past 20-30 μM, also indicating that other mutants of R1, such as Y731F and Y730F, containing 3-fluorotyrosine may be difficult to prepare for spectroscopic studies.

5.4 Conclusions

We hope that the developments presented in this Chapter will allow us to further clarify the radical initiation process of the class 1 RNRs. The ability to initiate PCET and nucleotide reduction synchronously with light should provide a means to kinetically resolve a long-range radical propagation for the first time in a physiologically-relevant protein system. The second-generation peptides incorporating 6-nitrotryptophan and Bpa, are ideal for our purposes as they can be excited away from the protein absorption envelope, and could also be used for site-specific modification of R2 by semi-synthetic methods. If we can generate •Y356 in a full-length R2, we also may be able to resolve the reverse PCET reaction to produce the stable •Y122. It would be interesting as well to examine PCET in an R1–R2 complex and to elucidate the directionality of radical propagation and its dependence on substrate and/or effector. It is possible that the radical equivalent would advance to •C439 in the presence of substrate and/or effector yet revert to •Y122 in their absence. Initial studies with photoaffinity labels derived from the R2 C-terminal peptide are quite promising as they show that R1 is indeed reactive toward the 3-azidotyrosine crosslinking group. Eventual identification of R1 adducts of the R2 peptide will help define the surfaces of R1 that contact Y356, and consequently the distance between Y356 and Y731 in R1 and W48 in R2. Global replacement of tyrosines in R1 with 3-fluorotyrosine provides a simple approach to distinguish •Y356 from •Y731 and •Y730 and may garner further insight into the proton dependence of PCET between Y356 and C439. Eventual efforts at R1 semi-synthesis or unnatural amino acid mutagenesis in
R1 using stop codon suppression will help further elucidate the mechanism of PCET within R1 by allowing controlled alterations of redox potential and $pK_a$ in the Y730–Y731 pathway.

5.6 Experimental methods

Materials. L-Tryptophan, 9-fluorenylmethyl chloroformate (Fmoc-Cl), diisopropylethylamine (DIEA), triisopropylsilane (TIS), peptide synthesis grade trifluoroacetic acid (TFA), TPCK-inhibited bovine trypsin (T-1426), Sephacryl 100 (S-100), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Fmoc-L-para-benzoyl-phenylalanine (Fmoc-Bpa-OH) was purchased from Advanced ChemTech (Louisville, KY). Fmoc-Cys('Bu)-OPfp was purchased from Novabiochem (San Diego, CA). Fmoc-Tyr('Bu)-OH, Fmoc-Ser(O'Bu)-OH, HATU, and 20% (v/v) piperidine in DMF were purchased from Applied Biosystems (Foster City, CA). Fmoc-3-nitrotyrosine, angiotensin I, and ACTH 18-39 was obtained from Bachem Chemicals (King of Prussia, PA). $[^3]$H-Acetic anhydride (Lot #030210, 100 mCi/mmol) was purchased from American Radiolabelled Chemicals (St. Louis, MO). 1,4-Dithiothreitol (DTT) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). Disposable columns were purchased from Bio-Rad Laboratories (Hercules, CA). Reverse-phase C-18 X Terra MS 5 μM columns were purchased from Waters Corporation (Milford, MA). Centricon and Centriprep concentrators were purchased from Millipore (Billerica, MA). Micro Bio-spin 6 columns and AG 50W-X8 resin were purchased from Bio-Rad Laboratories (Hercules, CA). Slide-a-Lyzer dialysis cassettes were obtained from Pierce (Rockford, IL). PM30 membranes were purchased from Millipore (Billerica, MA).

General method for manual addition of Fmoc-amino acids to Fmoc-R2C19 using HATU. Fmoc-R2C19 (100 mg, 0.025 mmol) was deprotected by $3 \times 7$ min treatments with 20% (v/v) piperidine containing 0.1 M HOBT in DMF in a 20 mL fritted column.
The reaction was mixed by a Vortex mixer using a foam adapter for 50 mL Falcon tubes, in which the column was placed. After washing 5 × DMF and 3 × acetone, Fmoc amino acids were added using a 0.9 HATU:2 DIEA activation scheme. Briefly, Fmoc amino acid and HATU were dissolved in 250 μL DMF to 0.5 M (0.125 mmol) and 0.45 M (0.113 mmol) respectively. All three components were combined and then activated by the addition of DIEA (44 μL, 0.25 mmol). The coupling was allowed to proceed for 1 hr before washing 5 × DMF. Removal of the N-terminal Fmoc was then carried out as described for Fmoc-R2C19 above.

**Isolation of crude peptide material.** Peptides were cleaved from the resin by vortexing for 3 h in a solution composed of 95% (v/v) TFA, 2.5% (v/v) TIS, and 2.5% (v/v) water. The peptide solution was collected by filtration and concentrated under a stream of N₂. The peptide was then precipitated by addition of diethyl ether. The suspension was incubated at −10 °C and pelleted by centrifugation. The peptide pellet was washed twice with ether and air-dried. The semi-dried pellet was solubilized in 0.1 M ammonium bicarbonate, pH 8.0.

**Semi-preparative reverse-phase HPLC (Gradient A).** Peptides were isolated by preparative HPLC was on an Waters C-18 XTerra MS 5 μM column (19 mm × 100 mm) using a linear gradient (22.5 mL/min) of 10% to 65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 45 min. Fractions were collected by hand and lyophilized.

**Analytical reverse-phase HPLC (Gradient D).** Peptides were analyzed by HPLC on a C-8 XTerra MS 5 μM column (4.6 mm × 100 mm) using a linear gradient (1.0 mL/min) of 10% to 65% acetonitrile versus 0.1 M ammonium bicarbonate, pH 8.0 over 45 min. Fractions were collected by hand and lyophilized.
Buffer exchange. Peptides were exchanged into phosphate buffer by reverse-phase HPLC on a C-18 XTerra MS 5 μM column (4.6 × 100 mM) in a linear gradient of 10% to 65% acetonitrile (1.0 mL/min) versus 4 mM potassium phosphate, pH 7.0 over 45 min. The peptide was then lyophilized and resuspended in water.

6-L-Nitrotryptophan (1). 6-L-nitrotryptophan was prepared from L-tryptophan by literature procedures [35]. $^1$H NMR (300 MHz, D$_2$O, 25 °C): $\delta$ = 7.93 (s, 1H, ArH), 7.67 (d, $J$ = 9.0 Hz, 4H, ArH), 7.49 (d, $J$ = 8.7 Hz, 4H, ArH), 3.54 (t, $J$ = 6.3 Hz, 1H, C$_6$H), 3.03 (ddd, $J_1$ = 27.0 Hz, $J_2$ = 14.7 Hz, $J_3$ = 5.7 Hz, 2H, C$_6$H$_2$). UV-Vis: $\lambda_{max}$ = 253 nm, 265 nm, 335 nm, 375 nm.

Fmoc-6-L-nitrotryptophan (2). Protection of the amino group of 6-nitrotryptophan with an Fmoc group was achieved by adaption of the reported procedure [4]. 2 (250 mg, 1.0 mmol) was dissolved in 10% sodium carbonate (3.0 mL). An additional 2 eq of sodium carbonate (212 mg, 2.0 mmol) were added to raise the pH to 10-11. Dioxane (3 mL) was then added to the reaction, which was allowed to equilibrate in an ice bath with stirring. Fmoc-Cl (259 mg, 1.0 mmol) was then dissolved in dioxane (1.25 mL) and added dropwise to the stirring reaction. The reaction was stirred for an additional 2 h in the ice bath and allowed to warm up to room temperature and to proceed overnight. The suspension was partitioned between water and diethyl ether and the aqueous layer extracted several times with diethyl ether. The aqueous layer was then acidified to pH 2.0 with 6 M hydrochloric acid, precipitating the yellow product in quantitative yield. $^1$H NMR (300 MHz, d$_6$-acetone, 25 °C): $\delta$ = 11.32 (s, broad, 1H, COOH), 10.84 (s, 1H, N$_{Trp}$H), 8.38 (d, $J$ = 1.2 Hz, 1H, Ar$_{Trp}$H), 7.93 (dd, $J_1$ = 8.7 Hz, $J_2$ = 1.4 Hz, 1H, Ar$_{Trp}$H), 7.82 (d, 2H, Ar$_{Fmoc}$H), 7.82 (d, 1H, Ar$_{Trp}$H), 7.63 (d, 1H, Ar$_{Trp}$H), 7.63 (d, 2H, Ar$_{Fmoc}$H), 7.38 (t, $J$ = 7.5 Hz, 2H, Ar$_{Fmoc}$H), 7.26 (t, $J$ = 7.5 Hz, 2H, Ar$_{Fmoc}$H), 6.75 (d, $J$ = 8.4 Hz, 1H, N$_{amids}$H), 4.8 (ddd, $J_1$ = 8.4 Hz, $J_2$ = 8.4 Hz, $J_2$ = 4.8 Hz, 1H, C$_6$H), 4.28 (m, 2H,
C_{FmocH_2}, 4.17 (t, J = 6.9 Hz, 1H, C_{FmocH}), 3.46 (dd, J_1 = 14.7 Hz, J_2 = 5.1 Hz, 1H, C_βH_1),
(dd, J_1 = 14.7 Hz, J_2 = 8.4 Hz, 1H, C_βH_2).

6-Nitrotryptophan-YLVGQIDSEVDTDLSNFQL (=NO_2WY-R2C19) (3). 3 was synthesized from R2C19 by the successive additions of Fmoc-Tyr(\(^i\)Bu)-OH and 2 using 0.9 HATU: 2.0 DIEA activation chemistry. The peptide was shielded from light using aluminum foil. 3 was then isolated by reverse-phase HPLC using gradient A. Purified 3 was analyzed by HPLC using gradient B. 3 was also exchanged into 5 mM potassium phosphate buffer, pH 7.0 by dialyzing against 2 \times 4000 mL of buffer using Spectra/Por 1000 MWCO tubing. HPLC: \(t_R = 19.2\) min. MALDI-TOF MS: \(MW_{\text{calc}} = 2501\) Da, \(MW_{\text{obs}} = 2502\) Da. UV-Vis: \(\lambda_{\text{max}} = 253\) nm, 265 nm, 335 nm (7,000 M\(^{-1}\)cm\(^{-1}\)), 375 nm.

N-Acetyl-para-benzoylphenylalanine-YLVGQIDSEVDTDLSNFQL (=Ac-BpaY-R2C19) (4). 4 was synthesized from R2C19 by the successive additions of Fmoc-Tyr(\(^i\)Bu)-OH and Fmoc-Bpa-OH using 0.9 HATU: 2.0 DIEA activation chemistry. The peptide was shielded from light using aluminum foil. N-terminal acetylation was achieved by incubation in a solution of 0.5 M acetic anhydride and 0.5 M DIEA (1.0 mL). 4 was then isolated by reverse-phase HPLC using gradient A. Purified 3 was analyzed by HPLC using gradient B and exchanged into potassium phosphate buffer. HPLC: \(t_R = 20.9\) min. MALDI-TOF MS: \(MW_{\text{calc}} = 2563\) Da, \(MW_{\text{obs}} = 2562\) Da. UV-Vis: \(\lambda_{\text{max}} = 264\) nm (18,000 M\(^{-1}\)cm\(^{-1}\)), 320 nm (200 M\(^{-1}\)cm\(^{-1}\)).

\[^3\text{H}\]-N-Acetyl-3-nitrotyrosine-LVQGIDSEVDTDLSFNQL (=Ac*-NO_2Y-R2C19) (5). 5 was synthesized by the coupling of Fmoc-3-nitrotyrosine with R2C19 using 0.9 HATU: 2.0 DIEA activation chemistry. The N-terminus was acetylated with \[^3\text{H}\]-acetic anhydride (52,500 cpm/nmol, 21 \(\mu\)L, 0.225 mmol) and DIEA (39 \(\mu\)L, 0.225 mmol) in 0.5 mL DMF for 45 minutes. The peptide was isolated by reverse-phase HPLC as described using gradient A. Purified 5 was analyzed by HPLC using gradient B. HPLC: \(t_R = 13.3\)
min. MALDI-TOF MS: \( MW_{\text{calc}} = 2358 \text{ Da} \), \( MW_{\text{calc}} = 2359 \text{ Da} \). UV-Vis: \( \lambda_{\text{max}} = 285 \text{ nm} \) (7,800 M\(^{-1}\)cm\(^{-1}\)), 428 nm (4,200 M\(^{-1}\)cm\(^{-1}\)). Specific activity (\(^3H\)) = 17,900 cpm/nmol.

\[^3H\]-N-Acetyl-3-aminotyrosine-LVGQIDSEVDLDLSFNQL (=Ac\(^*\)-NH\(_2\)Y-R2C19) (6). Peptide 5 (5 mg, 2 \( \mu \text{mol} \)) was converted quantitatively to the 3-aminotyrosine analogue 6 by hydrogenation. 10% Palladium/carbon (20 mg) was added to 5 in a 1:1 mixture of methanol and 0.1 M ammonium bicarbonate, pH 8.0 (10 mL) in a 200 mL round-bottom flask. The reaction was purged three times with hydrogen and stirred for 48 h with a balloon reservoir. The reaction was shielded from light using aluminum foil. Methanol was removed in vacuo before removing the Pd/C catalyst through a 0.2 \( \mu \text{M} \) syringe filter. The resulting clear liquid was lyophilized before purifying by reverse-phase HPLC using gradient A. Purified 6 was analyzed by HPLC using gradient B. HPLC: \( t_R = 12.7 \text{ min} \). MALDI-TOF MS: \( MW_{\text{calc}} = 2328 \text{ Da} \), \( MW_{\text{calc}} = 2329 \text{ Da} \). UV-Vis: \( \lambda_{\text{max}} = 289 \text{ nm} \) (2,800 M\(^{-1}\)cm\(^{-1}\)). Specific activity (\(^3H\)) = 15,200 cpm/nmol.

\[^3H\]-N-Acetyl-3-azidotyrosine-LVGQIDSEVDLDLSFNQL (=Ac\(^*\)-N\(_3\)Y-R2C19) (7). The lyophilized peptide 6 (1 mg, 0.4 \( \mu \text{mol} \)) was suspended in 0.1 M HCl (1.0 mL) in a 20 ml scintillation vial with sonication and placed in an ice bath. The reaction was shielded with aluminum foil and stirred for 10 min to allow the temperature to equilibrate. Upon addition of sodium nitrite (1.0 M, 60 \( \mu \text{L} \)), the suspension turned yellow and was stirred for an additional 20 min. Sodium azide (1.0 M, 75 \( \mu \text{L} \)) was then added; the reaction was removed from the ice bath and allowed to stir at room temperature for 4 h. Ammonium bicarbonate (0.1 M) was added until the suspension was dissolved and 7 was isolated immediately by semi-preparative reverse-phase HPLC by gradient A. The integration of the product peak showed that the conversion of 6 to 7 was approximately 75% using these conditions. Purified 7 was analyzed by HPLC using gradient B and exchanged into potassium phosphate buffer. ESI-MS was carried out on an untritiated version of 7. HPLC: \( t_R = 13.9 \text{ min} \). MALDI-TOF MS: \( MW_{\text{calc}} = 2354 \text{ Da} \), \( MW_{\text{obs}} = 2328 \)
Da. ESI MS: \( \text{MW}_{\text{calc}} = 2354 \text{ Da}, \text{MW}_{\text{obs}} = 2354 \text{ Da}. \lambda_{\text{max}} = 248 \text{ nm (5,700 M}^{-1}\text{cm}^{-1}), 296 \text{ nm (3,500 M}^{-1}\text{cm}^{-1}). \) Specific activity (\(^{3}\text{H}) = 16,600 \text{ cpm/nmol.}

\textbf{Cysteine(tert-butylthio)-Serine-3-nitrotyrosine-LVGQIDSEVDLDSFNQL} \ (= \text{Cys('Buthio)S-NO}_2\text{Y-R2C19}) (8). 8 \text{ was synthesized from R2C19 by the successive additions of Fmoc-3-nitrotyrosine, Fmoc-Ser(O'Bu)-OH, and Fmoc-Cys('Buthio)-OPfp to R2C19 as described previously [15]. The peptide was isolated as described by HPLC using gradient A. Purified 8 was analyzed by HPLC using gradient B. 8 (2.0 mL) was also exchanged into 50 mM Tris buffer, pH 8.0 buffer by dialyzing against 2 \times 2000 \text{ mL of buffer using Spectra/Por 1000 MWCO tubing. HPLC: } t_R = 15.6 \text{ min. MALDI-TOF MS: } \text{MW}_{\text{calc}} = 2595 \text{ Da}, \text{MW}_{\text{obs}} = 2595 \text{ Da. UV-vis: UV-Vis: } \lambda_{\text{max}} = 285 \text{ nm (7,800 M}^{-1}\text{cm}^{-1}), 428 \text{ nm (4,200).}

\textbf{Cysteine-Serine-3-nitro-Tyrosine-LVGQIDSEVDLDSFNQL} \ (=CS-NO}_2\text{Y-R2C19}) (9). The cysteiny1 protecting group was removed by treatment with DTT followed by dialysis, using published procedures [15]. MALDI-TOF MS: \text{MW}_{\text{calc}} = 2506 \text{ Da, } \text{MW}_{\text{obs}} = 2506 \text{ Da.}

\textbf{[^{3}\text{H]-N-Acetyl-para-benzoylelphenylalanine-SY-LVGQIDSEVDLDSFNQL}} \ (=Ac*-Bpa-SY-R2C19) (10). 10 \text{ was synthesized by the successive addition Fmoc-Tyr('Bu)-OH, Fmoc-Ser(O'Bu)-OH, and Fmoc-Bpa-OH to R2C19 (200 mg. 0.05 mmol) using 0.9 HATU: 2.0 DIEA activation chemistry. After addition of Fmoc-Bpa, the N-terminus was acetylated as described for 5. 10 \text{ was isolated by HPLC using gradient A. Purified 10 was analyzed by HPLC using gradient B and exchanged into potassium phosphate buffer. HPLC: } t_R = 21.0 \text{ min. MALDI-TOF MS: } \text{MW}_{\text{calc}} = 2650 \text{ Da, } \text{MW}_{\text{obs}} = 2651 \text{ Da. } \lambda_{\text{max}} = 264 \text{ nm (18,000 M}^{-1}\text{cm}^{-1}), 320 \text{ nm (200 M}^{-1}\text{cm}^{-1}). \) Specific activity (\(^{3}\text{H}) = 17,900 \text{ cpm/nmol.}

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2,3-fluorotyrosine (11). 2,3-Difluorophenol (10 mM, 5.2 g), pyruvic acid (60 mM, 26.4 g), pyridoxal phosphate (10 mg/L, 40 mg), β-mercaptoethanol (5 mM, 1.4 mL), and ammonium acetate (30 mM, 9.25 g) were dissolved in 4.0 L of water in a 4.0 L media bottle. The pH was adjusted to 8.0 with ammonium hydroxide before adding 4.0 mL of tyrosine phenol lyase (50 units). The yellow reaction was stirred in the dark for three days before adding more enzyme (50 units). After four days, the reaction was acidified to pH 3 with 6 M hydrochloric acid (~20 mL), turning the reaction clear. The acidified solution was filtered through a fritted funnel and then extracted 2.0 L at a time with 2 x 1.0 L of ethyl acetate to remove unreacted phenol. The aqueous layer was loaded onto a Dowex-50W column (20-50 mesh hydrogen form, 6.0 cm x 20 cm, 550 mL) equilibrated in water after prewashing with 2 V 6 M HCl followed by 2V of 6 N NaOH. The clear flow-through was checked by a ninhydrin test every 30 min (600 mL). The ninhydrin test was carried out by spotting the flow-through onto a silica gel TLC plate using a capillary and then overspotting with the ninhydrin solution, followed by heating with a heat gun. The column was then washed with 3.0 L of water before eluting with 10% (v/v) ammonium hydroxide. The pH of the eluent was checked using pH paper; once the eluent basified to pH 10 (500 mL), the ninhydrin test gave a strong positive. The bright yellow solution was collected (900 mL) and concentrated to 100 mL by rotary evaporation and lyophilized to a beige powder (3.0 g, 35%). 1H NMR (300 MHz, D2O, 25 °C): δ = 6.85 (dt, J1 = 8.4 Hz, J2 = 2.1 Hz, 1H, ArH), 6.71 (dt, J1 = 8.4 Hz, J2 = 1.8 Hz, 1H, ArH), 3.87 (dd, J1 = 7.8 Hz, J2 = 5.4 Hz, 1H, C6H), 3.20 (dd, J1 = 14.7 Hz, J2 = 5.7 Hz, 2H, CβH1), 3.01 (dd, J1 = 14.7 Hz, J2 = 7.8 Hz, 2H, CβH2).

Purification of tyrosine phenol lyase (TPL). The TPL preparation was modified from the literature methods [24] by replacing the octyl sepharose column with a DEAE anion exchange step due to high DNA content, which clogged the octyl sepharose column in previous preparation. E. coli SVS370 cells containing pTZTPL, the constitutive TPL
expression plasmid [36], overnight in 100 mL LB with 100 μg/mL ampicillin. The seed culture was pelleted at 5,000 rpm for 7 min and resuspended in LB to wash the cells. The cell suspension was then pelleted a second time and resuspended to inoculate 9.5 L LB medium containing 100 μg/mL ampicillin, which was incubated at 37 °C at 200 rpm for 20 h before harvesting. The resulting cell paste (50 g) was flash-frozen and stored at −80 °C. The cells paste was thawed and resuspended with homogenization in TPL buffer (100 mM potassium phosphate, 1 mM EDTA, 0.1 mM pyridoxal phosphate, 5 mM β-mercaptoethanol, pH 7.0) by adding 3 mL/g of cell paste. Cell lysis was carried out by one pass through a French pressure cell at 14,000 psi. A cleared lysate was obtained after centrifugation for 30 min at 14K rpm. DNA was precipitated by addition of 2% (w/v) protamine sulfate solution (25 mL, Lot # 119H1265) dropwise over 10 min at room temperature. After stirring for an additional 10 min at 4 °C, the sample was centrifuged for 30 min at 15K rpm. DNase I (150 μg, 225 units) was added to the supernatant and stirred for 10 min. Ammonium sulfate was added to 60% saturation (39 g/100 ml) over 30 min and stirred for an additional 20 min. The protein pellet was collected by centrifugation for 30 min at 14K rpm and resuspended in a minimal volume (60 mL). The absorbance maximum at this time was still at 260 nm, indicating incomplete removal of DNA from the protein solution. The protein solution was dialyzed 2 × 1.2 L TPL buffer at 4 °C. The dialysate (150 mL) was loaded onto a DEAE fast-flow sepharose column (150 mL). The yellow protein was eluted in a step gradient of 500 mM sodium chloride (350 mL) due to column clogging. The TPL was assayed using a lactate dehydrogenase-NADPH coupled spectrophotometric assay [36]. The final yield of enzyme was 3,500 units stored, which was flash-frozen in 1.5 mL aliquots at 21.5 units/mL.

**Synthetic Medium Preparation.** Amino acids were dissolved with sonication as a 20× solution in water and stored at 4 °C after filter-sterilization for up to 1 month.
<table>
<thead>
<tr>
<th>Glycerol/salt solution (per L)</th>
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<tr>
<td>glycerol</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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<th>Amino acids (per L)</th>
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<tr>
<td>alanine</td>
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<td>adenosine</td>
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<td>cytosine</td>
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<tr>
<th>Vogel-Bonner trace salt solution (per 100 mL of 1000x stock solution)</th>
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<tbody>
<tr>
<td>FeCl₃·6 H₂O</td>
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<tr>
<td>ZnSO₄·7H₂ O</td>
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<th>Vitamins (per L)</th>
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<td>biotin</td>
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Table 5.1 Growth medium for E. coli used for global incorporation of 3-fluorotyrosine into R1. Note that Vogel-Bonner salt solution is prepared separately and added as a 1:1000 dilution to the growth medium.
Nucleoside/nucleobases were prepared as a 4× solution in water and autoclaved. The resulting solution was stored overnight at 40 °C to avoid precipitation. The glycerol/salt solution was prepared as a 1.43× solution. Upon adjusting the pH to 7.0, salts were precipitated. However, the solution was autoclaved as a suspension and the supernatant poured off from the settled salt precipitate to use for media preparation. A Vogel-Bonner trace salt solution was made as a 1000× stock solution in 100 mL as indicated in Table 5.1 and autoclaved. The final media was assembled as follows (per L): 50 mL amino acid stock solution, 250 mL nucleoside/nucleobase stock solution, 700 mL glycerol/salt solution, 1.0 mL Vogel-Bonner stock solution, 25 mg thiamine, 5 mg biotin. Vitamins were added as solids just prior to inoculation.

**Growth of 3FY-R1 and 3FY-R1C225S.** Freshly transformed *E. coli* BL21(DE3) cells containing pMB1, the R1 overexpression plasmid, or pMB1C225S were grown overnight in 100 mL LB with 100 µg/mL ampicillin. The seed culture (20 mL per L media) was centrifuged at 5,000 rpm for 7 min and resuspended in 2.5 mL LB per 20 mL cell pellet. The cell suspension was then used to inoculate the synthetic medium containing 100 µg/mL ampicillin, which was incubated at 37 °C with shaking at 200 rpm. Upon reaching $A_{600\text{ nm}}$ of 0.7-0.8 OD (doubling time = 40-60 min), 3-fluorotyrosine (1.0 g/L) was added as a solid. The cell culture was induced by the addition of IPTG (0.5 mM final concentration) 15 min after the addition of $m$-fluorotyrosine ($A_{600\text{ nm}}$ of 1.0 OD). The cells were grown for another 4-5 h before harvesting.

**3FY-R1:** 9.0 g cell paste from 3.0 L synthetic medium.

**3FY-R1C225S:** 2.5 g cell paste from 1.0 L synthetic medium.

**Growth of 2,3FY-R1.** Freshly transformed *E. coli* BL21(DE3) cells containing pMB1, the R1 overexpression plasmid, or pMB1C225S were grown overnight in 100 mL LB with 100 µg/mL ampicillin. The seed culture (10 mL) was centrifuged at 5,000 rpm for 7
min and resuspended in 5 mL LB. The cell suspension was then used to inoculate the
minimal medium (500 mL; pH 7.2, pH 7.0, pH 6.3) containing 100 μg/mL ampicillin,
which was incubated at 37 °C with shaking at 200 rpm. Upon reaching A_{600 nm} of 0.8 OD,
2,3-difluorotyrosine (550 mg) was added as a solid. The cell culture was induced by the
addition of IPTG (0.5 mM final concentration) 15 min after the addition of 2,3-
fluorotyrosine (A_{600 nm} of 0.8 OD). The cells were grown for another 4-5 h before
harvesting. Typical yield of cell paste was 1.0-1.5 g.

Growth of R1C225S. Freshly transformed E. coli BL21(DE3) cells containing pMB1,
the R1 overexpression plasmid, or pMB1C225S were grown overnight in 100 mL LB
with 100 μg/mL ampicillin. The seed culture (20 mL per L media) was centrifuged at
5,000 rpm for 7 min and resuspended in 2.5 mL LB per 20 mL cell pellet. The cell
suspension was then used to inoculate the LB (3.0 L) containing 100 μg/mL ampicillin,
which was incubated at 37 °C with shaking at 200 rpm. Upon reaching A_{600 nm} of 1.0 OD,
the cell culture was induced by the addition of IPTG (0.5 mM final concentration). The
cells were grown for another 4-5 h before harvesting, yielding 9.7 g of cell paste.

Purification of R1s. The cells were resuspended with homogenization in R1 buffer (50
mM Tris, 15 mM magnesium acetate, 5 % (w/v) glycerol, pH 7.6) with DTT (4mM) and
PMSF (1.0 mM) by adding 3 mL/g of cell paste. Cell lysis was carried out in one pass at
14,000 psi in a French pressure cell. A cleared lysate was obtained after centrifugation for
10 min at 15K rpm. DNA was precipitated by dropwise addition of 0.2 volumes of a 6%
(w/v) streptomycin sulfate solution in R1 buffer over 10 min to a final concentration of
1.0%. After stirring for an additional 10 min, the sample was centrifuged for 30 min at
15K rpm. Ammonium sulfate was added to 66% saturation (39 g/100 mL) to the cleared
cell lysate over 20 min and stirred for an additional 20 min. The protein pellet was
collected by centrifugation for 1 h at 10K rpm and resuspended in a minimal volume. The
visibly brown protein fraction was collected in an Erlenmeyer flask from a desalting
Sephadex G-25 column. The sample was then loaded onto a dATP Sepharose affinity column (binding capacities: low-affinity~2 mg/mL, high-affinity~30 mg/mL) and washed until the A_{280 nm} was less than 0.05 OD before eluting with 10 mM ATP-2Na (pH 7.6) in R1 Tris with DTT (10 mM). Protein-containing fractions were identified by Bradford assay and pooled to concentrate by Amicon using a PM30 membrane. ATP was removed a Sephadex G-25 column (10:1 volume) after concentrating the R1 by Amicon with a PM30 membrane. Protein-containing fractions were pooled based on Bradford assay and concentrated again by Amicon and flash-frozen in 250 μL aliquots.

3FY-R1: Ammonium sulfate pellet (resuspended in 19 mL), Sephadex G-25 column (2.5 cm × 17 cm (85 mL) column, 100 mL protein solution eluted), low-affinity dATP Sepharose column (3.5 cm × 8 cm (80 mL) column, 160 mL protein solution eluted), Sephadex G-25 column (4.0 cm × 30 cm (350 mL) column, 6 mL protein solution loaded). Total yield: 58 mg stored at 7.5 mg/mL.

3FY-R1C225S: Ammonium sulfate pellet (resuspended in 2.5 mL), Sephadex G-25 column (1.0 cm × 15 cm (12 mL) column, 15 mL protein solution eluted), high-affinity dATP Sepharose column (1.0 cm × 2.0 cm (3 mL) column, 8 mL protein solution eluted), Sephadex G-25 (2.5 cm × 21 cm (100 mL) column). Total yield: 5 mg stored at 3.8 mg/mL.

RIC225S: Ammonium sulfate pellet (resuspended in 13 mL), Sephadex G-25 column (2.0 cm × 16 cm (50 mL) column, 60 mL protein solution eluted), high-affinity dATP Sepharose column (1.0 cm × 2.0 cm (3 mL) column, 11 mL protein solution eluted), Sephadex G-25 (2.5 cm × 25 cm (120 mL) column). Total yield: 76 mg stored at 7.6 mg/mL.

Characterization of 3FY-R1 and wt-R1. Proteins were passed through a Micro Bio-Spin 6 column pre-equilibrated with water. Proteins (20-50 μL) were submitted at a 15-
30 μM concentration for ESI-MS, MALDI-TOF MS, and N-terminal sequencing (MIT Biopolymers Laboratory).

3FY-R1: N-terminal sequencing: MNQNLVT KRDGSTERNLD, observed, NQNLVT KRDGSTERNLD, expected. MALDI-TOF MS (MH⁺): \( m/z_{\text{calc}} = 86,477 \) Da (+Met), \( m/z_{\text{obs}} = 86,424 \) Da. ESI MS (MH⁺): \( m/z_{\text{calc}} = 86,477 \) Da (+Met), \( m/z_{\text{obs}} = 86,411 \) Da.

wt-R1: ESI MS (MH⁺): \( m/z_{\text{calc}} = 85,555 \) Da and 85,739 Da (+Met), \( m/z_{\text{obs}} = 85,537 \) Da, 85,646 Da, and 85,776 Da.

**Peptide characterization by MALDI-TOF MS** The mass of peptides were determined using Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry on a Voyager workstation (PE Biosystems). The matrix consisted of a >10 mg/mL suspension of α-cyano-4-hydroxycinnamic acid in a solution of 50% acetonitrile (v/v), 49% (v/v) water, and 1% (v/v) TFA. The suspension was sonicated and centrifuged before spotting 1 μL of the supernatant with 1 μL of a salt-free sample dissolved in water onto the 100-well MALDI target. Mass spectra were collected in negative ion and linear mode using a 600-1000 MW cutoff. An external linear calibration was carried out before every use with angiotensin I (\( M_{\text{ave}} = 1295.5 \) Da), ACTH 18-39 (\( M_{\text{ave}} = 2464.7 \) Da), and oxidized bovine insulin (\( M_{\text{ave}} = 3495.9 \)).

**Characterization of Peptide Binding to R1.** The binding of peptides 3 and 4 to R1 was assessed by competitive inhibition of nucleotide reduction. R1 (0.1 μM), R2 (0.2 μM), thioredoxin (50 μM), thioredoxin reductase (1.0 μM), CDP (1.0 mM), ATP (1.6 mM), NADPH (0.2 mM) were incubated with varying concentrations of peptide (0-250 μM). Activity was measured by the decrease in \( A_{340 \text{ nm}} \). See Chapter 3 Experimental Methods for fitting routine for determination of the R1–peptide \( K_D \).
Crosslinking reactions and analysis. R1 was pre-reduced at room temperature for 30 min with DTT and hydroxyurea added to a final concentration of 20 mM. The R1 sample was then de-salted using a G-25 column and concentrated to 180 μM using a YM30 Centricon. The R1 was stored at stably at −80 °C. Immediately before running the crosslinking reaction, the R1 buffer was exchanged with 20 mM potassium phosphate, 5 mM magnesium chloride, pH 7.6 by passing the R1 sample through two consecutive Micro Bio-spin 6 columns in 90% yield. Crosslinking reactions were run under the following conditions in a 150 μL volume: R1 (100 μM), [3H]-peptide (either 6 or 10) (20 μM), CDP (1.0 mM), and ATP (1.6 mM) or TTP (100 μM). The samples were irradiated using a 1000 W Hg-Xe lamp (Oriel) with a 90% neutral density and 285 nm longpass filters for 25 min at 25 °C in a semi-micro 1 cm path-length quartz cuvette. The photolyzed samples were diluted five-fold to 750 μL and passed through a 50 mL Sephacryl 100 column (1.5 cm × 30 cm) with a 1:75 dilution factor. Fractions (40 drops, 1.2 mL) were collected using a fraction collector with eppendorf tubes on top of the glass tubes. Protein-containing fractions were pooled using a Bradford assay (95 μL) with 5 μL sample. Fractions (100 μL) were then counted to map the elution of the tritium-labeled peptide. The protein-containing fractions were pooled (~8 mL) and concentrated to ~1 mL using a YM50 Centriprep. Denaturation buffer was added to the sample (5 mL, 400 mm Tris, 6 M guanidinium chloride, 15 mM magnesium sulfate, 1 mM EDTA, pH 7.6) in a 25 mL pear-shaped flask. DTT was added as a solid to a final concentration of 15 mM and incubated at room temperature for 30 min. Iodoacetamide was then added as a solid to a final concentration of 100 mM, purged three times with argon, and incubated at room temperature for 3 h in the dark. The sample was then concentrated to ~2 mL using a YM50 Centriprep. The concentrated sample was then dialyzed 2 × 2.0 L water in a 10,000 MWCO Slide-a-lyzer (3.0 mL capacity). The protein precipitated within 1 h, but was allowed to dialyze for the full period. The suspension was removed using a 16 gauge syringe and pelleted by centrifugation for 1 min at 14 K rpm. The protein pellet was
suspended in 0.1 M ammonium bicarbonate, pH 8.0 (1.5 mL). TPCK-inhibited trypsin (100 µg, 1.4 µM) was added and the reaction was then incubated for 2.5 h at 37 °C. The tryptic digest was chromatographed on a C-18 XTerra MS 5 µM column (4.6 × 100 mM) in two different gradients. The first was a linear gradient of 0% to 50% acetonitrile (1.0 mL/min) versus 0.1% TFA over 75 min. The second was a linear gradient of 0% to 50% acetonitrile (1.0 mL/min) versus 0.1 M ammonium bicarbonate, pH 8.0 over 75 min. In both cases, 1.0 ml fractions were collected, 250 µL to 500 µL of which was counted for 5 min with 9.0 mL of Emulsifier-Safe scintillation fluid. The fractions containing tritium were partially lyophilized and analyzed using MALDI-TOF MS. Those fractions were also re-chromatographed by reverse-phase HPLC using either the first or second gradient.

Measurement of pH dependence of R1s. Buffers were made as follows: pH 7.0 (50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA), pH 7.6 (50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA), pH 8.0 (50 mM HEPES, 15 mM magnesium sulfate, 1 mM EDTA), pH 9.0 (50 mM TAPS, 15 mM magnesium sulfate, 1 mM EDTA). Aliquots of 50 µL were quenched by boiling for 1-2 min at 0, 2, 5, and 7 min time points. The conditions for wild-type R1 were: R1 (0.1 µM), R2 (0.5 µM), TR (100 µM), TRR (2.0 µM), ATP (1.6 mM), NADPH (1.0 mM), CDP (1.0 mM, 1413 cpm/nmol). The conditions for 3FY-R1 were: 3FY-R1 (0.5 µM), R2 (2.5 µM), TR (100 µM), TRR (2.0 µM), ATP (1.6 mM), NADPH (1.0 mM), CDP (1.0 mM, 1413 cpm/nmol). dCDP quantitation was carried out using the method of Steeper and Steuart [37].
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Outstanding Senior, Revelle College, University of California, San Diego 1997

University of California Regents’ Scholarship 1994–1997

Florence Riford Foundation Scholarship, La Jolla Rotary Association 1994–1997


Freshman Honors Program, Revelle College, University of California, San Diego 1994–1995

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