Photochemical Ribonucleotide Reductase for the Study of Proton-Coupled Electron Transfer

Steven Y. Reece
B.S., Chemistry (2002)
Davidson College

Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 2007

© 2007 Massachusetts Institute of Technology
All rights reserved

Signature of Author ____________________________
Department of Chemistry
June 8th 2007

Certified by ____________________________
Daniel G. Nocera
W. M. Keck Professor of Energy and Professor of Chemistry
Thesis Supervisor

Accepted by ____________________________
Robert W. Field
Haslam and Dewey Professor of Chemistry
Chairman, Department Committee on Graduate Studies
This doctoral thesis has been examined by a Committee of the Department of Chemistry as follows:

Professor Joseph P. Sadighi ____________________________________________ Associate Professor of Chemistry
Chairman

Professor Stephen J. Lippard ___________________________________________ Arthur Amos Noyes Professor of Chemistry

Professor Daniel G. Nocera ____________________________________________ W. M. Keck Professor of Energy and Professor of Chemistry
Thesis Supervisor

Professor JoAnne Stubbe ____________________________________________ Novartis Professor of Chemistry and Professor of Biology
Photochemical Ribonucleotide Reductase for the Study of Proton-Coupled Electron Transfer

by

Steven Y. Reece

Submitted to the Department of Chemistry on June 8th, 2007 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

ABSTRACT

Charge transport and catalysis in enzymes often rely on amino acid radicals as intermediates. The generation and transport of these radicals are synonymous with proton-coupled electron transfer (PCET), which intrinsically is a quantum mechanical effect as both the electron and proton tunnel. The caveat to PCET is that proton transfer (PT) is fundamentally limited to short distances relative to electron transfer (ET). This predicament is resolved in biology by the evolution of enzymes to control PT and ET coordinates on very different length scales. In doing so, the enzyme imparts exquisite thermodynamic and kinetic control over radical transport and radical-based catalysis at cofactor active sites.

New tools are needed to study PCET reactions of amino acid radical in biology. This thesis describes methods for photogeneration of amino acid radicals, with particular emphasis on tyrosine. Unnatural fluorotyrosine amino acids are developed to vary the driving force for proton and electron transfer in PCET reactions of tyrosyl radical (Y•), and to provide unique spectroscopic handles to study enzymes utilizing multiple Y•s. These tools allow for an in-depth study of the PCET mechanism of tyrosyl radical generation, both in solution and within the ribonucleotide reductase enzyme.

Enzymatic activity of class I E. coli ribonucleotide reductase requires the transport of charge from an assembled diiron-tyrosyl radical cofactor to the enzyme active site over 35 Å away via an amino acid radical hopping pathway spanning two protein subunits. To study the mechanism of this radical transport, we have developed photochemical RNRs wherein radical generation, transport, and enzymatic turnover can be initiated by UV-vis excitation of a peptide bound to the subunit containing the enzyme active site. This method allows us to observe Y•s competent for initiating turnover on the peptide bound to the protein subunit. Turnover assays with the wild-type and mutant proteins show that both the electron and proton move along a unidirectional pathway to affect radical transport in this subunit.

Thesis Supervisor: Daniel G. Nocera
Title: W. M. Keck Professor of Energy and Professor of Chemistry
To my parents for their love and support.
# Table of Contents

List of Tables
List of Charts
List of Schemes
List of Figures

Chapter I. Introduction

1.1 Fundamentals and Nomenclature
1.2 Theory of ET and PCET
1.3 PCET of Amino Acid Radicals in Biological Systems
   1.3.1 Amino Acid Radicals as Redox Active Cofactors
      1.3.1.1 Galactose Oxidase
      1.3.1.2 Cytochrome c Peroxidase
      1.3.1.3 Cytochrome c Oxidase
   1.3.2 Amino Acid Radicals as Charge Transport Intermediates
      1.3.2.1 Pyruvate Formate Lyase
      1.3.2.2 DNA Photolyase
      1.3.2.3 Photosystem II
1.4 Class I Ribonucleotide Reductase
1.5 Photochemical RNR
1.6 Scope of the Thesis
1.7 References

Chapter II. Trp as a Photochemical Radical Initiator & pH Dependence of Radical Transport in Trp–Tyr Dipeptides

2.1 Motivation
2.2 Background
2.3 Results and Discussion
2.4 Conclusions
2.5 Experimental Section
2.6 References
Chapter III. Investigations of Photochemical Oxidants of Tyrosine

3.1 Motivation 66
3.2 Ru(bpy)$_3^{2+}$ 66
  3.2.1 Background 66
  3.2.2 Results and Discussion 68
3.3 Pyrene 72
  3.3.1 Background 72
  3.3.2 Results and Discussion 73
3.4 Benzophenone and Anthraquinone 76
  3.4.1 Background 76
  3.4.2 Results and Discussion 77
3.5 Flavins 80
  3.5.1 Background 80
  3.5.2 Results and Discussion 82
3.6 Conclusions 84
3.7 Experimental Section 84
3.8 References 92

Chapter IV. Design of MLCT Excited States of Rhenium Polypyridyl Complexes for Direct Tyrosine Oxidation

4.1 Motivation 98
4.2 Background 98
4.3 Results 99
4.4 Discussion 108
4.5 Conclusion 109
4.6 Experimental Section 110
4.7 References 119

Chapter V. Bidirectional PCET of Tyrosine Oxidation: Buffer Effects and Parallel Mechanisms

5.1 Motivation 124
5.2 Background 124
Chapter IX. The Search for Intermediates in Photochemical RNRs with Re(bpy)(CO)₃CN as the Radical Initiator

9.1 Motivation and Background 192
9.2 Results 192
9.3 Discussion 202
9.4 Experimental Section 204
9.5 References 211

Chapter X. Towards Photoactive β2 Subunits

10.1 Motivation 214
10.2 [Re]-β2 215
  10.2.1 Background 215
  10.2.2 Results and Discussion 216
10.3 Y356C-BP β2 217
  10.3.1 Background 217
  10.3.2 Results and Discussion 218
10.4 Conclusions 220
10.5 Experimental Section 221
10.6 References 224

Acknowledgements 225

Biographical Note 229

Curriculum Vitae 231
List of Tables

Table 1.1 Various Nomenclatures Used to Describe PCET. 27

Table 3.1 Electrochemical Reduction and Oxidation Potentials of Ru Complexes. 69

Table 3.2 Ground and Excited State Reduction and Oxidation Potentials of Py-A. 75

Table 4.1 Spectroscopic and Photophysical Properties of the MLCT Excited State of ReI Polypyridyl Carbonyl Complexes in Aqueous Solution at Room Temperature 104

Table 4.2 Emission Spectral Parameters Obtained from Fitting Corrected Emission Band to Eq. (1) and Energy of the MLCT Excited State Calculated from Eq. (3) 104

Table 4.3 Differential Pulse Voltammetry and Excited State Reduction and Oxidation Potentials of Amino Acid-Derivatized ReI Polypyridyl Tricarbonyl Complexes 105

Table 4.4 Crystal data and structure refinement. 122

Table 5.1 Rate of the Tyrosine Oxidation in Three Separate Systems with Water or Water Containing HPO$_4^{2-}$. 127

Table 5.2 Thermodynamic and kinetic data for the series of bases used in the PCET oxidation of tyrosine in Re(P–Y). 129

Table 6.1 Fluorotyrosine derivatives studied here and their physical properties. 139

Table 6.2 Emission quenching and charge recombination kinetics data for [Re]-FnY complexes. 145

Table 6.3 Experimental Activation Energies and Calculated Reorganization Energies and Driving Forces for Charge Separation. 148
| **Table 6.4** | Crystal data and structure refinement for Re(bpy-COOH)(CO)_3CN. | 158 |
| **Table 7.1** | Characterization of Peptides | 160 |
| **Table 7.2** | Single Turnover Photo-initiated CDP Reduction Assay Data. | 165 |
| **Table 8.1** | Characterization of Pyrene Peptides | 182 |
| **Table 9.1** | Characterization of Peptides | 193 |
| Chart 3.1  | Chemical structures of flavins discussed herein. | 80 |
| Chart 3.2  | Redox and acid/base equilibria of flavin and flavin radicals. | 81 |
| Chart 5.1  | Compounds discussed in this chapter | 124 |
| Chart 6.1  | Structure of [Re]–F<sub>n</sub>Y complexes. | 144 |
List of Schemes

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme 1.1</td>
<td>Proposed mechanism of deoxynucleotide formation in E. coli class I RNR.</td>
<td>39</td>
</tr>
<tr>
<td>Scheme 4.1</td>
<td>Syntheses of amino acid containing Re I complexes.</td>
<td>100</td>
</tr>
<tr>
<td>Scheme 4.2</td>
<td>Electron shuttling mechanisms in oxidation of Y by $^3$MLCT excited states of Re I complexes</td>
<td>109</td>
</tr>
<tr>
<td>Scheme 5.1</td>
<td>Mechanisms of PCET, where B is solvent or base form of the buffer: Stepwise ETPT (pathway 1) or PTET (pathway 2) and CEP mechanisms.</td>
<td>125</td>
</tr>
<tr>
<td>Scheme 10.1</td>
<td>Synthesis of Cys-Pro([Re])-3,5-F$_2$Y-Leu-NH$_2$ peptide.</td>
<td>216</td>
</tr>
<tr>
<td>Scheme 10.2</td>
<td>Labeling of β2 with benzophenone derivative.</td>
<td>217</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1  Fundamentally distinct types of PCET 26
Figure 1.2  Square scheme for PCET. X is the ET and PT donor and Y is the acceptor. 26
Figure 1.3  Electronic (left) and nuclear (right) potential energy wells for an ET reaction. 29
Figure 1.4  Potential energy well for an ET reaction treated quantum mechanically. 30
Figure 1.5  Inverted region electron transfer. Area in red illustrates vibrational wavefunction overlap. 31
Figure 1.6  Two dimensional vibronic free energy surfaces for reactants (I, μ, blue) and products (II, ν, red) of a PCET reaction. 32
Figure 1.7  Crystal structure of the active site of GAO. 33
Figure 1.8  Crystal structure of yeast cyt. c peroxidase active site. 34
Figure 1.9  Crystal structure of bovine heart CCox heme a$_3$–Cu$_B$ site. 35
Figure 1.10  Crystal structure of PFL active site. Distances are in Å. 35
Figure 1.11  Crystal structure of DNA photolyase with the potential ET hopping pathway denoted by dashed lines. 36
Figure 1.12  Crystal structure of PSII oxidizing cofactors. Purple and green spheres represent a model for electron density corresponding to Mn and Ca, respectively, in the OEC. Orange sphere represents Mg and distances are in Å. 37
Figure 1.13  Conserved residues of class I RNR that compose the putative PCET pathway for radical transport from $\bullet$Y122 in $\beta_2$ to C439 in the $\alpha_2$ active site. Distances are from the separate crystal structures of the $\alpha_2$ and $\beta_2$ subunit from the E. coli enzyme. Residues where the radical has been directly observed or trapped via site-specific replacement with non-natural amino acid analogues are highlighted in green. Y356 is not
located in either the β2 or α2 crystal structures, hence its distance from W48 and Y731 is unknown.

**Figure 1.14**  Experimental design for studying the kinetics of radical transport along the •Y356 → Y731 → Y730 → C439 pathway. •Y356 is generated photochemically by a proximal photo-oxidant (red circle) on the R2C19 peptide. NDP, nucleoside diphosphate substrate; dNDP, deoxynucleoside diphosphate product; R2C19, 19-mer C-terminal peptide tail of β2.

**Figure 1.15**  Layout of the thesis designed towards the generation and study of photochemical RNRs.

**Figure 2.1**  Transient absorption spectrum following 266-nm laser excitation (fwhm = 3 ns) of W–Y solution at pH 7.8 at 2 (▬) and 20 (▬) μs. Insets: Single wavelength kinetics traces for the appearance of the 410 nm (Y•) signal and the disappearance of the 510 nm (W•) signal.

**Figure 2.2**  Transient absorption spectrum 2 μs after 266-nm laser excitation (fwhm = 3 ns) of Ac–W–Y solution at pH: 7.2 (▬), 4.3 (▬) and 3.1 (▬).

**Figure 2.3**  Rates of radical transport vs. pH for dipeptides: W•/[•WH]+–Y → W–Y• (○); Ac-W•/[Ac-•WH]+–Y → Ac-W–Y• (□); and W–Y• → W•–Y (●). The solid line is the ΔE = E_p(W•/W) – E_p(Y•/Y), calculated from the differential pulse voltammetry measurements shown in Figure 2.4.

**Figure 2.4**  E_p(W•/W) and E_p(Y•/Y) reduction potentials vs. pH as measured by differential pulse voltammetry of aqueous solutions of Ac-W–NH₂ and Ac-Y–NH₂.

**Figure 2.5**  The Y122↔W48↔Y356 pathway of β2 proposed to be important for the assembly of the diiron cofactor and radical transport leading to nucleotide reduction. The radical transfer pathway and distances are from the crystal structure of oxidized β2 at 1.4-Å resolution. The last 35 amino acids of the C-terminal tail of β2, in which Y356 resides, are
thermally labile and undetectable in available crystal structures.

**Figure 3.1** Cyclic voltammograms of (▬) Ru(bpy)$_2$(bpy–COOH)(PF$_6$)$_2$ and (▬) Ru(BTFMB)$_2$(bpy–COOH)(PF$_6$)$_2$ in MeCN with 0.1 M tetrabutylammonium hexafluorophosphate supporting electrolyte. The scan rate was 100 mV/s and the potentials are referenced to the Fc$^+$/Fc couple (0.65 V vs. NHE). The red voltammogram in the right panel was shifted to positive current values for illustration.

**Figure 3.2** Absorption and emission spectra of Ru(BTFMB)$_2$(bpy-F)(PF$_6$)$_2$ in 20 mM Tris buffer at pH 7.5.

**Figure 3.3** Plot of $k_q$ vs. concentration of [Ru$^{	ext{III}}$(NH$_3$)$_6$]Cl$_3$ quencher with linear fit for Ru(bpy)$_2$(bpy-(Y))$^{2+}$ (●,▬) and Ru(BTFMB)$_2$(bpy-(Y))$^{2+}$ (○,▬) in 20 mM Tris buffer at pH 7.5 in the presence of air.

**Figure 3.4** (Left) TA spectra of a solution of Ru(BTFMB)$_2$(bpy-F)$^{2+}$ obtained (▬) 115 ns, (▬) 1 and (▬) 10 μs after the laser flash. The * denotes scatter from the 355 nm laser. (Left Inset) Blow-up of the spectrum at 1 μs corresponding to the Ru$^{	ext{III}}$(bpy-F)$^{3+}$ flash quench product. (Right) TA spectra of a solution of Ru(BTFMB)$_2$(bpy-Y)$^{2+}$ obtained (▬) 115, (▬) 500 ns, (▬) 1, and (▬) 10 μs after the laser flash. (Right Inset) Blow-up of the spectrum at 1 μs showing the Y• product. Spectra obtained in 20 mM Tris buffer at pH 7.5 in the presence of 50 mM Ru$^{	ext{III}}$(NH$_3$)$_6$$^{3+}$ quencher.

**Figure 3.5** Plot of the absorption and emission spectra of Py-A and emission spectrum of Py-Y.

**Figure 3.6** Transient absorption spectra of a solution of Py-A in 10 mM KPi buffer at pH 7.5 obtained 1 μs after the laser flash in the presence (▬) and absence (▬) of 10 mM Co(III)(NH$_3$)$_6$Cl$_3$.

**Figure 3.7** Ground state UV-Vis absorbance spectra of BPA-Y-OMe•TFA and Anq-Y-OH.
Figure 3.8  Transient absorption spectra recorded at (▬) 100 ns, (▬) 400 ns, and (▬) 1 μs following 300 nm, 5 ns excitation of a 500 μM solution of BPA-Y-OMe buffered to pH 4.0 with 20 mM succinic acid. Inset: Single wavelength kinetics of the 547 nm absorption (○) and the single exponential decay fit (▬) to the data.

Figure 3.9  Left panel: Transient absorption spectra of Anq-F-OH obtained 65 (▬), 115 ns, 1, and 10 μs (▬) following 355 nm excitation. Right: Transient absorption spectra of Anq-Y-OH obtained 15 (▬), 115 ns, 1, and 10 μs (▬) following 355 nm excitation. Insets: Time-evolved absorbance data (○) with bi-exponential decay fit (▬) obtained at 410 and 520 nm.

Figure 3.10  Ground state UV-Vis absorbance and steady state emission spectra (450 nm excitation) of Fl-A in 10 mM KPi buffer (pH 7).

Figure 3.11  Transient absorption spectra obtained (▬) 115ns, (▬) 2μs, and (▬) 20μs following 355 nm ns-laser excitation of a solution of Fl-A in 10 mM KPi at pH 7.

Figure 3.12  Transient absorption spectra obtained (▬) 65ns, (▬) 115 ns, and (▬) 1 μs following 355 nm ns-laser excitation of a solution of Fl-Y in 10 mM KPi at pH 7.

Figure 4.1  Thermal ellipsoid plot of single isomer of [Re(phen)(PP)(CO)₂]PF₆ shown at 50% probability. The PF₆⁻ anion has been removed for clarity.

Figure 4.2  Thermal ellipsoid plot of single isomer of [Re(phen)(PP-Bn)(CO)₂]PF₆ • CDCl₃ shown at 50% probability. The PF₆⁻ anion, the solvent molecule and phosphine phenyl groups have been removed for clarity.

Figure 4.3  Normalized, corrected emission spectra for Re(bpy-COOH)(CO)₃(X) [X = CN⁻ (▬), Cl⁻ (▬), SCN⁻ (▬)] and [Re(phen)(P–F)(CO)₃](PF₆) (▬) in 10 mM phosphate buffer at pH 7 and [Re(phen)(dppe) (CO)₂](PF₆) (▬) in CH₃CN.

Figure 4.4  Top: TA spectra of a pH 7 solution of Re(bpy–Y)(CO)₃(CN) at 15 (▬),
65 (●), 115 (○), and 215 ns (● – ○) (Insets: Single wavelength kinetic traces and monoexponential fits for the disappearance of the 380 and 475 nm (3MLCT) signal). Bottom: Same experiment at pH 12 (Insets: Single wavelength kinetic traces and monoexponential fits for the disappearance of the 410 (Y•) and 500 nm (bpy•−) signal.

**Figure 4.5** Spectroelectrochemical UV/Vis absorption spectrum of Re(bpy−Y–OtBu)(CO)3(CN) at -0.75 V vs. Fe3+/Fe in DMF.

**Figure 5.1** Plot of the rate constant for emission quenching, k_{obs}, vs. pH for [Re(P−Y)(phen)(CO)3]PF6. Three pH regions are highlighted corresponding to the protonated (○) and deprotonated (●) forms of the tyrosine carboxylic acid (10 mM KPi buffer) and the 50 mM KPi high pH region (●).

**Figure 5.2** Top left: Phosphate buffer dependence of k_{obs} with fits to eq. 1 for Re(P−Y) at pH 4.5 (○), 6.1 (○), 7.5 (○), 8.3 (○), and 9.2 (○). Bottom left: Same experiment with RuesterY at pH 9.9 (●), 7.7 (●) and pH 6 (●). Right: Mole fraction of relevant buffer species as a function of pH.

**Figure 5.3** Phosphate buffer dependence of k_{obs}, for RuY and fits to eq. 1 at pH 7 (■,●), pH 9 (■,○) and pH 3 (■). Inset: pH dependence of k_{obs} in RuY at 0.5 mM buffer concentration (dashed line in main figure) with MES (●, pK_a = 6.2), borate (●, pK_a = 9.1), phosphate (●, pK_a = 7.2) and borate/phosphate mixture (●).

**Figure 5.4** (Left) Plot of the rate constant for emission quenching, k_q, vs. concentration of pyridine buffer for [Re(P−Y)(phen)(CO)3]PF6 at pH 3.75 (○), 4.65 (○), 5.35 (○), and 7.6 (○) (Right) Plot of, k_q, vs. concentration of imidazole buffers for [Re(P−Y)(phen)(CO)3]PF6 with 4-Br-Im (○), Im (○), and 4-Me-Im (○). The dotted line represents the limit of detection using the nanosecond pulsed laser for excitation.

**Figure 6.1** Reduction potential vs. NHE as a function of pH for Ac-F_n Y-NH_2 derivatives of Table 1: (▬) Ac-Y-NH_2, (▬) Ac-3-FY-NH_2, (▬) Ac-3,5-F_2 Y-NH_2, (▬) Ac-2,3-F_2 Y-NH_2, (▬) Ac-2,3,5-F_3 Y-NH_2, (▬) Ac-...
2,3,6-F₃Y-NH₂ and (_listing) Ac-F₄Y-NH₂.

**Figure 6.2** (Left) Plot of $E_p(\text{Ac-F}_n\text{Y}^-\cdot\text{NH}_2/\text{Ac-F}_n\text{Y}^-\cdot\text{NH}_2)$ vs. $E(\text{Ac-F}_n\text{Y}^-\cdot\text{NH}_2) - E(\text{Ac-F}_n\text{Y}^-\cdot\text{NH}_2)$, the total bonding energy difference between Ac-FₙY•-NH₂ and Ac-FₙY–-NH₂ as calculated by DFT, with linear fit (―). (Bottom) Plot of $-\ln(K_a)$ vs. $E(\text{Ac-F}_n\text{Y}^-\cdot\text{NH}_2) - E(\text{Ac-F}_n\text{Y}^-\cdot\text{NH}_2)$, the total bonding energy difference between Ac-FₙY–-NH₂ and Ac-FₙY-NH₂ as calculated by DFT, with linear fit (―). Color coded points correspond to data for (●) Ac-Y-NH₂, (●) Ac-3-FY-NH₂, (●) Ac-3,5-F₂Y-NH₂, (●) Ac-2,3-F₂Y-NH₂, (●) Ac-2,3,5-F₃Y-NH₂, (●) Ac-2,3,6-F₃Y-NH₂, (●) Ac-F₄Y-NH₂.

**Figure 6.3** Energy level diagram for frontier molecular orbitals of the phenolates: (―) Ac-Y–-NH₂, (―) Ac-3-FY–-NH₂, (―) Ac-3,5-F₂Y–-NH₂, (―) Ac-2,3-F₂Y–-NH₂, (―) Ac-2,3,5-F₃Y–-NH₂, (―) Ac-2,3,6-F₃Y–-NH₂, (―) Ac-F₄Y–-NH₂. Selected Kohn-Sham representations of orbitals for the tyrosine analog are shown at the 95% probability level. The Kohn-Sham representation of the HOMO orbital for Ac-3,5-F₂Y–-NH₂ is presented in the frame.

**Figure 6.4** The transient absorption spectrum of (―) BPA-Y-OMe, (―) BPA-3-FY-OMe, (―) BPA-3,5-F₂Y-OMe, (―) BPA-2,3-F₂Y-OMe, (―) BPA-2,3,5-F₃Y-OMe, (―) BPA-2,3,6-F₃Y-OMe and (―) BPA-F₄Y-OMe normalized to the peak at 547 nm obtained 100 ns after excitation of ~500 μM solutions of each dipeptide buffered to pH 4.0 with 20 mM succinic acid.

**Figure 6.5** X-Ray structure and atom labeling scheme for Re(bpy-COOH)(CO)₃CN•MeOH; solvent molecule not shown.

**Figure 6.6** Transient absorption spectra of [Re]–2,3,6-F₃Y observed (―) 15, (―) 65, (―) 115 and (―) 215 ns and following 355 nm, 3 ns laser irradiation. The Y• absorbance feature of the 15 ns trace is magnified. Inset: Single wavelength kinetics trace (○) obtained at 520 nm with bi-
exponential fit (—).

**Figure 6.7** Left: ln(k_CS) vs. –ΔG_CS° plot with linear (—) and parabolic (▬) fit. Right: ln(k_CR) vs. –ΔG_CR° plot with linear (—) fit.

**Figure 6.8** Temperature dependence of the rate of charge separation in [Re]-3,5-F₂Y⁻ and [Re]-F₄Y⁻ (○) with linear fit (—).

**Figure 7.1** Molecular structures of photooxidants used in this chapter.

**Figure 7.2** Transient absorption spectra of BPA-Y-R2C19 obtained 65 (—), 415 ns, 1, and 10 μs (▬) following 300 nm excitation. Insets: Time-evolved absorbance data (○) with mono-exponential decay fit (—) obtained at 410 and 550 nm.

**Figure 7.3** Left panel: Transient absorption spectra of Anq-Y-R2C19 obtained 115 ns (—), 1, and 10 μs (▬) following 355 nm excitation. Left and right insets: Single wavelength kinetics traces (○) with bi-exponential decay fit (—). Middle inset: Plot of ΔΔOD obtained by subtracting the normalized transients observed for Anq-Y-R2C19 and Anq-F-R2C19 at 100 ns. Bottom panel: Transient absorption spectra of Anq-F-R2C19 obtained 115 ns (—), 1, 10, and 100 μs (▬) following 355 nm excitation.

**Figure 7.4** Plots of relative RNR activity vs. peptide concentration in the competitive inhibition binding assay for Ac-BPA-Y-R2C19 (■), Ac-BPA-F-R2C19 (□), Anq-Y-R2C19 (●), and Anq-F-R2C19 (○) peptides.

**Figure 7.5** Light initiated, single turnover assays with Ac-BPA-(Y)-R2C19:α2, Anq-Y-R2C19:α2 and Ac-BPA-Y-R2C19:Y730F-α2. The bars refer to light (□) reactions and dark (■) controls. For 100% turnover, each dimer of α2 would produce 2 molecules of dCDP.

**Figure 7.6** SDS gel with molecular weight markers (lanes 1 & 5), α2 + Ac-BPA-Y-R219 in the dark at 2, 5, and 10 minutes (lanes 2-4), and in the light (lanes 6-8).
Figure 7.7  The crystal structure of the Y730F-α2 variant shows that mutation of Y730 to F730 interrupts the hydrogen bond network in α2 and significantly increases the proton tunneling distance. Y356 on the R2C20 peptide is not located in the structure and is represented here for illustrative purposes.

Figure 8.1  Spectrum obtained following excitation of a solution of Ac-BPA-F-R2C19 with pulsed, 300 nm laser light. Inset: Single wavelength absorbance kinetics trace monitored at 530 nm.

Figure 8.2  Light initiated, single turnover assays with Ac-BPA-F-R2C19:α2 and Anq-F-R2C19:α2. The bars refer to light (□) reactions and dark (■) controls. For 100% turnover, each dimer of α2 would produce 2 molecules of dCDP.

Figure 8.3  Plots of relative RNR activity vs. peptide concentration in the competitive inhibition binding assay for Py-Y-R2C19 (■) and Py-R2C19 (●).

Figure 8.4  Steady-state emission spectra of Py-R2C19 (3 μM) in the presence (▬) and absence (––) of α2 (30 μM).

Figure 8.5  Light initiated, single turnover assays with Py-R2C19 (200 μM), MV2+ (10 mM) and α2 (20 μM). The bars refer to light (□) reactions and dark (■) controls. For 100% turnover, each dimer of α2 would produce 2 molecules of dCDP.

Figure 8.6  Transient absorption spectra of a solution of 100 μM Py-R2C19 and 25 mM MV2+ in the absence (left) and presence (right) of 100 μM α2 obtained 235 ns (▬), 1 (▬), and 10 (▬) μs after a 355 nm ns-laser flash. Left and right insets for each panel correspond to single wavelength absorbance kinetics data (○) and exponential fit (▬) monitored at 396 and 455 nm, respectively.

Figure 9.1  Chemical structure of [Re]-3,5-F2Y-R2C19.
Figure 9.2  Emission spectra of [Re]-3,5-F₂Y-R2C₁₉ as a function of pH, which was titrated from 4.5 to 11.5. Inset: pH Titration curve of integrated emission intensity.

Figure 9.3  TA spectra of 100 µM solutions of [Re]-F-R₂C₁₉ (left) and [Re]-Y-R₂C₁₉ (right) in 50 mM Tris buffer (pH 7.6), 20% glycerol, 1 mM CDP, 3 mM ATP, and 15 mM MgSO₄ recorded at 65, 115, and 215 ns following a 355 nm laser pulse. Insets: Single wavelength kinetics traces (○) with single exponential fit (▬) recorded at 480 nm.

Figure 9.4  (Left) TA spectra of 100 µM [Re]-3,5-F₂Y-R₂C₁₉ obtained 65 and 215 ns after a 355 ns flash. (Right) TA spectrum of 100 µM [Re]-3,5-F₂Y-R₂C₁₉ in presence of 10 mM Ru(III)(NH₃)₆³⁺ obtained 65 ns after the 355 nm laser flash. Conditions: 50 mM Tris buffer (pH 8.5), 20% glycerol, 1 mM CDP, 3 mM ATP, and 15 mM MgSO₄. Insets: Single wavelength kinetics traces (○) with single exponential fit (▬) recorded at the wavelength indicated.

Figure 9.5  Competitive inhibition binding assay of [Re]-Y-R₂C₁₉ to α₂.

Figure 9.6  Emission spectra of [Re]-Y-R₂C₁₉ (5 µM) at pH 7.5 as the α₂ concentration is increased from 0→50 µM. Inset: Emission maximum as a function of α₂ concentration (○) with fit (▬) to binding model in eq 2.

Figure 9.7  Emission spectra of [Re]-3,5-F₂Y-R₂C₁₉ (5 µM) and α₂ (20 µM) as the pH is titrated from 9.0 to 6.3. The red spectra were recorded at pH 6.3 (lower spectrum) and pH 9.0 (upper spectrum) in the absence of α₂. Inset: Integrated emission intensity as a function of pH (○) with fit (▬) to monoprotic titration curve.

Figure 9.8  Amount of dCDP produced per α₂ under single turnover conditions after incubation with the labeled conditions. Photolysis was performed with λ>299 nm in a temperature controlled (25°C) bath.

Figure 9.9  Single turnover assays for α₂ with [Re]-(F/Y/3,5-F₂Y)-R₂C₁₉ peptides.
in 20% glyceral. Data was collected after 2, 5, and 10 minutes of photolysis with light of $\lambda > 348$ nm. Red and blue bars are at pH 8.2 and 7.5, respectively.

**Figure 9.10** TA spectra recorded for 100 $\mu$M solutions of [Re]-F-R2C19 (left) and [Re]-YR2C19 (right) in the presence of 135 $\mu$M $\alpha$2, 1 mM CDP, and 3 mM ATP in 20% glycerol at pH 7.5. Spectra are recorded at 65, 115, 215, and 415 ns (left) and at 115, 215, and 415 ns (right). Insets: Single wavelength absorbance kinetics data measured at 480 nm.

**Figure 9.11** TA spectra of a 100 $\mu$M solution of [Re]-3,5-F$_2$Y-R2C19 in the presence of 140 $\mu$M Y731F-$\alpha$2, 1 mM CDP, and 3 mM ATP in 20% glycerol at pH 8.2. Spectra were recorded at 65, 115, and 215 ns. Inset: Single wavelength absorbance kinetics recorded at 395 nm.

**Figure 9.12** TA spectra of a 100 $\mu$M solution of [Re]-3,5-F$_2$Y-R2C19 in the presence of 216 $\mu$M $\alpha$2, 1 mM CDP, and 3 mM ATP at pH 8.3. Spectra were recorded at (▬) 65, (▬) 215, and (▬) 415 ns.

**Figure 9.13** TA spectra of a 100 $\mu$M solution of [Re]-3,5-F$_2$Y-R2C19 with 10 mM Ru(III)(NH$_3$)$_6^{3+}$ (upper left) and 200 $\mu$M $\alpha$2 (upper right), 175 $\mu$M Y730F-$\alpha$2 (lower left), or 175 $\mu$M C439S-$\alpha$2 (lower right). Conditions: 1 mM CDP, 3 mM ATP, 15 mM MgSO$_4$ and 50 mM Tris buffer at pH 8.3. Spectra were recorded at (▬) 415 ns, (▬) 1, (▬) 2, and (▬) 10 $\mu$s.

**Figure 9.14** Normalized TA spectra of 100 $\mu$M solutions of [Re]-3,5-F$_2$Y-R2C19 containing 10 mM Ru(III)NH$_3^+$ (▬) and 200 $\mu$M $\alpha$2 (▬), 175 $\mu$M Y730F-$\alpha$2 (▬), or 175 $\mu$M C439S-$\alpha$2 (▬).

**Figure 9.15** X-Ray structure of $\alpha$2 with R2C20 peptide bound.

**Figure 9.16** DEAE ion exchange purification of [5-3H]-CDP. Fractions between the vertical lines were collected and pooled.

**Figure 9.17** Timing of the nanosecond TA instrument running at 10 Hz.

**Figure 9.18** Diagram of the nanosecond TA instrument running at 10 Hz.
Figure 10.1  Proposed photochemistry for [Re]-3,5-F2Y356-β2.

Figure 10.2  TA spectra recorded after photolysis of 100 μM solutions of 4-Ac-BP in the absence (left) and presence (right) of 10 mM L-tyrosine methyl ester with 320 nm pulsed laser light. Spectra in the left panel were recorded 100, 200 ns, 1, 2, and 5 μs after the laser pulse (top to bottom). Spectra in the right panel were recorded 200 ns, 1, and 5 μs after the laser pulse (top to bottom).

Figure 10.3  TA spectra recorded after 320 nm photolysis of 50 μM solutions of met-Y356C(BP)-β2 in the absence (A) and presence (B) of 70 μM α2, and 50 μM Y356C(BP)-β2 with 70 μM α2 (C). Spectra were recorded 150, 300 ns, 1 and 5 μs after the laser flash (top to bottom). Conditions: 50 mM Tris (pH 7.5), 15 mM MgSO4, 1 mM CDP, and 3 mM ATP.
Chapter I

Introduction

Parts of this chapter have been published:

1.1 Fundamentals and Nomenclature

Proton-coupled electron transfer (PCET)\textsuperscript{1,2} steps beyond simple electron transfer (ET) in the complexity associated with the two particle nature of this reaction. In a chemical context, ET is associated with redox events that are oftentimes influenced by the surrounding medium, while PCET is extended to combine redox and acid/base chemistry and encompasses charge transfer and bond-making/bond-breaking reactions. The electron and proton may be transferred together or separately in a concerted or stepwise fashion and the term PCET generally encompasses all of these reactions. Figure 1.1 presents an illustration of two fundamentally distinct types of PCET commonly encountered in chemistry and biology.\textsuperscript{3} The first type, termed unidirectional PCET, involves the transfer of both a proton and an electron from a single donor to a single acceptor. The proton and electron may be transferred together as a hydrogen atom, or separately involving electronic orbitals for ET and PT of different symmetry. In contrast, bidirectional PCET reactions invoke a single donor and two separate acceptors, one for ET and one for PT. Mechanistically, these PCET reactions have been treated within the context of “square schemes” as illustrated in Figure 1.2. The electron and proton may be transferred in a stepwise (ETPT or PTET) fashion.

\textbf{Figure 1.1.} \textit{Fundamentally distinct types of PCET.}

\textbf{Figure 1.2.} \textit{Square scheme for PCET. X is the ET and PT donor and Y is the acceptor.}
along the outside of the square, or in a concerted electron-proton transfer (CEP) along the
diagonal. Importantly, CEP is defined as occurring with a single transition state. Genuine
hydrogen atom transfer, HAT, can thus be classified as a type of CEP reaction.

Conflicting use of nomenclature by several authors has muddied the dissemination of
mechanistic insight obtained through recent investigations into PCET reactions. Table 1.1
presents a summary of the various nomenclatures used by prominent authors in this field. A
general agreement has been reached for the stepwise reactions, ETPT (electron transfer
followed by proton transfer) or PTET; both of which may be classified as a general PCET
reaction since both the electron and proton are transferred. The nomenclature for concerted
reactions (those occurring with a single transition state) is a bit more ambiguous.

**Table 1.1 Various Nomenclatures Used to Describe PCET.**

<table>
<thead>
<tr>
<th>Concerted Unidirectional PCET³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concerted Electron-Proton Transfer (CEP)⁴</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concerted Bidirectional PCET³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bidirectional Concerted Electron-Proton Transfer (CEP)⁴</td>
</tr>
</tbody>
</table>

Enzymes often rely on the coupling of electrons and protons to affect primary metabolic
steps involving charge transport and catalysis.⁹ Amino acid radical generation and transport is
synonymous with PCET¹⁰ as is the activation of most substrate bonds at enzyme active
sites.¹¹ PCET is especially prevalent at metallo-cofactors that activate substrates at carbon,
oxxygen, nitrogen, and sulfur atoms. PCET charge transport and activation events in enzymes
embody “quantum catalysis” inasmuch as PCET is intrinsically a quantum mechanical effect
because both the electron and proton tunnel.¹² The caveat to PCET is that the transfer of the
proton, as the heavier particle, is fundamentally limited to short distances whereas the
electron, as the lighter particle, may transfer over very long distances.¹³,¹⁴ When transport
distances are short, the electron and proton may transfer together. When they are long,
however, the predicament of the disparate transfer distances is resolved by the evolution of
enzymes to control proton-transfer (PT) and electron-transfer (ET) coordinates on very
different length scales. Adding to the challenge of effecting PCET over long distances with appreciable rates are the requirements that charge transport occur under physiological conditions, with minimal thermodynamic driving-force, with low over-potentials, and with specificity. To do so, enzymes impart exquisite thermodynamic and kinetic control over the electron and proton during radical transport and catalysis events. By discovering and understanding functional PCET mechanisms in these systems, we can begin to develop and test predictive theories for PCET reactions, in much the same way the ET field has developed.

1.2 Theory of ET and PCET

In 1956, a theory was developed by Marcus to treat the potential energy of the transition state of a fixed distance electron transfer reaction. ET occurs as a Franck-Condon transition with the nuclear coordinates frozen as the electron is transferred from donor to acceptor. Marcus’ theory addressed the electrostatic contribution associated with reorientation of solvent molecules to the free energy of formation of the transition state for ET. Later this theory was discussed in terms of potential energy surfaces and statistical mechanics.

Figure 1.3 presents examples of these potential energy surfaces as previously described. With the nuclear coordinates at the equilibrium position of the reactants (Figure 1.3, top), poor electronic overlap exists between the reactant and product electronic wavefunctions due to their offset in energy. The transition state occurs at the intersection of the reactant and product potential energy wells (Figure 1.3, middle), and, at this nuclear position, the reactant and product electronic wavefunctions are degenerate providing optimal overlap for electron tunneling through the barrier. Once the electron has been transferred, the nuclear coordinates relax to the equilibrium position of the product well (Figure 1.3, bottom).

Cast in this model, the expression for the rate and activation energy for ET was derived by Marcus:

\[ k_{ET} = A \exp \left( \frac{-\Delta G^\ddagger}{k_B T} \right) \]  

\[ \Delta G^\ddagger = \frac{(\lambda + \Delta G^\ddagger)^2}{4\lambda} \]
Figure 1.3. Electronic (left) and nuclear (right) potential energy wells for an ET reaction.
where $A$ is a prefactor that defines the frequency of the barrier crossing, $\Delta G^\dagger$ is the activation energy, $\Delta G^\circ$ is the free energy of the reaction, and $\lambda$ is the reorganization energy with contributions from both the inner sphere reactants and outer sphere solvent. Quantum mechanical treatments of the nuclear coordinates have resulted in the modified Marcus-Levich equation for ET reactions with very weak electronic coupling:

$$k_{ET} = H_{AB}^2 \frac{\pi}{h^2 \lambda k_B T} \exp\left(\frac{-(\lambda + \Delta G^\circ)^2}{4 \lambda k_B T}\right)$$

where $H_{AB}$ is the donor-acceptor electronic coupling.

Figure 1.4 presents a modified potential energy surface incorporating the quantum mechanical effect of electronic coupling. The reactant and product diabatic surfaces split near the intersection, often referred to as the “avoided crossing”, with the magnitude of the splitting equal to twice the electronic coupling. For an adiabatic ET reaction, $2H_{AB}$ is large, significantly decreasing the activation energy, and electron transfer occurs every time the reactants reach the transition state. For the nonadiabatic case, the electronic coupling is small and the reactants cross the transition state many times before the electron is transferred to the product energy well.

![Figure 1.4. Potential energy well for an ET reaction treated quantum mechanically.](image)

Marcus’ theory predicts a parabolic dependence of the $\ln(k_{ET})$ vs. $-\Delta G^\circ$: the rate increases to a maximum value where $-\Delta G^\circ = \lambda$ and decreases with further increase in driving force. The latter region is often referred to as the “inverted region” for electron transfer and has been experimentally observed many times. However, the decrease in rate with driving force is often less than parabolic. Nuclear tunneling through vibrational wavefunction overlap is usually coupled to inverted region ET, as illustrated in Figure 1.5. For inverted region ET, the equilibrium position of the reactant well is situated inside the product well, affording optimal overlap between the ground state reactant and excited state product vibrational wavefunctions (red region in Figure 1.5). Inverted region ET mediated by vibrational wavefunction overlap...
has been treated as a form of nonradiative decay in a modified version of the “energy gap law”: \[ 31-33 \]

\[
k_{\text{ET}} = \frac{2\pi}{\hbar} \frac{H_{AB}^2}{(\hbar\omega)(\Delta G^\circ - \lambda_o))^{1/2}} \exp \left[ -S - \frac{\gamma (\Delta G^\circ - \lambda_o)}{\hbar \omega} + \left( \frac{\gamma + 1}{\hbar \omega} \right)^2 \lambda_o k_B T \right]
\]  \hspace{1cm} (4)

\[
\gamma = \ln \left( \frac{\Delta G^\circ - \lambda_o}{\hbar \omega} \right) - 1
\]  \hspace{1cm} (5)

where \( \hbar \omega \) is the averaged energy of the accepting vibrational modes, \( \lambda_o \) is the outer sphere (solvent) reorganization energy, and \( S \) is the electron-vibrational coupling constant or Huang-Rhys factor\(^{34} \) which gives the extent to which the final and initial states coincide along the normal coordinate. Equation 4 is valid in the limit that \( \Delta G^\circ \gg S \hbar \omega \) and \( \hbar \omega \gg k_B T \) (low temperature and/or high energy acceptor mode) and predicts that \( \ln(k_{\text{ET}}) \) should vary linearly with \( -\Delta G^\circ \), rather than the parabolic dependence predicted by Marcus.

Theoretical treatments of PCET\(^{1,35-37} \) have more recently been developed and are based on the quantum mechanical results obtained for ET (eq. 3 and 4). These equations rely on the application of the Born–Oppenheimer and Condon approximations that allow for separation of the nuclear and electronic parts of the overall wavefunction. The validity of this approximation for PCET has yet to be verified.

Hammes-Schiffer has developed a PCET theory that treats potential energy surfaces as a pair of paraboloids, which are functions of the solvent response to changes in both electron (\( z_e \)) and proton (\( z_p \)) position as shown in Figure 1.6.\(^{36} \) In the limit of electronically adiabatic PT and electronically nonadiabatic ET, the following expression was derived:\(^{38} \)

\[
k = \frac{2\pi}{\hbar} \sum_{\mu} P_{\mu} \sum_{\nu} V_{\nu}^2 (4\pi\lambda_{\mu\nu} k_B T)^{-1/2} \exp \left[ -\frac{(\Delta G^\circ_{\mu\nu} + \lambda_{\mu\nu})^2}{4\lambda_{\mu\nu} k_B T} \right]
\]  \hspace{1cm} (6)

Figure 1.5. Inverted region electron transfer. Area in red illustrates vibrational wavefunction overlap.
where $\Sigma_{\mu}$ and $\Sigma_{\nu}$ indicate a sum over vibrational states associated with ET states I and II, respectively and $P_{I\mu}$ is the Boltzmann factor for state I\(\mu\). In this formalism, each vibrational mode or channel in the reactant well couples to the product well with a different electronic coupling, $V_{\mu\nu}$, and the overall rate for each vibrational mode is weighted by the Boltzmann factor for thermal population of that channel. Thus at extremely low temperatures or for high energy vibrations, only the ground state reactant vibrational mode is populated and contributes to the rate. An important difference between eqs. 3 and 6 is that the reorganization energies, equilibrium free energy difference, and couplings are defined in terms of the two-dimensional paraboloids for eq. 6 and depend on both the electron and proton position, as changes in both are coupled to solvent response. Through knowledge of the experimental rate constant and overall reaction driving force, the reorganization energies, electronic couplings, and percent contribution to the rate for each reactant-product vibrational channel can be computed theoretically. Although not predictive, this theory provides a framework with which to begin analysis of PCET reactions.

1.3 PCET of Amino Acid Radicals in Biological Systems

Enzymes often utilize PCET to effect bond making/breaking reactions and charge transport over long distances, therefore these biological systems serve as excellent mediums to discover, develop, and test PCET reaction mechanisms and theory. Amino acid radicals, in particular, have emerged prominently as vehicles for charge transport and cofactors for substrate activation. Oxidation of amino acids at physiological pH usually involves the loss of both a proton and electron, implicating PCET as the redox mechanism. Free amino acid radicals in solution typically have a micro-millisecond lifetime, yet Nature has evolved enzymes to manage both the proton and electron equivalents in harnessing the oxidative power of these intermediates for chemical transformations. Understanding these control
mechanisms may lead to the discovery of new reactivity as well as the development of drugs targeting specific enzymatic PCET steps.

1.3.1 Amino Acid Radicals as Redox Active Cofactors

1.3.1.1 Galactose Oxidase

Galactose oxidase (GAO) is a 68 kDa monomeric fungal copper metalloenzyme that is secreted by *Fusarium spp.* into the extracellular environment and functions as a broad-spectrum alcohol oxidation catalyst and a source of hydrogen peroxide. The two-electron nature of the substrate oxidation chemistry is interesting in that it occurs at a mononuclear copper active site, which typically functions as a one-electron redox agent. This quandary has been explained by the direct participation of the protein, in particular a tyrosine residue, in the redox steps. The X-ray crystal structure of GAO reveals that the Cu-atom in the active site is coordinated by two His residues, a Tyr residue, a water molecule, and a Cys-crosslinked-Tyr residue, as shown in Figure 1.7. Oxygen becomes reduced to hydrogen peroxide at the cuprous active site resulting in a cupric ion bound to a •Y272–C228 radical. In this way, the Y272–C228 residue serves as a redox active ligand allowing a single Cu center to perform multielectron redox chemistry. Substrate alcohol is believed to bind in place of the active site water molecule. No intermediates have been observed in the substrate oxidation process; however a mechanism has been proposed in which PT from the substrate hydroxyl group to Y495 activates the substrate for oxidation. Product formation involves the loss of two electrons and the cleavage of one C–H bond. The mechanism for this transformation is a subject of current debate, but may involve ET to Cu(II) followed by PCET/HAT from the alkoxy radical to •Y272–C228.
1.3.1.2 Cytochrome c Peroxidase

Cytochrome c peroxidase (CcP) is a Class I plant peroxidase whose main role is to remove hydrogen peroxide from the cell.\(^{47}\) Hydrogen peroxide is reduced to water at a heme active site in CcP using electrons from the cytochrome c redox substrate. Reduction of \(\text{H}_2\text{O}_2\) to water requires two electrons; however, cytochrome c is a one electron redox reagent. ENDOR studies have shown that \(\text{H}_2\text{O}_2\) binds to and oxidizes the ferric heme of CcP to an Fe(IV)=O, ferryl intermediate and a protein amino acid radical, most likely [\(\cdot\text{W191}\)]\(^+\).\(^{48}\) The high resolution crystal structure of CcP\(^{49}\) (Figure 1.8) reveals that this residue resides in the proximal pocket of the CcP heme. Electrons are therefore transferred to the [\(\cdot\text{W191}\)]\(^+\) and ferryl intermediate from two separate cytochrome c substrates in two separate steps, primarily along a CCP(heme)–Trp191–Gly192–Ala193–Ala194–Cyt. c (heme) ET pathway.\(^{50}\) Trp191 thus serves as a redox reservoir in the reduction of hydrogen peroxide to water.

1.3.1.3 Cytochrome c Oxidase

Cytochrome c oxidase (CcO) comprises the terminal component of the respiratory chain in aerobic organisms that couples NADPH oxidation to the reduction of oxygen to water.\(^{51}\) This process creates the necessary proton gradient across the membrane to drive the synthesis of ATP, the energy currency of biology.\(^{52,53}\) Reduction of oxygen to water at heme is a remarkably complicated process requiring precise timing of electrons and protons to prevent hydrogen peroxide formation.\(^{11}\) In CcO, oxygen reduction occurs at the Cu\(_B\)–heme \(a_3\) site of CcO and is mediated by electron transfer from a bound cytochrome c redox cofactor through a binuclear Cu\(_A\) cofactor. Cyt. c binds near Cu\(_A\) and electrons are transferred along a cyt c → Cu\(_A\) → heme \(a\) → Cu\(_B\)–heme \(a_3\) pathway.\(^{54,55}\) Oxygen binds to and is activated by the fully reduced ferrous heme \(a_3\)–cuprous Cu\(_B\) site. This process induces heterolytic O–O bond cleavage with the formation of an Cu(II), Fe(IV)=O intermediate\(^{56,57}\) and a protein derived radical.\(^{58}\) EPR experiments of the isotopically labeled enzyme reveal that the radical resides
on a tyrosine residue.\textsuperscript{59} Reaction of the radical with radioactive \textsuperscript{125}I followed by peptide mapping resulted in iodide labeling at Y244, although yields of the labeled protein were small.\textsuperscript{60} The crystal structure of the heme $a_3$–Cu\textsubscript{B} site in CcO (Figure 1.9) reveals the location of this residue in the distal pocket of heme $a_3$ and crosslinked to H240, a ligand to Cu\textsubscript{B}.\textsuperscript{61} Thus Y244 may serve as a redox reservoir in the reduction of oxygen to water in CcO in much the same as W191 in the reduction of hydrogen peroxide to water in CcP. Y244 is characterized as a deprotonated, neutral tyrosyl radical implicating PCET in the radical forming step; however, mechanistic details remain to be uncovered.

1.3.2 Amino Acid Radicals as Charge Transport Intermediates

1.3.2.1 Pyruvate Formate Lyase

As a participant in anaerobic glucose fermentation, pyruvate formate lyase (PFL) catalyzes the reversible conversion of pyruvate and coenzyme A to acetylCoA and formate. The enzyme relies on the formation of a stable glycyl radical at position 734 for activity,\textsuperscript{62} which is formed via a radical SAM mechanism\textsuperscript{63} utilizing a pyruvate formate lyase activating enzyme.\textsuperscript{64} The mechanism of action proposed for PFL is incredibly complicated involving radical transport from G734 to a pair of redox active cysteine residues, C418 and C419. The crystal structure of PFL with pyruvate substrate bound in the active site (Figure 1.10) reveals that these three
residues are within hydrogen bond contact. Thus hydrogen atom transfer (HAT) could operate as the radical transport mechanism.

Based on the work of Knappe and Kozarich, the conversion of pyruvate to formate and coenzyme A to acetylCoA can occur in two steps. In the first step, •C418 attacks the acetyl group of pyruvate, forming acylated C418, formate, and •C419, which relaxes back to •G734. In the second step, a transiently formed •C419 abstracts an H-atom from the thiol group of coenzyme A. The thiyl radical on coenzyme A then attacks the acetyl group of C418, forming acetylCoA and •C418, which relaxes back to •G734. When [14C]-pyruvate was incubated with C419S-PFL, acylated protein was not observed, consistent with the notion that radical transport from •G734→C419→C418 is required for the formation of the putative •C418 radical. Thiyl radical intermediates remain to be observed experimentally, inhibiting investigations into the mechanism of radical transport in PFL.

1.3.2.2 DNA Photolyase

Photolyases are monomeric proteins containing a flavin adenine dinucleotide (FAD) cofactor that is responsible for the photochemical repair of pyrimidine dimers in DNA. Upon cell lysis and isolation from E. coli, the FADH– cofactor is oxidized to the FADH• radical redox state. Exposure of this form of the enzyme to light in the presence of a reducing agent regenerates FADH– cofactor and reactivates the enzyme. Transient absorption spectroscopy revealed that the initially excited doublet state of FADH• converts to the lowest excited quartet state via intersystem crossing. This state then abstracts an electron from the apoenzyme, forming a neutral W• radical. Site directed mutagenesis studies revealed that the W306F mutant failed to be photochemically reactivated, suggesting that W306 was the site of the observed W•. The X-ray crystal structure of the enzyme (Figure 1.11), however, revealed that W306 was near the surface of the protein and remote from the FADH• cofactor. Picosecond transient absorption
studies produced a model wherein the excited FADH• chromophore initially oxidizes the proximate W382 residue; the radical then hops to the protein surface along a \( \text{W382}^+ \rightarrow \text{W359} \rightarrow \text{W306} \) pathway and the subsequently formed \( \text{W306}^+ \) radical deprotonates on the 300 ns timescale. Direct electron tunneling (superexchange) pathways from W306 to the excited FADH• chromophore have also been proposed. While interesting in its own right, this photochemical enzyme reactivation reaction likely has little physiological significance, as the necessary FADH– cofactor is stable in vivo.

1.3.2.3 Photosystem II

Photosystem II (PS II) is an integral component of photosynthesis, the process in plants that uses sunlight and water to drive NADPH and ATP production (which are then used to fix CO\(_2\) in the form of carbohydrates) with \( \text{O}_2 \) released as a byproduct. Chromophores in PS II harvest light and generate a charge separated state that is a weak reductant but a powerful oxidant capable of oxidizing \( \text{H}_2\text{O} \) to \( \text{O}_2 \). Light is harvested by various reaction center chromophores (antenna chlorophylls and carotenoids) that are electronically coupled to a chlorophyll \( \alpha \) component termed P680. The excited P680* then transfers an electron to a pheophytin \( \alpha \) cofactor and ultimately to a pair of quinone molecules termed QA and QB, the latter of which diffuses into the membrane toward the cytochrome \( \text{b}_{6f} \) complex. The oxidized P680+ transfers the electron-hole to the inorganic oxygen evolving complex (OEC) of PSII, which is responsible for water oxidation. Four flashes of light are required for \( \text{O}_2 \) evolution, and each flash steps the OEC through a different redox state, \( S_0 \) through \( S_4 \), with the \( S_4 \) to \( S_0 \) transition occurring in the dark with
thermal release of $O_2$. Time resolved X-ray absorption spectroscopy experiments have recently modified and enriched this $S$-state cycle.

In 1973, a dark-stable organic radical, denoted D, was observed in PSII by EPR experiments. Later, a light induced reversible phase of this EPR signal was also identified, denoted cofactor Z, and assigned to the physiological donor to P680$^+$. In 1987, EPR experiments identified the existence of a tyrosyl radical, $\cdot Y_D$, in PSII in dark adapted samples and suggested that the Z radical was also a tyrosyl radical, $\cdot Y_Z$. Site directed mutagenesis experiments isolated the radical to Y160 on the D2 polypeptide and suggested, by the pseudo-C$_2$ symmetry of the complex, that the Z radical corresponded to Y161 of the D1 polypeptide, which was later confirmed by mutagenesis studies of the D1 polypeptide.

More site directed mutagenesis studies revealed that the presence of H190 facilitated oxidation of $Y_Z$ by P680$^+$ by a factor of at least 200 in rate, implicating hydrogen bonding between these residues. PCET was then identified as the mechanism for $Y_Z$ oxidation with electron transfer to P680$^+$ and proton transfer to H190. One of the more recent crystal structures of PSII is presented in Figure 1.12 and clearly illustrates the hydrogen bond between $Y_Z$ and H190, along with the electron transfer tunneling distances along the P680$\rightarrow$Y$Z$$\rightarrow$OEC (Mn$_4$Ca) pathway. Thus, PSII is a clear example of bidirectional PCET in a biological system. The electron, as the lighter particle, can tunnel over much longer distances between the redox active cofactors, while proton tunneling is restricted to within the short distance provided by the H-bond between H190 and $Y_Z$.

1.4 Class I Ribonucleotide Reductase

Class I E. coli ribonucleotide reductase (RNR) plays a crucial role in DNA replication and repair by catalyzing the reduction of nucleoside diphosphates (NDPs) to deoxynucleoside diphosphates (dNDPs). The enzyme is composed of two homodimeric subunits designated $\alpha_2$ and $\beta_2$, and a complex between the two is required for activity. $\alpha_2$ houses the NDP binding sites and the binding sites for the effectors that control the specificity and rate of nucleotide reduction. $\beta_2$ harbors a diferric tyrosyl radical ($\cdot Y_{122}$) cofactor proposed to initiate nucleotide reduction by oxidizing a cysteine residue (C439) in the active site of $\alpha_2$.

The mechanism of deoxynucleotide formation has been the subject of intense investigation. Biochemical studies with radio-labeled substrates and mechanism based
inhibitors have resulted in Scheme 1.1 as the model for this chemical transformation. The transient •C439 generated in the active site of α2 abstracts the 3'-H atom from the ribose ring to initiate the reaction. The radical then migrates to the 2'-carbon with concomitant loss of water; E441 serves as a base to deprotonate the 3'-hydroxyl group forming the 3'-ketone. An active site cysteine residue (C462/C225) then reduces the 2'-radical with an H-atom, forming the disulfide radical anion. At this point, a PCET event may ensue with ET from the disulfide radical anion and PT to the 3'-ketone from E441, thereby regenerating the 3'-carbon centered radical. This radical then oxidizes C439 to complete the transformation and reform the thiol radical in the active site of α2. Following dNDP production, •C439 is reformed in the active site and the radical is transferred back to Y122 in β2; thus one β2 subunit can turnover multiple α2 subunits. Transport of the radical out of the active site of α2 prevents reaction of the sulfur centered thiol radical with oxygen, which is known to generate thiol peroxy and sulfinyl radicals.

The crystal structures of both subunits have been solved independently, and a docking model of the two proteins in a 1:1 complex has been generated based on their

Scheme 1.1. Proposed mechanism of deoxynucleotide formation in E. coli class I RNR.
complementary shapes and on knowledge of conserved residues. In this model, more than 35 Å separates the •Y122 on β2 from C439 in α2; this long distance has recently been verified by PELDOR studies of the mechanistically inhibited α2:β2 complex. Superexchange electron tunneling between •Y122 and C439 based on Marcus theory (k_{ET} = 10^{-6} \text{ s}^{-1} \text{ for } \beta = 1.2 \text{ Å}^{-1} \text{ under activationless conditions}) is too slow to account for a k_{cat} of ~2 to 10 s^{-1}. Thus, the radical generation process has been proposed to occur via a hopping mechanism involving conserved amino acid residues •Y122 → W48 → Y356 → Y731 → Y730 → C439 as shown in Figure 1.13. Site directed mutagenesis studies confirm that these residues are required for activity, however they do not provide additional information regarding the precise mechanism of radical transport.

Pre-steady state stopped flow experiments have been performed to probe the mechanism of radical transport in RNR. No changes in the •Y122 concentration could be detected upon mixing the α2 and β2 subunits under a variety of conditions. These data supported a model in which a rate-limiting physical or conformational step was required in the α2:β2 complex for radical transport and subsequent active site chemistry to occur.
Using intein protein ligation methods, semi-synthetic β2 subunits were generated with unnatural amino acids at position 356. In particular, a series of fluorinated tyrosine derivatives were generated with reduction potentials that vary from −50 mV to +270 mV relative to tyrosine over the accessible pH range for RNR and pKₐs that range from 5.6 to 9.9 (See Chapter VI). The pH rate profiles of deoxynucleotide production by these FnY356-β2s were reported, and the results suggested that the rate-determining step in RNR activity could be changed from the physical step to the radical propagation step, by altering the reduction potential of •Y356 with these analogues. These studies supported the model that Y356 is a redox active amino acid on the radical propagation pathway. Furthermore, several of the FnY356-β2s are deprotonated at pH > 7.5 and efficiently initiate nucleotide reduction. Thus a proton is not obligated to the pathway between W48 and Y356 of β2 and Y731 of α2, nor is H-atom transfer between these residues obligatory for radical propagation.

By incorporating 3,4-dihydroxyphenylalanine (DOPA) into position 356 in β2, the •Y122 radical can be trapped along the radical transport pathway at •DOPA356, but only in the presence of α2 and RNR substrate and effector. Further studies revealed the presence of back radical transport (•DOPA356→Y122) and an analysis of these reactions led to a new model for radical transport in RNR that questions the symmetry of the docking model. However, in all these studies catalytically competent radical intermediates have not been observed, preventing a detailed analysis of the PCET mechanism of radical transport along this conserved pathway.

1.5 Photochemical RNR

To address the challenge of kinetically resolving radical transport, we have developed photochemical RNRs: simplified RNR constructs utilizing the α2 subunit and the 19-mer C-terminal peptide tail (R2C19) of β2 as depicted in Figure 1.14. R2C19 contains the binding determinant of β2 to α2 and the redox active Y356 residue. Using solid-phase peptide synthesis, we can append a photo-oxidant proximate to Y356 (Figure 1.14, red circle) that, upon excitation, can produce •Y356, which can then be translated into the C439 active site of α2. The first experiments with this construct were performed using tryptophan as the phototrigger of •Y356. Excitation of the peptide-α2 complex in the presence of CDP substrate and ATP effector resulted in dCDP formation, demonstrating enzymatic activity was retained when the entire β2 subunit was replaced by a photoactive peptide. However a drawback
with tryptophan-based peptide constructs arose from the need for UV-light to excite tryptophan. Deep UV excitation profiles fall within the absorption envelope of RNR, thus presenting a significant problem for the direct kinetic analysis of α2-bound peptides owing to inner-filter optical effects and protein instability. Therefore, new photo-oxidants of tyrosine need to be developed.

1.6 Scope of the Thesis

This thesis explores the mechanisms of proton-coupled electron transfer (PCET) within the context of amino acid radical generation, with application to radical transport in the ribonucleotide reductase (RNR) enzyme. Figure 1.15 summarizes the thesis organization with regard to the study of photochemical RNR. Chapter II describes transient absorption (TA) studies of photochemical amino acid radical generation and transport in dipeptides containing W and Y, providing insight into the mechanism of radical generation in the W-based photochemical RNR and the mechanism of radical transport in the β2 subunit of RNR. In Chapter III, we explore a host of other organic and inorganic photochemical oxidants for the production of Y•. Chapter IV describes the design, synthesis, and photophysical characterization of new Re(I), MLCT excited states for photochemical Y• generation. Chapter V explores the mechanistic complexity of the PCET of Y• generation with one of these Re(I)
systems, and through comparison to other Ru(II) systems in the literature, presents a general scheme for the PCET of Y oxidation in aqueous solution. In Chapter VI, we develop the redox, acid-base, and spectroscopic characterization of a series of fluorotyrosine unnatural amino acids and their corresponding radicals as useful probes for the study of PCET in biological systems that utilize Y•. In Chapter VII, we characterize photochemical RNRs containing benzophenone and anthraquinone chromophores, and in Chapter VIII we show that Y356 is not required for activity of the photochemical RNRs. Chapter IX describes extensive work with Re(I) based photochemical RNRs in an attempt to characterize radical intermediates in α2, and Chapter X presents initial synthetic work towards developing photochemical β2 subunits, and preliminary TA experiments with benzophenone-labeled β2 to characterize radical transport in the full α2:β2 complex. The work in the first half of the thesis has general application to the study of PCET in enzymes that utilize amino acid radicals, while the latter half more specifically targets an understanding of the mechanism of radical transport in RNR.
1.7 References


93. Debus, R. J.; Barry, B. A.; Sithole, I.; Babcock, G. T.; McIntosh, L. *Biochemistry* 1988, 27, 9071.


Chapter II

Trp as a Photochemical Radical Initiator & pH Dependence of Radical Transport in Trp–Tyr Dipeptides

Parts of this chapter have been published:

2.1 Motivation

We wish to characterize the products of W and Y UV photolysis, as it has been used as a radical generator in the initial experiments with photochemical RNRS. We also wish to gain insight into the mechanism for radical transport in the \( \beta_2 \) subunit of class I RNR. This chapter describes characterization of radicals produced upon UV excitation of W-Y and N-terminal acetylated, Ac-W-Y, dipeptides using transient absorption spectroscopy. The pH dependence of the kinetics of radical transport between W and Y is also determined and provides a model for the mechanism of radical transport through W48 in \( \beta_2 \).

2.2 Background

Radical initiated electron transfer between tryptophan and tyrosine residues in proteins has been extensively studied,\(^1,2\) with particular focus on lysozyme enzymes.\(^3-5\) The observation of these two amino acids interacting with each other as part of a natural enzymatic electron transfer (ET) pathway, however, has been limited to only a few systems. \( A. \) nidulans photolyases possess a triad of Ws (W390, W367, and W314) through which a flavin generated electron hole passes to generate a tyrosine radical located at the surface of the protein.\(^6\) In the absence of exogenous reductants, Y• is generated in <500 ns, and reverse ET regenerates Y, suggesting regulated radical transport between Y and W.\(^7,8\) Y• hole transfer in the reverse direction is slowed in D\(_2\)O, suggesting the role of a proton in mediating the ET, though the basis for the increase in Y• lifetime has yet to be determined. Electron transfer between cytochrome \( c \) and cytochrome \( c \) peroxidase modified with a K\(^+\) binding site is also believed to be modulated by an equilibrium between tryptophan cation and tyrosyl radicals.\(^9\) Population of the K\(^+\) binding site shifts the equilibrium from [•WH]\(^+\) towards the Y•, which in turn retards the rate of cytochrome \( c \) oxidation by the Compound I intermediate of cytochrome \( c \) peroxidase.

Focusing on the redox interplay between Y and W in class I RNRS, oxidized W48 is proposed to be a key intermediate along the PCET radical hopping pathway from •Y122 in \( \beta_2 \) to C439 in \( \alpha_2 \) (see Chapter I).\(^10\) This residue is positioned between Y122, the initial site of radical generation in \( \beta_2 \), and Y356, the conduit for radical transport between \( \alpha_2 \) and \( \beta_2 \).\(^11\) Site directed mutagenesis studies suggest that W48 is needed for the forward propagation of the radical into the active site.\(^12\) This implicates oxidation of W48 by •Y122 to produce a radical,
which in turn is proposed to oxidize Y356. Moreover, studies of the assembly of the diiron cofactor of β2 provide direct spectroscopic evidence for a [•W48H]+ radical\textsuperscript{13-17} and suggest it to be directly involved in Y122 oxidation.\textsuperscript{14} Together these studies suggest that W↔Y electron transfer is needed for radical propagation in RNR and that the protonation state of W48 may be important in controlling the direction of radical propagation.

Tryptophan has also been used as a radical photoinitiator in turning on ribonucleotide reductase activity.\textsuperscript{18} In these experiments, the 20-residue, C-terminal peptide tail of β2, which contains the binding determinant of β2 to α\textsuperscript{219,20} and the redox active Y356 residue, was generated by solid phase peptide synthesis. W was appended immediately after the Y356 residue, the peptide bound to α2, and protein-peptide complex excited with light of λ > 285 nm in the presence of RNR substrate and effector. This construct resulted in ~20 % turnover, which decreased to ~2% upon omission of W from the peptide.\textsuperscript{18}

To gain insight into radical photo-generation and transport in systems containing W and Y, we have undertaken detailed time-resolved laser spectroscopy studies of the pH dependence of the rates of radical transfer between tryptophan and tyrosine in W–Y and Ac-W–Y dipeptides. The kinetics of W•–Y → W–Y• have previously been examined in dipeptides using either flash photolysis or pulsed radiolysis as the radical generator. At or near pH 7, the reaction rate is observed to occur with a rate constant of 9 ± 5 × 10\textsuperscript{4} s\textsuperscript{−1}.\textsuperscript{21-27} Studies of the intermolecular reaction of L-tryptophan and L-tyrosine in solution show that tyrosine radicals can oxidize tryptophan at pH 1.1 and possibly at 12.5, but, between these values, tryptophan radicals oxidize tyrosine.\textsuperscript{28} The pH dependence for radical transport through proline has been found to be constant between pH 6.5 and 11 and increase linearly below pH 6.\textsuperscript{29} We now show that the directionality of radical transport in W–Y is controlled by pH and suggest the importance of the W protonation state on the directionality of PCET in RNRs.

2.3 Results and Discussion

The transient absorption spectrum of laser excited (λ\textsubscript{exc} = 266 nm) solutions of W–Y at pH 7.8 is dominated by the dynamics of the decay of the \textsuperscript{3}W* excited state (λ\textsubscript{max} = 450 nm\textsuperscript{30}) at short times (10 ns -1 μs). After 2 μs, all triplet states have decayed to the ground state or reacted with dissolved O\textsubscript{2} to reveal the transient absorption spectrum shown in Figure 2.1. Two prominent transient signals are observed with λ\textsubscript{max} = 410 and 510 nm; these maxima
coincide with the maxima for Y• and W• radicals, respectively ($\varepsilon_{410\text{nm}}(Y\cdot)^{31} = 2750 \pm 200 \text{ M}^{-1}\text{cm}^{-1}$; $\varepsilon_{510\text{nm}}(W\cdot)^{32} = 1800 \pm 50 \text{ M}^{-1}\text{cm}^{-1}$). The time evolution of the absorption spectrum is revealed with single wavelength kinetics measurements. As shown in the inset, the W• radical decays with a rate constant of $8.6 \times 10^4 \text{ s}^{-1}$ and Y• appears on a commensurate time scale ($k_{X\cdot} = 1.1 \times 10^5 \text{ s}^{-1}$). Other intra- and intermolecular radical decay process of Y and W were examined by measuring the kinetics of control peptides Ac-F–Y and W–F, respectively. We find that the decay processes of photogenerated radicals in the control dipeptides are slow and do not contribute significantly to transient signals shown in Figure 2.1.

The rate constants for radical transport agree well with the rates previously observed for W•–Y $\rightarrow$ W–Y• transfer at neutral pH.21-27 A similar result is obtained for Ac-W•–Y $\rightarrow$ Ac-W–Y• with a slightly attenuated rate for radical transfer ($k_{X\cdot} = 1.8 \times 10^4 \text{ s}^{-1}$). Because radical transport is slower in the acetylated peptide, other radical decay processes become competitive for Y• decay but not W• decay as determined from the control peptides. For this reason, the kinetics of the Ac-W•–Y $\rightarrow$ Ac-W–Y• reaction (over all pH ranges) were determined from monitoring the 510-nm transient signal corresponding to the W•. In the case of the native W–Y dipeptide, the N-terminus is protonated and thus electron transport occurs across a favorable dipole. Ab initio calculations show that the removal of the positive charge at the N-terminus of dipeptides significantly reduces the dipole moment33; we therefore expect the electron transfer rate to be reduced, as observed, in the Ac-W–Y peptide. These observations are consistent with electron transfer in model peptides, which have been shown to exhibit enhanced rates for charge transfer when the direction of electron movement coincides favorably with the peptide dipole.34,35

The overall profile of the transient absorption spectrum and the kinetics for radical transport are maintained over a pH range of 6 to 10. Below pH 6, the transient signal for
tryptophan radical red-shifts from 510 nm to 560 nm as shown in Figure 2.2. At pH 3.1, the transient absorption spectrum is dominated by the 560-nm feature, which is consistent with the production of a protonated tryptophan radical, [•WH]⁺ (ε_{560nm} = 3000 ± 150 M⁻¹ cm⁻¹, pKa = 4.3) [36]. At intermediate pH, a signal is observed with a maximum that lies between the 510 and 560 nm signals for W• and [•WH]⁺, respectively. The transient signal at pH = 4.3 = pKa ([•WH]⁺) is shown in Figure 2.2 and is the superposition of nearly 1:1 contributions of the [•WH]⁺ and W• transient signals.

The rate for radical transport from W to Y in both W–Y and Ac-W–Y steadily increases as the pH is lowered from 6 to 2. However, below pH ~4, the radical transport in W–Y becomes fast enough to be convoluted and masked by the decay of 3W* . Owing to the slower radical transport in Ac–W–Y (vide supra), rate constants could be measured to pH ~2. At pH 2.35, Ac–[•WH]⁺–Y → Ac–W–Y• proceeds with rate constant of 1.5 × 10⁵ s⁻¹ as measured by the disappearance of the 560 nm component. For the control W–F peptide, the W decay is invariant with pH and slow with respect to this time scale. Thus specious radical decays do not interfere with these measurements. The observed steady increase in the rate of radical transport from W to Y with decreasing pH is consistent with similar previous studies in the Trp–Pro–Tyr peptide probed by pulse radiolysis [29].

At the other end of the pH scale, the radical transport rate constant decreases over the range 10 < pH < 11.6 for W–Y and 10 < pH < 12.2 for Ac-W–Y. The rate for radical transport becomes sufficiently slow that other first and second order radical decay processes, as measured for the control peptides, prevent extraction of an electron rate constant from the decay trace of the 510-nm transient. A further increase in pH beyond 12 is accompanied by opposite behavior in the time evolution of the transient absorption spectrum of W–Y. Namely, the 510-nm transient signal of W• appears with the concomitant disappearance of the 410 nm signal of Y•, indicating the reverse reaction, W–Y• → W•–Y–, where Y– represents the

Figure 2.2. Transient absorption spectrum 2 μs after 266-nm laser excitation (fwhm = 3 ns) of Ac–W–Y solution at pH: 7.2 ( ), 4.3 ( ) and 3.1 ( ).
deprotonated tyrosinate anion. The rate constant for this reverse reaction is $k_{\text{X}^\text{rev}} = 1.2 \times 10^5$ s$^{-1}$.

Figure 2.3 summarizes the pH dependence of the rates of the radical transport reactions of W–Y and Ac-W–Y. For both peptides, radical transport from W to Y is preferred for pH = 6-10 and the rate constant for the reaction is invariant over this pH range. At the endpoints of the pH plateau, the rate constant monotonically increases as the pH is lowered below 6 and decreases as the pH is raised above 10. For pH > 12, the reaction reverses and radical transport from Y to W is favored. This reversal of radical transport in W–Y dipeptides has escaped detection by laser photolysis and only been observed previously by pulse radiolysis at pH 13.\textsuperscript{28}

Insight into the rates and direction of radical transport between W and Y dipeptides is provided by analysis of the W•/W and Y•/Y reduction potentials. The solid line in Figure 2.3 is the difference in these reduction potentials, $\Delta E = E_p(W•/W) - E_p(Y•/Y)$, as calculated from the differential pulse voltammetry measurements of Tommos et al that are summarized in Figure 2.3.\textsuperscript{37} For pH > 6, the rate constants are approximated well by the driving force dependence of the radical transport. We ascribe the invariance of the rate constant for pH = 6 - 10 to the constant value of $\Delta E$ over this pH range. For pH > 10, the hydroxyl group of Y is deprotonated and $E_p(Y•/Y)$ becomes constant with pH whereas $E_p(W•/W)$ continues to decrease monotonically (see Figure 2.4). The confluence of these two trends causes the driving force for radical transfer between W• and Y to decrease in this pH range. The rate constant for radical transfer is expected to reflect this decrease until $E_p(Y•/Y) = E_p(W•/W)$ at pH 10.9; this is observed in Figure 2.3. For pHs > 10.9, the direction of radical transport reverses, as oxidation of W by Y• is preferred.
A perplexing increase in rate is observed for pH = 2 - 6. Over this pH range, protonation of W• to produce [•WH]+ is favorable and the reduction potential accordingly becomes pH independent (see Figure 2.4). The increased oxidizing power of the [•WH]+ radical, however, is offset by the increased difficulty in oxidizing Y, leading to a mild reduction in the driving force over this pH range. On this basis, the rate for Y oxidation by W•/[•WH]+ should slowly decrease, but this expectation is not met. The rate of Y oxidation increases with decreasing pH to pH = 2. We ascribe the rate enhancement in part to the further augmentation of the peptide dipole upon protonation of the tryptophan residue. The convergence of the Y•/Y and [•WH]+/W reduction potentials at pH 2.8 causes the driving force for radical transport to decrease to the point that the reaction becomes thermoneutral. A further decrease in the pH should shift the energetics to favor the W–Y• → W•–Y reaction. Indeed, radical transport from Y to W in at very acidic pH (=1) has been observed for the intermolecular reaction between W and Y in pulse radiolysis experiments.

2.4 Conclusions

The results reported herein reveal that W• can be effectively generated upon UV photolysis and that W• is capable of oxidizing Y under physiological pH. Thus W• generated in the W-based photochemical RNR is capable of oxidizing Y356 on the peptide or Y731 in α2. The data also have mechanistic implications in the radical-induced cofactor assembly and nucleotide reduction processes of RNR. Figure 2.5 shows the diiron cofactor and selected amino acid residues that comprise the beginning of the 35 Å radical transport pathway that spans β2 and α2 subunits of class I RNRS. The figure highlights the location of W48 between Y122 and Y356 and the proximity of W48 to D237.
The potential importance of W48 and [•W48H]⁺ was first noted by Nordlund and Eklund\(^{39,40}\) who proposed that the Fe₁→H₁₁₈→D₂₃₇→W₄₈ pathway in β₂ could be mechanistically important in cofactor assembly and nucleotide reduction, based on the structurally analogous and spectroscopically well-characterized Fe(heme)→H→D→W ET pathway in cytochrome c peroxidase. Subsequent studies have suggested that the PCET pathway in the assembly process is shared with part of the ET pathway in the nucleotide reduction process.\(^{10}\) Additionally, radical initiation at C₄₃₉ is thought to occur reversibly between the two subunits on every enzymatic turnover.\(^{41}\) The studies reported herein show that the protonation state of W₄₈ can control the rate and direction of radical transport by modulating the Y₁₂₂→W₄₈→Y₃₅₆ interactions. D₂₃₇ provides a site to establish PCET by coupling electron transfer along the radical transport pathway to proton transfer to or from W₄₈. As has been recently noted,\(^{42}\) a conserved residue, R₂₃₆, may also influence local pKa by interacting directly with D₂₃₇ or other redox active residues along the pathway.

By implicating D₂₃₇ in radical transport, a PCET pathway is constructed whereby proton transfer is orthogonal to the electron transfer pathway. Modulation of electron transport by a proton along an orthogonal path appears to be an emerging trend in the PCET events of biology. As described in Chapter I, Y₇ (Y₁₆₁) in Photosystem II (PSII) is the linchpin that manages electron and proton flow in the conversion of the primary light absorption event at the P₆₈₀ chlorophylls into the multielectron activation of water at the oxygen-evolving complex (OEC).\(^{4₃,4₄}\) Histidine H₁₉₀ positioned off of the ET pathway, provides a base function that is essential for effective oxidation of Y₇ by P₆₈₀\(^{+}\). The neutral radical, •Y₇,
which forms is the primary oxidant that steps the tetranuclear manganese cluster through each of the four S states of the Kok cycle.\textsuperscript{45,46} In RNR, PCET involves tyrosyl radicals produced from orthogonal proton and electron pathways; but unlike PS II the tyrosyl radical participation is not direct. Instead, W48 sandwiched between tyrosines provides the necessary coupling between the proton and electron. By enforcing the appropriate pKa on W48, the protein can control the direction of radical transport among tyrosines, which are proposed to be involved in radical initiation of nucleotide reduction and di-iron tyrosyl radical assembly.

2.5 Experimental Section

Materials. L-Tryptophanyl-tyrosine (W-Y), L-tryptophanyl-phenylalanine (W-F) (Sigma), N-\(\alpha\)-acetyl-L-tryptophan (Ac-Trp-OH), N-\(\alpha\)-acetyl-L-phenylalanine (Ac-F-OH), L-tyrosine tert-butyl ester (H-Tyr-O-t-Bu), 1-Hydroxybenzotriazole (HOBt) (NovaBiochem), 1-(3-Dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (WSC·HCl), and N-methylmorpholine (NMM) (Aldrich) and trifluoroacetic acid (TFA, 99.9%) (J. T. Baker) were used as received.

\textbf{N-\(\alpha\)-Acetyl-L-tryptophanyl-L-tyrosine (Ac-W-Y).} Ac-Trp-OH (301 mg, 1.22 mmol, 1.0 eq), H-Tyr-OtBu (290 mg, 1.22 mmol, 1.0 eq), WSC·HCl (258 mg, 1.34 mmol, 1.1 eq), HOBt (182 mg, 1.34 mmol, 1.1 eq) and NMM (550 μL, 4.88 mmol, 4.0 eq) were combined in a round bottom flask with 100 mL of chloroform and stirred overnight. The reaction solution was extracted in a separatory funnel with 2 × 50 mL 10 % citric acid, 1.0 M sodium bicarbonate, and water. The organic layer was dried over magnesium sulfate and the solvent removed in vacuo to yield a clear oil. The oil was dissolved in a minimal amount of ethyl acetate, loaded onto a chromatotron plate (silica gel, 2mm), and eluted with ethyl acetate. Three bands were observed under a UV lamp (254 nm). The first band to elute was collected and the solvent removed in vacuo to yield a clear oil. The oil was dissolved in 1% MeOH/10%TFA/CH\(_2\)Cl\(_2\) (100 mL) and stirred for one hour during which time the solution turned pink. Solvent was removed in vacuo resulting in a tan oil that was placed under high vacuum overnight. It was then dissolved in a minimal amount of ethyl acetate and loaded onto another chromatotron plate (silica gel, 2 mm). The plate was allowed to dry to remove the ethyl acetate and was then eluted with 5 - 6% MeOH/CH\(_2\)Cl\(_2\) (1% acetic acid). The most intense band was collected and solvent removed under high vacuum. The tan oil was dissolved in 150 mL ethyl acetate, washed with 2 × 75 mL water to remove any remaining
Acetic acid, dried over MgSO₄, and the solvent removed in vacuo to yield a tan oil that solidified over a few days (205 mg, 0.500 mmol, 41 %). ¹H NMR (300 MHz, CD₃OD, 25 °C) δ = 7.93 (d, 1H, N-H), 7.57 (d, 1H, indole-H), 7.30 (d, 1H, ind-H), 7.15 - 6.61 (m, 7H, ind-H and phenol-H), 4.67 (m, 1H, W-CH), 4.56 (m, 1H, Y-CH), 3.28 - 2.80 (m, 4H, W-CH₂ and Y-CH₂), 1.86 (s, 3H, Ac-CH₃). Anal. Calcd for C₂₂H₂₃N₃O₅: C, 64.54; H, 5.66; N, 10.26. Found: C, 64.47; H, 5.71; N, 10.15.

*N-α-Acetyl-L-phenylalanyl-L-tyrosine.* Ac-Phe-OH (291 mg, 1.40 mmol, 1.0 eq), H-Y-O-t-Bu (333 mg, 1.40 mmol, 1.0eq), WSC·HCl (296 mg, 1.54 mmol, 1.1 eq), HOBt (208 mg, 1.54 mmol, 1.0 eq) and NMM (631 μL, 5.60 mmol, 4.0 eq) were dissolved in chloroform (125 mL) and stirred overnight in a round bottom flask. The reaction solution was extracted in a separatory funnel with 2 × 50 mL 10 % citric acid, 1.0 M sodium bicarbonate, and water. The organic layer was dried over magnesium sulfate and the solvent removed under rotary evaporation to yield a clear oil that eluted as one spot by TLC (Si gel, ethyl acetate). The oil was dissolved in 1% MeOH / 10 % TFA / CH₂Cl₂ (100 mL) and stirred for one hour. The solvents were removed in vacuo and the remaining residue was placed under high vacuum overnight to yield a clear oil which crystallized from ethyl acetate as a white solid (450 mg, 1.26 mmol, 90 %). ¹H NMR (300 MHz, CD₃OD, 25 °C) δ = 8.08 (d, 1H, N-H), 7.21 (m, 5H, F-C₆H₅), 7.02 (m, 2H, Phenol-H), 6.67 (m, 2H, Phenol-H), 4.58 (m, 2H, F-CH and Y-CH), 3.16-2.71 (m, 4H, F-CH₂ and F-CH₂), 1.84 (s, 3H, Ac-CH₃). Anal. Calcd for C₁₉H₂₀N₂O₅: C, 64.04; H, 5.66; N, 7.86. Found: C, 64.15; H, 5.61; N, 7.76.

**Physical Measurements.** ¹H NMR spectra were recorded on a Varian 300 MHz NMR at the MIT Department of Chemistry Instrumentation Facility (DCIF). UV-vis absorption spectra were recorded on a Cary 17D spectrophotometer. Pump light for transient absorption (TA) measurements was provided by an Infinity Nd:YAG laser (Coherent) running at 20 Hz. The set-up of the instrument was slightly modified from that previously described.⁴⁷ The second harmonic was frequency doubled with an XPO-UV frequency doubling crystal (Coherent) to produce a 266 nm laser pulse with beam diameter of 4 mm, 2.5 ns pulse width, and pulse energy of ~500 μJ. A 75-W Xe arc lamp (unpulsed, PTI) provided the probe light. For photochemical measurements, a sample ground state absorbance of 1 at 266 nm in a 1 cm cuvette (~0.3 mM) was used in order to maximize signal and minimize first and second order radical reactions. Aqueous samples were prepared in deionized water with 10 mM phosphate,
pH adjusted with 1 M NaOH or 1 M HCl, and flowed through a 1-cm cell with continual oxygen bubbling.
2.6 References


Chapter III

Investigations of Photochemical Oxidants of Tyrosine

Parts of this chapter have been published:


3.1 Motivation

To study the light initiated, amino acid radical hopping in the photochemical RNRs described in Chapter I, photo-oxidants that absorb light of wavelengths outside of the protein absorption envelope (λ > 290 nm) need to be synthetically incorporated into peptides and their competence for photochemical tyrosine oxidation demonstrated. The excited states of these oxidants should react with tyrosine rapidly (ideally sub-nanosecond) to avoid convolution of the initial, •Y356-forming step with radical hopping into R1. The •Y356-reduced-oxidant charge separated state should also persist for long times (μs to ms) to allow for charge injection from •Y356 on the peptide into R1 to occur. Finally, the reduced oxidant needs to be spectrally distinguished from •Y356 to allow for spectroscopic characterization of the radical formation and propagation steps. Two basic types of photo-oxidants have been explored: (1) organic and inorganic compounds known to undergo electron transfer reactions from their excited states, and (2) organic photo-oxidants that are capable of accepting both an electron and a proton in their excited state. This chapter describes the synthesis and photophysical characterization of these excited state oxidants covalently appended to tyrosine in model systems designed for photochemical tyrosine radical generation.

3.2 Ru(bpy)_3^{2+}

3.2.1 Background

Metal-to-ligand charge transfer (MLCT) excited states of metal complexes can be used to initiate biological redox processes using visible light. The MLCT excited state of Ru" bipyridyl (bpy) and its derivatives have been especially useful for redox processes involving metallo-cofactors. The excited state reduction and oxidation potentials for Ru(bpy)_3^{2+} are modest: E°(Ru"*/I) = 1.1 V and E°(Ru"*/III) = 0.7 V vs. NHE. Therefore, application of the Ru" polypyridyl MLCT excited states is best-suited for the study of ET reactions involving metal containing cofactors with mild reduction potentials such as hemes, iron-sulfur clusters, and blue copper proteins. Extension of this methodology to oxidation of protein derived organic cofactors, such as amino acids, is more difficult; for example the MLCT excited state of Ru(bpy)_3^{2+} is not kinetically capable of direct tyrosine oxidation at neutral pH. The utility of the Ru" polypyridyl MLCT excited state may be expanded when implemented as part of the flash quench method. With this technique, the more powerfully
reducing Ru\textsuperscript{I} or oxidizing Ru\textsuperscript{III} centers are photogenerated by bimolecularly quenching the MLCT excited state with an external reductant or oxidant, respectively. The Ru\textsuperscript{I} or Ru\textsuperscript{III} complex persists with sufficient lifetime, owing to a slow bimolecular back-reaction or irreversible quenching step, to enable it to initiate a subsequent redox process on timescales longer than that for the decay of the corresponding MLCT excited state. Owing to the greater oxidizing and reducing power of the flash-quenched products, as compared to that of the Ru\textsuperscript{II} MLCT excited state, redox processes of non-metal based cofactors may be initiated. The approach has been extended to amino acid radicals by Hammarström and co-workers\textsuperscript{6} who, using the flash-quench generated Ru\textsuperscript{III}(bpy)\textsubscript{3}\textsuperscript{3+}, observed oxidation of a tyrosine residue covalently bound to one of the bpy ligands. The overall reaction is depicted in the reaction Scheme below.

\[ \begin{align*}
\text{Ru}^{\text{II}}(\text{bpy})\textsubscript{2}(\text{bpy–Y})\textsuperscript{2+} & \xrightarrow{h\nu} \text{MLCT} \quad \text{Ru}^{\text{III}}(\text{bpy})\textsubscript{2}(\text{bpy–Y})\textsuperscript{2+} \quad (1) \\
\text{Ru}^{\text{III}}(\text{bpy})\textsubscript{2}(\text{bpy–Y})\textsuperscript{2+} & \xrightarrow{k_{\text{PCET}}} \text{Ru}^{\text{II}}(\text{bpy})\textsubscript{2}(\text{bpy–Y})\textsuperscript{2+} \quad (2)
\end{align*} \]

The rate of charge transport, \( k_{\text{PCET}} \), is \( 5 \times 10^4 \text{ s}^{-1} \) at neutral pH and tyrosyl radical formation occurs by a PCET mechanism with proton loss to bulk solvent (see Chapter V for a detailed discussion of the PCET mechanism of Y oxidation in this system).\textsuperscript{9} The rate of tyrosine oxidation in this system relatively slow, owing to the PCET nature of the reaction and the significant driving force required for Y oxidation (\( E^\circ(Y–\text{OH}^+/\text{Y–OH}) = 1.35 \text{ V vs. NHE} \)). Application of this system to PCET studies with RNR would likely be inhibited by convolution of the initial rate of radical formation with the rate of radical hopping into R1.

Radical generation can be accelerated by increasing the oxidizing strength of the flash-quenched, Ru\textsuperscript{III}, product. When two of the bipyridine ligands are substituted with ethyl esters (2,2'-bipyridine-4,4'-diethyl ester), the Ru\textsuperscript{III/III} reduction potential is increased by 0.24 mV, thereby increasing the driving force for tyrosine oxidation upon flash quench generation of the Ru\textsuperscript{III} intermediate.\textsuperscript{11} This increase in driving force results in an increase in the rate for intramolecular tyrosine oxidation to \( 5 \times 10^5 \text{ s}^{-1} \) at pH 7, corresponding to a 2 \( \mu \text{s} \) lifetime for radical generation.\textsuperscript{11} To make this system amenable for application to PCET studies in RNR,
we decided to further increase the electron-withdrawing nature of the bipyridine ligands using the 4,4′-bis(trifluoromethyl)-2,2′-bipyridine (BTFMB) ligand in hopes that a more strongly oxidizing Ru\textsuperscript{III} flash-quenched product would expedite tyrosine oxidation.

3.2.2 Results and Discussion

The \([\text{Ru(BTFMB)}\textsubscript{2}(\text{bpy-(Y/F)})](\text{PF}_\text{6})\textsubscript{2}\) complexes could be synthesized from the \text{Ru(BTFMB)}\textsubscript{2}Cl\textsubscript{2} starting material by coordination of bpy-COOH, followed by coupling of the appropriate tert-butyl protected amino acid to the free carboxylic acid of the bpy ligand. Deprotection of the tert-butyl group in TFA affords the free carboxylic acid compound which is soluble in water.

Figure 3.1 plots the cyclic voltammograms obtained for the \text{Ru(bpy)}\textsubscript{2}(bpy-COOH)(\text{PF}_\text{6})\textsubscript{2} and \text{Ru(BTFMB)}\textsubscript{2}(bpy-COOH)(\text{PF}_\text{6})\textsubscript{2} compounds. Standard reduction potentials \((E^\circ)\) were obtained from the half-wave potential values \((E_{1/2})\)\textsuperscript{13} and are listed in Table 3.1, along with the corresponding values for the amino acid conjugated \text{Ru(BTFMB)}\textsubscript{2}(bpy-F)(\text{PF}_\text{6})\textsubscript{2} complex. Each reduction or oxidation wave was either reversible or quasi-reversible under the conditions used with anodic and cathodic peak currents separated by less than 100 mV. Oxidation of the Ru\textsuperscript{II} complex occurs upon scanning to positive potentials (Figure 3.1, left panel). The electron is removed from the highest occupied molecular orbital (HOMO), which is mainly comprised of metal-centered d-orbitals.\textsuperscript{14} Substitution of bpy at the 4,4′ positions

![Figure 3.1. Cyclic voltammograms of (\textbullet\textsuperscript{ }) \text{Ru(bpy)}\textsubscript{2}(bpy–COOH)(\text{PF}_\text{6})\textsubscript{2} and (\textbullet\textsuperscript{ }) \text{Ru(BTFMB)}\textsubscript{2}(bpy–COOH)(\text{PF}_\text{6})\textsubscript{2} in MeCN with 0.1 M tetrabutylammonium hexafluorophosphate supporting electrolyte. The scan rate was 100 mV/s and the potentials are referenced to the Fc\textsuperscript{+/−}/Fc couple (0.65 V vs. NHE).\textsuperscript{12} The red voltammogram in the right panel was shifted to positive current values for illustration.

68
with trifluoromethyl groups results in a 280 mV increase in the Ru<sup>III/II</sup> redox couple, reflecting the electron-withdrawing nature of the BTFMB ligands. Three reduction events are observed upon scanning to negative potentials (Figure 3.1, right panel), and these events are assigned to the Ru<sup>III/II</sup>, Ru<sup>I/0</sup>, and Ru<sup>0/-1</sup> redox couples. Reduction occurs primarily to orbitals of π*-symmetry centered on the bpy ligands. The Ru<sup>II/I</sup> and Ru<sup>I/0</sup> couples increase by 470 mV upon bpy fluorination, while the Ru<sup>0/-1</sup> exhibits a more moderate increase (170 mV). Importantly, the Ru<sup>III/II</sup> couple is unaffected by coupling of F to bpy-COOH, thereby showing that we have effectively increased the driving force for tyrosine oxidation in the flash quench generated Ru<sup>III</sup>(BTFMB)<sub>2</sub>(bpy-<sup>-Y</sup>)<sup>3+</sup> compared to Ru<sup>III</sup>(bpy)<sub>2</sub>(bpy-<sup>-Y</sup>)<sup>3+</sup>.

Figure 3.2 shows the absorption and emission spectra of the [Ru(BTFMB)<sub>2</sub>(bpy-F)](PF<sub>6</sub>)<sub>2</sub> control complex in 20 mM Tris buffer at pH 7.5. The absorption spectrum exhibits several broad, overlapping peaks from 400-500 nm corresponding to the MLCT transitions and a sharp, intense transition with λ<sub>max</sub> = 295 nm corresponding to the π → π* transitions of the bipyridine ligands. The emission band is broad and featureless with λ<sub>max</sub> = 655 nm, typical of room temperature 3MLCT emission. The emission band was fit with a standard Franck-Condon analysis<sup>15-17</sup> to yield the energy of
the $^3$MLCT excited state ($\Delta G^0_{\text{MLCT}}$) as 2.1 eV. The room temperature emission lifetimes of the Ru(BTFMB)$_2$(bpy-(F/Y))$^{2+}$ complexes were also measured under the same conditions yielding values of 424 ns and 410 ns for the F and Y-containing complexes, respectively. These values are virtually identical, within 5% experimental uncertainty; therefore tyrosine does not substantially quench the $^3$MLCT excited state directly under these conditions.

A sacrificial oxidant is required in solution for bimolecular oxidation of the $^3$MLCT excited state to form the Ru$^{III}$ intermediate in the flash quench reaction. [Ru$^{III}$(NH$_3$)$_6$]Cl$_3$ was chosen as the bimolecular oxidant due to its relatively high reduction potential (0.214 V vs. NHE) and its prevalence in the flash quench literature.

Figure 3.3 plots the emission quenching rate, $k_q$, versus the concentration of the [Ru$^{III}$(NH$_3$)$_6$]Cl$_3$ quencher in solution for both Ru(bpy)$_2$(bpy-(Y))$^{2+}$ and Ru(BTFMB)$_2$(bpy-(Y))$^{2+}$ complexes. The slope of the linear fits to these data provides the bimolecular quenching rate constants. The rate constant for [Ru$^{III}$(NH$_3$)$_6$]Cl$_3$ quenching of the $^3$[Ru(bpy)$_2$(bpy-(Y))$^{2+}$] excited state (1.5 × 10$^9$ M$^{-1}$s$^{-1}$) is a factor of 22 larger than for the $^3$[Ru(BTFMB)$_2$(bpy-(Y))$^{2+}$] excited state (6.8 × 10$^7$ M$^{-1}$s$^{-1}$). Since both complexes have similar excited state lifetimes under these conditions, we obtain 22 times more Ru$^{III}$ flash quenched product per laser flash with the bpy complex vs. the BTFMB complex. The slow bimolecular quenching rate for the BTFMB complex necessitates using high concentrations of the Ru$^{III}$(NH$_3$)$_6$$^{3+}$ quencher for an appreciable yield of the Ru$^{III}$(bpy-Y)$^{3+}$ flash quenched product.

We undertook transient absorption (TA) studies to characterize any tyrosine oxidation in the Ru$^{III}$(BTFMB)$_2$(bpy-Y)$^{3+}$ complex. Figure 3.4 (left) plots the TA spectra obtained upon photolysis of a solution of the Ru(BTFMB)$_2$(bpy-F)$^{2+}$ control complex in the presence of 50 mM Ru$^{III}$(NH$_3$)$_6$$^{3+}$ quencher at 115 ns, 1 and 10 $\mu$s after the laser flash. The spectrum at 115 ns contains a strong peak with $\lambda_{\text{mas}} = 375$ nm and a broad ground state absorbance bleach with
Figure 3.4. (Left) TA spectra of a solution of Ru(BTFMB)$_2$(bpy-F)$^{2+}$ obtained (▬) 115 ns, (▬) 1 and (▬) 10 μs after the laser flash. The * denotes scatter from the 355 nm laser. (Left Inset) Blow-up of the spectrum at 1 μs corresponding to the Ru$^{III}$(bpy-F)$^{3+}$ flash quench product. (Right) TA spectra of a solution of Ru(BTFMB)$_2$(bpy-Y)$^{2+}$ obtained (▬) 115, (▬) 500 ns, (▬) 1, and (▬) 10 μs after the laser flash. (Right Inset) Blow-up of the spectrum at 1 μs showing the Y• product. Spectra obtained in 20 mM Tris buffer at pH 7.5 in the presence of 50 mM Ru$^{III}$(NH$_3$)$_6$$^{3+}$ quencher.

$\lambda_{max} \sim 465$ nm. This spectrum corresponds to the unquenched $^3$MLCT excited state of the Ru$^{II}$(bpy-F)$^{2+}$ complex which has a lifetime of 140 ns from time resolved emission studies. By 1 μs, the peak at 375 nm has decayed, leaving a spectrum with only the ground state absorbance bleach (expanded in the left inset of Figure 3.4). This spectrum can been attributed to the Ru$^{III}$(bpy-F)$^{3+}$ flash quench product. At 10 μs, we observe a spectrum with a broad absorbance at $\lambda_{max} \sim 463$ nm and bleach at 540 nm. We attribute this spectrum at long times to a product of photo-induced ligand exchange reactions of the 50 mM Ru$^{III}$(NH$_3$)$_6$$^{3+}$ quencher.

Figure 3.4 (right) plots the TA spectra obtained after photolysis of a solution of the Ru(BTFMB)$_2$(bpy-Y)$^{2+}$ complex in the presence of 50 mM Ru$^{III}$(NH$_3$)$_6$$^{3+}$ quencher at 115, 500 ns, 1 and 10 μs after the laser flash. The spectrum at 115 ns again resembles the unquenched $^3$MLCT excited. Some of the unquenched $^3$MLCT absorbance remains in the spectrum recorded at 500 ns, however a new feature appears with $\lambda_{max} = 410$ nm. By 1 μs, all of the $^3$MLCT has decayed and only the feature at 410 nm remains, shown in the blow-up in the right inset of Figure 3.4. We attribute this feature to Y• ($\varepsilon_{410} = 2750 \pm 200$ M$^{-1}$cm$^{-1}$),$^{19}$ which is the result of tyrosine oxidation by the flash quench Ru$^{III}$(bpy-Y)$^{3+}$ intermediate. Unfortunately, the kinetics of Y• formation (and Ru$^{III}$(bpy-Y) decay) are masked by the decay
of the unquenched $^3$MLCT excited state. However, we note that in none of the spectra is the Ru$^{III}$(bpy-Y) intermediate clearly visible (the bleach at 463 nm is always accompanied by the absorbance at 375 nm, corresponding to the unquenched $^3$MLCT state). Therefore, tyrosine oxidation must occur concomitant with or faster than the decay of the $^3$MLCT state ($\tau = 140$ ns).

Ineffective quenching of the Ru$^{II}$ excited state by Ru$^{III}$(NH$_3$)$_6$$^{3+}$ masks the kinetics of Y$\bullet$ formation due to the dynamics of the unquenched excited state and ultimately results in a reduced quantum yield of Y$\bullet$ production. The low quantum yield of the Ru$^{III}$(BTFMB)$_2$(bpy- (Y))$^{3+}$ flash quenched product arises from the lower driving force for electron transfer from the excited state to the quencher, due to the higher Ru$^{III/II}$ reduction potential for the BTFMB ligated complex vs. bpy. This presents an inherent downfall to this approach, as it is this higher Ru$^{III/II}$ reduction potential that we initially sought for increased driving force for Y oxidation. In an effort to circumvent this problem, we searched for other quenchers that could more effectively oxidize the $^3$MLCT excited state. These oxidants should be transparent between 355-420 nm to prevent absorption of the probe light by quencher in the region for tyrosyl radical detection. The quenchers we did investigate included methyl viologen, Co$^{III}$(NH$_3$)$_6$$^{3+}$, Ru$^{III}$(tris-ethylenediamine)$_3$$^{3+}$, Ru$^{III}$(EDTA)Cl, and decamethylferrocinium tetrafluoroborate. In all cases, the quenching was either less effective than for [Ru$^{III}$(NH$_3$)$_6$]Cl$_3$, or the Ru$^{III}$(bpy-F)$^{3+}$ flash quench product was not observed following decay of the quenched excited state owing to fast back electron transfer. Tropylium tetrafluoroborate proved an effective irreversible quencher, however the reduced product (presumably ditropyl, C$_{14}$H$_{14}$) precipitated from the solution hampering data acquisition in the absence of a flow cell.

3.3 Pyrene

3.3.1 Background

Pyrene, Py, is a highly fluorescent, blue emitting organic chromophore that exhibits marked vibronic spectral structure, which is sensitive to the polarity of the environment.$^{20}$ As such it has found application as a fluorescent probe in biological media and as a dynamic probe of protein interactions and conformations. In addition, the emissive $\pi\pi^*$ singlet excited state of pyrene is a powerful one-electron oxidant and reductant. Of relevance to light initiated
amino acid radical generation, this $^1\pi\pi^*$ excited state was found to oxidize indole bimolecularly in solution$^{21}$ and was used as an oxidant of tryptophan, forming the [WH•]$^+$ radical cation and the Py•$^-$ radical anion, for the study of electron transfer in peptides.$^{22-24}$ Additional reactivity can be achieved via the flash quench method, in which the singlet excited state of pyrene is irreversibly quenched by a sacrificial oxidant in solution forming the Py•$^+$ radical cation. This procedure has been used to study the oxidation of Y$^{25}$ and W$^{25-27}$ in peptides.

The ground state of pyrene exhibits a large absorption cross section to the red of the protein absorption envelope ($\varepsilon_{\text{max}} \approx 30,000 \text{ M}^{-1}\text{cm}^{-1}$) and the absorption spectra of both the radical cation and radical anion$^{28}$ do not significantly overlap that of Y•. Given these characteristics and the previously observed photochemistry with amino acid radical generation, we therefore thought that pyrene would be a prime candidate for initiating and probing the radical hopping chemistry in RNR upon excitation with light. The synthesis and photophysical characterization of pyrene incorporated into “dipeptides” with alanine and tyrosine are now described.

### 3.3.2 Results and Discussion

Pyrene was incorporated into dipeptides, Py-A and Py-Y, by coupling the commercially available 1-pyrenecarboxylic acid with the tert-butyl protected amino acids (alanine and tyrosine), followed by deprotection of the protecting group with TFA to afford the free carboxylic acid. Figure 3.5 plots the ground state absorption spectrum of Py-A in 10 mM KPi buffer at pH 7.5. As previously observed for other pyrene derivatives,$^{20,29}$ the absorption spectrum of Py-A exhibits three bands with $\varepsilon(375 \text{ nm}) = 1600 \text{ M}^{-1}\text{s}^{-1}$, $\varepsilon(340 \text{ nm}) = 29,000 \text{ M}^{-1}\text{s}^{-1}$.

![Figure 3.5. Plot of the absorption and emission spectra of Py-A and emission spectrum of Py-Y.](image-url)
and ε(275 nm) = 35,000 M\(^{-1}\)s\(^{-1}\) corresponding to excitation to the first, second, and third excited singlet state, respectively, with the second and third bands exhibiting marked vibronic fine structure. The first band has a low extinction coefficient because the corresponding transition is symmetry forbidden.\(^{20}\)

Pyrene fluorescence typically occurs from the lowest excited singlet state.\(^{20}\) Figure 3.5 plots the emission spectra of Py-A and Py-Y in 10 mM KPi buffer at pH 7.5. The spectra of both the Y and A derivatives are similar, exhibiting vibronic fine structure with λ\(_{\text{max}}\) = 384 and 398 nm. However, the fluorescence of Py-Y (Φ\(_{\text{Fl}}\) = 0.097) is 87% quenched compared to that of the A derivative (Φ\(_{\text{Fl}}\) = 0.73). The lifetimes of the Py-A and Py-Y derivatives were measured as 24.7 ± 0.5 and 3.4 ± 0.1 ns, respectively, confirming that the pyrene singlet excited state is 87% quenched when appended to tyrosine and revealing the dynamic nature of this emission quenching.

We next measured the excited state reduction and oxidation potential of the pyrene chromophore in the “dipeptide” for thermodynamic considerations of the mechanism of pyrene fluorescence quenching in the Py-Y system. Table 3.2 presents the ground state reduction potentials for the Py\(^+\)/Py and Py/Py\(^-\) redox couples in the Py-A control compound. Both pyrene reduction and oxidation events occur as irreversible waves in cyclic voltammetry, CV, experiments with 100 mV/s scan rate; the peak potential for these events was more accurately measured using differential pulse voltammetry, DPV. The potentials match closely those previously observed for the quasi-reversible Py\(^+\)/Py and Py/Py\(^-\) redox couples measured for 1-pyrenebutanoic acid ethyl ester using CV at higher scan rates.\(^{30}\) Table 3.2 also presents the measured singlet excited state energy, \(E_{00}(1\text{Py}^*)\), and the calculated excited state reduction and oxidation potentials. These data confirm that \(1\text{Py}^*\) is both a powerful one electron reductant and oxidant, owing primarily to the high energy of the singlet excited state.

Experiments with nanosecond transient absorption spectroscopy were carried out to characterize the nature of the emission quenching in Py-Y. We first wished to characterize the absorption spectra of the pyrene excited states and radical anions with our TA setup. Figure 3.6 plots the TA spectra obtained after flashing a solution of Py-A in 10 mM KPi buffer at pH 7.5. The black spectrum was obtained 1 μs after the laser flash and contains a peak with λ\(_{\text{max}}\) ~ 420 nm with a shoulder to the red at 510 nm. This spectrum corresponds to the triplet
excited state of pyrene, $^3\text{Py}^*$, ($\varepsilon(414 \text{ nm}) = 16700 \pm 4500 \text{ M}^{-1}\text{cm}^{-1}$ for triplet 1-pyrenebutanoic acid in water\textsuperscript{31}) which has a previously measured lifetime of 31 $\mu$s\textsuperscript{25} in aqueous solution in the absence of triplet-triplet annihilation (i.e. at low concentrations).

The red spectrum in Figure 3.6 was obtained under the same conditions but in the presence of 10 mM Co$^{\text{III}}$(NH$_3$)$_6$Cl$_3$, which serves as an irreversible flash-quench oxidant. This spectrum exhibits a sharp peak with $\lambda_{\text{max}} = 456$ nm, consistent with that previously observed for Py$^*^+$ ($\varepsilon(457 \text{ nm}) = 13000 \pm 3000 \text{ M}^{-1}\text{cm}^{-1}$ for the radical cation of 1-pyrenebutanoic acid in water\textsuperscript{31}). The radical anion of pyrene is expected to have a peak at 490 nm ($\varepsilon \sim 49,000 \text{ M}^{-1}\text{cm}^{-1}$).\textsuperscript{28}

Upon flashing a solution of Py-Y, only very weak signals corresponding to $^3\text{Py}$ were observed on the nanosecond timescale. The decreased intensity of these signals, compared to that observed for Py-A, is a result of the previously described quenching of the singlet excited

Table 3.2. Ground and Excited State Reduction and Oxidation Potentials of Py-A.*

<table>
<thead>
<tr>
<th>$E_p(\text{Py}/\text{Py}^-)^a$</th>
<th>$E_p(\text{Py}^+^*/\text{Py})^a$</th>
<th>$E_0(1\text{Py}^*)^b$</th>
<th>$E^o(1\text{Py}/\text{Py}^-)^c$</th>
<th>$E^o(1\text{Py}/\text{Py}^+)^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>-2.16</td>
<td>0.92</td>
<td>3.28</td>
<td>1.77</td>
<td>1.71</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Peak potential from DPV reported in V vs. Fc$^-$/Fc with 0.1 M (Bu$_4$N)PF$_6$ supporting electrolyte in CH$_3$CN. \textsuperscript{b}Reported in eV from the intersection of the normalized absorption and emission bands. \textsuperscript{c}$E^o(1\text{Py}/\text{Py}^-) = E_0(1\text{Py}^*) + E_p(\text{Py}/\text{Py}^-)$ and reported vs. NHE. \textsuperscript{d}$E^o(1\text{Py}/\text{Py}^+^*) = E_0(1\text{Py}^*) - E_p(\text{Py}/\text{Py}^+^*)$ and reported vs. NHE.
state (and thus lower quantum yield for triplet formation). Importantly, no transients corresponding to Y• or Py•− could be detected. Therefore, if tyrosine oxidation is occurring from the pyrene singlet excited state, then back electron transfer must be very rapid to deplete the charge transfer state on the < 10 ns timescale. Nanosecond TA experiments with Py-Y were also carried out with 10 mM CoIII(NH3)6Cl in an effort to generate Py-Y• via the flash quench method, however the short fluorescence lifetime of this compound inhibited buildup of the critical Py•+ -Y intermediate.

3.4 Benzophenone and Anthraquinone

3.4.1 Background

The preceding sections described oxidants which only manage the electron transfer part of tyrosine oxidation. Recent work has shown, however, that tyrosine oxidation can be expedited by forming a hydrogen bond between the phenol and a base32-34 (see also Chapter V), thus switching the photo-oxidation from ET to PCET.35 Following this lead, we sought to develop photo-oxidants that could fulfill the dual role of electron and proton acceptor in the rapid tyrosine oxidation required for phototriggering RNR and at the same time could be excited by light outside the RNR absorption manifold.

Both benzophenone (BP)36-38 and anthraquinone (Anq)39-44 are strong triplet nπ* excited state oxidants with the ability to oxidize amines, aliphatic alcohols, and even water, though sluggishly. Studies on the mechanism of phenol oxidation by carbonyl triplet excited states have resulted in a model wherein PCET occurs within a hydrogen-bonded exciplex.45 In this model, electron transfer from the phenol to the electronically excited carbonyl is kinetically coupled to proton transfer between the oxygen atoms of phenol and the photo-oxidant. In fact, 3BP* has been shown to be a competent, bimolecular oxidant of tyrosine46 with a bimolecular rate constant of 2.6 ± 0.2 × 109 M−1s−1. The triplet excited state of Anq was used to create organic radicals upon oxidation of the nucleo-base of guanine and, as such, was used to study long distance hole transport in DNA.47 We therefore thought that BP and Anq would serve as a prime candidates for rapid formation of •Y356 on the R2C20 peptide. We sought to first demonstrate this in dipeptides containing the BP and Anq chromophores and tyrosine.
3.4.2 Results and Discussion

Figure 3.7 shows the low energy region of the absorption spectra of BPA-Y-OMe•TFA and Anq-Y-OH for wavelengths to the red of the protein absorption envelope ($\lambda > 300$ nm). The BPA chromophore exhibits a weak low energy feature for $\lambda > 300$ nm corresponding to the $n\pi^*$ transition of the BPA chromophore, while the Anq chromophore has a much larger absorption cross section for $\lambda > 300$ nm. Therefore, in the transient absorption experiments, BPA must be excited with the 300 nm, frequency-doubled OPO laser output, to afford enough excited state for a good signal-to-noise ratio while Anq can be more conveniently excited with the third harmonic of our Nd:YAG laser (355 nm).

Excitation of a solution of BPA-Y-OMe at pH 4.0 with a 300 nm nanosecond laser pulse produces a species with the transient absorption spectrum shown in Figure 3.8 at 100 ns. The strong, sharp peak with $\lambda_{\text{max}} = 334$ nm and broad peak at $\lambda_{\text{max}} = 547$ nm with a shoulder to the blue are the spectral signatures of the reduced benzophenone ketyl radical $^{48}$ ($\varepsilon_{550\text{ nm}(\text{BPK}\cdot)} = 3300 \pm 700 \text{ M}^{-1}\text{cm}^{-1}$ in cyclohexane), $^{49}$ whereas the absorption bands at $\lambda_{\text{max}} = 390$ and 407 nm are

![Figure 3.7. Ground state UV-Vis absorbance spectra of BPA-Y-OMe•TFA and Anq-Y-OH.](image)

![Figure 3.8. Transient absorption spectra recorded at (●) 100 ns, (■) 400 ns, and (−−−) 1 μs following 300 nm, 5 ns excitation of a 500 μM solution of BPA-Y-OMe buffered to pH 4.0 with 20 mM succinic acid. Inset: Single wavelength kinetics of the 547 nm absorption (○) and the single exponential decay fit (●) to the data.](image)
characteristic of Y• ($\varepsilon_{410}(Y\cdot) = 2750 \pm 200$). The Y• peak is slightly blue-shifted from that of free Y• owing to their overlap with the tailing absorption of the 334 nm band of the ketyl radical of BPA (BPA•). The subsequent gray traces recorded at 400 ns and 1 μs show that the overall profile shape does not change with time. The transient signals decayed concomitantly with a rate constant of $5.6 \times 10^6$ s$^{-1}$; single wavelength kinetics data recorded at 547 nm is shown in the inset of Figure 3.8. This unimolecular decay is consistent with charge recombination between BPA• and Y•. The kinetics were unaffected by the presence or absence of oxygen in solution. A minor residual absorbance (<10%), which persists for tens of microseconds, is likely due to a small fraction of product formed in the bimolecular reaction between $^3$BPA-Y-OMe and ground state BPA-Y-OMe. This reaction is favored at the high concentrations of dipeptide required for the TA experiment.

Figure 3.9 (left) shows the absorption spectrum obtained 50 ns after excitation of the control “dipeptide” Anq-F-OH with a 355 nm nanosecond laser pulse. The transient contains a broad feature with $\lambda_{max} \approx 417$ nm and an even broader and weaker shoulder with $\lambda_{max} \approx 530$ nm. Additional spectra obtained at 100 ns, 1, and 10 μs show that the overall profile remains fairly constant with time. Single wavelength kinetics obtained at 410 nm revealed that the spectrum has a non-exponential decay, and does not decay to baseline on the μs-ms range.

**Figure 3.9.** Left panel: Transient absorption spectra of Anq-F-OH obtained 65 ( ), 115 ns, 1, and 10 μs ( ) following 355 nm excitation. Right: Transient absorption spectra of Anq-Y-OH obtained 15 ( ), 115 ns, 1, and 10 μs ( ) following 355 nm excitation. Insets: Time-evolved absorbance data (○) with bi-exponential decay fit (▬) obtained at 410 and 520 nm.
timescales. These spectra are not the result of a reaction of a photoreaction with the buffer, as similar spectra and kinetics were recorded in Tris buffer (pH 7.6) and in neat water. The triplet excited state of 9,10-anthraquinone-2-sulfonate, $^3$AQS, was previously shown to react with water and/or OH$,^-$, although the exact photoproducts and reaction mechanism remain unclear.$^{44}$ Given this information, along with the lack of any observable triplet phosphorescence which is normally observed for $^3$Anq in organic solvents, it is likely that the spectrum in the top panel of Figure 3.9 is due to an unidentified photoproduct of the reaction between $^3$Anq and water.

The right panel of Figure 3.9 displays the results for the same experiment performed on Anq-Y-OH. The first transient (50 ns) is markedly different from that obtained for Anq-F-OH and contains features with $\lambda_{\text{max}} \sim 520$ nm and 410 nm and a shoulder at 395 nm. We ascribe the 520-nm absorption to the anthraquinone semiquinone radical anion, Anq$•^-$, based on the similarity to absorption spectra of the native 9,10-anthraquinone semiquinone radical anion ($\varepsilon_{395} = 7800$ M$^{-1}$cm$^{-1}$ and $\varepsilon_{480} = 7300$ M$^{-1}$cm$^{-1}$ at pK$\text{a} = 5.3$)$^{40}$ and anthraquinone-2-sulfonate semiquinone radical anion ($\varepsilon_{400} = 8000$ M$^{-1}$cm$^{-1}$, $\varepsilon_{500} = 8000$ M$^{-1}$cm$^{-1}$ at pK$\text{a} = 3.25$).$^{50}$ The higher energy TA band is a superposition of two absorptions at 394 nm and 408 nm; these wavelengths are commensurate with the absorptions of Y$•$ ($\varepsilon_{410}$ nm = 2750 $\pm$ 200),$^{19}$ and Anq$•^-$ of the diradical, Anq$•^-\text{-Y}•\text{-OH}$, which we propose to result from Y oxidation by the triplet n$\pi^*$ excited state of Anq, $^3$Anq. This photochemistry is consistent with the known reactivity of $^3$Anq with alcohols.$^{39,41}$ The concomitant disappearance of the 410 and 520 nm transient absorptions (Figure 3.9, right insets) are indicative of radical recombination. A small residual absorbance, evident at times >10 $\mu$s, is similar to the transient features observed with Anq-F-OH (Figure 3.9, left panel). Single wavelength kinetics obtained at 410 nm and 520 nm are best fit to a bi-exponential decay function with a short ($\tau_1 = 370 \pm 20$ ns, 80%) and long ($\tau_2 > 10$ $\mu$s, 20%) components. The major, short component of the decay is assigned to the radical recombination between Anq$•^-$ and Y$•$ in Anq$•^-\text{-Y}•\text{-OH}$, proceeding at $2.7 \pm 0.1 \times 10^6$ s$^{-1}$. The minor, long component is assigned to the decay of the Anq-solvent photoproduct identified in the TA spectrum of the Anq-F-OH dipeptide $(\text{vide supra})$.  

79
3.5 Flavins

3.5.1 Background

Flavins have a rich excited state photochemistry\textsuperscript{51} with lumiflavin (LF) and riboflavin (RF) (Chart 3.1) being particularly well characterized owing to the role of riboflavin as a redox active cofactor in biological enzymes. Both LF and RF can exhibit redox activity from their singlet and triplet excited states. The singlet excited state is highly fluorescent ($\Phi_f = 0.26$),\textsuperscript{51} thus steady-state and time-resolved emission spectroscopy can be used as a probe for its reactivity. On the other hand, flash photolysis or flash EPR methods must be used to probe reactions with the dark (at room temperature) triplet excited state. The singlet excited state converts to the triplet on the order of nanoseconds,\textsuperscript{51} therefore most of the redox activity reported in the literature deal with the triplet excited state of LF or RF.

The triplet excited state of LF/RF can act as a strong oxidant\textsuperscript{52,53} and reductant.\textsuperscript{54,55} The photochemistry is complicated by the observation of different protonation states of the oxidized,\textsuperscript{55} reduced,\textsuperscript{56} and even triplet excited state (pKa = 4.45),\textsuperscript{57} as well as bimolecular reactivity of the triplet excited state with itself (triplet-triplet quenching) and with another ground state molecule.\textsuperscript{53,58} The redox and acid/base equilibria of the ground state flavin and flavin radicals described here are presented in Chart 3.2. From these data, two protonation forms of the oxidized radical, Fl$_{ox}^+$ and Fl$_{ox}^\text{-H}$, and reduced radical, Fl$_{red}^\bullet$ and Fl$_{red}^\text{-}$, could play a role near neutral pH. Bimolecular oxidation of tryptophan and tyrosine with the triplet excited state of flavins was reported to forming the reduced FlH$.\textsuperscript{51,59}$ We sought to extend this photochemistry to dipeptides containing the Fl unnatural amino acid shown in Chart 3.1

![Chart 3.1. Chemical structures of flavins discussed herein.](image-url)
Chart 3.2. Redox and acid/base equilibria of flavin and flavin radicals. \(^a\)Ref 55 \(^b\)Ref 51 \(^c\)Ref 56.
and tyrosine. The synthesis of Fl was accomplished using the method of Carrel and et al., and dipeptides with tyrosine and alanine were synthesized using standard peptide coupling methods. The photophysics and photochemistry of these dipeptides is now described.

3.5.2 Results and Discussion

Figure 3.10 shows the ground state UV-vis absorption and fluorescence emission spectra of Fl-A. The Fl chromophore exhibits a strong absorption feature in the visible with $\lambda_{\text{max}} = 370, 442$ ($\varepsilon_{445\text{ nm}} = 10930 \text{ M}^{-1}\text{cm}^{-1}$ for LF) and a strong emission peak with $\lambda_{\text{max}} = 530$ nm. Time-resolved fluorescence ($\lambda_{\text{exc}} = 400$ nm) was measured in 10 mM phosphate buffer at pH 7 for both Fl-A and Fl-Y dipeptides, yielding fluorescence lifetimes of 5.25 and 2.95 ns, respectively. The quenched fluorescence lifetime for Fl-Y compared to the Fl-A control compound could be due to oxidation of tyrosine from the singlet excited state, although other mechanisms such as stacking of the aromatic phenol with the isoalloxazine ring or hydrogen bonding from the phenol cannot be ruled out.

Nanosecond transient absorption spectroscopy was employed to probe the mechanism of the

Figure 3.10. Ground state UV-Vis absorbance and steady state emission spectra (450 nm excitation) of Fl-A in 10 mM KPi buffer (pH 7).

Figure 3.11. Transient absorption spectra obtained (---) 115ns, (---) 2µs, and (---) 20µs following 355 nm ns-laser excitation of a solution of Fl-A in 10 mM KPi at pH 7.
Figure 3.12. Transient absorption spectra obtained (▬) 65ns, (▬) 115 ns, and (▬) 1μs following 355 nm ns-laser excitation of a solution of Fl-Y in 10 mM KP$_i$ at pH 7.

fluorescence quenching, as well potential reactions of the dark triplet excited state. Figure 3.11 shows the transients observed following 355 nm, ns laser excitation of Fl-A in 10 mM phosphate buffer at pH 7. At the earliest time point (115 ns) the spectrum contains peaks at 395, 660 and ~715 nm and a ground state bleach at 450 nm, resembling that of the triplet excited state, $^3$LF.$^{58}$ The low energy region of the spectrum with the peak at 715 nm is particularly informative in distinguishing $^3$LF from the reduced and oxidized radical forms of Fl. The spectrum at 115 ns decays on the microsecond timescale to another transient with a broad ground state bleach for 450 nm < $\lambda$ < 500 nm and a broad absorption between 500 and 650 nm that persists into the hundreds of microseconds timescale. This spectrum resembles that of Fl$_{\text{redH}•}$$^{56}$ superimposed on the ground state bleach. From the literature, the decay of $^3$LF is complex with rates for first-order nonradiative decay and concentration dependent triplet-triplet and triplet-ground state quenching, in some cases yielding Fl$_{\text{redH}•}$ and Fl$_{\text{ox}(-H)}$ or Fl$_{\text{ox}+}$$^{53,58}$.

In contrast to the spectra observed for Fl-A, the transient observed 65 ns following excitation of Fl-Y (Figure 3.12) does not contain the low energy absorption feature at 715 nm. Instead this spectrum features a broad ground state bleach at $\lambda = 450$ nm and a broad, tailing absorption for $\lambda > 500$ nm that decays on the nanosecond timescale. This spectrum also resembles that of Fl$_{\text{redH}•}$ superimposed on the ground state bleach and could be due to reduction of the $^3$Fl by Y to form the $\text{•HFl}_{\text{red-Y}•}$ charge separated state. However, we would also expect to see an absorption feature near 410 nm due to Y•. Unfortunately, the ground state of Fl absorbs strongly in this region, thus the ground state bleach of Fl$_{\text{redH}•}$ in the TA spectra inhibits direct detection of the Y• signal. Owing to the fact that the Y• signal is obscured and that the Fl photophysics are so
complex, we abandoned Fl early on as a potential excited state oxidant of Y for the proposed studies with RNR.

3.6 Conclusions

A Ru(bpy)$_3^{2+}$ derivative, pyrene, benzophenone, anthraquinone, and flavin were investigated as excited state oxidants of tyrosine in model “dipeptides.” All chromophores show some evidence for tyrosine oxidation from their excited states, however each has a drawback with regard to application to the RNR system. For the Ru(BTFMB)$_2$(bpy)$_2^{2+}$ system, the high Ru$^{III/II}$ potential inhibited significant buildup of the flash-quench Ru$^{III}$ product required for Y oxidation. In the pyrene dipeptides, tyrosine oxidation may occur from the singlet excited state, however no buildup of the charge separated state could be observed on the nanosecond timescale. For benzophenone, the ketyl radical is clearly distinguished from the tyrosyl radical in the TA spectrum of the •BPA-Y•-OMe diradical state. However, the low absorption cross section of the benzophenone chromopore necessitates excitation at $\lambda \leq 300$ nm. There is some overlap with the tailing absorption of RNR in this region, creating difficulties in obtaining good signal-to-noise ratios at the peptide concentrations required for peptide:α2 experiments. The benzophenone aromatic ring could be substituted with electron donating groups to shift the absorption spectrum farther to the red, which we investigate in Chapter X. Both the Anq and Fl chromophores overcome this absorption cross section problem with higher molar absorptivities in the near UV and visible regions of the spectrum, respectively. Both chromophores are competent for Y• generation, but the reduced Anq$^-$ and Fl$_{red}$H• TA signals significantly overlap that of Y•, thereby obscuring any potential reactivity of •Y356 with α2. Nonetheless, since BPA and Anq exhibited the cleanest photochemistry with regard to Y oxidation that could be characterized with nanosecond TA, these chromophores were incorporated into Y-R2C19, the C-terminal peptide tail of β2, for light initiated activation of α2. Characterization of the ensuing photochemistry of the chromophore on the peptide and light-initiated turnover assays of these peptides bound to α2 is discussed in Chapter VII.

3.7 Experimental Section

**Materials.** L-Tyrosine tert-buty 1 ester (Y–O$\text{tBu}$), 4-benzoyl-N-[(1,1-dimethylethoxy) carbonyl]-L-phenylalanine (Boc-BPA-OH), 1-hydroxybenzotriazole (HOBt) (NovaBiochem), L-alanine tert-buty 1 ester hydrochloride (A–O$\text{tBu}$•HCl), L-phenylalanine tert-buty 1 ester
hydrochloride (F–O\textsubscript{T}Bu•HCl), 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) (Advanced ChemTech), magnesium sulfate (MgSO\textsubscript{4}) (EMD), Anthraquinone-2-carboxylic acid (Anq-COOH), 4-dimethylaminopyridine (DMAP), 1-pyrenecarboxylic acid (Py-COOH), 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl), N-methylmorpholine (NMM), 60 % hexafluorophosphoric acid (HPF\textsubscript{6}), hexaaminecobalt(III)chloride (Co\textsuperscript{III}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}) (Aldrich) and trifluoroacetic acid (TFA, 99.9%) (J. T. Baker) were used as received. 4´-Methyl-2,2´-bipyridine-4-carboxylic acid (bpy–COOH) was prepared as described.\textsuperscript{63} Bis(4,4´-bis(trifluoromethyl)-2,2´-bipyridine)ruthenium\textsuperscript{II} dichloride (Ru(BTFMB)\textsubscript{2}Cl\textsubscript{2}) was a gift from Prof. Masaoki Furue.\textsuperscript{64} Bis(bipyridine)(4-methyl-2,2´-bipyrindle-4´-carboxylic acid)ruthenium\textsuperscript{II} hexafluorophosphate ([Ru(bpy)\textsubscript{2}(bpy-COOH)](PF\textsubscript{6})\textsubscript{2}) was prepared as previously described.\textsuperscript{65} The flavin-containing unnatural amino acid, (S)-2-amino-N\textalpha-(tert-butyloxycarbonyl)-6-(7',8'-dimethylisoalloxazin-10'-yl)hexanoic Acid (Boc-Fl-OH), was synthesized by the method of Carell et al.\textsuperscript{60} \[
\text{[Ru(BTFMB)\textsubscript{2}(bpy-COOH)](PF\textsubscript{6})\textsubscript{2}}. \]
Ru(BTFMB)\textsubscript{2}Cl\textsubscript{2} (200 mg, 0.264 mmol, 1 eq) and bpy-COOH (60 mg, 1.06 eq) were combined in a 100 mL round bottom flask along with 50 mL of 70% ethanol. The mixture was heated at reflux for one hour, at which point 60 mg more of the bpy-COOH ligand was added. The mixture was heated at reflux for seven more hours and the solvent removed in vacuo. The residue was dissolved in ~30 mL of water, acidified to pH 1 with 60% HPF\textsubscript{6}, and solid NH\textsubscript{4}PF\textsubscript{6} was added to induce precipitation. The dark red/purple precipitate was isolated by filtration over a fine glass frit and washed with cold water and ether. The product was recrystallized from acetone/ether and isolated as a red solid (260 mg, 83 % yield). \textsuperscript{1}H NMR (300 MHz, d-acetone, 25 °C) δ = 2.63 (s, 3H, bpy-CH\textsubscript{3}), 7.44-9.52 (m, 18H, bpy aromatic region).

\textit{[Ru(BTFMB)\textsubscript{2}(bpy-Y)](PF\textsubscript{6})\textsubscript{2}.} [Ru(BTFMB)\textsubscript{2}(bpy-COOH)](PF\textsubscript{6})\textsubscript{2} (100 mg, 0.084 mmol, 1 eq), Y-O\textsubscript{T}Bu (22 mg, 1.1 eq), HCTU (52 mg, 1.5 eq), and HOBt (17 mg, 1.5 eq) were combined in a 100 mL round bottom flask with 10 mL of dimethylformamide. NMM (37 μL, 4 eq) was added and the reaction was stirred for 5 hours. The reaction was diluted with 50 mL of methylene chloride and extracted with 3 x 30 mL of water, 10 % citric acid, 1 M NaHCO\textsubscript{3}, and water in that order. The organics were dried over MgSO\textsubscript{4}, the drying agent removed by filtration, and the solvent removed in vacuo. The red residue was chromatographed on a 1 mm alumina Chromatotron plate using 3% MeOH / CH\textsubscript{2}Cl\textsubscript{2} as the eluant. The desired product was
isolated as a red band and the solvents removed in vacuo. The resulting t-butyl ester protected product was dissolved in 10 mL of 1:1 CH₂Cl₂ / TFA and stirred for one hour at room temperature. The solvents were evaporated under a stream of N₂ and the product precipitated upon addition of ether. The resulting red solid was isolated by filtration (62 mg, 55% yield). ¹H NMR (300 MHz, d-acetone, 25 °C) δ = 2.62 (s, 3H, bpy-CH₃), 3.05-3.29 (m, 2H, CβH₂), 4.84 (m, 1H, CαH), 6.73 (d, 7.7 Hz, 2H, phenol CH), 7.18 (d, 8.8 Hz, 2H, phenol CH), 7.40-9.50 (m, 19H, bpy aromatic + amide NH region). MALDI-TOF Calcd. (Found): [M – PF₆]⁺ 1207.8 (1208.1); [M – 2 PF₆]⁺ 1063.1 (1063.1).

[Ru(BTFMB)₂(bpy-F)](PF₆)₂. This compound was prepared by the same method as [Ru(BTFMB)₂(bpy-Y)](PF₆)₂, except that F-OrBu•HCl was used instead of Y-OrBu (60 mg, 53%). ¹H NMR (300 MHz, d-acetone, 25 °C) δ = 2.61 (s, 3H, bpy-CH₃), 3.13-3.43 (m, 2H, CβH₂), 4.94 (m, 1H, CαH), 7.15-9.50 (m, 24H, bpy aromatic + phenyl aromatic + amide NH region). MALDI-TOF Calcd. (Found): [M – PF₆]⁺ 1192.1 (1192.0); [M – 2 PF₆]⁺ 1047.1 (1047.1).

[Ru(bpy)₂(bpy-Y)](PF₆)₂. [Ru(bpy)₂(bpy-COOH)](PF₆)₂ (300 mg, 0.322 mmol, 1eq), Y-OrBu (115 mg, 1.5 eq), HOBt (65 mg, 1.5 eq), and WSC•HCl (123 mg, 2 eq) were combined in a round bottom flask with 20 mL of DMF. NMM (220 μL, 6 eq) was added and the solution was stirred overnight at room temperature. The solvent was removed in vacuo and the residue was taken up in 100 mL of CH₂Cl₂ and extracted with 2 x 50 mL of water, 10 % citric acid, 1 M NaHCO₃, and water in that order. The organics were dried over MgSO₄, the drying agent removed by filtration, and the solvent removed in vacuo. The red residue was chromatographed on a 1 mm alumina Chromatotron plate using 3% MeOH / CH₂Cl₂ as the eluant. The desired product was isolated as a red/orange band and the solvent removed in vacuo. The resulting t-butyl ester protected product was dissolved in 10 mL of 1:1 CH₂Cl₂ / TFA and stirred for one hour at room temperature. The solvents were evaporated under a stream of N₂ and the product precipitated upon addition of ether. The resulting red/orange solid was isolated by filtration. ¹H NMR (300 MHz, d-acetone, 25 °C) δ = 2.62 (s, 3H, CH₃), 3.05 (m, 1H, CβH), 3.26 (m, 1H, CβH), 4.88 (m, 1H, CαH), 6.73 (m, 2H, phenol CH), 7.16 (m, 2H, phenol CH), 7.42 – 9.00 (m, 23 H, bpy aromatic & amide NH).

[Ru(bpy)₂(bpy-F)](PF₆)₂. This compound was prepared by the same method as [Ru(bpy)₂(bpy-Y)](PF₆)₂, except that F-OrBu•HCl was used instead of Y-OrBu. ¹H NMR (300 MHz, d-acetone, 25 °C) δ = 2.61 (s, 3H, bpy-CH₃), 3.13-3.43 (m, 2H, CβH₂), 4.94 (m, 1H, CαH), 7.15-9.50 (m, 24H, bpy aromatic + phenyl aromatic + amide NH region). MALDI-TOF Calcd. (Found): [M – PF₆]⁺ 1192.1 (1192.0); [M – 2 PF₆]⁺ 1047.1 (1047.1).
MHz, d-acetone, 25 °C) \(\delta = 2.61\) (s, 3H, CH\(_3\)), 3.12 (m, 1H, C\(\beta\)H), 3.37 (m, 1H, C\(\beta\)H), 4.96 (m, 1H, C\(\alpha\)H), 7.17-7.37 (m, 5H, phenyl CH), 7.44-8.96 (m, 23 H, bpy aromatic & amide NH).

1-Pyrenecarboxyl-L-tyrosine [Py-Y]. Py-COOH (200 mg, 0.812 mmol, 1 eq), Y-O\(\text{tBu}\) (193 mg, 1eq), WSC•HCl (171 mg, 1.1 eq), and HOBt (121 mg, 1.1 eq) were combined in a round bottom flask with 25 mL of CH\(_2\)Cl\(_2\). NMM (370 \(\mu\)L, 4.0 eq) was added and the solution was stirred overnight. The 75 mL of CH\(_2\)Cl\(_2\) was added and the solution was extracted with 2 x 50 mL of 10% citric acid, 1 M NaHCO\(_3\) and water. The organics were dried over MgSO\(_4\), the drying agent removed by filtration and the solvents removed to yield the \(\text{t}-\text{butyl ester protected product as a yellow foam. The foam was dissolved in 10 mL of 1:1 TFA/CH}_2\text{Cl}_2\) with 1% MeOH and stirred for two hours. The solvents were removed under a stream of nitrogen and the resulting yellow powder was dried under vacuum (199 mg, 60 %). \(^1\)H NMR (300 MHz, d-acetone, 25 °C) \(\delta = 3.14\) (dd, 9.8 Hz, 14.0 Hz, 1H, C\(\beta\)H), 3.40 (dd, 4.6 Hz, 14.0 Hz, 1H, C\(\beta\)H), 5.11 (m, 1H, C\(\alpha\)H), 6.84 (d, 8.5 Hz, 2H, phenol CH), 7.27 (d, 8.5 Hz, 2H, phenol CH), 7.89 (d, 7.8 Hz, 1H, amide NH), 8.03-8.44 (m, 9H, pyrene aromatic CH).

1-Pyrenecarboxyl-L-alanine [Py-A]. This compound was made by the same method as Py-Y, except A-O\(\text{tBu•HCl}\) was used instead of Y-O\(\text{tBu}\). (176 mg, 68 %). \(^1\)H NMR (300 MHz, d-acetone, 25 °C) \(\delta = 1.63\) (d, 7.2 Hz, 3H, CH\(_3\)), 4.87 (qd, 7.5 Hz, 8.1 Hz, 1H, C\(\alpha\)H), 8.01-8.35 (m, 9H, pyrene aromatic CH), 8.73 (d, 9.4 Hz, 1H, amide NH).

4-Benzoyl-L-Phenylalan-Tyrosyl Methyl Ester Trifluoroacetic Acid (BPA-Y-OMe\(\text{CF}_3\text{COOH}\)) Y-OMe (0.432 mmol, 1.0 eq), Boc-BPA-OH (160 mg, 0.432 mmol, 1.0 eq), N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride (91 mg, 0.475 mmol, 1.1 eq), and 1-hydroxybenzotriazole (HOBt) (64 mg, 0.475 mg, 1.1 eq) were combined in a 50 mL round bottom flask with 20 mL of methylene chloride. N-methylmorpholine (NMM) (175 \(\mu\)L, 1.73 mmol, 4 eq) was added and the solution was stirred overnight at room temperature. The solution was diluted to 75 mL with methylene chloride and washed with 2 \(\times\) 30 mL of 10% citric acid solution. The organic layer was dried over MgSO\(_4\) and the solvent removed in vacuo. The resulting clear oil was dissolved in a few mLs of methylene chloride and loaded onto a 1 mm thick silica gel Chromatotron plate. The product was eluted with 3% methanol/methylene chloride and the solvent was removed in vacuo. The resulting clear oil was dissolved in 1:1 TFA:CH\(_2\)Cl\(_2\) and stirred for 20 min at room temperature. The solvents were then evaporated under a stream of \(\text{N}_2(\text{g})\) and the resulting clear oil was dried under high vacuum. Trituration with
CH₂Cl₂ followed by the dropwise addition of ether resulted in the formation of white crystals, which were cooled to –20 °C and isolated by filtration. 

**1H NMR (300 MHz, (CD₃)₂CO, 25 °C)** δ = 2.88-3.18 (m, 2H, C₁β-H), 3.37 (m, 2H, C₁β-H), 3.68 (s, 3H, -OCH₃), 4.62 (m, 1H, C₁α-H), 4.88 (m, 1H, C₁α-H), 6.78 (m, 2H, phenol CH), 7.15 (m, 2H, phenol CH), 7.30 (d, 2H, C₆H₄OC₆H₅, 7.7 Hz), 7.55 (m, 2H, C₆H₄OC₆H₅), 7.67 (m, 3H, C₆H₄OC₆H₅), 7.76 (m, 2H, C₆H₄OC₆H₅).

**N-[(9,10-Dihydro-9,10-dioxo-2-anthracenyl)carbonyl]-L-tyrosine (Anq-Y-OH).** Anq-COOH (300 mg, 1.19 mmol, 1.0 eq), Y-OᵗBu (282 mg, 1.0 eq), WSC·HCl (250 mg, 1.1 eq), and HOBt (177 mg, 1.1 eq) were combined in a 100 mL flask with 30 mL of DMF. NMM (530 μL, 4.0 eq) was added and the solution was stirred overnight. The solvent was removed in vacuo, and the resulting oil was dissolved in 80 mL of methylene chloride. The organics were washed with 2 × 50 mL of water, dried over MgSO₄, and filtered to remove the drying agent. The solvent was removed in vacuo to yield a yellow oil, which was dissolved in a few mL’s of methylene chloride and loaded onto a Chromatotron plate (Si gel, 2mm). The product was eluted with 2% MeOH/ CH₂Cl₂. The solvent was removed in vacuo to yield a yellow oil, which was redissolved in 10 mL of 1:1 TFA/ CH₂Cl₂ and stirred for one hour. The solution was concentrated to < 1mL under a stream of N₂(g) and taken up in ether. The resulting yellow precipitate was isolated by filtration and dried in vacuo to yield 500 mg of the title compound as a yellow powder (95 %). 

**1H NMR (300 MHz, CD₃OD, 25 °C)** δ = 3.05 (m, 1H, C₁β-H), 3.26 (m, 1H, C₁β-H), 4.83 (m, 1H, C₁α-H), 6.72 (m, 2H, phenol- CH), 7.13 (m, 2H, phenol CH), 7.85 (m, 2H, Anq. arom. CH), 7.97 (m, 1H, NH), 8.12 (m, 1H, Anq. arom. CH), 8.27 (m, 3H, Anq. arom. CH), 8.58 (d, 1H, NH, 1.6 Hz). ESI-MS Calc’d (Found): [M+H]⁺ 416.11 (416.11), [2M+H]⁺ 831.22 (831.22)

**N-[(9,10-Dihydro-9,10-dioxo-2-anthracenyl)carbonyl]-L-phenylalanine (Anq-F-OH).** The title compound was prepared by the same method as that used for Anq-Y-OH, except that F-OᵗBu·HCl was used in place of Y-OᵗBu (63%). 

**1H NMR (300 MHz, (CD₃)₂CO, 25 °C)** δ = 3.14 (m, 1H, C₁β-H), 3.40 (m, 1H, C₁β-H), 4.93 (m, 1H, C₁α-H), 7.17-7.35 (m, 5H, F phenyl-CH), 7.87 (m, 2H, Anq. arom. CH), 8.11 (m, 1H, Anq. arom. CH), 8.29 (m, 3H, Anq. arom. CH), 8.58 (d, 1H, Anq. arom. CH, 1.6 Hz), 9.00 (d, 1H, NH, 8.3 Hz). ESI-MS Calc’d (Found): [M+H]⁺ 400.12 (400.12), [2M+H]⁺ 799.23 (799.22)

**Fl-A•CF₃COOH.** Boc-Fl-OH (394 mg, 0.835 mmol, 1 eq), A-OᵗBu·HCl (152 mg, 1eq), WSC·HCl (177 mg, 1.1 eq), and HOBt (125 mg, 1.1 eq) were combined in a round bottom flask with 50 mL of chloroform. NMM (256 μL, 3.3 eq) and DMAP (10.2 mg, 0.1 eq) were added
and the solution was stirred overnight. The reaction was diluted with 100 mL of chloroform and extracted with $3 \times 75$ mL of 10% citric acid, water, 5% sodium bicarbonate, and water again in that order. The organic layer was dried over MgSO$_4$, filtered to remove the drying agent, and the solvent removed in vacuo. The resulting reddish orange residue was purified by column chromatography (Si gel) with 5% MeOH/chloroform as the eluant. The product was isolated as a reddish orange band and the solvent removed in vacuo. The resulting residue (Boc-Fl-A-OtBu) was dissolved in 10 mL of 1:1 TFA/CH$_2$Cl$_2$ and stirred for 2 hours. The solvents were evaporated under a stream of N$_2$ and the product was precipitated by the addition of ether as the trifluoroacetate salt. The resulting orange precipitate was isolated by filtration, washed with ether, and dried in vacuo.

$^1$H NMR (300 MHz, CD$_3$OD, 25°C) $\delta = 1.43$ (d, 3H, A-CH$_3$), 1.68 (m, 2H, Fl-C$_\gamma$H$_2$), 1.84-2.15 (m, 4H, Fl-C$_\beta$H$_2$, Fl-C$_\delta$H$_2$) 2.45 (s, 3H, Fl-CH$_3$), 2.58 (s, 3H, Fl-CH$_3$), 3.93 (m, 1H, Fl-C$_a$H), 4.41 (m, 1H, A-C$_a$H), 4.74 (m, 2H, Fl-C$_e$H$_2$), 7.74 (s, 1H, Fl-arom. CH), 7.89 (s, 1H, Fl-arom. CH), 8.72 (br. d, amide CH). ESI-FT MS Calcd. (Found): [M + H]$^+$ 443.2043 (443.2021)

**Fl-Y•CF$_3$COOH.** This compound was prepared as described for Fl-A-OH•CF$_3$COOH, except that Y-OtBu was used instead of A-OtBu•HCl. $^1$H NMR (500 MHz, CD$_3$OD, 25°C) $\delta = 1.61$ (m, 2H, Fl-C$_\gamma$H$_2$), 1.90-2.10 (m, 4H, Fl-C$_\beta$H$_2$, Fl-C$_\delta$H$_2$), 2.48, (s, 3H, Fl-CH$_3$), 2.59 (s, 3H, Fl-CH$_3$), 2.92 (m, 1H, Y-C$_\beta$-H), 3.15 (m, 1H, Y-C$_\beta$-H), 3.88 (t, 1H, Fl-C$_a$H, 6.5 Hz), 4.61 (td, 1H, Y- C$_a$H, 4.9 Hz, 4.9 Hz), 4.76 (m, 2H, C$_e$H$_2$), 6.68 (d, 2H, phenol CH, 8.0 Hz), 7.81 (d, 2H, phenol CH, 8.5 Hz), 8.58 (br. d, amide CH). ESI-FT MS Calcd. (Found): [M + H]$^+$ 543.2227 (543.2321)

**Physical Measurements.**

*NMR Spectroscopy and Mass Spectrometry.* $^1$H NMR spectra were recorded on a Varian Mercury 300 MHz or Varian Inova 500 MHz spectrometer at the MIT Department of Chemistry Instrumentation Facility (DCIF) and externally referenced to tetramethylsilane. MALDI-TOF mass spectrometry was performed on a Bruker Omniflex instrument in the DCIF using dithranol as the matrix. The instrument was calibrated with a quadratic polynomial using a mixture of bradykinin fragment 1-7 (757.3997), angiotensin II (1046.5423), and P14R synthetic peptide (1533.8582) (Sigma) with α-cyano-4-hydroxycinnamic acid as the matrix. ESI-FT mass spectrometry was performed with an Bruker Daltonics APEXII instrument housed in the DCIF.
**Electrochemistry.** Electrochemical measurements were performed using a Bioanalytical Systems (BAS) Model CV-50W potentiostat/galvanostat. Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) was performed in dry MeCN with 0.1 M tetrabutylammonium hexafluorophosphate (Bu₄N)PF₆ as the supporting electrolyte. A platinum disk working, Ag/AgNO₃ nonaqueous (0.1 M (Bu₄N)PF₆ / MeCN) reference, and a platinum wire auxiliary electrodes were used. Solutions were bubbled with Ar to remove dissolved O₂. All potentials are reported vs. $E^o(Fc^+/Fc) = 0.65$ V vs. NHE in CH₃CN.12

**Absorption and Emission Spectroscopy.** UV-vis absorption spectra were recorded on a Cary 17D modified by On-Line Instrument Systems (OLIS) to include computer control or a Spectral Instruments 440 spectrophotometer. Steady state emission spectra were recorded on an automated Photon Technology International (PTI) QM 4 fluorimeter equipped with a 150-W Xe arc lamp and a Hamamatsu R928 photomultiplier tube. The $E_00$ for the $^1$Py* excited state was measured from the intersection of the normalized lowest energy singlet absorption peak and the normalized highest energy emission peak. Both spectra were plotted in wavenumbers (cm⁻¹), and the intensity values converted to units of quanta ($I \times \lambda^2$) by the correction of Parker and Rees.66 Fluorescence quantum yields for Py-A and Py-Y were measured using the equation:

$$\Phi_{sam} = \Phi_{ref} \left( \frac{A_{ref}}{A_{sam}} \right) \left( \frac{I_{sam}}{I_{ref}} \right) \left( \frac{\eta_{sam}}{\eta_{ref}} \right)^2$$  (3)

where $\Phi_{ref}$ is the quantum yield of the reference actinometer quinine sulfate in 1N H₂SO₄ ($\Phi_{ref} = 0.55$).67 $A$ is the measured absorbance, $\eta$ is the refractive index of the solvent, and $I$ is the integrated emission intensity of the corrected emission band plotted in wavenumbers. The measured quantum yields were identical, within 5% experimental error, in the presence and absence of oxygen indicating that O₂ does not react with the singlet excited state of pyrene in these dipeptides to a significant extent. The Franck-Condon analysis method 15-17 for fitting the $^3$MLCT emission band of Ru(BTFMB)$_2$(bpy-F)(PF₆)$_2$ to obtain $\Delta G_{MLCT}^\circ$ is described in detail in Chapter 4.

**Transient Absorption Spectroscopy.** Transient absorption (TA) measurements were made with pump light provided by the third harmonic (355 nm) of an Infinity Nd:YAG laser (Coherent) running at 20 Hz as previously described.68 In the case of the BPA-Y-OMe, 300 nm pump light was used as previously described owing to the lack of absorption at 355 nm for this chromophore. For this task, the third harmonic (355 nm, 200 mJ per pulse) was used to pump a
Type II XPO (Coherent). The 600 nm signal beam was isolated and frequency doubled in a XPO-UV frequency doubling crystal (Coherent) to produce a slightly divergent 300 nm beam with a ~5 ns pulse width and a pulse energy of ~500 μJ. This beam was then passed through a slow focusing lense and through the sample at a ~160°, producing a beam spot of 2 mm diameter on the sample. A 75 W Xe arc lamp (unpulsed, PTI) provided the probe light, which was focused onto the sample, overlapped with the pump beam, re-collimated after the sample, and focused onto the entrance slit of a Triax 320 spectrometer.

Transient absorption experiments for BPA and Anq compounds were performed in a 2 mm cuvette under ambient conditions at 350 μM concentration for the Anq and 500 μM for BPA-Y-OMe. Anq dipeptides were buffered to pH 7.5 with 20 mM Tris buffer, while BPA-Y-OMe was buffered to pH 4 with 20 mM succinic acid. For transient absorption experiments involving Anq-Y-OH, 10 mL of solution were continuously flowed through a 2 mm cuvette during data collection to ensure that fresh sample was present for each laser shot. Time resolved experiments on Fl compounds were performed in a 1 cm cuvette with compounds at an optical concentration of 0.4 OD. These solutions were prepared with 10 mM KPi buffer at pH 7 and degassed on a high vac line to 10⁻⁶ torr.
3.8 References


Chapter IV

Design of MLCT Excited States of Rhenium Polypyridyl Complexes for Direct Tyrosine Oxidation

Parts of this chapter have been published:

4.1 Motivation

We wish to develop electron transfer photo-oxidants with large absorption cross sections for \( \lambda > 300 \) nm and that are capable of oxidizing tyrosine directly from their excited state with charge separated states that persist into the nanosecond regime. Success in this area will allow the radical chemistry of RNR to be initiated with light in the absence of sacrificial electron acceptors, which can interfere with the RNR biochemistry. This chapter describes the synthesis and photophysical characterization of \( \text{Re}^I \) polypyridal complexes designed for direct Y photo-oxidation from their MLCT excited state.

4.2 Background

In Chapter III, we discussed the utility of the \( ^3\text{MLCT} \) excited state of \( \text{Ru(bpy)}_3^{2+} \) in photochemical oxidation of tyrosine. Tyrosine oxidation in these systems requires the flash-quench generation of the more oxidizing \( \text{Ru}^{III} \) intermediate.\(^1\) Unfortunately, the metallo-based quenchers (\( \text{Ru(NH}_3)_6^{3+} \) and \( \text{Co(NH}_3)_6^{3+} \)) commonly used in this chemistry can inactive RNR (see Chapter IX). Other quenchers, such as methyl viologen, are strongly absorbing in their one-electron reduced state, thereby obscuring the spectral features of \( \text{Y}^* \).\(^2\) The flash-quench method of generating \( \text{Y}^* \) can be circumvented by the design of long-lived, highly oxidizing \( ^3\text{MLCT} \) excited states, which can directly oxidize tyrosine.

In this regard, \( \text{Re}^I(\text{NN})(\text{CO})_3(\text{L}) \) (\( \text{NN}= \text{bpy}, \text{phen}; \text{L}= \text{halogen}, \text{pyridine}, \text{imidazole}, \) or phosphine) compounds should prove useful. These complexes typically have MLCT bands that can be excited near 355 nm and their \( ^3\text{MLCT} \) excited states are more oxidizing than that of \( \text{Ru(bpy)}_3^{2+} \) by \( \sim0.4-0.7 \) V.\(^3,4\) These complexes also afford a certain degree of synthetic flexibility in that either the NN ligand\(^5\) or \( \text{L} \) may be functionalized with amide bonds, allowing for coupling of tyrosine and intramolecular \( \text{Y}^* \) generation upon excitation with light. The excited state energies and lifetimes can be modulated by changes in both the NN and L ligand.\(^3,4,6\) We now present a foray into the synthesis and spectroscopy of \( \text{Re}^I \) complexes designed for oxidation of tyrosine directly from their \( ^3\text{MLCT} \) excited state.
4.3 Results

Synthesis. Scheme 1 highlights the two approaches undertaken in the syntheses of the amino acid containing Re\(^{1}\) complexes. The Re(bpy–AA–OrBu)(CO)\(_3\)(X) complexes (AA = amino acid, A and Y; X = Cl\(^-\), SCN\(^-\)) were prepared by pre-coupling the tert-butyl ester protected amino acid to the carboxylic acid of the bpy ligand using standard peptide coupling conditions. This modified bpy ligand was then added to the appropriate Re\(^{1}\) synthon, followed by deprotection of the amino acid with trifluoroacetic acid. We note that these deprotection conditions led to scrambling of the \(^1\)H NMR peaks in the bpy-aryl region and the observation of biexponential emission decay lifetimes. These results are consistent with an exchange of the X\(^-\) ligand by the deprotected carboxylate of the coupled amino acid. Similar complexes, Re(phen)(CO)\(_3\)(L\(^+\)) (L = pyridine, imidazole, His), have previously been shown to exchange L for carboxylate in reactions with acetic acid and/or synthetically appended carboxylate groups.\(^7\) For the preparation of Re(bpy–AA–OrBu)(CO)\(_3\)(CN) (AA = F and Y), exchange of X = Cl\(^-\) by CN\(^-\) did not proceed cleanly in the pre-coupling synthesis scheme and thus this method was abandoned. Instead, we found that a post-coupling strategy proved more synthetically feasible, in which bpy–COOH was first complexed to a Re\(^{1}\) synthon employing methods used to obtain other Re(NN)(CO)\(_3\)Cl (NN = polypyridyl) complexes.\(^8\) The Cl\(^-\) anion was then exchanged for CN\(^-\) and the amino acid coupled to the Re(bpy–COOH)(CO)\(_3\)(CN) complex. Unlike the X = Cl\(^-\) and SCN\(^-\) derivatives, the X = CN\(^-\) derivative proved to be inert to carboxylate exchange under the conditions of amino acid deprotection, thus enabling the isolation of pure Re(bpy–AA)(CO)\(_3\)(CN) compounds for spectroscopic study.

The amide linkage required for amino acid modification can also be furnished from carboxylic acid containing phosphine ligands of the analogous monodentate (P) Re(NN)(P)(CO)\(_3\)\(^+\) and bidentate (PP) Re(NN)(PP)(CO)\(_2\)\(^+\) complexes. The dppe analog can be derivatized by introducing carboxylic acid functionality on the ethylene backbone in the protected form as the 2,3-bis(diphenylphosphino)maleic anhydride (bma). The anhydride can be allowed to react with the ReBr(CO)\(_3\)(THF)\(_2\) synthon to form Re(bma)(CO)\(_3\)Br.\(^{14}\) Reaction of the anhydride with methanol yields the asymmetric methyl ester, carboxylic acid functionalized PP ligand.\(^{15}\) However, the free carboxylic acid and methyl ester groups are heat sensitive. Attempts to chelate phen to this complex at the high temperatures required for CO dissociation yielded the parent complex [Re(phen)(dppe)(CO)\(_2\)](PF\(_6\)), originally
synthesized by Schutte et al., which was obtained in single crystal form, and structurally characterized by X-ray diffraction (Figure 4.1).

An amide bond linkage can be established through the nucleophilic attack of benzyl amine on bma. This amide functionalized ligand was adapted to Re coordination chemistry by reacting it with Re(CO)Cl and subsequently chelating with phen to yield the model complex, [Re(phen)(PP–Bn)(CO)PF]. The extra carboxylic acid group, which remained on the Bn–PP–COOH ligand upon ring opening the anhydride, was observed to decarboxylate with the application of the heat required for subsequent PP and phen chelation. The crystal structure of one geometric isomer of [Re(phen)(PP–Bn)(CO)PF], shown in Figure 4.2, confirms the formation of the crucial amide bond to one carboxylic acid and the decarboxylation of the other. The two CO ligands adopt a cis stereochemistry and the phosphorus atom on the derivatized side of the chelating ligand is trans to CO. The other isomer, which did not crystallize, has this phosphorus trans to the nitrogen of phen. The bite angles of the PP–Bn and phen ligands (P–Re–P) =

![Figure 4.1. Thermal ellipsoid plot of single isomer of [Re(phen)(PP)(CO)]PF shown at 50% probability. The PF anion has been removed for clarity.](image)

Scheme 4.1. Syntheses of amino acid containing Re complexes.
80.72° and $\angle N(1)$–Re(1)–N(2) = 75.37°) create a distorted octahedral coordination geometry. The Re–C bond distances are slightly longer (d(Re–C) = 1.902 and 1.928 Å) and the C–O bond distances slightly shorter (d(C–O) = 1.169 and 1.164 Å) than a crystallographically characterized relative, Re(CO)$_2$(POEt)$_3$(PPh$_3$) (4,4’-Me$_2$-2,2’-bpy)$^+$, which features two monodentate phosphine ligands trans to one another (d(Re–C) = 1.84(3) and 1.88(3) Å; d(C–O) = 1.18(4) and 1.17(3) Å).¹¹

The carboxylic acid derivatives of the monodentate and bidentate phosphines can be modified with amino acids. We present the chemistry of only the former owing to their greater water solubility and higher MLCT energy (vide infra). The [Re(phen)(P–AA)(CO)$_3$](PF$_6$) (AA = F and Y) complexes were synthesized using the AA-ligand post-coupling strategy of Scheme 1. These compounds were inert to carboxylate exchange upon amino acid deprotection, as exhibited by the single, downfield shifted peak in the $^{31}$P NMR spectrum (versus free phosphine), satisfactory MALDI-TOF and elemental analysis, and the single exponential behavior of the emission decay (vide infra).

Photophysics. The spectroscopic properties for the Re$^1$ polypyridyl carbonyl complexes are summarized in Table 4.1. The MLCT transition for the Re$^1$ polypyridyl carbonyl complexes appears as a shoulder on the strong $\pi \rightarrow \pi^*$ absorption profile of the polypyridyl ligand. As has been observed previously,¹² the MLCT absorption band of the biscarbonyl complexes is red-shifted from that of their tricarbonyl counterparts. Excitation into the MLCT absorbance manifold produces the broad, structureless emission bands shown in Figure 4.3, where the intensity has been scaled in quanta ($I \times \lambda^2$) and normalized.

The emission maximum ($E_{em}$) of Re(bpy-COOH)(CO)$_3$(X) decreases across the series X = CN$^-$ > Cl$^-$ > SCN$^-$. Derivatization of the bpy ligand with an amino acid causes a red-shift of the MLCT emission as evidenced from the comparison of the Re(bpy–COOH)(CO)$_3$(CN) and Re(bpy–F)(CO)$_3$(CN) emission maxima in Table 4.1. Numerous studies of the room
temperature emission properties of Re\(^1\)(NN)(CO\(_3\))L\(^+\) complexes establish the emission to be of \(^3\)MLCT parentage.\(^{16}\) A more detailed analysis of Re\(^1\)(NN)(CO\(_3\))Cl photophysics reveals that the MLCT emission arises from three states of triplet orbital parentage in thermal equilibrium at room temperature.\(^{17}\) The situation may be further complicated by the presence of an intraligand \(^3\)ππ* excited state that is energetically proximate to the \(^3\)MLCT excited state, as observed for Re\(^1\)(s-phen)(CO\(_3\))Cl complexes (s-phen = 4,7-Me\(_2\)-1,10-phen, 5,6-Me\(_2\)-1,10-phen, 3,4,7,8-Me\(_2\)-1,10-phen).\(^{18}\) The case of energetically proximate intraligand and charge transfer excited states, however does not appear to be pertinent to Re\(^1\)(phen)(CO\(_3\))(phosphine)+ complexes because the room temperature emission does not exhibit vibronic structure, which is a signature of \(^3\)ππ* emission from transition metal polypyridyl complexes.\(^{19}\) Accordingly, we ascribe the emission band of Re\(^1\)(phen)(CO\(_3\))(P–F)+ shown in Figure 4.3 to a predominantly \(^3\)MLCT parentage, as assigned for other Re(phen)(CO\(_3\))(L)+ (L ≠ phosphine) complexes with long lifetimes and similar emission envelopes.\(^{20}\) As noted previously, removal of a CO ligand to form Re\(^1\)(NN)(dicarbonyl)+ complexes lowers the energy of the \(^3\)MLCT excited state relative to their tricarbonyl counterparts. In accordance with these observations, the emission band maximum of [Re(phen)(dppe)(CO\(_2\))](PF\(_6\)) is 2800 cm\(^{-1}\) to the red of [Re(phen)(P–F)(CO\(_3\))](PF\(_6\)).

The precise determination of the \(^3\)MLCT excited state energy of the Re\(^1\) polypyridyl carbonyl complexes is problematic for several reasons. Location of the \(E_{00}\) for the emitting state cannot be deduced from the overlap of the absorption and emission bands, as predicted by the Franck-Condon principle, because absorption occurs into the \(^1\)MLCT state and emission occurs from the corresponding \(^3\)MLCT state. Moreover, the room temperature emission bands of d\(^6\) polypyridyl complexes are usually broad and featureless and thus the \(\nu' = 0 \rightarrow \nu = 0\) transition cannot be directly observed within the emission envelope. Evaluation
of the emission energy from low temperature glasses of Re\textsuperscript{I} complexes is further complicated by the rigidochromic effect.\textsuperscript{36,21}

In the absence of a direct measure of the excited state energy, a Frank-Condon analysis of the emission band shapes was employed. The values of $E_0$, $S$, frequency ($\hbar \omega$), and band width ($\Delta \bar{v}_{1/2}$) listed in Table 4.2 were obtained by fitting the emission spectra shown in Figure 4.3 to eq (3) (see Experimental Section). The $E_0$ and $\Delta \bar{v}_{1/2}$ values may be used calculate the free energy of the MLCT excited state, $\Delta G_{\text{MLCT}}^0$, according to the following:\textsuperscript{41,42}

$$\Delta G_{\text{MLCT}}^0 = E_0 + \frac{(\Delta \bar{v}_{1/2})^2}{16 k_B T \ln(2)} \quad (1)$$

The values of $\Delta G_{\text{MLCT}}^0$ are listed in Table 4.2. Within the homologous polypyridyl series, Re(bpy)(CO)\textsubscript{3}X, the decrease in $\Delta G_{\text{MLCT}}^0$ across the series $X = \text{CN}^- > \text{SCN}^- > \text{Cl}^-$ is paralleled by a decrease in the emission quantum yields ($\Phi_{\text{em}}$) and lifetimes ($\tau_{\text{em}}$) listed in Table 4.1. This behavior is in accordance with the predictions of the energy gap law.\textsuperscript{16} There are several other notable features of the $\Phi_{\text{em}}$ and $\tau_{\text{em}}$ listed in Table 4.1: (1) [Re(phen)(dppe)(CO)\textsubscript{2}](PF\textsubscript{6}) in CH\textsubscript{3}CN exhibits a slightly faster $\tau_{\text{em}}$ and lower $\Phi_{\text{em}}$ than that previously reported in the less polar CH\textsubscript{2}Cl\textsubscript{2} solvent.\textsuperscript{13} (2) Solutions of the benzyl amide conjugate, [Re(phen)(PP–Bn)(CO)\textsubscript{2}](PF\textsubscript{6}), exhibit bi-exponential emission decay behavior ($\tau_1 = 1.1$ $\mu$s and $\tau_2 = 4.3$ $\mu$s) as a result of a mixture of geometric isomers. A crystal of the isomer characterized by X-ray diffraction (shown in Figure 4.1) dissolved in CH\textsubscript{3}CN exhibits a monoexponential decay ($\tau = 0.96$ $\mu$s) equivalent to $\tau_1$ of the isomer mixture. We therefore ascribe the short lifetime decay to the excited state of the isomer shown in Figure 4.1 and the longer lifetime decay to the other isomer. (3) The [Re(phen)(CO)\textsubscript{3}(P–F)](PF\textsubscript{6}) complex exhibits the longest $\tau_{\text{em}}$ and highest $\Phi_{\text{em}}$ and $\Delta G_{\text{MLCT}}^0$ of all the complexes in this study. The more energetic excited state and/or disposition of a phosphine trans to a CO may be responsible for a photolysis reaction that we observe for this complex upon extensive laser irradiation. A similar photochemical behavior observed for Re\textsuperscript{I}(NN)(CO)\textsubscript{3}(PR\textsubscript{3})\textsuperscript{+} complexes upon irradiation with light ($\lambda > 320$ nm) has been attributed to loss of a CO ligand.\textsuperscript{12}
### Table 4.1. Spectroscopic and Photophysical Properties of the MLCT Excited State of Re\(^{I}\) Polypyridyl Carbonyl Complexes in Aqueous Solution at Room Temperature

<table>
<thead>
<tr>
<th>Complex</th>
<th>(E_{\text{abs}}(\text{cm}^{-1}))</th>
<th>(E_{\text{em}}(\text{cm}^{-1})^a)</th>
<th>(\Phi_{\text{em}}^a)</th>
<th>(\tau_{\text{em}}(\text{ns})^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re(bpy–COOH)(CO)(_3)Cl</td>
<td>∼28500</td>
<td>15700</td>
<td>1.1 × 10(^{-3})</td>
<td>5.5</td>
</tr>
<tr>
<td>Re(bpy–COOH)(CO)(_3)SCN</td>
<td>27400</td>
<td>15100</td>
<td>1.6 × 10(^{-3})</td>
<td>12.0</td>
</tr>
<tr>
<td>Re(bpy–COOH)(CO)(_3)CN</td>
<td>28600</td>
<td>16400</td>
<td>1.4 × 10(^{-2})</td>
<td>86.5</td>
</tr>
<tr>
<td>Re(bpy–F)(CO)(_3)CN</td>
<td>27900</td>
<td>15800</td>
<td>–</td>
<td>59.4</td>
</tr>
<tr>
<td><a href="PF(_6)">Re(phen)(P–F)(CO)(_3)</a></td>
<td>27100</td>
<td>19100</td>
<td>9.4 × 10(^{-2})</td>
<td>3900</td>
</tr>
<tr>
<td><a href="PF(_6)">Re(phen)(dppe)(CO)(_2)</a>(^b)</td>
<td>∼27000</td>
<td>16300</td>
<td>7.8 × 10(^{-2})</td>
<td>3200</td>
</tr>
<tr>
<td><a href="PF(_6)">Re(phen)(PP–Bn)(CO)(_2)</a>(^b,c)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>940</td>
</tr>
</tbody>
</table>

\(^a\) \(\lambda_{\text{exc}} = 355\) nm. \(^b\) CH\(_3\)CN solvent. \(^c\) Isomer shown in Figure 1. Isolated solids from synthesis exhibit biexponential behavior arising from the presence of the two isomers (see text).

### Table 4.2. Emission Spectral Parameters Obtained from Fitting Corrected Emission Band to Eq. (3) and Energy of the MLCT Excited State Calculated from Eq. (1)

<table>
<thead>
<tr>
<th>Compound</th>
<th>(E_0) cm(^{-1})</th>
<th>(S)</th>
<th>(h\omega) cm(^{-1})</th>
<th>(\Delta \bar{\nu}_{1/2}) cm(^{-1})</th>
<th>(\Delta G^e_{\text{MLCT}}) eV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re(bpy–COOH)(CO)(_3)Cl</td>
<td>16400</td>
<td>1.0</td>
<td>1900</td>
<td>2800</td>
<td>2.47</td>
</tr>
<tr>
<td>Re(bpy–COOH)(CO)(_3)SCN</td>
<td>16300</td>
<td>1.5</td>
<td>1700</td>
<td>3000</td>
<td>2.52</td>
</tr>
<tr>
<td>Re(bpy–COOHOH)(CO)(_3)CN</td>
<td>16800</td>
<td>0.8</td>
<td>2000</td>
<td>3100</td>
<td>2.60</td>
</tr>
<tr>
<td>Re(bpy–F)(CO)(_3)CN</td>
<td>16500</td>
<td>0.9</td>
<td>1800</td>
<td>3200</td>
<td>2.59</td>
</tr>
<tr>
<td><a href="PF(_6)">Re(phen)(P–F)(CO)(_3)</a></td>
<td>20300</td>
<td>1.8</td>
<td>1200</td>
<td>1800</td>
<td>2.70</td>
</tr>
<tr>
<td><a href="PF(_6)">Re(phen)(dppe)(CO)(_2)</a>(^a)</td>
<td>16700</td>
<td>0.7</td>
<td>1700</td>
<td>2600</td>
<td>2.42</td>
</tr>
</tbody>
</table>

\(^a\) Solvent is CH\(_3\)CN
The high values of $\Delta G_{\text{MLCT}}^0$ for the amino acid conjugates, $[\text{Re}(\text{phen})(\text{CO})_3(\text{P–F})](\text{PF}_6)$ and $\text{Re(bpy–F)(CO)}_3\text{CN}$, make these platforms excellent candidates for promoting the oxidation of tyrosine directly from the excited state. The excited state reduction potentials for the Re I polypyridyl tricarbonyl complexes are listed in Table 4.3 along with the modified Latimer diagram from which they were determined. The portion of the diagram relevant to tyrosine oxidation is highlighted in black. The ground state $E^0(\text{Re}^\text{I}/0)$ and $E^0(\text{Re}^\text{II}/0)$ reduction potentials were determined from differential pulse voltammetry (DPV) measurements. Reversible electrochemical behavior is observed for the $E^0(\text{Re}^\text{I}/0)$ redox process, whereas the $E^0(\text{Re}^\text{II}/0)$ redox couple is quasi-irreversible and is therefore reported as a peak potential, $E_p$, in Table 4.3.

Photochemical Y• generation in Re I Complexes. With the excited state reduction potentials in hand, we next investigated the facility of tyrosine to quench the emission of these powerful complexes.

### Table 4.3. Differential Pulse Voltammetry$^a$ and Excited State Reduction$^b$ and Oxidation Potentials$^c$ of Amino Acid-Derivatized Re I Polypyridyl Tricarbonyl Complexes

<table>
<thead>
<tr>
<th>Complexes</th>
<th>$E_p(\text{Re}^{\text{II}/0})$</th>
<th>$E^0(\text{Re}^{\text{I}/0})$</th>
<th>$E^0(\text{Re}^\text{I*//0})$</th>
<th>$E^0(\text{Re}^{\text{I*//II}})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re(bpy–A–OtBu)(CO)$_3$Cl</td>
<td>0.94</td>
<td>–1.58</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Re(bpy–F)(CO)$_3$CN</td>
<td>1.09</td>
<td>–1.65</td>
<td>1.59</td>
<td>0.85</td>
</tr>
<tr>
<td><a href="PF$_6$">Re(phen)(P–F)(CO)$_3$</a></td>
<td>1.52</td>
<td>–1.57</td>
<td>1.78</td>
<td>0.53</td>
</tr>
</tbody>
</table>

$^a$ Reported in V vs. Fe$^{3+}$/Fe with 0.1 M (Bu$_4$N)PF$_6$ supporting electrolyte in CH$_3$CN. $^b$ $E^0(\text{Re}^\text{I*//0}) = \Delta G_{\text{MLCT}}^0 + E^0(\text{Re}^{\text{I}/0})$ vs. NHE. $^c$ $E^0(\text{Re}^{\text{I*//II}}) = \Delta G_{\text{MLCT}}^0 - E^0(\text{Re}^{\text{I//1}})$ vs. NHE.
excited state oxidants. A comparison of the luminescence properties of Re(bpy–AA)(CO)3CN (AA = Y and F) complexes in 10 mM aqueous phosphate buffer at pH 7 established no phosphorescence quenching of the tyrosine complex. However, deprotonation of the tyrosine phenol (pK_a = 10.1 for L-tyrosine) at pH 12 induces significant emission quenching. A rate constant for this process may be determined from the excited state lifetimes in the presence and absence of quenching,

\[
k_q = \frac{1}{\tau_Y} - \frac{1}{\tau_F}
\]

where \(\tau_Y\) and \(\tau_F\) are the lifetimes of emission decay for the Y and F derivatives, respectively. The measured lifetime of \(\tau_Y = 1.2\) ns for Re(bpy–Y)(CO)3CN yields a \(k_q = 8.2 \times 10^8\) s\(^{-1}\).

In contrast to the pH behavior of Re(bpy–Y)(CO)3CN quenching, the emission from [Re(phen)(P–Y)(CO)3](PF6) was quenched at all pHs. At pH 7 in 10 mM phosphate buffer, \(\tau_Y = 2.2\) μs and \(\Phi_{em,Y} = 0.059\) for the tyrosine derivative whereas \(\tau_F = 3.9\) μs and \(\Phi_{em,F} = 0.094\) for the phenylalanine derivative (\(\tau_F\) and \(\Phi_{em,F}\) are independent of pH), yielding a quenching rate constant of \(k_q = 2.0 \times 10^5\) s\(^{-1}\). As noted above, the [Re(phen)(P–AA)(CO)3](PF6) complexes are subject to CO dissociation under prolonged irradiation. We contend that the kinetics for CO dissociation upon complex excitation are the same for the F and Y derivatives and do not vary with pH. Under this assumption, eq. (2) isolates the quenching process due to Y, and we assign this process to the PCET oxidation of Y by the \(^3\)MLCT excited state.

Figure 4.4 Top: TA spectra of a pH 7 solution of Re(bpy–Y)(CO)3(CN) at 15 (●), 65 (□), 115 (– – ), and 215 ns (* – *) (Insets: Single wavelength kinetic traces and monoexponential fits for the disappearance of the 380 and 475 nm (\(^3\)MLCT) signal). Bottom: Same experiment at pH 12 (Insets: Single wavelength kinetic traces and monoexponential fits for the disappearance of the 410 (Y•) and 500 nm (bpy•–) signal.)
Chapter V will present a detailed analysis of the PCET mechanisms involved with this reaction.

Nanosecond transient absorption spectroscopy (TA) was then performed to characterize the electron transfer products of these photochemical reactions. The TA profiles following 355 nm excitation for Re(bpy–Y)(CO)₃(CN) in pH 7 and 12 solutions are presented in Figure 4.4. The $\Delta OD$ spectrum at pH 7 (Figure 4.4, top panel) is the same as that obtained for Re(bpy–F)(CO)₃(CN) (not shown) and consists of maxima at 380 and ~475 nm and a minimum at 400 nm. These features are in accord with that observed for the $^3$MLCT state of Re$(^1$NN)(CO)$_3$L complexes.$^{16,23}$ In support of a similar assignment here, the single wavelength transient signals obtained at 380 and 475 nm shown in the top insets of Figure 4.4 decay to baseline with time constants (60.6 and 59.8 ns, respectively) identical to the emission lifetime of the $^3$MLCT excited state (60.4 ns). In contrast, the time-resolved absorption spectra at pH 12 (Figure 4.4, bottom panel) are not that of the $^3$MLCT excited state. The transient profile is dominated by absorptions at 410 and 520 nm. The 410 nm feature coincides with the absorption maximum of Y•$^{24}$ and the 520 nm feature resembles the absorption maxima of bpy•$^{25,26}$ For comparison, the spectroelectrochemical UV/Vis absorption spectrum of the reduced Re(bpy–Y–OtBu)(CO)₃(CN) in DMF is shown Figure 4.5. The spectrum contains a maximum at 530 nm with vibronic fine structure similar to that observed in the TA spectra at the bottom of Figure 4.4. These results provide direct evidence for the charge separated state, Re(bpy•–Y•)(CO)₃(CN). The time-evolved gray traces establish that the overall profile does not change with time. The single wavelength transient signals obtained at 410 and 500 nm and shown in the bottom insets decay concomitantly with a rate constant of $k_{CR} = 2.2 \times 10^7$ s$^{-1}$, which we attribute to charge recombination. Unfortunately, the [Re(phen)(P–Y)(CO)$_3$](PF$_6$) was not sufficiently soluble in aqueous solution to allow for TA experiments.

**Figure 4.5** Spectroelectrochemical UV/Vis absorption spectrum of Re(bpy–Y–OtBu)(CO)$_3$(CN) at $-0.75 \, V$ vs. Fc$/^+/Fc$ in DMF.
4.4 Discussion

*Electron Shuttling in Y Oxidation.* Intramolecular shuttling of electrons between tyrosine and the MLCT excited state may occur along two fundamentally different pathways, designated in Scheme 4.2 as pop-pop and pop-fizz. The latter describes the electron transfer events induced by excitation of typical Re(NN)(CO)_3X complexes, in which the donor is attached to the polypyridyl ligand, as is the case for Re(bpy–Y)(CO)_3CN. Photon absorption (“pop”) forms the \(^3\text{Re}^{II}(\text{bpy}^-\text{–}Y)(\text{CO})_3\text{CN}\) MLCT state. Subsequent tyrosine oxidation occurs by a contraposed electron flow, whether through the bpy or through space. The pathway is anticipated to incur several barriers to \(Y^*\) generation. Oxidation of tyrosine brings the electron through the reduced bpy ligand. Though the effect of superexchange through a reduced polypyridyl ligand has not been investigated critically, general theory suggests that the increased energy gap between reduced donor and oxidized acceptor levels would retard the tunneling rate for \(Y^*\) generation.\(^{27}\) More problematic is the favored back reaction resulting from the disposition of a neighboring hole and electron on \(Y^*\) and \(\text{bpy}^*\) respectively. The correlation between the back electron transfer rate and proximity of hole/electron pairs has been examined in a comparative study of \(\text{Re}^I(\text{bpy})(\text{CO})_3(\text{py})^+\) complexes containing a phenothiazine (PTZ) electron donor coupled to the pyridine ligand in \(\text{Re}(4,4'\text{-Me}_2\text{-bpy})(\text{CO})_3(\text{py–PTZ})^+\), and bipyridine ligand in \(\text{Re}(\text{PTZ–CH}_2\text{-bpy})(\text{CO})_3(4\text{-Etpy})^+\).\(^{28}\) An accelerated back electron transfer rate was established for the former, which exhibits a \(\text{Re}(\text{PTZ}^+\text{–CH}_2\text{-bpy}^*\text{–})(\text{CO})_3(4\text{-Etpy})^+\) charge distribution upon intramolecular quenching of the MLCT excited state. A similar charge distribution is retained in the charge separated state of the tyrosine-modified \(\text{Re}^I\) polypyridyl complex, \(\text{Re}^I(\text{bpy}^*\text{–}Y^*)(\text{CO})_3\text{CN}\), thus accounting for its short lifetime of 45 ns (“fizz”).

The hole/electron pair is optimally separated by appending the tyrosine ligand to the sixth ligand, away from the polypyridyl ligand. We chose phosphine ligands as the scaffold for the amino acid owing to the high energies and long lifetimes of \(\text{Re}(\text{NN})(\text{PP})(\text{CO})_2^+\) and \(\text{Re}(\text{NN})(\text{P})(\text{CO})_3^+\) model compounds. Of these two platforms, the latter was more ideally suited to \(Y^*\) generation owing to a greater water solubility and intrinsically higher MLCT excited state energy. The architecture of the \(\text{Re}^I(\text{phen})(\text{CO})_3(\text{P–Y})^+\) complex permits the charge distribution in Scheme 4.2 to be established. Excitation pops the electron to the phen ligand in the MLCT state to produce \(\text{Re}^{II}(\text{phen}^*\text{–})(\text{CO})_3(\text{P–Y})^+\), which then allows for the
electron to flow from the phosphine ligand, quenching the Re$^{II}$ hole and furnishing the Re$^I$(phen$^-$)(CO)$_3$(P–Y$^-$)$^+$ radical intermediate. The electron and hole are disposed at maximal distance within the primary coordination sphere of the complex, presumably retarding the rate of charge recombination.

4.5 Conclusions

Rhenium(I) polypyridyl complexes provide suitable platforms for the intramolecular photogeneration of tyrosyl radicals owing to the significant oxidizing power of their MLCT excited states. The excited state of Re(bpy)(CO)$_3$CN complex can oxidize Y, but only at pHs where the tyrosine phenol is deprotonated. For this complex to find application to our work with photochemical RNRS and to generate Y$^\cdot$ at near neutral pH, we need to develop a method to lower the pKa of the tyrosine phenol. Unnatural amino acids can prove useful in this regard and are discussed in Chapter VI.

On the other hand, phosphine-containing Re$^I$ polypyridyl complexes are particularly useful excited state oxidants of Y for three reasons. First, the MLCT excited state is highly energetic and presents a significant overpotential for tyrosine oxidation. The additional energy of the MLCT excited state is crucial for amino acid oxidation because, unlike a simple ET quenching process, it is needed to overcome the barrier for proton transfer as well.$^{29,30}$ Second, the phosphine complexes exhibit a dramatically extended lifetime of the MLCT excited state, allowing for a higher Stern-Volmer constant ($SV = k_q\tau_o$) for quenching.$^{31}$ Finally, by appending the tyrosine to an ancillary ligand, remote from the electron accepting polypyridyl ligand in MLCT excitation, the intramolecular shuttling occurs via a
unidirectional electron cascade for the efficient generation and preservation of the photogenerated Y•.

The Re¹ phosphine scaffolds presented here provide a convenient method for investigating radical-based mechanisms in biology because, unlike previous approaches, the amino acid radical can be generated directly and on microsecond timescales without the need for external oxidants or reductants. Moreover, unlike previous Re¹ complexes used to photogenerate amino acid radicals, such as Re¹(phen)(CO)₃(His)⁺, 32-34 the Re¹ phosphine complexes are resistant to carboxylate exchange. Thus these MLCT states may be used to initiate and study radical reactions from peptides or within proteins containing multiple aspartate or glutamate residues. Finally, we note that this method is not necessarily specific to tyrosine but can be extended to other amino acids by simple derivatization of the monophosphine.

4.6 Experimental Section

Materials and Methods. L-Tyrosine tert-butyl ester (Y–OtBu), L-alanine tert-butyl ester hydrochloride (A–OtBu•HCl), 1-hydroxybenzotriazole (HOBt) (NovaBiochem), L-phenylalanine tert-butyl ester hydrochloride (F–OtBu•HCl) (Advanced ChemTech), magnesium sulfate (MgSO₄) (EMD), thallium hexafluorophosphate (TIPF₆) (Strem), potassium thiocyanate (KSCN), sodium cyanide (NaCN), 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (WSC•HCl), dimethylaminopyridine (DMAP), N-methylmorpholine (NMM), pentacarbonylchlororhenium (Re(CO)₅Cl), 1,10-phenanthroline (phen), 4-diphenylphosphinobenzoic acid (Ph₂PPh–COOH) (Aldrich) and trifluoroacetic acid (TFA, 99.9%) (J. T. Baker) were used as received. 4´-Methyl-2,2´-bipyridine-4-carboxylic acid (bpy–COOH), 35 cis-1,2-(bisdiarylphosphino)ethylene)(1,10-phenanthroline)(dicarboxyl)rhenium(I) hexafluorophosphate ([Re(phen)(dppe)(CO)₂](PF₆)), 13 and tricarbonylchloro(1,10-phenanthroline)rhenium(I) (Re-(phen)(CO)₃Cl) 36 were prepared as previously described. cis-1-Carboxy-2-(N-benzylcarbamoyl)-1,2-bis(diphenylphosphino)ethene (HOOC–PP–Bn) 37 was prepared from 2,3-bis(diphenylphosphino)maleic anhydride (bma) 38 and benzylamine (Aldrich).

Tricarbonylchloro(4´-methyl-2,2´-bipyridine-4-carboxylic acid)rhenium(I) (Re(bpy–COOH)(CO)₃Cl). Re(CO)₅Cl (347 mg, 0.962 mmol, 1.0 eq) and bpy–COOH (206 mg, 0.962 mmol, 1.0 eq) were added to toluene (25 mL) contained in a round bottom flask and the mixture was heated at reflux overnight. The bright orange solution was cooled to room
temperature, and the yellow/orange solid was isolated by filtration, washed with ether, and
dried under vacuum (470 mg, 94%). \(^1\)H NMR (300 MHz, d-acetone, 25 °C) \(\delta = 2.67\) (s, 3H, bpy-CH\(_3\)), 7.66 (m, 1H, bpy-H), 8.21 (m, 1H, bpy-H), 8.81 (s, 1H, bpy-H), 8.95 (d, 1H, bpy-H), 9.00 (s, 1H, bpy-H), 9.29 (d, 1H, bpy-H). \(^1\)H NMR (300 MHz, CD\(_3\)OD, 25 °C) \(\delta = 2.62\) (s, 3H, bpy-CH\(_3\)), 7.55 (m, 1H, bpy-H), 8.13 (m, 1H, bpy-H), 8.57 (s, 1H, bpy-H), 8.87 (d, 1H, bpy-H), 8.98 (s, 1H, bpy-H), 9.19 (d, 1H, bpy-H).

**Tricarbonylthiocyanato(4′-methyl-2,2′-bipyridine-4-carboxylic acid)rhenium(I)** (Re(bpy–COOH)(CO)\(_3\)SCN). Re(bpy–COOH)(CO)\(_3\)Cl (200 mg, 0.385 mmol, 1.0 eq) and KSCN (3.73 g, 38.5 mmol, 100 eq) were combined in a flask with 20 mL of 1:1 95% ethanol/water and refluxed overnight under \(\text{N}_2\). The ethanol was removed in vacuo, and the mixture was extracted with ethyl acetate. The various extracts were combined, dried over MgSO\(_4\), and the solvent was removed in vacuo to furnish a red powder (136 mg, 65%). \(^1\)H NMR (300 MHz, d-acetone, 25 °C) \(\delta = 2.70\) (s, 3H, bpy-CH\(_3\)), 7.73 (d, 1H, bpy-H), 8.28 (m, 1H, bpy-H), 8.87 (s, 1H, bpy-H), 8.99 (d, 1H, bpy-H), 9.07 (s, 1H, bpy-H), 9.33 (d, 1H, bpy-H). Anal. Calcd for C\(_{16}\)H\(_{10}\)N\(_3\)O\(_5\)ReS: C, 35.42; H, 1.86; N, 7.75; S, 5.91. Found: C, 35.35; H, 1.81; N, 7.71; S, 5.97.

**Tricarbonylcyano(4′-methyl-2,2′-bipyridine-4-carboxylic acid)rhenium(I)** (Re(bpy–COOH)(CO)\(_3\)CN). Re(bpy–COOH)(CO)\(_3\)Cl (200 mg, 0.385 mmol, 1.0 eq) and NaCN (1.89 g, 38.5 mmol, 100 eq) were combined in a round bottom flask with 40 mL of 1:1 95% ethanol/water, and the mixture was heated at reflux for 6 h. The ethanol was removed in vacuo yielding a bright yellow precipitate, which was isolated by filtration, washed with copious amounts of water and ether, and dried under vacuum (168 mg, 86%). \(^1\)H NMR (300 MHz, d-CD\(_3\)OD, 25 °C) \(\delta = 2.63\) (s, 3H, bpy-CH\(_3\)), 7.54 (d, 1H, bpy-H), 8.03 (m, 1H, bpy-H), 8.49 (s, 1H, bpy-H), 8.89 (m, 2H, bpy-H), 9.08 (d, 1H, bpy-H). Anal. Calcd for C\(_{16}\)H\(_{10}\)N\(_3\)O\(_5\)Re: C, 37.65; H, 1.97; N, 8.23. Found: C, 37.53; H, 2.06; N, 8.20.

**4′-Methyl-2,2′-bipyridine-4-alanine tert-butyl ester** (bpy–A–OtBu). Bpy–COOH (0.502 g, 2.34 mmol, 1.0 eq), A–OtBu•HCl (0.425 g, 2.34 mmol, 1.0 eq), WSC•HCl (0.493 g, 2.57 mmol, 1.1 eq), and HOBT (0.374 g, 2.57 mmol, 1.1 eq) were combined in a round bottom flask. CH\(_2\)Cl\(_2\) 100 mL and NMM (1 mL, 9.4 mmol, 4 eq) were added and the reaction was stirred overnight. The reacted solution was washed in a separatory funnel with 2 × 50 mL of water, 10% citric acid, and 1.0 M NaHCO\(_3\) and the organic layer was collected and dried.
over MgSO₄. The solvent was removed in vacuo to yield an oil, which solidified under high vacuum as a white mass (0.5947 g, 57%). ¹H NMR (300 MHz, CDCl₃, 25 °C) δ = 1.47 (m, 12H, tBu and A-CH₃), 2.45 (s, 3H, bpy-CH₃), 4.72 (m, 1H, A-CH), 7.07 (d, 1H, N-H), 7.15 (d, 1H, bpy-H), 7.70 (d, 1H, bpy-H), 8.25 (s, 1H, bpy-H), 8.55 (d, 1H, bpy-H), 8.70 (s, 1H, bpy-H), 8.78 (d, 1H, bpy-H).

4´-Methyl-2,2´-bipyridine-4-tyrosine tert-butyl ester (bpy–Y–O₄Bu). Bpy–COOH (0.750 g, 3.50 mmol, 1.0 eq), Y–O₄Bu (0.830 g, 3.50 mmol, 1.0 eq), WSC·HCl (0.738 g, 3.85 mmol, 1.1 eq), HOBt (0.520 g, 3.85 mmol, 1.1 eq) and DMAP (43 mg, 0.35 mmol, 0.1 eq) were combined in a round bottom flask. Methylene chloride (CH₂Cl₂, 200 mL) and NMM (1.7 mL, 15.4 mmol, 4.4 eq) were added and the reaction was stirred overnight. The reacted solution was washed in a separatory funnel with 4 × 100 mL of water and the organic layer was collected and dried over MgSO₄. The solvent was removed in vacuo to yield a yellow oil, which under high vacuum produced an off-white solid. The solid was dissolved in a few mL of ethyl acetate, loaded onto a Chromatotron plate (alumina, 2mm), and eluted with ethyl acetate. The first band to elute was collected and the solvent removed in vacuo to yield an oil, which solidified under high vacuum as an off-white solid (0.5921 g, 39%). ¹H NMR (300 MHz, CDCl₃, 25 °C) δ = 1.47 (s, 9H, tBu), 2.45 (s, 3H, bpy-CH₃), 3.16 (m, 2H, Y-CH₂), 4.96 (m, 1H, Y-CH), 6.68 (d, 2H, phenol-H), 7.02 (m, 2H, phenol-H and bpy-H), 7.17 (d, 1H, N-H), 7.70 (m, 1H, bpy-H), 8.23 (s, 1H, bpy-H), 8.53 (d, 1H, bpy-H), 8.63 (s, 1H, bpy-H), 8.78 (d, 1H, bpy-H).

Tricarbonylchloro(4´-methyl-2,2´-bipyridine-4-alanine tert-butyl ester) rhenium(I) (Re(bpy–A–O₄Bu)(CO)₃Cl. Re(CO)₅Cl (500 mg, 1.38 mmol, 1.0 eq) and bpy–A–O₄Bu (471 mg, 138 mmol, 1.0 eq) were combined in a round bottom flask with 50 mL of toluene and the mixture heated at reflux overnight. The yellow suspension was cooled in an ice bath and the yellow solid was isolated by filtration, washed with ether and dried under vacuum (722 mg, 81%). ¹H NMR (300 MHz, d-acetone, 25 °C) δ = 1.47 (m, 12H, tBu), 2.59 (s, 3H, bpy-CH₃), 4.41 (m, 1H, Y-CH), 7.61 (d, 1H, bpy-H), 8.02 (m, 1H, bpy–H), 8.70 (s, 1H, bpy-H), 8.85 (d, 1H, bpy-H), 8.95 (s, 1H, bpy-H), 9.13 (d, 1H, bpy-H), 9.31 (m, 1H, N-H) Anal. Calcd for C₂₂H₂₃ClN₃O₆Re: C, 40.83 H, 3.58; N, 6.49. Found: C, 40.96; H, 3.72; N, 6.52.

Tricarbonylchloro(4´-methyl-2,2´-bipyridine-4-tyrosine tert-butyl ester) rhenium(I) (Re(bpy–Y–O₄Bu)(CO)₃Cl. Re(CO)₅Cl (467 mg, 1.29 mmol, 1.0 eq) and bpy–Y–O₄Bu (560 mg, 2.50 mmol, 1.0 eq) were combined in a round bottom flask with 50 mL of toluene and the mixture heated at reflux overnight. The yellow suspension was cooled in an ice bath and the yellow solid was isolated by filtration, washed with ether and dried under vacuum (722 mg, 81%). ¹H NMR (300 MHz, d-acetone, 25 °C) δ = 1.47 (m, 12H, tBu), 2.59 (s, 3H, bpy-CH₃), 4.41 (m, 1H, Y-CH), 7.61 (d, 1H, bpy-H), 8.02 (m, 1H, bpy–H), 8.70 (s, 1H, bpy-H), 8.85 (d, 1H, bpy-H), 8.95 (s, 1H, bpy-H), 9.13 (d, 1H, bpy-H), 9.31 (m, 1H, N-H) Anal. Calcd for C₂₂H₂₃ClN₃O₆Re: C, 40.83 H, 3.58; N, 6.49. Found: C, 40.96; H, 3.72; N, 6.52.
mg, 1.29 mmol, 1.0 eq) were combined in a round bottom flask with 50 mL of toluene and the mixture heated at reflux overnight. The yellow suspension was cooled in an ice bath and the yellow solid was isolated by filtration, washed with ether and dried under vacuum (668 mg, 70%). \( ^1 \)H NMR (300 MHz, d-acetone, 25 °C) \( \delta = 1.36 \) (s, 9H, tBu), 2.58 (s, 3H, bpy-CH\(_3\)), 3.01 (m, 2H, Y-CH\(_2\)), 4.57 (m, 1H, Y-CH), 6.66 (d, 2H, phenol-H), 7.08 (d, 2H, phenol-H), 7.61 (d, 1H, bpy-H), 7.97 (m, 1H, bpy-H), 8.65 (s, 1H, bpy-H), 8.86 (m, 2H, bpy-H), 9.17 (m, 1H, bpy-H), 9.33 (m, 1H, N-H) Anal. Calcd for C\(_{28}\)H\(_{27}\)ClN\(_3\)O\(_7\)Re: C, 45.50; H, 3.68; N, 5.68. Found: C, 45.36; H, 3.75; N, 5.74.

**Tricarbonylthiocyanato(4’-methyl-2,2’-bipyridine-4-tyrosine tert-butyl ester)rhenium (I)** (Re(bpy–Y–O\(_{t\text{Bu}}\))(CO)\(_3\)SCN). Re(bpy–Y–O\(_{t\text{Bu}}\))(CO)\(_3\)Cl (111 mg, 0.150 mmol, 1.0 eq) and KSCN (1.46 g, 15 mmol, 100 eq) were combined in a round bottom flask with 40 mL of 1:1 95% ethanol/water and heated at reflux overnight. The ethanol was removed in vacuo and the red precipitate was isolated by filtration, washed with copious amounts of water, and dried under high vacuum (91 mg, 80%). \( ^1 \)H NMR (300 MHz, d-acetone, 25 °C) \( \delta = 1.45 \) (s, 9H, tBu), 2.70 (s, 3H, bpy-CH\(_3\)), 4.87 (m, 1H, Y-CH), 6.79 (m, 2H, phenol-CH\(_2\)), 7.19 (m, 2H, phenol-CH\(_2\)), 7.73 (m, 1H, bpy-H), 8.12 (m, 1H, bpy-H), 8.46 (d, 1H, N-H), 8.69 (m, 1H, bpy-H), 8.90 (m, 1H, bpy-H), 8.98 (m, 1H, bpy-H), 9.25 (m, 1H, bpy-H).

**Tricarbonylcyan(4’-methyl-2,2’-bipyridine-4-phenylalanine)rhenium(I)** (Re(bpy–F)-(CO)\(_3\)CN). Re(CO)\(_3\)(bpy–COOH)(CN) (130 mg, 0.321 mmol, 1.0 eq), F–O\(_{t\text{Bu}}\)•HCl (72 mg, 0.321 mmol, 1.0 eq), WSC•HCl (53 mg, 0.353 mmol, 1.1 eq) and HOBt (37 mg, 0.353 mmol, 1.1 eq) were combined in a round bottom flask with 25 mL of DMF. NMM (0.12 mL, 4.0 eq) was added and the mixture was stirred overnight. The solvent was removed in vacuo, and the resulting yellow oil was dissolved in 100 mL CH\(_2\)Cl\(_2\) and washed with 2 × 25 mL water, 10% citric acid, 1.0 M NaHCO\(_3\) and water in that order. The organic phase, isolated with a separatory funnel, was dried over MgSO\(_4\) and the solvent was removed in vacuo. The resulting yellow solid was dissolved in 10 mL of 1:1 TFA/CH\(_2\)Cl\(_2\) and stirred for 1 h. The solution was concentrated to <1 mL under a stream of N\(_2\) and triturated with ether. The bright yellow solid that precipitated was isolated by filtration, washed with copious amounts of ether and dried under vacuum (82 mg, 39%). \( ^1 \)H NMR (300 MHz, d-CD\(_3\)OD, 25 °C) \( \delta = 2.64 \) (s, 3H, bpy-CH\(_3\)) 3.13 (m, 2H, F-CH\(_2\)), 4.96 (m, 1H, F-CH), 7.30 (m, 5H, F-ph), 7.57 (m, 1H, bpy-H), 7.86 (m, 1H, bpy-H), 8.49 (m, 1H, bpy-H), 8.79 (m, 1H, bpy-H), 8.89 (m, 1H, bpy-}
Tricarbonylcyanomethyl-2,2'-bipyridine-4'-tyrosine)rhenium(I) (Re(bpy–Y)(CO)3-(CN). The synthesis of the tyrosine derivative was the same as that for the phenylalanine derivative, except that Re(CO)3(bpy–COOH)(CN) (193 mg, 0.377 mmol, 1.0 eq), WSC•HCl (80 mg, 0.415 mmol, 1.1 eq), and HOBT (43 mg, 0.415 mmol, 1.1 eq) were combined with Y–O–Bu (90 mg, 0.377 mmol, 1.0 eq), instead of F–O–Bu•HCl (49%). 1H NMR (300 MHz, d-CD3OD, 25 °C) δ = 2.64 (s, 3H, bpy-CH3), 3.03 (m, 2H, Y-CH2), 4.87 (m, 1H, F-CH), 6.71 (d, 2H, phenol-H), 7.11 (d, 2H, phenol-H), 7.57 (m, 1H, bpy-H), 7.87 (m, 1H, bpy-H), 8.48 (m, 1H, bpy-H), 8.76 (m, 1H, bpy-H), 8.89 (m, 1H, bpy-H), 9.15 (m, 1H, bpy-H) Anal. Calcd for C25H19N4O7Re: C, 44.42; H, 2.76; N, 8.17.

Tricarbonyl(acetonitrile)(1,10-phenanthroline)rhenium(I) hexafluorophosphate, ([Re(phen)(CO)3(CH3CN)](PF6)). Re(phen)(CO)3Cl (400 mg, 0.823 mmol, 1.0 eq) and TlPF6 (374 mg, 1.07 mmol, 1.3 eq) were combined in a round bottom flask with 40 mL of CH3CN and heated at reflux in the dark overnight. The solvent was removed in vacuo leaving a yellow oil, which was dissolved in CH2Cl2. Any remaining solids were filtered off, and ether was slowly dripped into the solution to produce long yellow/green luminescent crystals, which were isolated by filtration, washed with ether and dried under vacuum (450 mg, 86%). 1H NMR (300 MHz, d-acetone, 25 °C) δ = 2.17 (s, 3H, CH3CN-Re), 8.27 (m, 2H, phen-H), 8.42 (m, 2H, phen-H), 9.11 (d, 2H, phen-H), 9.63 (d, 2H, phen-H). Anal. Calcd. C17H11F6N3O3PRe: C, 32.08; H, 1.74; N, 6.60. Found: C, 32.15; H, 1.66; N, 6.47.

Tricarbonyl(diphenylphosphinobenzoic acid)(1,10-phenanthroline)rhenium(I) hexafluorophosphate, ([Re(phen)(Ph2PPh-COOH)(CO)3](PF6)). [Re(phen)(CO)3(CH3CN)](PF6) (877 mg, 1.37 mmol, 1.0 eq) and Ph2PPh-COOH (633 mg, 2.07 mmol, 1.5 eq) were combined in a round bottom flask with 40 mL of N2 purged acetone. The solution was heated at reflux overnight under an N2 atmosphere in the dark. The solvent was removed in vacuo without heating and the yellow solid was taken up in CH2Cl2. The resulting light yellow precipitate was isolated by filtration and washed with CH2Cl2 (1.118 g, 90%). 1H NMR (300 MHz, d-acetone, 25 °C) δ = 7.05-7.80 (m, 14H, Ph2PPh-R), 8.02 (m, 2H, phen-H), 8.27 (s, 2H, phen-H), 8.90 (d, 2H, phen-H), 9.44 (d, 2H, phen-H) MALDI-TOF
Calcd. (Found): [M – PF$_6$]$^+$ 757.09 (757.03); [M – CO$_2$PF$_6$]$^+$ 729.10 (729.05). Anal. Calcd. C$_{34}$H$_{23}$F$_6$N$_2$O$_5$P$_2$Re: C, 45.29; H, 2.57; N, 3.11. Found: C, 45.21; H, 2.63; N, 3.06.

$[^{1}]$Re(phen)(P–Y)(CO)$_3$(PF$_6$). Re(phen)(CO)$_3$(Ph$_2$PPh–COOH)](PF$_6$) (200 mg, 0.222 mmol, 1.0 eq), Y–O$^-$tBu (53 mg, 0.222 mmol, 1.0 eq), WSC•HCl (47 mg, 0.244 mmol, 1.1 eq), HOBT (33 mg, 0.244 mmol, 1.1 eq), and NMM (100 μL, 0.888 mmol, 4.0 eq) were combined in a round bottom flask with 20 mL of CH$_2$Cl$_2$ and stirred overnight. The solution was diluted with 50 mL of CH$_2$Cl$_2$, washed with 2 × 25 mL of water, and dried over MgSO$_4$. The solution was concentrated to a few mL in vacuo and loaded onto a silica gel Chromatotron plate (2 mm). The highly emissive band that eluted with 5% MeOH/CH$_2$Cl$_2$ was collected and the solvent removed in vacuo to yield a yellow solid, which was dissolved in 10 mL of 1:1 TFA/CH$_2$Cl$_2$ and stirred for 1 h. The solution was concentrated under a stream of N$_2$. Addition of ether caused a yellow solid to precipitate; the solid was isolated by filtration and dried under vacuum (140 mg, 59%).$^{1}$H NMR (300 MHz, d-acetone, 25 °C) $\delta = 3.18$ (m, 2H, Y-CH$_2$), 4.89 (s, 1H, Y-CH), 6.85 (d, 2H, phenol-H), 7.20 (d, 2H, phenol-H), 7.27-7.85 (m, 14H, Ph$_2$P-Ph-R), 7.98 (m, 2H, phen-H), 8.14 (s, 2H, phen-H), 8.80 (m, 2H, phen-H), 9.42 (m, 2H, phen-H) $^{31}$P NMR (300 MHz, d-acetone, 25 °C) $\delta = 19.5$ (s, Re-P) MALDI-TOF Calcd. (Found): [M – PF$_6$]$^+$ 920.15 (920.05); [M – CO$_2$PF$_6$]$^+$ 892.16 (892.07). Anal. Calcd. C$_{43}$H$_{32}$F$_6$N$_3$O$_7$P$_2$Re: C, 48.50; H, 3.03; N, 3.95. Found: C, 48.42; H, 2.95; N, 4.06.

$[^{2}]$Re(phen)(P–F)(CO)$_3$(PF$_6$). The synthesis of the phenylalanine derivative was the same as that for the tyrosine derivative, except that F–O$^-$tBu•HCl was used instead of Y–O$^-$tBu (74%).$^{1}$H NMR (300 MHz, d-acetone, 25 °C) $\delta = 3.29$ (m, 2H, F-CH$_2$), 4.93 (m, 1H, F-CH), 6.72-7.54 (m, 19H, F-ph and Ph$_2$PPh-R), 7.97 (m, 2H, phen-H), 8.14 (s, 2H, phen-H), 8.81 (m, 2H, phen-H), 9.43 (m, 2H, phen-H). $^{31}$P NMR (300 MHz, d-acetone, 25 °C) $\delta = 19.3$ (s, Re-P) MALDI-TOF Calcd. (Found): [M – PF$_6$]$^+$ 904.16 (903.94); [M – CO$_2$PF$_6$]$^+$ 876.16 (875.98). Anal. Calcd. C$_{43}$H$_{32}$F$_6$N$_3$O$_6$P$_2$Re: C, 49.24; H, 3.03; N, 4.01. Found: C, 49.18; H, 3.04; N, 3.96.

Re(PP–Bn)(CO)$_3$Cl. Re(CO)$_3$Cl (126 mg, 0.14 mmol, 1.0 eq) and HOOC–PP–Bn (200 mg, 0.174 mmol, 1.0 eq) were combined in a flask with 25 mL of N$_2$-purged toluene and the mixture was heated at reflux under an N$_2$ overnight. The solution was concentrated to a few mL in vacuo and triturated with hexanes. The white precipitate was isolated by filtration,
washed with hexanes and dried under vacuum (0.258 g). The crude product, which contained some Re(HOOC–PP–Bn)(CO)₃Cl, was used in the next step without further purification. MALDI-TOF Calcd. (Found): [M – Cl]^+ 800.11 (800.22).

\[\text{Re(phen)(PP–Bn)(CO)₃Cl} \rightarrow \text{Re(PP–Bn)(CO)₃Cl} + \text{phen}\]

Re(PP–Bn)(CO)₃Cl (150 mg, 0.171 mmol, 1.0 eq), phen (34 mg, 0.188 mmol, 1.1 eq) and TlPF₆ (64 mg, 0.188 mmol, 1.1 eq) were combined in a round bottom flask with 25 mL of anhydrous o-dichlorobenzene. The mixture was bubbled with N₂ for 0.5 min and heated at reflux for 5 h under N₂. The dark yellow solution was filtered over Celite, which was rinsed with CH₂Cl₂. The solvent was then removed from the filtrate in vacuo; the residue was dissolved in a minimal volume of CH₂Cl₂ and loaded onto a Chromatotron plate (alumina, 1 mm). The product eluted with 10% CH₃CN/CH₂Cl₂ as a golden yellow band. The solvent was removed in vacuo yielding a yellow solid, which was characterized as a mixture of two isomers. ¹³¹P NMR (300 MHz, CDCl₃, 25 °C) δ = 35.3 (d), 44.0 (d), 49.9 (d), 60.9 (d). MALDI-TOF Calcd (Found): [M – PF₆]^+ 952.19 (952.25); [M – CO,PF₆]^+ 924.19 (924.25).

**X-ray Crystal Structure of [Re(phen)(dppe)(CO)₂](PF₆) and [Re(phen)(PP–Bn)(CO)₂](PF₆)•CDCl₃.** Single crystals of the complexes were grown by slow evaporation from a concentrated solution in CDCl₃. A crystal was removed from the supernatant, coated with Paratone N oil, and mounted on a glass fiber. X-ray diffraction data were collected at –123 °C on a Siemens diffractometer equipped with a CCD detector, using the Mo Kα radiation. The data were integrated to hkl-intensity and the unit cell calculated using the SAINT v.4.050 program from Siemens. Solution and refinement were performed by direct methods (SHELXTL v.6.10, Sheldrick, G. M., and Siemens Industrial Automation, Inc., 2000). The structures were solved by the Patterson method, non-hydrogen atoms were refined anisotropically, and hydrogen atoms were placed at calculated positions. Table 4.4 contains details regarding the refined data, cell parameters and results of the solution of the X-ray diffraction data for [Re(phen)(dppe)(CO)₂](PF₆) and [Re(phen)(PP–Bn)(CO)₂](PF₆)•CDCl₃.

**Physical Measurements.** ¹H and ³¹P NMR spectra were recorded on a Varian Mercury 300 MHz NMR at the MIT Department of Chemistry Instrumentation Facility (DCIF) and externally referenced to tetramethylsilane (¹H) and phosphoric acid (³¹P). MALDI-TOF mass spectrometry was performed on a Bruker Omniflex instrument in the DCIF using dithranol as the matrix. The instrument was calibrated with a quadratic polynomial using a mixture of...
bradykinin fragment 1-7 (757.3997), angiotensin II (1046.5423), and P14R synthetic peptide (1533.8582) (Sigma) with α-cyano-4-hydroxycinnamic acid as the matrix. Elemental analysis was performed by H. Kolbe Mikroanalytisches Laboratorium in Mülheim an der Ruhr, Germany.

UV-vis absorption spectra were recorded on a Cary 17D modified by On-Line Instrument Systems (OLIS) to include computer control or a Spectral Instruments 440 spectrophotometer. Steady state emission spectra were recorded on an automated Photon Technology International (PTI) QM 4 fluorimeter equipped with a 150-W Xe arc lamp and a Hamamatsu R928 photomultiplier tube. Time resolved emission and transient absorption (TA) measurements on the >20 ns timescale were made with pump light provided by the third harmonic (355 nm) of an Infinity Nd:YAG laser (Coherent) running at 20 Hz as previously described. Time resolved emission measurements on the <20 ns timescale were made with the frequency doubled (400 nm) pump light provided by a Ti:sapphire laser system (100 fs pulsewidth) and collected on a Hamamatsu C4334 Streak Scope streak camera as previously described.

The corrected emission spectra were analyzed by a standard Franck-Condon analysis:

\[
I(\nu) = \sum_{\nu'} \left[ \frac{E_0 - \nu h \omega}{E_0} \right]^3 \left[ \frac{S\nu'}{\nu!} \right] \exp \left[ -4 \ln 2 \left( \frac{\nu - E_0 + \nu h \omega}{\Delta\nu_{1/2}} \right)^2 \right]
\]

(3)

in which \( I(\nu) \) is the corrected emitted intensity in quanta \( (I(\lambda) \times \lambda^2) \). The other parameters of eq. (3) have been described elsewhere. The \( E_0 \), \( S \), frequency \( (h \omega) \), and band width \( (\Delta\nu_{1/2}) \) values for the emission spectra were obtained by fitting the emission bands to eq. (3) using Microcal Origin. Without a direct and independent measure of these parameters, the fit is overparametrized and the values obtained are at best an approximation. Relative quantum yields of samples, \( \Phi_{\text{sam}} \), were calculated using [Ru(bpy)₃](PF₆)₂ in CH₃CN and water as the reference actinometer according to:

\[
\Phi_{\text{sam}} = \Phi_{\text{ref}} \left( \frac{A_{\text{ref}}}{A_{\text{sam}}} \right) \left( \frac{I_{\text{sam}}}{I_{\text{ref}}} \right) \left( \frac{\eta_{\text{sam}}}{\eta_{\text{ref}}} \right)^2
\]

(4)
Φ_{ref} is the emission quantum yield of the Ru(bpy)$_3^{2+}$ reference taken to be 0.062 in CH$_3$CN$^{44}$ and 0.053 in water.$^{45}$ $A$ is the measured absorbance, $\eta$ is the refractive index of the solvent, and $I$ is the integrated emission intensity, which was obtained by plotting and integrating the entire emission band fitted using eq. (3). Samples for phosphorescence quantum yield and time resolved spectroscopic measurements were freeze-pump-thaw degassed for 5 cycles to $10^{-6}$ torr. Unless otherwise noted, all spectroscopy was performed in 10 mM aqueous phosphate buffer with the pH adjusted using 1 M NaOH or HCl. In cases of limited solubility, the samples were sonicated in a Branson 3200 sonicator and filtered through a 0.2 micron Gelman Acrodisc polypropylene syringe filter prior to measurement. pD measurements were made with a glass pH electrode that had been calibrated in standard H$_2$O buffers by applying the standard correction of +0.4 to the meter reading of the D$_2$O solution.$^{46}$

Electrochemical measurements were performed using a Bioanalytical Systems (BAS) Model CV-50W potentiostat/galvanostat. Differential pulse voltammetry (DPV) was performed using a platinum disk working electrode, a Ag/AgCl reference electrode, and a platinum wire auxiliary electrode. DPV experiments were performed in dry CH$_3$CN with 0.1 M tetrabutylammonium hexafluorophosphate (Bu$_4$N)PF$_6$ as the supporting electrolyte, and solutions were bubbled with Ar to remove dissolved O$_2$. All potentials are reported vs. $E^\circ$$(\text{Fc}^+/$Fc$) = 0.65$ V vs. NHE in CH$_3$CN.$^{47}$ Spectroelectrochemistry was performed in dry DMF with 0.1 M (Bu$_4$N)PF$_6$ as the supporting electrolyte and Pt gauze as the working electrode in a 2 mm pathlength quartz optical cell. A blank spectrum was recorded on the solution prior to electrolysis at -0.75 V vs. Fe$^+/\text{Fc}$. Spectra were recorded every 10 seconds for 5 minutes.
4.7 References


Table 4.4. Crystal data and structure refinement.

<table>
<thead>
<tr>
<th>Compound</th>
<th><a href="PF6">Re(phen)(PP–Bn)(CO)2</a> • CDCl3</th>
<th><a href="PF6">Re(phen)(dppe)(CO)2</a>.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C_{49}H_{37}DCl_{3}F_{6}N_{3}O_{3}P_{3}Re</td>
<td>C_{40}H_{30}F_{6}N_{2}O_{2}P_{3}Re</td>
</tr>
<tr>
<td>Formula weight</td>
<td>1217.33</td>
<td>963.77</td>
</tr>
<tr>
<td>T (K)</td>
<td>150(2)</td>
<td>150(2)</td>
</tr>
<tr>
<td>λ (Å)</td>
<td>0.71073</td>
<td>0.71073</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Triclinic</td>
<td>Monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P1</td>
<td>P2(1)/n</td>
</tr>
<tr>
<td>a (Å)</td>
<td>13.1590(14)</td>
<td>15.1299(10)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>13.9281(15)</td>
<td>15.6153(11)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>15.1161(16)</td>
<td>16.7302(12)</td>
</tr>
<tr>
<td>α (°)</td>
<td>73.916(2)</td>
<td>90</td>
</tr>
<tr>
<td>β (°)</td>
<td>64.282(2)</td>
<td>109.328(2)</td>
</tr>
<tr>
<td>γ (°)</td>
<td>83.849(2)</td>
<td>90</td>
</tr>
<tr>
<td>V (Å³)</td>
<td>2398.1(4)</td>
<td>3729.9(4)</td>
</tr>
<tr>
<td>Z</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>ρ_{calc} (Mg/m³)</td>
<td>1.683</td>
<td>1.716</td>
</tr>
<tr>
<td>Abs. coeff (mm⁻¹)</td>
<td>2.870</td>
<td>3.456</td>
</tr>
<tr>
<td>θ range for data collection (°)</td>
<td>1.52 to 23.27</td>
<td>1.83 to 23.15</td>
</tr>
<tr>
<td>Index ranges</td>
<td>–14 ≤ h ≤ 14, −15 ≤ k ≤ 11, −16 ≤ ℓ ≤ 11</td>
<td>−16 ≤ h ≤ 15, 0 ≤ k ≤ 17, 0 ≤ ℓ ≤ 18</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>13421</td>
<td>5287</td>
</tr>
<tr>
<td>Independent reflns (R_{int})</td>
<td>6833 (0.0316)</td>
<td>5287</td>
</tr>
<tr>
<td>Completeness to θ_{max}</td>
<td>98.7%</td>
<td>99.8%</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>6833 / 0 / 613</td>
<td>5287 / 0 / 487</td>
</tr>
<tr>
<td>GOFa on F²</td>
<td>1.038</td>
<td>0.970</td>
</tr>
<tr>
<td>R1b</td>
<td>0.0348</td>
<td>0.0546</td>
</tr>
<tr>
<td>wR2c</td>
<td>0.0904</td>
<td>0.0981</td>
</tr>
<tr>
<td>Largest diff. peak, hole</td>
<td>1.053, −1.688 eÅ⁻³</td>
<td>1.074 and −0.672 eÅ⁻³</td>
</tr>
</tbody>
</table>

\(^a\)GOF = (Σ w(F_o^2 − F_c^2)^2/(n − p))^{1/2} where n is the number of data and p is the number of parameters refined. \(^b\)R1 = Σ||F_o − |F_c||/Σ|F_o|. \(^c\)wR2 = (Σ(w(F_o^2 − F_c^2)^2)/Σ(w(F_o^2)^2))^{1/2}. 
Chapter V

Bidirectional PCET of Tyrosine Oxidation: Buffer Effects and Parallel Mechanisms

Parts of this chapter have been published or submitted for publication:


5.1 Motivation

We wish to understand the PCET mechanism of Y oxidation in the [Re(phen)(CO)\textsubscript{3}(P–Y)](PF\textsubscript{6}), \textbf{Re(P–Y)}, complex presented in Chapter IV. This reaction has relevance to the radical chemistry of ribonucleotide reductase and Photosystem II, as discussed in Chapter I. In this chapter we present a detailed pH and buffer dependent analysis of the emission quenching in \textbf{Re(P–Y)} and, in comparison with other Ru\textsuperscript{II} systems in the literature, present a unified picture of the parallel PCET mechanisms that facilitate tyrosine oxidation.

5.2 Background

Proton-coupled electron transfer (PCET) in reactions of amino acid radicals are of great interest as they are frequently encountered in biological systems.\textsuperscript{1-3} The proton and the electron need not transfer as a hydrogen atom to be coupled.\textsuperscript{4} An example of this type of mechanism, in which the proton and electron are transferred between one donor and separate acceptors, has been termed MS-EPT\textsuperscript{5} or bidirectional PCET.\textsuperscript{4,6} This reaction is exemplified by Y\textsubscript{Z} in Photosystem II, in which tyrosine oxidation is coupled to proton transfer to a hydrogen bonded H190 residue (see Chapter I).\textsuperscript{7} Y\textsubscript{Z} oxidation could proceed by either a stepwise (ETPT or PTET; Scheme 1) or concerted electron-proton transfer (CEP) mechanism, the latter defined as occurring with a single transition state. Previous studies on systems with Y appended to Ru(bpy)\textsubscript{3}\textsuperscript{2+} (\textbf{RuY}),\textsuperscript{8} Ru(bpy-4,4’-COOEt)\textsubscript{2}(bpy)\textsuperscript{2+} (\textbf{RuesterY}),\textsuperscript{9} and Re(phen)(CO)\textsubscript{3}(PPh\textsubscript{3})\textsuperscript{+} (\textbf{Re(P-Y)})\textsuperscript{10} (see Chart 5.1 for chemical structures) showed a pH-dependent rate constant for the PCET oxidation of Y. The nature of this pH-dependence has recently been of some debate.\textsuperscript{5,11}

\begin{center}
\includegraphics[width=\textwidth]{chart51.png}
\end{center}

\textbf{Chart 5.1} Compounds discussed in this chapter.

For the \textbf{RuY} system, the data was analyzed within a Marcus framework for electron transfer (ET)\textsuperscript{12} with the driving force defined including the pH dependent Y•/Y reduction potential:
\[ E^\circ(Y^*/Y) = E^\circ(Y^*/Y^-) + 59 \text{ mV} \log(1 + (10^{-pH}/10^{-pK_a(Y)})). \]

This analysis yielded a slope of 0.4 - 0.5 in the \( \log(k_{\text{PCET}}) \) vs. pH plot, which was interpreted as a CEP mechanism with PT to bulk solvent.\(^8\) However, a pH dependence in Y oxidation may also be explained by a PCET reaction with PT to OH\(^-\) or the basic form of buffer,\(^5,11\) which may call into question previous interpretations.\(^8,9\) In this chapter, we clearly distinguish the contributions from these two mechanisms from the rate of Y oxidation in \( \text{RuY, Ru}_{\text{ester}}Y, \) and \( \text{Re(P-Y)} \). We explicitly show that a pH dependence in the rate of tyrosine oxidation can arise in the absence of buffer and that, at high buffer concentrations, the basic form of the buffer acts as a proton acceptor. Analysis of the series of compounds reveals parallel PCET mechanisms, the relative contribution of which varies with the oxidant strength.

5.3 Results

Laser flash photolysis and/or time resolved emission spectroscopy was used to directly measure the rate of Y oxidation in the \( \text{RuY, Ru}_{\text{ester}}Y, \) and \( \text{Re(P-Y)} \) systems. Figure 5.1 plots \( \ln(k_{\text{obs}}) \) vs. pH for the \( \text{Re(P-Y)} \) system, where \( k_{\text{obs}} \) is the emission quenching rate constant calculated by comparison to the \( \text{Re(P-F)} \) control (see Chapter IV). The points are separated into three color coded regions. The black and red points were collected in 10 mM KP\(_i\) buffer and correspond to the protonated and deprotonated forms of the tyrosine carboxylic acid, respectively. The green points are in a higher pH region where KP\(_i\) is not as effective a buffer, and, for this reason, 50

**Scheme 5.1** Mechanisms of PCET, where \( B \) is solvent or base form of the buffer: Stepwise ETPT (pathway 1) or PTET (pathway 2) and CEP mechanisms.

![Scheme 5.1](image)

**Figure 5.1.** Plot of the rate constant for emission quenching, \( k_{\text{obs}} \), vs. pH for \([\text{Re(P–Y)}(\text{phen})(\text{CO})_3]PF_6\). Three pH regions are highlighted corresponding to the protonated (○) and deprotonated (○) forms of the tyrosine carboxylic acid (10 mM KP\(_i\) buffer) and the 50 mM KPi high pH region (○).
mM KP$_i$ was used. Above pH 10, the quenching of the excited state increases significantly. At pH 12, effectively no emission was visible at the concentrations used for the experiment. We attribute this quenching to direct ET from the deprotonated phenol at high pH. Unfortunately, the low solubility of Re(P-Y) in water prevented further investigation of the emission quenching by transient absorption methods.

We next investigated the effect of the phosphate buffer on $k_{obs}$ for the Re(P-Y) system.

![Figure 5.2](image)

**Figure 5.2.** Top left: Phosphate buffer dependence of $k_{obs}$ with fits to eq. 1 for Re(P-Y) at pH 4.5 (○), 6.1 (○), 7.5 (○), 8.3 (○), and 9.2 (○). Bottom left: Same experiment with Ru$_{ester}$Y at pH 9.9 (●), 7.7 (●) and pH 6 (●). Right: Mole fraction of relevant buffer species as a function of pH.

Figure 5.2 (upper left panel) shows a plot of the rate of Y oxidation, $k_{obs}$, in Re(P-Y) as a function of phosphate buffer concentration and pH. The rate is independent of buffer at low concentrations (<10$^{-3}$ M) and increases linearly with buffer at high concentrations. The buffer concentration dependence becomes more pronounced as the pH is increased from 4.5 to 9.2. The right panel of Figure 5.2 shows the pH titration of H$_2$PO$_4^-$/HPO$_4^{2-}$ with pK$_a$ = 7.2. These data are consistent with HPO$_4^{2-}$ acting as the proton acceptor at high buffer concentrations, as previously described for Y oxidation in other systems.$^5$ The entire data set can be fit to eq 1:

$$k_{obs} = k_w + f_b[\text{Buffer}]k_b$$

where $k_w$ is the PCET rate obtained in the absence of buffer (with solvent as proton acceptor), $f_b$ is the fraction of the basic form of the buffer in solution (HPO$_4^{2-}$), and $k_b$ is the bimolecular PCET rate with proton transfer to HPO$_4^{2-}$. The bottom left panel of Figure 5.2 plots $k_{obs}$ in
**Ru** 

vs. buffer concentration following flash-quench generation of the RuIII intermediate and indicates a similar trend to that observed for Re(P-Y).

Figure 5.3 shows a plot of $k_{obs}$ for the RuY system as a function of phosphate buffer concentration at pH 7 and 9. The fit of these data to eq. 1, however, results in a pH-dependent $k_w$, as illustrated by the offset in the pH 9 (blue line) and pH 7 (green line) data. The inset of Figure 5.3 plots the pH dependence of $k_{obs}$ at 0.5 mM concentration of different buffers with various pKas. The main figure indicates an independence of $k_{obs}$ on the buffer at this concentration (dashed line). The linear correlation in the inset shows a genuine pH dependence of $k_w$ that is independent of buffer identity and pKas. The rate does not level out at pH > pKa of 2-(N-morpholino)ethanesulfonic acid (MES) and [H2PO4 –], as would be the case if the base form of the buffer were the proton acceptor. The pH-dependent data at 0.5 mM are in good agreement with our previously reported data at 10 mM. Note that even without buffer, the data in Figure 5.3 span the same range of rates between pH 3 and 9.

Deuterium isotope effect studies were undertaken to further probe the PCET mechanism in these systems. In D2O the pKa of D2PO4 – shifts to 7.814 while we measure a shift of the tyrosine pKa to 10.6. Both the pKas shift by the same amount, therefore, the driving force for deuteron

---

### Table 5.1. Rate of the Tyrosine Oxidation in Three Separate Systems with Water or Water Containing HPO4 2–.

<table>
<thead>
<tr>
<th></th>
<th>$k_w$ / $10^5$ s$^{-1}$</th>
<th>$k_H/k_D$ (H2O)</th>
<th>$k_b$ / $10^7$ M$^{-1}$s$^{-1}$</th>
<th>$k_H/k_D$ (HPO4 2–)</th>
<th>$k_{ET2}$ / $10^7$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RuY</td>
<td>0.1$^{a,b}$</td>
<td>2.2-2.5$^c$</td>
<td>0.3</td>
<td>1.8-2.0</td>
<td>5$^b$</td>
</tr>
<tr>
<td>RuesterY</td>
<td>4.4$^a$</td>
<td>2$^c$, &gt;10$^e$</td>
<td>3.0</td>
<td>N/A</td>
<td>&gt;10$^d$</td>
</tr>
<tr>
<td>Re(P-Y)</td>
<td>1.0</td>
<td>&lt; 3</td>
<td>1.7</td>
<td>3.0</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

$^a$ pH-dependent, at pH 7; $^b$ Ref. 8; $^c$ Ref. 2; $^d$ Ref 9; $^e$ pH 10
transfer should be the same as for proton transfer. The bimolecular rate for quenching of \( \text{Re(P–Y)} \) emission with DPO₄²⁻ in D₂O was measured as \( 5.7 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \), yielding a deuterium isotope effect (\( k_{H}/k_{D} \)) of 3.0. For \( \text{RuY} \) and phosphate buffer \( k_{H}/k_{D} = 1.8 \) to 2.0 in the buffer-dependent region (ca. 50 mM). The \( k_{w}, k_{b} \) and deuterium isotope effects for each rate and for each system are summarized in Table 5.1.

We can extend the \( \text{Re(P–Y)} / \text{HPO}_{4}^{2–} \) system to other “buffers” to systematically change the driving force for proton transfer along with the hydrogen bond geometry between the tyrosine phenol and hydrogen bonded base. As a first step towards this study, we show that pyridine and imidazoles are also effective bases in enhancing the emission quenching in \( \text{Re(P–Y)} \) in aqueous solution. Figure 5.4 (left) plots the rate of emission quenching vs. the total concentration of added pyridine at different pHs. The “buffer effect” is turned on at higher pH as proton transfer occurs to pyridine in the PCET oxidation of tyrosine. The effect is similar to that observed for phosphate, however now the bimolecular rate constant for quenching increases as the pH is titrated from 3.8-7.6 (instead of pH 4.5-9 as observed for phosphate). This is as expected since the pKa of pyridinium is lower than that of \( \text{H}_{2}\text{PO}_{4}^{2–} \) (see Table 5.2). The data were analyzed with equation 1 to yield a value of \( 1.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \) for \( k_{b} \). Figure 5.4 (right) plots the rate of emission quenching vs. the concentration of added imidazole for a series of imidazoles with different pKₐs for their conjugate acids. The data were collected at a pH where the imidazole was deprotonated.

![Figure 5.4](image-url)

**Figure 5.4.** (Left) Plot of the rate constant for emission quenching, \( k_q \), vs. concentration of pyridine buffer for \([\text{Re(P–Y)}(\text{phen})(\text{CO})_3]PF_6\) at pH 3.75 (○), 4.65 (○), 5.35 (○), and 7.6 (○) (Right) Plot of, \( k_q \), vs. concentration of imidazole buffers for \([\text{Re(P–Y)}(\text{phen})(\text{CO})_3]PF_6\) with 4-Br-Im (○), Im (○), and 4-Me-Im (○). The dotted line represents the limit of detection using the nanosecond pulsed laser for excitation.
Table 5.2. Thermodynamic and kinetic data for the series of bases used in the PCET oxidation of tyrosine in Re(П–Y).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pK$_a$(BH)$^+$</th>
<th>$k_b$ / 10$^7$ M$^{-1}$s$^{-1}$</th>
<th>$k_{H}/k_{D}$</th>
<th>$K_C$ / M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPO$_4^{2-}$</td>
<td>7.2$^a$</td>
<td>1.7 ± 0.1</td>
<td>3.0</td>
<td>0.5$^d$</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.23$^b$</td>
<td>1.1 ± 0.1</td>
<td>2.9</td>
<td>0.6-0.7$^e$</td>
</tr>
<tr>
<td>4-Me-Im</td>
<td>7.45$^c$</td>
<td>24.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Im</td>
<td>6.95$^c$</td>
<td>10.3</td>
<td>1.8</td>
<td>-</td>
</tr>
<tr>
<td>4-Br-Im</td>
<td>3.7$^c$</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Ref.13; $^b$Ref.15; $^c$Ref.16; $^d$Ref.17; $^e$Ref.18. $k_b$ is the second-order rate constant for buffer-assisted PCET; $k_{H}/k_{D}$ is the kinetic isotope effect; $K_C$ is the association constant for the tyrosine-buffer complex (Scheme 5.1).

The bimolecular $k_b$ rate constants for each imidazole were determined from the slopes of the lines in Figure 5.4 (right).

Table 5.2 presents a summary of the data for the pyridine, phosphate, and imidazole systems including the measured deuterium isotope effects and $k_b$, and the literature values for the pKa of the conjugate acid of the base and the association constant of the base to tyrosine or phenol. Some striking observations can be made from this data: (1) the bimolecular $k_b$ rate does not correlate with the pKa of the conjugate acid of the base across the pyridine, phosphate, imidazole series; (2) within the series of imidazoles $k_b$ does correlate with pKa of the conjugate acid of the base; (3) an inverse relationship may exist between the measured deuterium isotope effect and $k_b$.

5.4 Discussion

5.4.1 pH Dependence of $k_{obs}$ in the Absence of Buffer

The pH-dependence for RuY in the absence of buffer cannot be explained by a PTET mechanism to H$_2$O or OH$^-$ (Scheme 5.1), or by a reversible PCET reaction as we now describe: PCET via an ETPT mechanism.
As the pKₐ for the tyrosine radical is c.a. –2, its deprotonation in aqueous media is very rapid, on the order of 100 fs. Therefore, the ETPT reaction will be ET-limited, and not expected to depend on either pH or buffer concentration.

**PCET via CEP or PTET, in the absence of buffer**

A pH dependence for tyrosine oxidation in absence of buffer may arise via PCET reactions with proton transfer to a single molecular acceptor, such as OH⁻. For concerted (CEP) or stepwise PTET mechanism, both are predicted to be first-order in [OH⁻]:

For PTET with OH⁻ as acceptor, the initial PT step will be rate-determining in the pH-interval examined, as the following ET is comparatively rapid: \( k_{ET2} \geq 5 \times 10^7 \text{ s}^{-1} \) for the complexes examined here (see Table 5.1). Therefore both PTET and CEP with OH⁻ as acceptor will be limited by diffusional encounter with OH⁻ and show the same rate dependence on [OH⁻].

Thus, if OH⁻ is the proton acceptor, the log(\( k_{obs} \)) vs. pH plot should have a slope of 1 for both mechanisms, rather than the slope of 0.4-0.5 observed for RuY. The limiting rate at pH = 7 is given by the diffusional encounter between tyrosine and OH⁻, and is expected to give an upper limit for the pseudo first order rate of 10³ s⁻¹, which is significantly lower than what we observe for any of the systems at pH 7.

If a single H₂O is the proton acceptor in an irreversible reaction, the driving force for proton transfer depends on the difference in pKₐ for tyrosine and H₃O⁺, and therefore we would not expect a pH dependence in \( k_{obs} \) for either PTET or CEP. In addition, for PTET the rate constant for deprotonation of tyrosine (pKₐ = 10) to H₂O is very small: \( \sim 10 \text{ s}^{-1} \).

We now consider a reversible PCET with proton transfer to a single H₂O:

\[
\begin{align*}
A & \rightleftharpoons B + H_3O^+ \\
& \quad \text{where } A = [M^{n^+}]–\text{TyrOH and } B = [M^{(n-1)^+}]–\text{TyrO}^\bullet.
\end{align*}
\]

The observed rate law for this equilibrium reaction is:
\[
\text{rate} = \frac{-d[A]}{dt} = k_f [A] - k_b [B] [H_3O^+] \\
[B] = [A]_o - [A]
\]
then
\[
[A] = [A]_o \left( \frac{k_f [H_3O^+]}{k_f + k_b [H_3O^+]} + \frac{k_b [H_3O^+]}{k_f + k_b [H_3O^+]} e^{-(k_f + k_b [H_3O^+])t} \right)
\]
assuming that [H_3O^+] is constant throughout the reaction. The observed rate constant, \(k_{obs} = k_f + k_b [H_3O^+]\), gives a slope of –1 in the \(\log(k_{obs})\) vs pH plot in the region where \(k_b [H_3O^+] >> k_f\) and a slope of zero in the other limit. Note that the slope would never be positive, as observed experimentally.

The mechanistic treatment above does not explain the pH dependence of \(k_{obs}\) observed for RuY and RuesterY in the absence of buffer and at low buffer concentrations. The pH-dependence of the rate constant cannot be explained by a single molecular species as proton acceptor for the CEP reaction in a rate-determining step. We therefore assign the observed slope of ca. 0.5 in the \(\ln(k_{obs})\) vs. pH plot as arising from CEP reaction with proton transfer to the solvent that is affected by the bulk pH.\(^8,9\) For stronger oxidants, as in Re(P-Y) the pH independence of \(k_w\) is consistent with an ETPT mechanism. In RuesterY, with intermediate oxidant strength, \(k_w\) is first pH-independent but becomes pH-dependent at high pH when the CEP mechanism dominates\(^9\) (see data at pH 9.9 in Figure 5.2). ETPT has lower reorganization energy compared to CEP;\(^9,20\) thus the ETPT rate increases more steeply with increasing \(-\Delta G^0\) and can out-compete CEP for stronger oxidants.\(^9\) The higher kinetic isotope effect \((k_H/k_D)\) for the CEP reactions (RuY and RuesterY, pH 10, Table 5.1) compared to the pure ETPT reactions with tyrosine (RuesterY, pH 7 and Re(P-Y), Table 5.1) or ET from tyrosinate\(^8\) support these mechanistic assignments.

### 5.4.2 Buffer Dependence of \(k_{obs}\)

At higher phosphate buffer concentrations (>10 mM) the rate is first order in [HPO_4^{2-}] indicating a buffer-assisted PCET. Several mechanisms were considered within the framework of Scheme 5.1:

**PTET with proton transfer to buffer**

We first consider PTET with a rate limiting ET:
with a diffusion-controlled deprotonation of the buffer by TyrO^−, \( k_{PT} \approx 1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \), and an equilibrium constant \( (k_{PT} / k_{-PT}) = 10^{-\Delta pK_a} \) where \( \Delta pK_a = pK_a(\text{TyrOH}) - pK_a(\text{H}_2\text{PO}_4^-) = 2.8 \). A rate-limiting ET requires that \( k_{ET} \ll k_{-PT}[\text{HB}^+] \). As \( k_{-PT}[\text{HB}^+] \approx 1 \times 10^7 \text{ s}^{-1} \) and \( k_{ET} \geq 5 \times 10^7 \text{ s}^{-1} \) (\( k_{ET2} \) in Table 5.1) this condition does not apply for any of the systems studied.

Secondly, for PTET with a rate limiting deprotonation, the observed rate constant is equal to \( k_{PT} = 1.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) (assuming \( k_{-PT} \approx 1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \)), which is comparable to the values of \( k_b \) that we measure for Re(P-Y) and RuesterY. For the imidazoles and pyridine instead, the predicted values of \( k_{PT} \) are much lower than the observed values of \( k_b \) (Table 5.2). For 4-Me-Im, with \( \Delta pK_a = 2.55 \), would give \( k_{PT} \approx 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) while we observe \( k_b = 2.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \). For 4-Br-Im we predict \( k_{PT} \approx 5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \) while the observed rate \( k_b = 3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \). This excludes the PTET mechanism for the imidazole or pyridine as proton acceptor and suggests that the buffer-assisted PCET reaction is a CEP, with proton transfer to these bases.

**PTET within a H-bond complex**

We furthermore consider the reaction scheme in which PCET occurs within a hydrogen bonded complex as depicted in Scheme 5.1. For PTET (pathway 2), the steady-state approximation can be used to derive an expression of the rate constant for the reaction:

\[
k_b = \frac{k_d \cdot k_{PT2} \cdot k_{ET2}}{k_{-PT2} \cdot k_d - k_{ET2} \cdot k_{PT2}}
\]

The diffusion controlled complexation rate, \( k_d = 1 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1} \), and association constant, \( K_C = 0.5 \text{ s}^{-1} \) (Table 5.2) gives a dissociation rate \( k_d = 2 \times 10^{10} \text{ s}^{-1} \). The protonation rate of tyrosine within the complex \( (k_{PT2}) \) cannot be faster than the collision frequency factor of \( 6 \times 10^{12} \text{ s}^{-1} \), given by absolute rate theory. Assuming that \( \Delta pK_a = 2.8 \) does not change within the complex (given the weak association constant, we expect the hydrogen bond to be weak and not significantly shift the pKa of the phenol or the base), this gives the rate of deprotonation \( k_{PT2} = 1 \times 10^{10} \text{ s}^{-1} \), and finally \( k_{ET2} = 5 \times 10^7 \text{ s}^{-1} \) for RuY. Using these values for the rate constants in the equation above, \( k_b \) is estimated to \( 4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) which is two orders of magnitude slower than the observed rate of \( 3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \) for RuY. Due to the similar behavior of the three systems
studied, RuY, RuYester and Re(P-Y), we have no reason to believe that the mechanism for PCET within a complex between Y and B would be different for the three systems.

We therefore assign the buffer-assisted PCET to a CEP mechanism with electron transfer to metal oxidant and proton transfer to basic form of the buffer. For Re(P-Y) and RuesterY we only have limiting values for \( k_{PTET} \), but we measure kinetic isotope effects that are larger than expected for a PTET reaction as \( \Delta pK_a \) was found to be the same in H\(_2\)O and D\(_2\)O. The driving force for the primary PCET step is increased with a buffer molecule as the proton acceptor compared to a single water molecule (\( pK_a(H_3O^+) \approx -1.5 \)), or to ET oxidation forming Y•\(^+\) (\( E'(Y^{+}/Y) = 1.46 \) V vs. NHE\(^9\)). Hydrogen bonding bases may also further enhance the rate of CEP reactions in aqueous media by increasing the proton vibrational wavefunction overlap and/or by decreasing the reorganization energy for CEP.\(^{20}\)

5.5 Conclusions

We have revealed a pattern of reactivity in the RuY, RuesterY, and Re(P-Y) series with at least three competing PCET mechanisms: (1) CEP with the solvent as proton acceptor, which does indeed show a pH-dependent rate that is independent of buffer; (2) pH-independent ETPT; (3) a buffer-assisted CEP that is a general phenomenon at higher buffer concentrations (Figures 5.2 and 5.3). The pH-dependence of (3) follows the titration of the buffer. The relative importance of (2) increases systematically with oxidant strength.

The pH-dependence of CEP with proton transfer to water, identified for the first time for RuY,\(^8\) has been questioned on theoretical grounds,\(^{11,21}\) and proposed to arise from reactions with the buffer.\(^5\) Here we experimentally confirm that the pH-dependence is genuine and cannot be explained by buffer-assisted reactions or simple reaction schemes with first-order dependencies on OH\(^-\) or H\(_3\)O\(^+\). While the rate-dependence on pH phenomenologically follows the Marcus equation for pure electron transfer,\(^8,9\) a microscopic model to explain this type of CEP reaction remains to be developed. The free energy of the overall PCET process may depend on a weighted average of microconfigurations, the distribution of which is pH dependent. It remains to be seen if the driving force dependence is coincidental or if the factors determining the rate-dependence on pH are directly connected to the free energy of the overall process. Our results underpin the mechanistic richness of PCET one may encounter and serve as a model for discussion of PCET reactions in more complex radical enzymes such as ribonucleotide reductase.
5.6 Experimental Section

Materials. RuY, RuesterY, and Re(P–Y) were available from previous studies. Potassium phosphate buffer (Mallinkrodt) was used as received. Pyridine (Sigma Aldrich) was distilled from and stored over KOH. Imidazoles (Sigma Aldrich) were purified by sublimation.

Time Resolved Emission and Transient Absorption Spectroscopies. Time resolved emission experiments with solutions of Re(P–Y) were performed as described in Chapter IV. Transient absorption experiments with RuY and RuesterY were performed as previously described.
5.7 References


Chapter VI

Unnatural Fluorotyrosine Amino Acids as Probes of PCET Mechanism in Reactions of Tyrosyl Radicals

Parts of this chapter have been published:


6.1 Motivation

We wish to develop methods for probing the mechanisms of PCET reactions of tyrosyl radicals in enzymes. Furthermore, we wish to distinguish between radical hops along the Y356→Y731→Y730 pathway in RNR. For these reasons, we have synthesized a series of fluorotyrosine unnatural amino acids, FₙY, and studied the effect of fluorination on both the pKₐ of the phenolic proton and the reduction potential of the oxidized radical. This chapter describes density functional theory calculations that explain the trends in reduction potential of these radicals. Additionally, FₙYs are incorporated into dipeptides with benzophenone to characterize the transient absorption spectrum of each fluorotyrosyl radical. Finally, the FₙYs are appended to the Re(bpy)(CO)₃CN complex for a Marcus-type driving force analysis of charge separation and recombination reactions involving the deprotonated, FₙY⁻. These data show that FₙY can be used to vary the driving force for both the proton and the electron part of PCET reactions involving tyrosyl radical, that the spectra of FₙY• are significantly shifted to distinguish it from Y•, and that ET reactions involving FₙY⁻ are well described by Marcus’ theory for ET.

6.2 Background

Of the amino acid radicals participating in biological charge transfer processes, tyrosyl radical (Y•) is pre-eminent.1-4 Nature employs tyrosine to fulfill two enzymatic criteria: (1) as a charge transport relay and (2) to transport both a proton and an electron for the activation of substrates at an enzyme active site. This one-electron, protein-based oxidant has been identified as both a stable cofactor in deoxynucleotide biosynthesis (ribonucleotide reductase)5 and as a transient intermediate with directed reactivity in photosynthetic O₂ evolution (Photosystem II),6 prostaglandin biosynthesis (prostaglandin synthase),7 alcohol oxidation (galactose oxidase)8 and reduction of oxygen to water (cytochrome c oxidase) (see Chapter I).9 Despite extensive knowledge about the structure of the Y•s in these systems, the precise mechanism of their formation and reactivity at a functional biological level has, for the most, part remained unknown. However, at a mechanistic level, it is well established that oxidation of tyrosine under physiological conditions requires the loss of both a proton and an electron, implicating proton-coupled electron transfer (PCET) in the radical forming mechanism.4,6,10 Therefore, the study of tyrosine radicals in the initiation and propagation of
charge in biology will necessarily demand that new tools are needed to control both the
electron and proton for Y• formation within the enzyme. This is especially pertinent to our
studies of class I ribonucleotide reductase (RNR).4,11 Radical migration over the
unprecedented distance between the β2 •Y122 cofactor and the α2 active site has been
suggested to involve a radical hopping pathway spanning both subunits with three other
tyrosine residues, Y356 (β2), 731 (α2) and 730 (α2), as discussed in Chapter I. Moreover, the
mechanism of formation of the stable Y122•, which involves a putative di-iron, FeIII/FeIV
intermediate as the oxidant, also remains unknown.12- 16

Substitution of tyrosine with other redox active amino acids can provide insight into the
mechanism of charge transport in enzymes. However, only the natural amino acids of
tryptophan and cysteine have reduction potentials close to that of tyrosine, and they are poor
structural analogues. For this reason, we have turned our attention to unnatural amino acids,
specifically, fluorotyrosines because they offer minimal structural perturbations compared to
tyrosine. They are isosteric with tyrosine and the van der Waals
radius of fluorine is only 0.15 Å
larger than that of hydrogen,17 thus
representing the most conservative
substitution barring isotopic
replacement. Fluorination at various
points on the phenol ring affects a
large range of pKa values for the
unprotected amino acid (FnYs).18 A
similar variation in the tyrosyl
radical reduction potentials upon
fluorine substitution would provide a
systematic variation in ΔG°ET and
ΔG°PT, the factors that control the
thermodynamics of PCET reactions.

Table 6.1. Fluorotyrosine derivatives studied here
and their physical properties.

<table>
<thead>
<tr>
<th>Fluorotyrosine</th>
<th>pKa</th>
<th>E°(Y•/Y−) / mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Y-NH2</td>
<td>9.9</td>
<td>642</td>
</tr>
<tr>
<td>Ac-3,5-F2Y-NH2</td>
<td>7.2</td>
<td>755</td>
</tr>
<tr>
<td>Ac-2,3-F2Y-NH2</td>
<td>7.8</td>
<td>810</td>
</tr>
<tr>
<td>Ac-2,3,5-F3Y-NH2</td>
<td>6.4</td>
<td>853</td>
</tr>
<tr>
<td>Ac-2,3,6-F3Y-NH2</td>
<td>7.0</td>
<td>911</td>
</tr>
<tr>
<td>Ac-F4Y-NH2</td>
<td>5.6</td>
<td>968</td>
</tr>
</tbody>
</table>

The synthesis and physical characterization of the N-acetyl and C-amide protected
fluorotyrosines, Ac-FnY-NH2s, shown in Table 6.1 have been reported from our labs.19 The
methodologies for determining the $\Delta G^\circ_{ET}$ and $\Delta G^\circ_{PT}$ for the half of the PCET reaction involving fluorotyrosine oxidation have been determined by measuring both the reduction potentials for the radicals, $E_p(F_nY•/F_nY)$, and the $pK_a$ of the phenolic protons for Ac-F$_n$Y-NH$_2$s. The $pK_a$ varies from 5.6 to 8.4 and the reduction potential varies over 320 mV in the pH region accessible to most proteins, as shown by the plot of $E_p(F_nY•/F_nY)$ vs. pH in Figure 6.1. The utility of F$_n$Ys in providing insight into the mechanism of tyrosine oxidation in biological systems were also demonstrated in our labs by incorporating them site-specifically at position 356 in the β2 subunit of E. coli RNR and correlating RNR activity to $pK_a$ and reduction potentials of the Ac-F$_n$Y-NH$_2$s presented here.

This chapter describes density functional theory calculations that explain the trends in reduction potential of these radicals. The UV/Vis absorbance spectra of the F$_n$Y•s in dipeptides is also presented using transient laser absorption spectroscopy with benzophenone as the photo-oxidant. The kinetics for photogeneration of F$_n$Y• attached to a Re$^1$ polypyridyl complex and the lifetime of the radical in the charge separated state are also described. The excited state of Re(bpy)(CO)$_3$CN is shown to directly oxidize deprotonated F$_n$Y•, and the kinetics for charge separation are in the normal Marcus regime whereas charge recombination occurs in the inverted region.

6.3 Results and Discussion

6.3.1 Electronic Structure Calculations of Ac-F$_n$Y-NH$_2$s.

The Ac-F$_n$Y•-NH$_2$/Ac-F$_n$Y$^-$-NH$_2$ redox couple was found to generally increase with the number of fluorine substituents on the phenol ring (Table 6.1 and Figure 6.1). Within the
Figure 6.2. (Left) Plot of $E_p(Ac-F_nY\cdot-NH_2/Ac-F_nY^-NH_2)$ vs. $E(Ac-F_nY\cdot-NH_2) - E(Ac-F_nY^-NH_2)$, the total bonding energy difference between Ac-F_nY•-NH_2 and Ac-F_nY–-NH_2 as calculated by DFT, with linear fit ($\blacktriangle$). (Bottom) Plot of $-\ln(K_a)$ vs. $E(Ac-F_nY^-NH_2) - E(Ac-F_nY-NH_2)$, the total bonding energy difference between Ac-F_nY–-NH_2 and Ac-F_nY-NH_2 as calculated by DFT, with linear fit ($\blacktriangle$). Color coded points correspond to data for (●) Ac-Y-NH_2, (●) Ac-3-FY-NH_2, (●) Ac-3,5-F_2Y-NH_2, (●) Ac-2,3-F_2Y-NH_2, (●) Ac-2,3,5-F_3Y-NH_2, (●) Ac-2,3,6-F_3Y-NH_2, (●) Ac-F_4Y-NH_2.

A series of di- and trifluorotyrosines, the couples follow a trend: Ac-2,3-F_2Y-NH_2 > Ac-3,5-F_2Y-NH_2 and Ac-2,3,6-F_3Y-NH_2 > Ac-2,3,5-F_3Y-NH_2 that is captured computationally. Geometry optimizations on Ac-F_nY-NH_2, Ac-F_nY•-NH_2, and Ac-F_nY−NH_2 were performed while keeping the amino acid backbone in the same conformation.

Figure 6.2 (left) compares the difference in total bonding energy between the Ac-F_nY•-NH_2 and Ac-F_nY−NH_2 redox states as calculated by DFT to the experimentally determined $E_p$s ($Ac-F_nY\cdot-NH_2/Ac-F_nY^-NH_2$). The linear correlation between observed and calculated results validates the quality of the computation. The electronic structure calculation summarized in Figure 6.3 shows that the observed trend in the reduction potential arises primarily from stabilization of the HOMO and HOMO-1 orbitals of the phenolate with increasing fluorination of the aromatic ring of tyrosine. This stabilization of HOMO and HOMO-1 levels is most pronounced for Ac-F_nY−NH_2; these levels exhibit only a modest shift for Ac-F_nY•-NH_2 (and Ac-F_nY-NH_2). The Kohn-Sham representations of the frontier molecular orbitals, shown for tyrosinate in Figure 6.3, reveals that the HOMO orbital carries significant π-electron density at the 3 and 5 positions of the aromatic ring whereas the...
HOMO-1 has $\pi$-electron density localized on the orbital of the oxygen of the phenol, in an in-plane antibonding interaction with the aromatic ring. The interaction of the HOMO and HOMO-1 ring orbitals with the $p\pi$-orbitals of the fluorine atom substituents allows subtle variations in the reduction potential among members of the phenolate series to be understood. For instance, substitution of fluorine at the 2,3 vs 3,5 ring positions results in a 55 mV increase in the reduction potential of the radical. The HOMO, which is presented in the framed box of Figure 6.3, exhibits an antibonding interaction between the $\pi$-orbitals of the fluorine atoms and the 3,5-carbon $p\pi$ orbitals of the aromatic ring. By moving a fluorine from a 5- to a 2-position, this antibonding interaction is relaxed significantly, resulting in stabilization of the HOMO. A similar effect is observed for 2,3,5- and 2,3,6-trifluorotyrosines.

A parallel calculation was performed to assess the difference between the observed $\ln(K_a)$ and the total bonding energy difference of protonated and deprotonated Ac-F$_n$Y-NH$_2$s. The

Figure 6.3. Energy level diagram for frontier molecular orbitals of the phenolates: (▬) Ac-Y-NH$_2$, (▬) Ac-3-FY-NH$_2$, (▬) Ac-3,5-F$_2$Y-NH$_2$, (▬) Ac-2,3-F$_2$Y-NH$_2$, (▬) Ac-2,3,5-F$_3$Y-NH$_2$, (▬) Ac-2,3,6-F$_3$Y-NH$_2$ and (▬) Ac-F$_4$Y-NH$_2$. Selected Kohn-Sham representations of orbitals for the tyrosine analog are shown at the 95% probability level. The Kohn-Sham representation of the HOMO orbital for Ac-3,5-F$_2$Y-NH$_2$ is presented in the frame.
calculation predicts the general trend that increasing the number of fluorine substituents decreases the pKₐ of the phenolic proton (Figure 6.2, right), however a poorer correlation is observed as compared to that for the reduction potentials. The anomaly is most obvious for Ac-2,3-F₂Y-NH₂ and Ac-3,5-F₂Y-NH₂, which are calculated to have similar pKₐs despite an observed difference of 0.6 pKₐ units. We believe that such deviations arise from the deficiency of gas phase DFT calculations in modeling specific hydrogen bonding interactions that are important in determining the overall pKₐ of the fluorotyrosines.²¹-²³

6.3.2 Spectroscopic Characterization of FₙY•.

As presented in Chapter III, the triplet excited state of benzophenone in the BPA-Y-OMe dipeptide is competent for oxidation of tyrosine, rapidly forming the •BPA-Y•-OMe diradical state which persists for hundreds of nanoseconds. The absorption spectrum of the benzophenone ketyl radical, produced upon tyrosine photo-oxidation, does not significantly overlap with that of the fluorotyrosyl radical analogue. Therefore, the BPA photo-oxidant provides an excellent means for measuring the absorption spectra of the fluorotyrosyl radical analogues when photolysed in the BPA-FₙY-OMe dipeptides.

Figure 6.4 shows the TA spectra obtained 100 ns after 300 nm excitation of each of the BPA-FₙY-OMe dipeptides. The amount of BPA•-FₙY•-OMe produced in each experiment varies due to changes in pump-probe beam overlap and sample concentration. Therefore, to allow for comparison of the FₙY• absorption maxima between experiments, the spectra in

![Figure 6.4](image.png)

**Figure 6.4.** The transient absorption spectrum of (▬) BPA-Y-OMe, (▲) BPA-3-FY-OMe, (▼) BPA-3,5-F₂Y-OMe, (▶) BPA-2,3-F₂Y-OMe, (♦) BPA-2,3,5-F₃Y-OMe, (♣) BPA-2,3,6-F₃Y-OMe and (—is) BPA-F₄Y-OMe normalized to the peak at 547 nm obtained 100 ns after excitation of ~500 μM solutions of each dipeptide buffered to pH 4.0 with 20 mM succinic acid.
Figure 6.5 are normalized to the 547 nm peak of the BPA• ketyl radical. The feature is invariant for each of the dipeptides whereas the absorption band of each F_nY• species varies in its maximum between 395 and 415 nm. An interesting comparison can be made between the F_nY•s and Y• absorbance features. The 3-FY•, 2,3-F_2Y•, 2,3,6-F_3Y• and F_4Y• all have the familiar double hump absorbance feature of Y•, though they are slightly broader and red-shifted. Of particular note are the single peaks at 395 and 400 nm for 3,5-F_2Y• and 2,3,5-F_3Y•, respectively, which are significantly blue-shifted from the larger 407 nm absorbance peak of Y•.

6.3.3 Marcus Analysis of ET in [Re]-F_nY.

The complex, Re(bpy)(CO)_3CN (= [Re]), is a powerful excited state oxidant ($E^\circ(Re^{1+}/0) = 1.6$ V vs. NHE). When the native amino acid, Y, is appended to the bpy ligand in [Re]–Y (n = 0, Chart 6.1), excited state quenching ensues and Y• forms if the tyrosine phenol is deprotonated. We prepared the series of [Re]–F_nY presented in Chart 6.1 to determine how variation in the F_nY•/F_nY– reduction potential affects the rate of radical formation and charge recombination. The compounds were obtained by coupling the previously reported series of fluorotyrosine methyl esters with Re(bpy-COOH)(CO)_3CN using standard amide bond peptide coupling chemistry in modest yield (25 - 50%). In the course of this work, single crystals of Re(bpy-COOH)(CO)_3CN were obtained. The solution of the X-ray crystal structure is presented in Figure 6.5; this is the first reported structure of a mononuclear Re(bpy)(CO)_3CN complex. Of note, the weaker field CN– ligand results in a longer Re—C
bond length of 2.17 Å compared to the shorter Re—C bond lengths of the stronger field, 
facial CO ligands (1.92 - 1.95 Å).

Ultrafast time-resolved emission and nanosecond transient absorption spectroscopies were 
undertaken on [Re]–FₙY⁻ complexes to characterize kinetically any photoinduced electron 
transfer. For each [Re]–FₙY⁻, the excited state [Re] emission was quenched compared to Re– 
Y at pH 7 and could be fit to a single exponential decay function. The emission quenching is 
due to electron transfer²⁴ to the excited Re complex from FₙY⁻ and the rate of charge 
separation, \( k_{CS} \), could be determined from:

\[
  k_{CS} = \frac{1}{\tau([\text{Re}]-\text{F}_n\text{Y}^-)} - \frac{1}{\tau([\text{Re}]-\text{Y})}
\]

where \( \tau([\text{Re}]-\text{F}_n\text{Y}^-) \) is the emission lifetime of the quenched complex at pH 12 and \( \tau([\text{Re}]-\text{Y}) \) 
is the lifetime of the unquenched control at pH 7. These data are presented in Table 6.2; \( k_{CS} \) 
varies from 89.2 - 2.15 × 10⁷ s⁻¹ depending on the FₙY employed.

Nanosecond transient absorption spectroscopy confirms that the emission quenching is 
due to electron transfer in the [Re]–FₙY⁻. Figure 6.6 shows the transient spectra observed 
following 355 nm (FWHM = 3 ns) excitation of [Re]–2,3,6-F₃Y⁻. The peak at 520 nm 
corresponds to the reduced bpy•⁻ ligand²⁴ and the peak at 418 nm corresponds to the 
absorption feature for 2,3,6-F₃Y•. The spectra at 15 and 65 ns are somewhat different, 
however, as the ratio of the 520/480 nm peaks change slightly. These dynamics are due to the 
decay of the quenched ³MLCT state which occurs with a time constant of 14.5 ns obtained 
from the ultrafast emission quenching described above. As such, the single wavelength 
kinetics trace obtained at 520 nm in the inset of Figure 6.6 (and for the other FₙY⁻ with n ≥ 2)

| Table 6.2. Emission quenching and charge recombination kinetics 
data for [Re]-FₙY complexes |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>( \tau([\text{Re}]-\text{F}_n\text{Y}) )</td>
<td>( k_{ET} ) (10⁷ s⁻¹)</td>
<td>( k_{CR} ) (10⁷ s⁻¹)</td>
</tr>
<tr>
<td>[Re]–Y</td>
<td>1.10</td>
<td>89.2</td>
<td>2.17</td>
</tr>
<tr>
<td>[Re]–3-FY</td>
<td>1.84</td>
<td>52.7</td>
<td>1.96</td>
</tr>
<tr>
<td>[Re]–3,5-F₂Y</td>
<td>3.7</td>
<td>25.3</td>
<td>1.79</td>
</tr>
<tr>
<td>[Re]–2,3-F₂Y</td>
<td>6.3</td>
<td>14.2</td>
<td>1.89</td>
</tr>
<tr>
<td>[Re]–2,3,5-F₃Y</td>
<td>11.1</td>
<td>7.31</td>
<td>1.67</td>
</tr>
<tr>
<td>[Re]–2,3,6-F₃Y</td>
<td>14.5</td>
<td>5.20</td>
<td>1.69</td>
</tr>
<tr>
<td>[Re]–F₄Y</td>
<td>26.0</td>
<td>2.15</td>
<td>1.54</td>
</tr>
</tbody>
</table>
was fit starting at 20 ns with a sum of two exponentials: a fast growth component whose time constant was fixed to that obtained in the ultrafast emission experiment and a slow decay component. The latter component corresponds to charge recombination (CR), the rate constants for which, $k_{CR}$, are presented in Table 6.2. For $F_nY^-$ with $n < 2$, the quenched $^3$MLCT state decayed within the instrument response and thus the 520-nm traces could be fit to a single exponential.

With the rates for charge separation and recombination in $[Re]^-F_nY^-$ in hand, we next chose to analyze the driving force dependence of these rates within the Marcus-Levich framework for ET described by eq. 6.2:

$$k_{ET} = \sqrt{\frac{\pi}{\hbar^2 \lambda k_B T}} \left| H_{AB} \right|^2 \exp \left[ -\frac{(\Delta G^\circ + \lambda)^2}{4\lambda k_B T} \right]$$

where $H_{AB}$ is the electronic coupling, $\lambda$ is the reorganization energy, and $\Delta G^\circ$ is the free energy driving force for the electron transfer. The driving force for the CS and CR reactions, $\Delta G_{CS}^\circ$ and $\Delta G_{CR}^\circ$, were derived from eqs. 6.3 and 6.4, respectively,

$$\Delta G_{CS}^\circ = E^\circ(ReI^*/0) - E_p(F_nY^*/F_nY^-)$$

$$\Delta G_{CR}^\circ = E^\circ(ReI^*/0) - E_p(F_nY^*/F_nY^-)$$

where $E^\circ(ReI^*/0)$ and $E^\circ(ReI^*/0)$ are the excited and ground state Re$^I$ reduction potentials and $E_p(F_nY^*/F_nY^-)$ is the peak potential for the $F_nY^*/F_nY^-$ couple (Table 6.1). Figure 6.7 (left) plots ln($k_{CS}$) vs. $-\Delta G_{CS}^\circ$, and shows that the data are well fit by both a linear and parabolic Marcus relation. These data indicate that CS is strongly activated and occurs in the normal region for Marcus’ ET. In the limit that $4\lambda >> \Delta G^\circ$, eq. 2 predicts that the ln($k_{CS}$) vs. $-\Delta G_{CS}^\circ$ plot should be linear with a slope of $0.5/(k_B T)$. From the linear fit in Figure 6.7 (left), we...
obtain $0.3/(k_B T)$. The parabolic fit (eq. 2) yields an approximate value of $\lambda$ for this system of 1.9 eV, indicating that $4\lambda$ is about an order of magnitude larger than $\Delta G^\circ$. 

The lack of curvature in the plot in Figure 6.7 (left) introduces a large degree of error in the calculation of $\lambda$ from eq. 6.2 for CS. To provide a more accurate measurement of $\lambda$, the temperature dependence of $k_{CS}$ for [Re]-3,5-F$_2$Y and [Re]-F$_4$Y was measured. The data were analyzed as $\ln(k_{CS} \cdot T^{1/2})$ vs. $T^{-1}$ plots (Figure 6.8) and fit to eq. 6.5:

$$\ln(k_{CS} \cdot T^{1/2}) = A \frac{\Delta G^\ddagger}{k_B T} \quad (6.5)$$

where $\Delta G^\ddagger$ is the activation energy, $A$ is a pre-factor, and $k_B$ is the Boltzmann constant. The reorganization energy, $\lambda$, could then be calculated with eq. 6.6:

$$\Delta G^\ddagger = \frac{(\lambda + \Delta G^\circ_{CS})^2}{4\lambda} \quad (6.6)$$

The calculated values of $\Delta G^\ddagger$ and $\lambda$ are listed below in Table 6.3. The calculated values of $\lambda$ from the temperature dependence experiments are in agreement with the value calculated from the driving force plot in Figure 6.8 (left).

**Figure 6.7.** Left: $\ln(k_{CS})$ vs. $-\Delta G_{CS}^\circ$ plot with linear (▬) and parabolic (▬) fit. Right: $\ln(k_{CR})$ vs. $-\Delta G_{CR}^\circ$ plot with linear (▬) fit.

**Figure 6.8.** Temperature dependence of the rate of charge separation in [Re]-3,5-F$_2$Y and [Re]-F$_4$Y (○) with linear fit (▬).
Figure 6.7 (right) plots $\ln(k_{CR})$ vs. $-\Delta G_{CR}^\circ$ and, in contrast to CS, shows that CR is weakly dependent on driving force and occurs in the inverted region for Marcus’ ET. Previous work has shown that inverted region ET often does not exhibit the predicted parabolic dependence on driving force due to electron coupling to vibrational modes that assist the reaction (see Chapter I). Under such conditions, a modest linear decrease in $\ln(k_{CR})$ with $-\Delta G_{CR}^\circ$ is predicted by the energy gap law. The scatter in these data is likely due to experimental precision (2 ns error shown in plot) and small differences in electronic coupling and/or frequency of the accepting vibrational modes for each of the $F_nY$s.

### 6.4 Conclusions

The mechanistic complexity arising from the coupling of electron and proton transfers in charge transport processes involving amino acid radicals requires the development of new tools and methods for their study. The results reported and discussed herein show that $F_nY$s will be useful in the study of biological charge transport mechanisms involving tyrosine for the following reasons. (1) They provide a range of $pK_a$s for the phenolic proton and $E_{ps}$ for the $Y^*/Y$ couple, which allows for tuning of the $\Delta G_{PT}^\circ$ and $\Delta G_{ET}^\circ$ for the PCET reaction. UV-Vis and DPV analysis illustrate that the Ac-$F_nY$-$NH_2$s offer a range of almost 5 units in $pK_a$ and 320 mV in redox potential within the pH range of 6-9 where proteins can be studied. (2) The oxidized forms of $F_nY^*$s possess unique spectral signals allowing a distinction between $Y^*$ and $F_nY^*$ in the same system. The time-resolved absorption spectra of BPA-$F_nY$-OMe dipeptides show that the $\lambda_{max}$ for $F_nY^*$ absorption can be shifted by ~10 nm relative to $Y^*$. (3) The variation in $E_{ps}$ for the $F_nY^*/F_nY^-$ couple directly correlates to the rate of electron transfer in reactions involving $F_nY^*$ as predicted by the driving force dependence of ET from Marcus Theory. The correlation between ET rate and driving force also validates the DPV method of measuring the reduction potentials for $F_nY^*$.

The $F_nY$s will be useful for a variety of mechanistic studies in biology, in which tyrosine is integral to radical initiation and transport. We have demonstrated one example of this utility.
by site-specifically replacing Y356 of R2 with the FₙYs presented here. A study of the pH dependent activity of each of these new semi-synthetic enzymes cast with the backdrop of the Ac-FₙY-NH₂S pKₐ and Ac-FₙY•-NH₂S reduction potential data discussed above have allowed for mechanistic details of the PCET reactivity at this site to be unveiled.

Furthermore, with the basic characterization of FₙY• and the of electron transfer reactions in [Re]–FₙY in hand, we can now focus on incorporation of this system into RNR for the study of photoinitiated radical transport. Radical transport in the enzyme likely occurs via a PCET mechanism as amino acid oxidation is coupled to changes in protonation state. Upon substituting FₙY for Y, we can now predict what affect this will have on the ET part of the reaction, allowing us to gauge the degree of proton coupling to the radical transport process.

The ability to phototrigger radical formation is a powerful tool for the study of radical based enzymes. Previous methods to generate the radicals by metal complexes have relied on indirect bimolecular methods (flash-quench) owing to insufficient oxidizing power of the direct excited state. As we show here, this is not the case for Re¹ polypyridyl excited states. Transient spectroscopic results show that tyrosyl radical is generated promptly upon photoexcitation and it is of sufficient lifetime to promote the investigation of radical transport. One important reason for the prompt photogeneration is that the non-natural FₙY amino acids are deprotonated at physiological pHs so that radical initiation occurs by simple electron transfer. Using previously described peptide methods, the [Re]–FₙY constructs may be incorporated into the PCET network of RNR, thus allowing the kinetics for radical transport to be probed.

6.5 Experimental Section

Materials. N-acetyl-L-tryosinamide, 3-fluoro-L-tyrosine, trifluoroacetic acid (TFA), thionyl chloride, 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (WSC•HCl) and N-methylmorpholine (NMM) were purchased from Sigma-Aldrich. L-Tyrosine methyl ester hydrochloride (Y-OMe•HCl) and 4-benzoyl-N-[(1,1-dimethylethoxy) carbonyl]-L-phenylalanine (Boc-BPA-OH) were from Advanced ChemTech. 1-Hydroxybenzotriazole (HOBt) was obtained from NovaBiochem. All chemicals were used as received. Fluorotyrosines (FₙYs) and rhenium(I)(4-methyl-2,2´-bipyridine-4´-carboxylic acid)tricarbonyl cyanide (Re(bpy-COOH)(CO)₃CN) were prepared as previously described.
**Fₙ-Tyrosine Methyl Ester Hydrochloride (FₙY-OMe·HCl).** In a typical synthesis, the diammonium salt of the FₙY (0.6 mmol) was combined with 20 mL of methanol in a 50 mL round bottom flask. Thionyl chloride (400 μL, 5.5 mmol) was added dropwise and the solution was stirred for three days at room temperature. The solvent was removed *in vacuo* and the resulting white solid was dissolved in a minimal amount of methanol. Ether was added dropwise to precipitate NH₄Cl, which was removed by filtration. Further dropwise addition of ether induced crystallization of the title compound. After cooling to –20°C, the white crystals were isolated by filtration and dried *in vacuo* providing product in 70 - 88% yield.

3-FY-OMe·HCl: $^1$H NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 3.02$ (m, 2H, Cβ-H), 3.68 (s, 3H, -OCH₃), 4.22 (m, 1H, Cα-H), 6.80 (m, 1H, arom. C-H), 6.90 (m, 1H, arom. C-H), 7.02 (m, 1H, arom. C-H), 8.56 (b.s., 3H, -NH₃⁺), 9.88 (b.s., 1H, PhO-H). $^{19}$F NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 27.3$ (t, 1F, 11.1 Hz).

3,5-F₂Y-OMe·HCl: $^1$H NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 3.05$ (m, 2H, Cβ-H), 3.70 (s, 3H, -OCH₃), 4.27 (m, 1H, Cα-H), 6.94 (d, 2H, arom. C-H, 8.8 Hz), 8.63 (b.s., 3H, -NH₃⁺), 10.15 (s, 1H, PhO-H). $^{19}$F NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 32.1$ (d, 2F, 11.1 Hz).

2,3-F₂Y-OMe·HCl: $^1$H NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 3.09$ (m, 2H, Cβ-H), 3.65 (s, 3H, -OCH₃), 4.14 (m, 1H, Cα-H), 6.78 (m, 1H, arom. C-H), 6.89 (m, 1H, arom. C-H), 8.64 (b.s., 3H, -NH₃⁺), 10.51 (s, 1H, PhO-H). $^{19}$F NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = -0.5$ (d, 1F, 22.2 Hz), $\delta = 19.5$ (d, 1F, 22.2 Hz).

2,3,5-F₃Y·HCl: $^1$H NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 3.12$ (m, 2H, Cβ-H), 3.69 (s, 3H, -OCH₃), 4.22 (m, 1H, Cα-H), 7.10 (m, 1H, arom. C-H), 8.70 (b.s., 3H, -NH₃⁺), 10.83 (b.s., 1H, PhO-H). $^{19}$F NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 5.5$ (d, 1F, 16.7 Hz), 15.8, (s, 1F), 24.5 (s, 1F).

2,3,6-F₃Y·HCl: $^1$H NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 3.07$ (m, 2H, Cβ-H), 3.64 (s, 3H, -OCH₃), 4.07 (m, 1H, Cα-H), 6.73 (m, 1H, arom. C-H), 8.70 (b.s., 3H, -NH₃⁺), 11.13 (s, 1H, PhO-H). $^{19}$F NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = -4.8$ (s, 1F), 22.3, (d, 1F, 22.2 Hz), 40.9 (m, 1F).

2,3,5,6-F₄Y·HCl: $^1$H NMR (300 MHz, (CD₃)₂SO, 25 °C) $\delta = 3.16$ (m, 2H, Cβ-H), 3.66 (s, 3H, -OCH₃), 4.14 (m, 1H, Cα-H), 8.75 (b.s., 3H, -NH₃⁺), 11.56 (b.s., 1H, PhO-H). $^{19}$F NMR
The compounds were prepared as described in Chapter 3 for BPA-Y-OMe•CF₃COOH, except that FₙY-OMe•HCl was used instead of Y-OMe (45-65% yield).

BPA-3-FY-OMe•CF₃COOH: ¹H NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = 2.90 - 3.20 (m, 2H, Cβ-H), 3.32-3.48 (m, 2H, Cβ-H), 3.69 (s, 3H, -OCH₃), 4.65 (m, 1H, Cα-H), 4.95 (m, 1H, Cα-H), 7.03 (m, 2H, phenol-H), 7.33 (d, 2H, C₆H₄OC₆H₅, 8.3 Hz), 7.56 (m, 2H, C₆H₄OC₆H₅), 7.62-7.85 (m, 5H, C₆H₄OC₆H₅). ¹⁹F NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = 26.6 (s, 2F), 85.6 (s, 3F).

BPA-2,3-F₂Y-OMe•CF₃COOH: ¹H NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = 2.93 - 3.49 (m, 4H, Cβ-H), 3.69 (s, 3H, -OCH₃), 4.68 (m, 1H, Cα-H), 4.95 (m, 1H, Cα-H), 6.80 (m, 1H, phenol-H), 7.01 (m, 1H, phenol-H), 7.39 (d, 2H, C₆H₄OC₆H₅, 7.7 Hz), 7.56 (m, 2H, C₆H₄OC₆H₅), 7.63 - 7.86 (m, 5H, C₆H₄OC₆H₅). ¹⁹F NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = −1.7 (m, 1F), 18.3 (m, 1F), 85.6 (s, 3F).

BPA-2,3,5-F₃Y-OMe•CF₃COOH: ¹H NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = 2.93-3.51 (m, 4H, Cβ-H), 3.70 (s, 3H, -OCH₃), 4.68 (m, 1H, Cα-H), 5.04 (m, 1H, Cα-H), 7.11 (m, 1H, phenol-H), 7.41 (d, 2H, C₆H₄OC₆H₅, 7.7 Hz), 7.55 (m, 2H, C₆H₄OC₆H₅), 7.62 - 7.85 (m, 5H, C₆H₄OC₆H₅). ¹⁹F NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = 7.93 (d, 1F, 22.2 Hz), 18.56 (m, 1F), 26.1 (s, 1F), 89.9 (s, 3F).

BPA-2,3,6-F₃Y-OMe•CF₃COOH: ¹H NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = 3.00-3.26 (m, 2H, Cβ-H), 3.44 (m, 2H, Cβ-H), 3.67 (s, 3H, -OCH₃), 4.66 (m, 1H, Cα-H), 5.12 (m, 1H, Cα-H), 6.76 (m, 1H, phenol-H), 7.41-7.85 (m, 9H, C₆H₄OC₆H₅). ¹⁹F NMR (300 MHz, (CD₃)₂CO, 25 °C) δ = −1.86 (m, 1F), 25.2 (d, 1F, 22.2 Hz), 44.1 (m, 1F), 90.0 (s, 3F).
BPA-2,3,5,6-F4Y-OMe•CF3COOH: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta$ = 3.10 - 3.52 (m, 4H, C$_{\beta}$-H), 3.68 (s, 3H, -OCH$_3$), 4.65 (m, 1H, C$_{\alpha}$-H), 5.05 (m, 1H, C$_{\alpha}$-H), 7.46 - 7.85 (m, 9H, C$_6$H$_4$OC$_6$H$_5$). $^{19}$F NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta$ = −2.42 (d, 2F, 22.2 Hz), 14.6 (d, 2F, 22.2 Hz), 85.6 (s, 3F).

[Re]–FnY. In a typical synthesis Re(bpy-COOH)(CO)$_3$CN (195 mg, 0.383 mmol, 1 eq), FnY–COOMe•HCl (1 eq), WSC•HCl (147 mg, 0.766 mmol, 2 eq), and HOBt (104 mg, 0.766 mmol, 2 eq) were combined in a 50 mL round bottom flask with 20 mL of dimethylformamide. NMM (255 $\mu$L, 2.30 mmol, 6 eq) was added and the solution stirred for 2.5 hours at room temperature. Upon addition of NMM in the synthesis of [Re]-2,3-F$_2$Y, the reaction mixture initially turned green, but changed back to yellow as the reaction progressed. The solvent was removed in vacuo and the residual yellow/orange oil was dissolved in 75 mL of CH$_2$Cl$_2$. The organics were washed with 2 × 30 mL of water and dried over MgSO$_4$. The solvent was removed in vacuo and the resulting yellow solid was dissolved in a few mLs of 5% MeOH/CH$_2$Cl$_2$ and loaded onto a silica gel Chromatotron plate (2 mm). The product eluted as a yellow band with 5% MeOH/CH$_2$Cl$_2$ and the solvent was removed in vacuo yielding a yellow solid that was triturated with ether and isolated by filtration (25 - 50 % yield).

[Re]–Y: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta$ = 2.67 (d, 3H, bpy-CH$_3$), 3.02 - 3.27 (m, 2H, C$_{\beta}$-H$_2$), 3.72 (d, 3H, Y-OCH$_3$), 4.88 (m, 1H, C$_{\alpha}$-H), 6.77 (m, 2H, phenol C-H), 7.16 (m, 2H, phenol C-H), 7.66 (m, 1H, bpy-H), 8.03 (m, 1H, bpy-H$_2$), 8.25 (s, 1H, phenol O-H), 8.53 (m, 1H, amide N-H), 8.66 (m, 1H, bpy-H), 8.85 (m, 1H, bpy-H), 8.97 (d, 1H, bpy-H), 9.24 (m, 1H, bpy-H).

[Re]–3-FY: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta$ = 2.65 (d, 3H, bpy-CH$_3$), 3.05-3.30 (m, 2H, C$_{\beta}$-H$_2$), 3.74 (d, 3H, Y-OCH$_3$), 4.91 (m, 1H, C$_{\alpha}$-H), 6.94 (m, 2H, phenol C-H), 7.12 (m, 1H, phenol C-H), 7.66 (m, 1H, bpy-H), 8.03 (m, 1H, bpy-H$_2$), 8.58 (m, 2H, phenol O-H, amide N-H), 8.65 (m, 1H, bpy-H), 8.84 (m, 1H, bpy-H), 8.98 (d, 1H, bpy-H), 9.25 (m, 1H, bpy-H). $^{19}$F NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta$ = 23.25 (m, 1F).

[Re]–3,5-F$_2$Y: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta$ = 2.65 (d, 3H, bpy-CH$_3$), 3.08-3.30 (m, 2H, C$_{\beta}$-H$_2$), 3.74 (d, 3H, Y-OCH$_3$), 4.95 (m, 1H, C$_{\alpha}$-H), 7.01 (m, 1H, phenol C-H), 7.66 (m, 1H, bpy-H), 8.03 (m, 1H, bpy-H$_2$), 8.63 (m, 2H, amide N-H, bpy-H), 8.86 (m, 2H,
bpy-H, phenol O-H), 8.98 (d, 1H, bpy-H), 9.26 (m, 1H, bpy-H). $^{19}$F NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = 26.85$ (d, 1F).

[Re]–2,3-F$_2$Y: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = 2.67$ (s, 3H, bpy-CH$_3$), 3.10-3.42 (m, 2H, C$_\beta$-H$_2$), 3.74 (s, 3H, Y-OCH$_3$), 4.97 (m, 1H, C$_\alpha$-H), 6.75 (m, 1H, phenol C-H), 6.99 (m, 1H, phenol C-H), 7.67 (m, 1H, bpy-H), 8.04 (m, 1H, bpy-H$_3$), 8.65 (m, 2H, amide N-H, bpy-H), 8.88 (m, 1H bpy-H), 8.97 (d, 1H, bpy-H), 9.06 (s, phenol O-H), 9.24 (m, 1H, bpy-H). $^{19}$F NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = –1.52$ (m, 1F), 18.40 (m, 1F).

[Re]–2,3,5-F$_3$Y: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = 2.64$ (d, 3H, bpy-CH$_3$), 3.05-3.44 (m, 2H, C$_\beta$-H$_2$), 3.76 (d, 3H, Y-OCH$_3$), 4.97 (m, 1H, C$_\alpha$-H), 7.05 (m, 1H, phenol C-H), 7.66 (m, 1H, bpy-H), 8.05 (m, 1H, bpy-H$_3$), 8.68 (m, 2H, amide N-H, bpy-H), 8.89 (m, 1H bpy-H), 8.98 (m, 1H, bpy-H), 9.26 (m, 1H, bpy-H), 9.42 (s, 1H, phenol O-H). $^{19}$F NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = 8.00$ (m, 1F), 18.67 (m, 1F), 26.26 (m, 1F).

[Re]–2,3,6-F$_3$Y: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = 2.66$ (s, 3H, bpy-CH$_3$), 3.17-3.44 (m, 2H, C$_\beta$-H$_2$), 3.74 (d, 3H, Y-OCH$_3$), 4.91 (m, 1H, C$_\alpha$-H), 6.60 (m, 1H, phenol C-H), 7.65 (m, 1H, bpy-H), 8.06 (m, 1H, bpy-H$_3$), 8.65 (m, 1H, bpy-H), 8.74 (m, 1H, amide N-H), 8.90 (m, 1H bpy-H), 8.98 (m, 1H, bpy-H), 9.27 (m, 1H, bpy-H), 9.63 (s, 1H, phenol O-H). $^{19}$F NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = –1.59$ (m, 1F), 25.34 (m, 1F), 44.13 (m, 1F).

[Re]–F$_4$Y: $^1$H NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = 2.66$ (s, 3H, bpy-CH$_3$), 3.26-3.48 (m, 2H, C$_\beta$-H$_2$), 3.76 (d, 3H, Y-OCH$_3$), 4.99 (m, 1H, C$_\alpha$-H), 7.67 (m, 1H, bpy-H), 8.05 (m, 1H, bpy-H$_3$), 8.64 (m, 1H, bpy-H), 8.73 (m, 1H, amide N-H), 8.88 (m, 1H bpy-H), 8.98 (m, 1H, bpy-H), 9.26 (m, 1H, bpy-H), 10.0 (bs, 1H, phenol O-H). $^{19}$F NMR (300 MHz, (CD$_3$)$_2$CO, 25 °C) $\delta = –2.30$ (d, 2F), 14.75 (d, 2F).

**NMR Methods.** $^1$H and $^{19}$F NMR spectra were recorded on a Varian 300 MHz NMR at the MIT Department of Chemistry Instrumentation Facility (DCIF). Aqueous samples for $^1$H NMR and $^{19}$F NMR were carried out in D$_2$O with 3-(trimethylsilyl)-propionic acid-d$_4$ (TSP) as an internal standard and CFCl$_3$ as an external standard, respectively. Nonaqueous $^1$H and $^{19}$F NMR were externally referenced to tetramethylsilane and CFCl$_3$, respectively.

**X-Ray Crystal Structure of Re(bpy-COOH)(CO)$_3$CN•MeOH.** Single crystals of the complex were grown by slow evaporation of a saturated solution in methanol. X-ray diffraction data were collected at 100 K on a Siemens diffractometer equipped with a CCD
detector, using the Mo Kα radiation. The data were integrated to hkl-intensity and the unit cell calculated using the SAINT v.4.050 program from Siemens. Solution and refinement were performed by direct methods (SHELXTL v.6.10, Sheldrick, G. M., and Siemens Industrial Automation, Inc., 2000). The structures were solved by the Patterson method, non-hydrogen atoms were refined anisotropically, and hydrogen atoms were placed at calculated positions. Details regarding the refined data, cell parameters and results of the solution are presented at the end of this chapter (Table 6.4).

**Computational Methods.** Density functional theory calculations (DFT) were performed using the Amsterdam Density Functional (ADF2002.02) program a home-built Linux cluster comprising 60 Intel processors organized in groups of 12 running in parallel. The generalized gradient approximation was used as implemented in ADF by the Becke-88 functional for exchange, and the Perdew-Wang-91 functional for correlation. A basis set of triple-ζ Slater-type functions augmented by a polarization set (TZP) was used for all atoms; spin restriction was lifted for all Ac-FnY•-NH2 radicals. Energies reported are gas-phase internal energies at 0 K, without zero-point vibrational energy, of the geometry optimized structure. Each structure was optimized from the same amino acid backbone configuration, which was kept nearly constant throughout the calculation.

**Transient Absorption (TA) Spectroscopy.** Laser radiation (400 nm, ~100 fs) was used as the pump source in the time-resolved emission experiments and the data were collected on a streak camera as previously described. TA measurements were performed with pump light provided by an Infinity Nd:YAG laser (Coherent) running at 20 Hz, slightly modified from that described in Chapter II. For studies with [Re]-FnY, the third harmonic (355 nm) provided a convenient pump source, however due to low absorption of BPA-FnY-OMe at 355 nm, 300 nm pump light was used for excitation as described in Chapter III. Aqueous BPA-FnY-OMe samples were prepared at 200-500 μM concentrations in deionized water buffered to pH 4 with 20 mM succinic acid. All TA measurements were performed in a 2 mm quartz optical cell. For BPA-3-FY-OMe, 266 nm pump light was used as described in Chapter II and the sample was flowed through a 2 mm cell to ensure fresh sample for each laser shot. For [Re]-FnY, all samples were dissolved in aqueous solutions of 10 mM KOH (pH 12) in the concentration range of 50 - 100 μM and freeze-pump-thaw degassed to 10⁻⁵ torr on a high-vac line.
Temperature Dependent Time-Resolved Emission Spectroscopy. The emission lifetime of [Re]-3,5-F₂Y⁻ and [Re]-F₄Y⁻ were measured in 10 mM KOH as a function of temperature over the range 320-260 K. The emission lifetime of the control complex, [Re]-Y-COOH,²⁴ ([Re]-Y with a free carboxylic acid) was also measured in 20 mM Tris buffer at pH 7.5 over the same temperature range. At this pH the tyrosine phenol is protonated and tyrosine oxidation does not occur, thus allowing for measurement of the intrinsic temperature dependence of the ³MLCT excited state. Each sample was freeze-pump-thaw degassed to 10⁻⁵ torr in a glass vial to remove traces of dissolved oxygen. The samples were then sealed in the glass vial under vacuum and placed in a modular cryostat system equipped with glass windows to allow for excitation of the sample and collection of the emitted light. The [Re]-Y-COOH control complex was excited with 355 nm, nanosecond laser light and the emitted light monitored with a photomultiplier tube. The [Re]-3,5-F₂Y and [Re]-F₄Y complexes were excited with 400 nm, ~100 femtosecond laser light and the emitted light collected with a streak scope. Data were collected at 5 K intervals and the sample was allowed to equilibrate for 15 minutes at the set temperature before collection.

The overall photochemical sequence of events is:

\[
[\text{Re}^I]–F_nY– \xrightarrow{\text{hv(MLCT)}} [\text{Re}^I]^*–F_nY– \xrightarrow{k_{CS}} [\text{Re}^0]–F_nY^* \xrightarrow{k_{CR}} [\text{Re}^I]–F_nY–
\]

Rate constants for charge separation at a given temperature were calculated with the eq. 6.7:

\[
k_{CS}(T) = \tau^{-1}([\text{Re}–F_nY](T)) – \tau^{-1}([\text{Re}–Y-COOH](T))
\]

where \( \tau ([\text{Re}–F_nY](T)) \) and \( \tau ([\text{Re}–Y-COOH](T)) \) are the emission lifetimes of the [Re]-FₙY⁻ and [Re]-Y-COOH at a given temperature.
6.6 References


### Table 6.4. Crystal data and structure refinement for Re(bpy-COOH)(CO)$_3$CN.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical formula</td>
<td>C$<em>{17}$H$</em>{14}$N$_3$O$_6$Re</td>
</tr>
<tr>
<td>Formula weight</td>
<td>542.51</td>
</tr>
<tr>
<td>T (K)</td>
<td>100(2)</td>
</tr>
<tr>
<td>$\lambda$ (Å)</td>
<td>0.71073</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>$P\bar{1}$</td>
</tr>
<tr>
<td>a (Å)</td>
<td>8.3707(5)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>10.4102(6)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>11.8772(7)</td>
</tr>
<tr>
<td>$\alpha$ (°)</td>
<td>104.8670(10)</td>
</tr>
<tr>
<td>$\beta$ (°)</td>
<td>96.5250(10)</td>
</tr>
<tr>
<td>$\gamma$ (°)</td>
<td>106.2030(10)</td>
</tr>
<tr>
<td>V (Å$^3$)</td>
<td>941.28(10)</td>
</tr>
<tr>
<td>Z</td>
<td>2</td>
</tr>
<tr>
<td>$\rho_{\text{calcd}}$ (Mg/m$^3$)</td>
<td>1.914</td>
</tr>
<tr>
<td>Abs. coeff (mm$^{-1}$)</td>
<td>6.493</td>
</tr>
<tr>
<td>$\theta$ range for data collection (°)</td>
<td>1.81 to 28.29</td>
</tr>
<tr>
<td>Index ranges</td>
<td>$-11 \leq h \leq 4, -13 \leq k \leq 13, -14 \leq \ell \leq 15$</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>5964</td>
</tr>
<tr>
<td>Independent reflns ($R_{\text{int}}$)</td>
<td>4430 (0.0221)</td>
</tr>
<tr>
<td>Completeness to $\theta_{\text{max}}$</td>
<td>94.9%</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>4430 / 0 / 260</td>
</tr>
<tr>
<td>GOF$^a$ on $R^2$</td>
<td>1.055</td>
</tr>
<tr>
<td>$R1^b$</td>
<td>0.0297</td>
</tr>
<tr>
<td>wR2$^c$</td>
<td>0.0743</td>
</tr>
<tr>
<td>Largest diff. peak, hole</td>
<td>1.928, $-1.457$ eÅ$^{-3}$</td>
</tr>
</tbody>
</table>

---

$^a$ GOF = $(\Sigma w(F_o^2 - F_c^2)^2/(n - p))^{1/2}$ where $n$ is the number of data and $p$ is the number of parameters refined.  

$^b$ $R1 = \Sigma ||F_o - |F_c||/\Sigma |F_o|$.  

$^c$ wR2 = $(\Sigma (w(F_o^2 - F_c^2)^2)\Sigma (w(F_o^2))^{1/2}$.
Chapter VII

PhotoRNRs with Benzophenone and Anthraquinone
Excited States as Radical Generators

Parts of this Chapter have been published:

Reece, S. Y; Seyedsayamdost, M. R.; Stubbe, J.; Nocera, D. G. “Photoactive Peptides for Light Initiated Tyrosyl Radical Generation and Transport into Ribonucleotide Reductase”

7.1 Motivation and Background

Having developed methods for photochemical Y• generation (Chapters III and IV), a basic understanding of PCET mechanisms of Y• formation (Chapter V), and tools to probe the PCET reactions involving Y• in enzymes (Chapter VI), we decided to apply these methodologies to the study of PCET in RNR. This chapter presents photochemical RNR systems based on the benzophenone and anthraquinone (Figure 7.1) excited state oxidants of Y•. These molecules can be excited with UV light of wavelengths outside of the protein absorption envelope and their corresponding excited states are competent for rapid (sub-ns) tyrosine oxidation (see Chapter III). We now append these photooxidants to the end of the Y–R2C19 peptide (see Chapter I, Figure 1.14), bind them to the α2 subunit, and photolyze them in the presence of RNR substrate and effector. The RNR turnover data obtained, in comparison to that for the Y730F-α2 variant, provide insight into the mechanism of radical transport in α2.

7.2 Results

*Synthesis.* Chromophore-(F/Y)-R2C19 peptides were synthesized by extending the Fmoc-R2C19-PEG-PS resin bound peptide, first with Fmoc-(F/Y)-OH, followed by the appropriate

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$t_R$ (min)$^a$</th>
<th>MW Calcd (m/z)</th>
<th>MW Found (m/z)$^b$</th>
<th>$IC_{50}^c$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-BPA-Y-R2C19</td>
<td>16.7</td>
<td>2563</td>
<td>2564</td>
<td>15</td>
</tr>
<tr>
<td>Ac-BPA-F-R2C19</td>
<td>18.3</td>
<td>2547</td>
<td>2548</td>
<td>30</td>
</tr>
<tr>
<td>Anq-Y-R2C19</td>
<td>17.1</td>
<td>2504</td>
<td>2507</td>
<td>4</td>
</tr>
<tr>
<td>Anq-F-R2C19</td>
<td>19.9</td>
<td>2488</td>
<td>2489</td>
<td>7</td>
</tr>
</tbody>
</table>

$^a$Retention time ($t_R$) from analytical HPLC trace. $^b$MALDI-TOF MS in negative ion mode. $^c$Concentration of peptide at 50% RNR inhibition.
carboxylic acid containing chromophore (Fmoc-BPA-COOH or Anq-COOH) using standard Fmoc-SPPS conditions. Table 7.1 lists the analytical HPLC retention times and MALDI-TOF mass spectral data for each of the peptides synthesized.

Time-resolved Spectroscopy. The BPA and Anq dipeptides and full-length peptides were weakly emissive or non-emissive in aqueous solutions. Accordingly, the formation and reaction of the chromophore excited states and photoproducts were characterized by transient absorption spectroscopy. The BPA-Y-OMe dipeptide produces the •BPA-Y•-OMe diradical state upon excitation with $\lambda_{\text{exc}} = 300$ nm. Radical recombination occurs with a rate constant of $5.6 \times 10^6$ s$^{-1}$ (Chapter III).\(^1\) The transient absorption and kinetics traces shown in Figure 7.2 establish a similar photochemistry for the full length peptide, Ac-BPA-Y-R2C19. Transient absorption features at 337, 410 and 550 nm, obtained 65 ns following 300 nm excitation, are consistent with the formation of Y• ($\varepsilon_{410}$ nm = 2750 ± 200)\(^2\) and the benzophenone ketyl radical ($\varepsilon_{330}$ nm = 22000 M$^{-1}$ cm$^{-1}$ and $\varepsilon_{540}$ nm = 2800 M$^{-1}$ cm$^{-1}$).\(^3\) The overall absorption profile does not change over the time course of 415 ns, 1 μs and 10 μs. Time-evolved absorption kinetics obtained at 410 and 550 nm (inset of Figure 7.2) can both be fit to a mono-exponential decay function with a time constant of 490 ± 20 ns, thus yielding a rate constant for radical recombination of $2.0 \pm 0.1 \times 10^6$ s$^{-1}$. The direct and concomitant return of the transient signals of •BPA and •Y to baseline indicates that radical recombination proceeds without the production of additional intermediates. Moreover, the similarity of the transient data for the dipeptide and full-length peptide indicates that the BPA reacts solely with Y and not other residues on the R2C19 peptide.

We next investigated the photochemistry of the Anq chromophore appended to the Y-R2C19 peptide against the backdrop of the dipeptide results already discussed in Chapter III.

\[\text{Figure 7.2. Transient absorption spectra of BPA-Y-R2C19 obtained 65 (---), 415 ns, 1, and 10 μs (----) following 300 nm excitation. Insets: Time-evolved absorbance data (○) with mono-exponential decay fit (---) obtained at 410 and 550 nm.}\]
Figure 7.3. Left panel: Transient absorption spectra of Anq-Y-R2C19 obtained 115 ns (▬), 1, and 10 μs (▬) following 355 nm excitation. Left and right insets: Single wavelength kinetics traces (○) with bi-exponential decay fit (▬). Middle inset: Plot of ΔΔOD obtained by subtracting the normalized transients observed for Anq-Y-R2C19 and Anq-F-R2C19 at 100 ns. Bottom panel: Transient absorption spectra of Anq-F-R2C19 obtained 115 ns (▬), 1, 10, and 100 μs (▬) following 355 nm excitation.

Figure 7.3 (left) shows the transients obtained 100 ns, 1 μs and 10 μs after excitation of Anq-Y-R2C19 with 355 nm laser pulse. The spectra are similar to those observed for the Anq-Y-OH dipeptide and are ascribed to the Anq•–-Y•-R2C19 diradical state of the peptide. Single wavelength kinetics obtained at 410 nm and 520 nm (Figure 7.3, left and right insets) could be fit to a bi-exponential decay with short, major and long, minor components similar to that for Anq-Y-OH (Chapter III): 410 nm, $\tau_1 = 540 \pm 30$ ns (80%), $\tau_2 = 2.1 \pm 0.3$ μs (20%); 510 nm, $\tau_1 = 630 \pm 70$ ns (80%), $\tau_2 = 2.1 \pm 0.3$ μs (20%). The shorter components are the same within the error of the measurement, and, as with Anq-Y-OH, are attributed to radical recombination between Anq• and Y• on the peptide with a rate constant of $1.7 \pm 0.3 \times 10^6$ s⁻¹. As with Anq-Y-OH, we attribute the slower component to the decay of the Anq-solvent photoproduct.

To further confirm Y• formation in Anq-Y-R2C19, the control peptide Anq-F-R2C19 (as with the dipeptides) was examined in an effort to uncover photochemical reactivity arising from that other than Y356 as a number of other residues are potential reductants of $^3$Anq. $^3$AQS has been shown to oxidize the carboxylate-containing residues of glycy1-glycine dipeptides leading to the formation of the AQS•– semiquinone anion radical and the decarboxylated amidomethylene radical. There are five carboxylic acid containing Asp/Glu residues on the R2C19 peptide. In addition, $^3$Anq has also been shown to oxidize alcohols; both hydroxyl-containing Ser and Thr residues are present on R2C19 as well. The right panel
of Figure 7.3 shows the transients observed upon excitation of the Anq-F-R2C19 peptide with 355 nm excitation at 100 ns, 1, 10, and 100 μs. These transients observed at 100 ns do not resemble that for Anq-F-OH (Chapter III, Figure 3.8, left panel). The peaks at 410 and 520 nm resemble that of Anq•-. The decay of the signals at 410 and 520 nm in the right panel of Figure 7.3 are complex, decaying on both the hundreds of nanoseconds and microsecond timescales, with a Anq•- remaining after 100 μs. Because Anq•- should be the only absorbing species in the 375 - 450 nm spectral region upon excitation of Anq-F-R2C19, this spectrum may be used for the normalization and subtraction of the transients following excitation of Anq-Y-R2C19 and Anq-F-R2C19 at 100 ns. The inset in the left of Figure 7.3 (middle inset) plots the ΔΔOD for this subtraction. The distinctive Y• absorption profile, with at peak at 410 nm and a shoulder to the blue (for reference, compare to Figure 7.2 with the Ac-BPA-Y-R2C19 system), is obtained. This difference spectrum further supports the contention that Y• is produced promptly after excitation of Anq-Y-R2C19.

Peptide Binding to α2. To study PCET in the photochemical RNR system, the chromophore-(Y/F)-R2C19 peptides need to bind to α2. A protocol has been developed by Climent and coworkers7,8 to asses binding of β2 C-terminal tail peptides of various lengths to α2. These studies found that (1) the C-terminal peptide tail of β2 accounts for most of the binding interaction between this subunit and α2; (2) these peptides are inhibitors of RNR activity by competing with β2 for binding to α2; (3) addition of an N-terminal tyrosine to Ac-R2C19 to form Ac-Y-R2C19 results in stronger binding by a factor of 2 (Ki = 40.0 and 20.0 μM, respectively); and (4) further addition of residues at the N-terminus, up to R2C37, did not significantly enhance binding. We were interested to know if the addition of a chromophore to the N-terminus of Y-R2C19 significantly perturbs binding from that of the native peptide studies of Climent. To address this issue, we used Climent’s methods7 to asses binding of the Ac-BPA- and Anq-containing peptides.

α2 and β2 (0.1 and 0.2 μM, respectively) were combined and the relative RNR activity was measured as the peptide concentration was increased from 0 to 80 μM. Under these conditions, the peptide concentrations at 50% inhibition, IC50, can be used to approximate Ki.7 IC50s were estimated from the plots of relative RNR activity vs. peptide concentration shown in Figure 7.4 and are listed in Table 7.1. These data show that the presence of the chromophore does not weaken binding. In fact, the Anq-containing peptides bind more
strongly by a factor of ~4 compared to the BPA containing peptides suggesting a hydrophobic interaction between the planar aromatic chromophore and the surface of α2. Furthermore, replacement of the F residue for Y at position 356 on peptides of the same length results in a doubling of the binding strength, suggesting a specific interaction between the hydroxyl group of Y356 and residues in α2.

Photoinitiated Nucleotide Reduction. Having established the Y• photogeneration and α2 binding, light-initiated nucleotide reduction assays were examined to test whether charge transport occurs across the non-covalent peptide/α2 complex. To quantitate the maximum amount of dCDP that could be formed under single turnover conditions, α2 was incubated with β2 and CDP substrate for 10 min. To determine if the peptide deactivates the enzyme in the dark, α2 was also incubated with the Ac-BPA-Y-R2C19 peptide in the dark for 2 min, followed by 10 min incubation with β2 and CDP substrate. Both experiments resulted in 2.1 eq of dC per dimer of α2 (Table 7.2); the photoassay data are therefore reported as a percent turnover per eq of monomer, α.

Photoinitiated nucleotide reduction was performed under conditions where all the α2 was bound to peptide. The calculation of the percent α2 bound treats the α2 dimer as two separate monomers, with each binding one peptide with the same Kd. By following the protocol of Climent and coworkers,7 the IC50 values in Table 7.1 may be used to approximate Kd for the peptide-α2 complex. Under the conditions of the single turnover assays for photoinitiated CDP reduction, α2 is 92% and 98% bound by Ac-BPA-Y-R2C19 and Anq-Y-R2C19 peptides, respectively.
The results of the photo-assay for α2:Ac-BPA-Y-R2C19 and α2:Anq-Y-R2C19 protein:peptide complexes are presented in Figure 7.5 and in Table 7.2. Turnover for wt-α2 was complete by the time the first data point was collected (2 minutes), and therefore data collected at subsequent times (i.e., 5 and 10 minutes) were invariant. Accordingly, the data for wt-α2 correspond to the average of 9 data points (3 × 2, 5, and 10 minutes). The Ac-BPA-Y-R2C19 and Anq-Y-R2C19 peptides turnover α2 under irradiation at 12.0 ± 0.6 % and 13.1 ± 0.6 %, respectively (indicated by the open bars in Figure 7.2), which are well above the background turnover (grey bars) that arises from β2 contamination and radiochemical

### Table 7.2. Single Turnover Photo-initiated CDP Reduction Assay Data.

<table>
<thead>
<tr>
<th>Sample Condition</th>
<th>Counts / cpm</th>
<th>dC / % per α</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromophore and Y356 Dependence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-BPA-Y-R2C19 + α2</td>
<td>hv</td>
<td>367 ± 17</td>
</tr>
<tr>
<td>Anq-Y-R2C19 + α2</td>
<td>hv</td>
<td>395 ± 16</td>
</tr>
<tr>
<td><strong>α2 Pathway Dependence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-BPA-Y-R2C19 + Y730F-α2</td>
<td>hv</td>
<td>55 ± 2</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-BPA-Y-R2C19 + α2</td>
<td>dark</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>Anq-Y-R2C19 + α2</td>
<td>dark</td>
<td>60 ± 3</td>
</tr>
<tr>
<td>Ac-BPA-Y-R2C19 + Y730F-α2</td>
<td>dark</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>α2</td>
<td>dark</td>
<td>57 ± 2</td>
</tr>
<tr>
<td><strong>Quantitation of Total α2 Turnovers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2 + β2</td>
<td>dark</td>
<td>5887b</td>
</tr>
<tr>
<td><strong>Inactivation of α2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-BPA-Y-R2C19 + α2</td>
<td>dark</td>
<td>3341d</td>
</tr>
<tr>
<td>Ac-BPA-Y-R2C19 + α2</td>
<td>hv</td>
<td>133.5d</td>
</tr>
</tbody>
</table>

*aUsing [14C]-CDP with activity of 5455 cpm/nmol unless otherwise noted. bUsing [3H]-CDP with activity of 11100 cpm/nmol. cα2 incubated with peptide for 2 min, followed by incubation with β2 and CDP for 10 min. dUsing [14C]-CDP with activity of 6330 cpm/nmol.

The results of the photo-assay for α2:Ac-BPA-Y-R2C19 and α2:Anq-Y-R2C19 protein:peptide complexes are presented in Figure 7.5 and in Table 7.2. Turnover for wt-α2 was complete by the time the first data point was collected (2 minutes), and therefore data collected at subsequent times (i.e., 5 and 10 minutes) were invariant. Accordingly, the data for wt-α2 correspond to the average of 9 data points (3 × 2, 5, and 10 minutes). The Ac-BPA-Y-R2C19 and Anq-Y-R2C19 peptides turnover α2 under irradiation at 12.0 ± 0.6 % and 13.1 ± 0.6 %, respectively (indicated by the open bars in Figure 7.2), which are well above the background turnover (grey bars) that arises from β2 contamination and radiochemical
impurities. In an attempt to understand why turnover was limited to ~13%, the activity of α2 was assayed before and after photolysis with the Ac-BPA-Y-R2C19 peptide. At 2 minutes, α2 photolyzed in the presence of peptide was found to be completely inactive for nucleotide reduction with β2 (Table 7.2), whereas the α2 from the dark reaction maintained activity up to the final time point of 10 minutes. SDS gels of the dark and light reactions (Figure 7.6) reveal that the α2 band from the light reaction smeared compared to the dark control, consistent with multiple cross-links of the peptide to α2 and degradation of the protein. These data indicate that either the 3BPA or radical photo-products can deactivate the enzyme by direct reaction with α2.

Figure 7.5. Light initiated, single turnover assays with Ac-BPA-(Y)-R2C19:α2, Anq-Y-R2C19:α2 and Ac-BPA-Y-R2C19:Y730F-α2. The bars refer to light (□) reactions and dark (■) controls. For 100% turnover, each dimer of α2 would produce 2 molecules of dCDP.

Figure 7.6. SDS gel with molecular weight markers (lanes 1 & 5), α2 + Ac-BPA-Y-R219 in the dark at 2, 5, and 10 minutes (lanes 2-4), and in the light (lanes 6-8).

The photoactivity of the α2:Ac-BPA-Y-R2C19 protein:peptide complex is perturbed significantly by the replacement of Y730 with phenylalanine. Assuming that the $K_d$ for the Y730F-α2 peptide complex is the same as with wt-α2, Y730F-α2 is 80% bound by the Ac-BPA-Y-R2C19 peptide under the conditions of the assay. However, the turnover measured for Y730F-α2 bound by Ac-BPA-Y-R2C19 after 2 minutes of photolysis is within the background levels of the assay (see Figure 7.5), indicating that the mutant is inactive towards photoinitiated dCDP production.
7.3 Discussion

The excited states of BPA and Anq are rapid (> $10^8$ s$^{-1}$) phototriggers of Y• when appended to the 20-mer peptide C-terminus of β2. The modified Y-R2C19 peptides bind to α2 with comparable or larger affinity than Y-R2C19 itself, suggesting a hydrophobic interaction between the chromophore and α2. Light excitation of the α2:X-Y-R2C19 (X = Ac-BPA or Anq) complex triggers RNR activity, as determined by single turnover assays of α2 in the presence of substrate and effector. In this way, the entire β2 subunit of the Class I enzyme can be replaced by a small, redox active peptide for •C439 radical generation in the active site, in much the same way as β2 is replaced by the small adenosylcobalamin cofactor in the Class II RNR enzyme.9

The BPA and Anq add to the growing repertoire of Y• phototriggers of RNR. Tryptophan may also effect light-initiated activity of RNR,10 however, the process does not occur through a discrete excited state. W• radicals are obtained through irreversible photo-ionization of W, which leads to peptide decomposition.11 The TA spectra shown in Figures 7.2 and 7.3 establish that Y• is transiently generated in BPA- and Anq-containing peptides and that the radical decays ($τ \sim 500$ ns) via charge recombination to reform the ground state products. Thus, in the absence of α2, the peptide is stable to excitation. However, we note that benzophenone is commonly used as a photoactive cross-linker for peptide and protein ligation.12 Peptide crosslinking to α2 would compete with Y oxidation on Y-R2C19 and may provide an explanation for the limiting turnover of 12 - 13%.

Radical transport from Y356 to C439 in the active site of α2 has been proposed to occur through Y731 and Y730 residues.13 The crystal structure of α2 (Figure 1.13) shows the Y731-Y730-C439 triad to be within hydrogen bonding contact distances, with Y731 at the surface of α2.14 Site directed mutagenesis studies showed the Y730F and Y731F-α2 variants to be catalytically inactive and structural studies15 of these mutants revealed that the π –π interaction was maintained upon mutation of either of the Y residues to F; thus the hydrogen bond network between Y730-Y731-C439 was proposed to be critical for radical transport in α2.16 Work with W as the photochemical radical generator on the Y-R2C19 peptide bound to α2 supported this contention, however turnover yields were too low for further analysis of the radical transport mechanism.10
The peptide region of β2 containing Y356 is particularly mobile in that it is not located in the crystal structure of β2 nor in the structure of α2 with the Y-R2C19 peptide bound. Thus the photo-oxidant or a mobile •Y356 residue photo-generated on the BPA/Anq-Y-R2C19 peptide bound to α2 could, in theory, interact with Y731. In either case, we can probe the mechanism of radical transport along the Y731-Y730-C439 pathway.

We consider three distinct mechanisms for radical transport in α2; these are summarized in Figure 7.7: (1) direct ET tunneling via superexchange coupling between C439 and •Y731; (2) ET hopping in which the electron hole tunnels from •Y731 → Y730 → C439, forming the protonated Y730•+ radical cation as an intermediate; and (3) PCET hopping, in which both an electron and a proton tunnel between the •Y731 → Y730 → C439 residues, forming the neutral •Y730 radical as an intermediate. The Y730F-α2 variant provides a convenient way to evaluate these potential mechanisms by independently tuning the electron and proton transfer distances. As described above, the X-ray crystal structure of the Y730F-α2 variant shows that mutation of Y730 to F730 interrupts the hydrogen bond network in α2 and significantly increases the proton tunneling distance. Y356 on the R2C20 peptide is not located in the structure and is represented here for illustrative purposes.

Figure 7.7. The crystal structure of the Y730F-α2 variant shows that mutation of Y730 to F730 interrupts the hydrogen bond network in α2 and significantly increases the proton tunneling distance. Y356 on the R2C20 peptide is not located in the structure and is represented here for illustrative purposes.
superexchange coupling since the distance between the aromatic residues is preserved. Accordingly, the rates for radical transport, and consequently turnover, would not be expected to differ greatly in wt-α2 or Y730F-α2. However, Figure 7.5 clearly shows that photoinitiated turnover (and thus radical transport) is perturbed in the mutant.

Mechanism (2) relies on ET hopping among aromatic residues, which can prevail for long distance charge transfer in proteins and DNA. For this case, the “hop” in the Y730F mutant entails ET tunneling from C439 to Y731. The hopping distance increases by ~2.5 Å with the replacement of Y730 with phenylalanine. Using a $\beta = 1.1 \text{Å}^{-1}$ for tunneling through a 3-ring pathway, the increased distance in Y730F-α2 should reduce the rate to ~6% that of wild type (assuming all parameters equal except for distance). Based on this ET-hopping radical transport rate, an overall activity of 0.8% would be expected. From the data in Figure 7.5 and Table 7.2, turnover with Y730F-α2 is $0.4 \pm 0.1\%$, which is less than that expected for a direct tunneling or hopping pathway. We note that the 0.1% error is the lower limit of our detection. As can be seen from the data for the α2 dark control experiment, the 0.4% activity includes counts from contaminating background radiation (most likely due to radiological impurities in CDP). If the activity is normalized to this background, the Y730F-α2 mutant is essentially inactive.

Of relevance to radical transport by Mechanism (3), the self-exchange reaction between phenoxy radical and phenol has recently been studied computationally. The reaction occurs within a hydrogen bonded complex and proceeds via a PCET mechanism in which the electron is transferred between orbitals of $\pi$ symmetry on the phenol and the proton is transferred between distinct orbitals of $\sigma$(O–H) symmetry. As has been discussed, since the electron and proton transport is of different origins, the PCET is not derived from a genuine hydrogen atom transfer but rather from the coupling between the electron and proton. Inasmuch as the electron and proton tunnel for such transfers, the reaction rates are predicted to be highly dependent on tunneling distance and, as has been emphasized, the decoupling of electron transfer and proton transfer distances is crucial to probing PCET mechanisms. The Y730F mutation increases the tunneling distance for the proton, assuming that non-crystallographically identified water molecules are not present in the structure, while maintaining the electron transfer distance. The O---S distance from Y731 to C439 is 5.9 Å in Y730F-α2, while in wt-α2 the longest distance between phenol-O and thiol-S
atoms is 3.4 Å. The attenuation of turnover is consistent with the interruption of the proton transfer pathway, as indicated in Figure 7.7.

The foregoing analysis points toward a proton-dependent hopping mechanism for radical transport in α2. Higher turnover numbers with wt-α2 compared to that for Y730F-α2 will be critical to further distinguishing between ET (Mechanism (2)) and PCET (Mechanism (3)) hopping mechanisms. We note that the active RNR complex may be an asymmetric dimer, and hence the activity of the peptide-α2 may be limited to 50% owing to half-site reactivity in α2. To overcome this complication, we will next focus on direct spectroscopic detection of radicals along the pathway in α2. To this end, the current BPA and Anq containing peptides set a benchmark inasmuch as Y• may be generated with excitation light that lies to the red spectral side of the protein absorption envelope, and, for BPA, the Y• absorption feature can be clearly detected. The disadvantages of the BPA and Anq chromophores lie in their complicated photochemistry and propensity towards protein crosslinking/degradation. Future experiments will take advantage of the lower energy metal-to-ligand charge transfer excited states of metal complexes discussed in Chapter IV that are competent for radical generation in conjunction with fluorotyrosine unnatural amino acids (Chapter VI).

7.4 Experimental Section

Materials. Anthraquinone-2-carboxylic acid (Anq-COOH), reduced β-nicotinamide adenine dinucleotide phosphate (NADPH), N-hydroxyurea, deoxycytidine (dC), cytidine-5′-diphosphate (CDP), trifluoroacetic acid (TFA), adenosine 5′-triphosphate (ATP), 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl), piperidine, diisopropylethylamine (DIPEA), triisopropylsilane and N-methylmorpholine (NMM) were purchased from Sigma-Aldrich. 4-Benzoyl-N-[9H-fluoren-9-ylmethoxy]carbonyl]-L-phenylalanine (Fmoc-BPA-COOH) and L-phenylalanine tert-butyl ester hydrochloride (F-OtBu•HCl) were from Advanced ChemTech. 1-Hydroxybenzotriazole (HOBt) and L-tyrosine tert-butyl ester (Y-OtBu) were obtained from NovaBiochem. O-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), O-(1,1-dimethylethyl)-N-[9H-fluoren-9-ylmethoxy]carbonyl]-L-tyrosine (Fmoc-Y(tBu)-OH), and N-[9H-fluoren-9-ylmethoxy]-carbonyl]-L-phenylalanine (Fmoc-F-OH) were purchased from Applied
biosystems. [2-14C]-CDP was purchased from Moravek Biochemicals and diluted to a specific activity of 5455 cpm/nmol. Calf-intestine alkaline phosphatase (20 U/μL) was purchased from Roche. All chemicals were used as received, except for piperidine, which was freshly distilled from KOH under N2 prior to use. 4-Benzoyl-L-phenylalanyl-tyrosine methyl ester trifluoroacetic acid (BPA-Y-OMe•TFA) was available from a previous study.29 E. coli thioredoxin (TR, SA of 40 U/mg), E. coli thioredoxin reductase (TRR, SA of 1800 U/mg) and E. coli β2 (SA of 6800 nmol/min mg) were isolated as previously described.30

Synthesis of Chromophore-(Y/F)LVGQIDSEVTDDLSNFQL [Chromophore-(Y/F)-R2C19]. Solid phase peptide synthesis (SPPS) using Fmoc-protected amino acids was employed to extend the Fmoc-R2C19-PEG-PS resin bound peptide, which was available from a previous study.31 For the syntheses of the chromophore-(Y/F)-R2C19 peptides, typically 125 mg of the Fmoc-R2C19-PEG-PS resin bound peptide (0.2 mmol/g) was loaded into a 10 mL Bio-Rad Poly-Prep column containing a porous 30 μm polyethylene bed in the bottom to hold the resin. The N-terminal Fmoc protecting group was cleaved by shaking the resin in a solution of 1.8 mL 0.1 M HOBt in 20% piperidine/DMF for 3 × 8 min using a FisherScientific Vortex Genie 2 (VWR). The resin was then washed with 3 × 2 mL of DMF and CH2Cl2. Fmoc-Y(tBu)-OH/ Fmoc-F-OH was then coupled to the free N-terminus by shaking the resin for 2 × 70 - 80 min in a solution of 0.5 M amino acid, 0.45 M HATU and 1 M DIPEA; the volume was adjusted accordingly such that the amino acid was in 6 - 8 fold excess. The chromophore-COOH (chromophore = Anq and Fmoc-BPA) compounds were then coupled in a similar manner with the following modifications. In the case of the Anq chromophore, the coupling solution consisted of 4 eq of Anq-COOH suspended/dissolved in 300 - 600 μL of DMF containing 0.45 M HATU and 1 M DIPEA due to limited solubility of the chromophore. In the case of Fmoc-BPA, after chromophore coupling, the Fmoc protecting group was cleaved as described above and the N-terminus acetylated by shaking the resin in a 0.5 M acetic anhydride, 0.5 M DIPEA in DMF for 1 hour. Cleavage of the peptide from the resin was carried out by shaking in 2.5 mL of 95% TFA, 2.5% triisopropylsilane, 2.5% water
for 4 h. The resin was then washed for 2 × 1 min with 2 mL of TFA. The cleavage cocktail and washings were combined, evaporated under a stream of N₂, and taken up in 15 mL of ether to precipitate the crude peptide, which was pelleted in a centrifuge and the ether decanted. The precipitate was dissolved in 0.1 M ammonium bicarbonate HPLC buffer via sonication over several minutes.

**Peptide Purification and Characterization.** Peptides were purified by reversed phase HPLC as previously described.¹⁰ The HPLC system consisted of a Waters 600 controller and a Waters 996 photodiode array detector, which were interfaced to and controlled by a computer using Waters’ Millenium 32 software. Samples were manually injected onto a semi-preparative Waters XTerra MS C-18 column (19 × 100 mm), which had been previously equilibrated with 10% acetonitrile/0.1 M ammonium bicarbonate (pH 8). A linear gradient of 10 → 65 % acetonitrile vs. ammonium bicarbonate over 45 min at a flow rate of 5 mL/min was used to elute the peptides. The eluant absorbance was monitored at the λ_max of the chromophore and at 210 nm, where the peptide amide bond absorbs. Fractions were collected by hand, lyophilized, taken up in 50 mM Tris buffer at pH 7.5, combined, and stored at –80 °C. Analytical HPLC was employed to confirm the purity of the peptides. The samples were manually injected onto an analytical Waters XTerra MS C-8 (4.6 × 100 mm) column and eluted with the same gradient used for purification at a flow rate of 1 mL/min. The molecular weight (MW) of the peptide was characterized by MALDI-TOF mass spectrometry as described below. The HPLC retention times (t_R) and MALDI-TOF m/z ratios for each peptide are listed in Table 7.1. Analytical HPLC traces for each peptide were recorded at 210 nm to confirm purity.

**Isolation, Purification and Pre-reduction of α2.** *E. coli* α2 was isolated by standard procedures.³⁰ To remove contaminating β2, it was further purified using a POROS HQ/20 anion exchange column (Applied Biosystems) on a BIOCAD Sprint Perfusion Chromatography System (Applied Biosystems). The column was loaded with α2 (10 - 15 mg) and washed with Tris buffer (50 mM, pH 7.6) for 5 min. α2 was eluted with a linear gradient
of 0 - 700 mM NaCl over 30 min at a flow rate of 4 mL/min. Fractions were collected by hand and concentrated on a YM-30 membrane (Millipore). This procedure reduced background turnover of CDP 7 - 8 fold. To pre-reduce α2, ~30 mg were incubated with 30 mM DTT for 30 min at room temperature. Hydroxyurea, ATP and CDP were added to final concentrations of 30 mM, 3 mM and 1 mM, respectively, and the incubation continued for an additional 20 min. Another 10 mM DTT was added, and the mixture incubated for 10 min and desalted on a G-25 Sephadex column (~35 mL, 1.5 × 23 cm) pre-equilibrated in 50 mM Tris, 15 mM MgSO₄, pH 7.6.

**α2 Activity Assay and Competitive Inhibition Assay for Binding of Peptide to α2.** α2 (0.1 μM, specific activity = 1900 nmol min⁻¹ mg⁻¹), β2 (0.2 μM, specific activity = 6800 nmol min⁻¹ mg⁻¹), TR (30 μM), TRR (0.5 μM), NADPH (0.2 mM), CDP (1 mM), and ATP (1.6 mM) were combined in 50 mM HEPES, 15 mM MgSO₄, 1 mM EDTA buffer at pH 7.6. Enzyme activity was measured by the consumption of NADPH, which was monitored by the decrease in absorbance at 340 nm. This was then repeated with peptide concentrations ranging from 2 - 80 μM.

**Single Turnover Assays for Photoinitiated Nucleotide Reduction in Peptide-α2 Complexes.** The assays were performed by a method modified from that previously reported. FPLC purified, pre-reduced α2 (20 μM, specific activity = 1800 nmol min⁻¹ mg⁻¹), peptide (200 μM), ATP (3.0 mM), and [2-¹⁴C]-CDP (1.0 mM, 5445 cpm nmol⁻¹) in 50 mM Tris, ~7 mM MgSO₄ buffer at pH 7.5 were added to a 1 cm quartz micro-cuvette with total solution volume of 200 μL. Care should be taken in preparation of the peptide stock solutions as they are sensitive to the concentration of MgSO₄ and the peptide can precipitate upon addition of solid MgSO₄ to the solutions. Typically, the peptide stock solutions were prepared in 50 mM Tris buffer (pH 7.5) and diluted into the reaction mixture containing millimolar concentrations of Mg²⁺. The reaction was initiated by the addition of [2-¹⁴C]-CDP (97 mM), quickly mixed, spun down in a minicentrifuge (< 10 s, 2000 g) to consolidate the liquid, and pipetted into the cuvette. Samples were irradiated at room temperature with the focused light
from a 1000 W Xe arc lamp equipped with a 299 nm long pass and IR filters. Fractions (60 μL) were removed at 2, 5 and 10 min intervals and immediately quenched by heating in a boiling water bath for 2 min. The precipitated protein was then spun down for 10 min in a minicentrifuge at 20,000 g. The supernatant (50 of the 60 μL) was transferred to a new Eppendorf tube, diluted with 14 units of alkaline phosphatase and 120 nmol of carrier dC to a final volume of 170 μL, and incubated at 37 °C for 2 h in a sealed Eppendorf vial. dC was then separated from C and quantitated as previously described. Each reaction was repeated twice along with a dark α2 control and a dark α2-peptide control to quantify background counts arising from contaminating β2 that co-purifies with α2 and from radiochemical impurities in the stock [2-14C]-CDP that elute with the product. Background counts were low in these experiments, as shown in Table 7.2. For reactions involving the Y730F-α2 mutant, 90 μM peptide and 20 μM protein were used, with all other conditions the same as for wt-α2.

**Physical Measurements.** 1H NMR spectra were recorded on a Varian Mercury 300 MHz NMR at the MIT Department of Chemistry Instrumentation Facility (DCIF) and externally referenced to tetramethylsilane. ESI-FT mass spectrometry was performed with a Bruker Daltonics APEXII instrument housed in the DCIF. MALDI-TOF mass spectrometry was performed with a Bruker Omniflex instrument in the DCIF using α-cyano-4-hydroxycinnamic acid as the matrix. The instrument was calibrated in positive ion mode with a quadratic polynomial using a mixture of angiotensin II (1046.5423), P14R synthetic peptide (1533.8582), and ACTH fragment 18-39 (2465.1989) (Sigma). All peptides synthesized were analyzed in negative ion mode due to the large number of carboxylate containing residues.

UV-vis absorption spectra were recorded on a Cary 17D modified by On-Line Instrument Systems (OLIS) to include computer control or a Spectral Instruments 440 spectrophotometer. TA measurements were made with pump light provided by the third harmonic (355 nm) of an Infinity Nd:YAG laser (Coherent) running at 20 Hz as previously described. In the case of the BPA, 300 nm pump light was used as previously described owing to the lack of
absorption at 355 nm for this chromophore. Transient absorption experiments for all compounds were performed in a 2 mm cuvette in 20 mM Tris buffer at pH 7.5 at 350 µM for Anq dipeptides/Y(F)-R2C19 peptides and 500 µM for the BPA dipeptides/Y-R2C19 peptide. Sample volumes of 200 µL were employed for all the peptide spectroscopy experiments reported herein. All experiments were performed under ambient conditions.
7.5 References


177
Chapter VIII

Turnover of α2 by Peptide-Based Photooxidants in the Absence of “Y356”
8.1 Motivation and Background

The experiments with the anthraquinone and benzophenone based photochemical RNRs prompt the question of whether the photooxidant on the Y-R2C19 peptide can interact with Y731 in α2 directly. To test this hypothesis, we repeated the photochemical RNR experiments with the (Ac-BPA/Anq)–F-R2C19 peptides, keeping in mind that these photooxidants can accept both a proton and an electron. We also developed peptides that could oxidize Y731 directly, but could only accept an electron. Pyrene (Py) functionalized peptides are ideal for this application, as this chromophore has a large molar absorptivity in the near UV/vis spectral region (to the red of the protein absorption envelope) and, under certain conditions, is capable of oxidizing Y. This chapter presents experiments with the BPA, Anq, and Py-modified R2C19 peptides that either contain F or the photooxidant itself in the place of “Y356” on the peptide. Photochemical assay results are presented for these peptides in complex with α2 in the presence of RNR substrate and effector, along with initial transient absorption (TA) experiments to investigate radical transport in the Py-R2C19:α2 complex.

8.2 Results

8.2.1 BPA and Anq Based Systems

We first wished to characterize the products produced upon photolysis of the (Ac-BPA/Anq)-F-R2C19 peptides. In Chapter VII we performed this experiment for the Anq-F-R2C19 peptide and found that the 3Anq excited state is capable of oxidizing other residues on the peptide, forming the •Anq− semiquinone radical anion. For the Ac-BPA-F-R2C19 peptide, photolysis with 300 nm initiates an irreversible photo-reaction that depletes the starting material; therefore a fresh sample of peptide was required for each experiment. Figure 8.1 shows the transient absorbance trace obtained immediately following (50 ns) the nanosecond laser pulse. The feature at 525 nm is
consistent with that previously observed for the triplet excited state of benzophenone \( (\varepsilon_{520\text{nm}}(^{3}\text{BP}) = 5800 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}) \).\(^1\) Single wavelength kinetics obtained at this wavelength (Figure 8.1, inset) can be fit to a single exponential decay with a time constant of 1.2 \( \mu \text{s} \), which is similar to the observed lifetime of triplet benzophenone in water.\(^2\)

As discussed in Chapter VII, the (Ac-BPA/Anq)-F-R2C19 peptides were observed to bind to \( \alpha_2 \) with micromolar affinities, thus allowing for formation of a peptide: \( \alpha_2 \) complex for photochemical RNR studies. Photoinitiated nucleotide reduction was therefore performed under identical conditions to that reported for the (Ac-BPA/Anq)-Y-R2C19 systems in Chapter VII: \( \alpha_2 \) was photolysed in the presence of 10-fold excess of peptide, 3mM ATP effector, and 1 mM [2-\(^{14}\text{C}\)]-CDP substrate. Figure 8.2 plots the percent of deoxycytidine that was formed in these experiments per \( \alpha \) subunit. For the Ac-BPA-F-R2C19: \( \alpha_2 \) system, turnover was complete after 2 minutes with 7.5 \( \pm \) 0.5 \% dC formed per \( \alpha \). Photolysis of the Anq-F-R2C19: \( \alpha_2 \) system resulted in a time-dependent turnover that generated up to 7.7 \( \pm \) 0.3 \% dC per \( \alpha \) after 10 minutes. The counts measured for radioactive dC product in these photolysis experiments was well above the background limit of detection, as determined by the Ac-BPA-F-R2C19: \( \alpha_2 \) and Anq-F-R2C19: \( \alpha_2 \) dark controls (0.2 \( \pm \) 0.1 and 0.1 \( \pm \) 0.1 \% dC per \( \alpha \), respectively). Thus the Ac-BPA-F-R2C19: \( \alpha_2 \) and Anq-F-R2C19: \( \alpha_2 \) systems are competent for photochemical RNR turnover, although not as efficient as their Y-containing counterparts.

8.2.2 Pyrene Based Systems

Synthesis. Py-R2C19 and Py-Y-R2C19 peptides were generated using solid-phase peptide synthesis to extend the Fmoc-R2C19-PEG-PS resin bound peptide with Fmoc-Y-OH (in the case of the latter) and pyrene-1-carboxylic acid as described for the Anq- and BPA-containing peptides in Chapter VII. The full length peptide was stable to the peptide cleavage and
purification conditions. Table 1 presents the analytical HPLC retention time and MALDI-TOF mass spectrometry characterization data for these peptides.

*Spectroscopy.* Py-Y-R2C19 was synthesized in hopes of characterizing photochemical Y• formation on the full length peptide using pyrene as the excited photooxidant. The fluorescence decay of Py-R2C19 (control peptide without tyrosine) was single exponential with $\tau_{\text{Py-R2C19}} = 14.7$ ns, while a biexponential function was needed to fit the fluorescence decay of Py-Y-R2C19, with $\tau_1(\text{Py-Y-R2C19}) = 4.3$ ns (65%) and $\tau_2(\text{Py-Y-R2C19}) = 9.3$ ns (45%). Both lifetime components are quenched compared to the Py-R2C19 control peptide; the shorter component is similar to the lifetime of the Py-Y dipeptide reported in Chapter III. We assign the biexponential emission lifetime of the Py-Y-R2C19 sample to two different conformations of the peptide in solution.

Transient absorption experiments of Py-Y-R2C19 were performed to probe the nature of the emission quenching in this peptide. Nanosecond TA required the use of low laser powers (50 $\mu$J per pulse, 355 nm) to avoid the formation of eximer excited states. Under these conditions, only weak absorptions corresponding to unquenched $^3\text{Py-Y-R2C19}$ (not shown) were observed following laser excitation, similar to that obtained for $^3\text{Py-Y-OH}$. Therefore, as with the Py-Y dipeptide, no products of the fluorescence quenching could be observed on the nanosecond timescale.

*RNR binding.* The competitive inhibition assay described in Chapter VII was used to characterize binding of the Py-R2C19 and Py-Y-R2C19 peptides to $\alpha_2$. Figure 8.3 plots the relative RNR activity vs. concentration of

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$t_R$ (min)$^a$</th>
<th>MW Calc’d (m/z)</th>
<th>MW Found (m/z)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Py-Y-R2C19</td>
<td>19.3</td>
<td>2498</td>
<td>2501</td>
</tr>
<tr>
<td>Py-R2C19</td>
<td>20.1</td>
<td>2334</td>
<td>2338</td>
</tr>
</tbody>
</table>

$^a$Retention time ($t_R$) from analytical HPLC trace. $^b$MALDI-TOF MS in negative ion mode

Figure 8.3. Plots of relative RNR activity vs. peptide concentration in the competitive inhibition binding assay for Py-Y-R2C19 (■) and Py-R2C19 (●).
peptide for the assay experiment with both Py-R2C19 and Py-Y-R2C19 peptides. We again observe an approximate two-fold increase in binding with the “Y356”-containing peptide (Py-Y-R2C19, \( IC_{50} \approx 3 \, \mu M \)) compared to the peptide without this residue (Py-R2C19, \( IC_{50} \approx 6 \, \mu M \)). As with the Anq-containing peptides in Chapter VII, the hydrophobic pyrene moiety results in a ~4 fold stronger binding affinity of the peptide to \( \alpha_2 \) compared to peptides without this chromophore.\(^3\,4\) Steady-state emission spectroscopy was also used to characterize binding of a pyrene-containing peptide to \( \alpha_2 \). Figure 8.4 plots the normalized emission spectrum of a 3 \( \mu M \) solution of Py-R2C19 in the presence and absence of 30 \( \mu M \) \( \alpha_2 \). In the absence of \( \alpha_2 \) the spectrum is broad and lacks the vibrational fine structure observed for the simpler “dipeptides” (Figure 3.4). Upon binding to \( \alpha_2 \), the vibrational fine structure reappears. Pyrene emission is known to be sensitive to environment polarity and, as such, has found application as a probe of protein-protein interactions.\(^5\) We therefore attribute the dynamics in Figure 8.4 to an ordering of the peptide structure upon binding to \( \alpha_2 \).

*Photoinitiated Nucleotide Reduction.* Since we could not observe \( Y^* \) photoproducts in the nanosecond transient absorption experiments with Py-Y-R2C19, we decided to generate the pyrene cation radical on Py-R2C19 and use this species as the radical initiator in the photochemical RNR experiment. As characterized by Figure 3.5, the Py\(^*\) radical can be formed upon pyrene excitation in the presence of a sacrificial electron acceptor. We used methyl viologen, MV\(^{2+}\), as the acceptor for the photoinitiated deoxynucleotide production.

**Figure 8.4.** Steady-state emission spectra of Py-R2C19 (3 \( \mu M \)) in the presence (▬) and absence (■) of \( \alpha_2 \) (30 \( \mu M \)).

**Figure 8.5.** Light initiated, single turnover assays with Py-R2C19 (200 \( \mu M \)), MV\(^{2+}\) (10 mM) and \( \alpha_2 \) (20 \( \mu M \)). The bars refer to light (□) reactions and dark (■) controls. For 100% turnover, each dimer of \( \alpha_2 \) would produce 2 molecules of dCDP.
Figure 8.5 plots the percent dC formed per α monomer for both light and dark reactions of Py-R2C19:α2 with 10 mM MV2+. The samples turned blue during photolysis indicating the presence of the reduced MV•+ radical. For the light induced reaction, we measured 14.5 ± 1.0 % turnover per α, which is well above the activity of the dark, control experiments (1.3 ± 0.7 % for Py-R2C19:α2 and 0.5 ± 0.1 % for α2 alone). This activity is also in the range of that measured for the Anq-(Y/F)-R2C19 and Ac-BPA-(Y/F)-R2C19 peptides.

**TA Studies of the Py-R2C19:α2 complex.** Nanosecond transient absorption spectroscopy was used to characterize the reaction of •Py-R2C19 + bound to α2. These experiments were conducted in 20 mM Tris buffer at pH 7.5 with 100 μM concentration of Py-R2C19 and α2, 1 mM CDP, 3 mM ATP, and 25 mM MV2+ as the sacrificial electron acceptor.

The left panel of Figure 8.6 plots the absorbance traces obtained 230 ns, 1, and 10 μs after the 355 nm nanosecond laser pulse in 20 mM Tris buffer at pH 7.5 with 100 μM of Py-R2C19, 25 mM MV2+, 1 mM CDP, and 3 mM ATP. The spectra show features at 396 and 455 nm, which are assigned to the electron transfer products MV•+ (ε396 nm = 42100 ± 800)⁶ and Py•+ (see Figure 3.5), respectively. The broad absorbance cut off at the red edge in this spectrum also corresponds to MV•+ (ε606 nm = 13700 ± 300).⁶ Single wavelengths kinetics recorded at 396 and 455 nm (Figure 8.6, left panel insets) can be fit to a single exponential decay with commensurate time constants (8.4 and 11.8 μs, respectively); this decay is...
therefore assigned to charge recombination between $\text{MV}^\bullet$ and $\text{Py}^\bullet$. The right panel of Figure 8.6 plots the absorbance traces for the same experiment, but in the presence of 100 μM $\alpha_2$. Again, absorption peaks corresponding to the $\text{MV}^\bullet$ and $\text{Py}^\bullet$ are observed; however these features do not decay concomitantly (Figure 8.6, right panel insets). The 455 nm, $\text{Py}^\bullet$ time-evolved absorbance can be fit to a bi-exponential decay with time constants, $\tau_1 = 2.5$ μs (58 %) and $\tau_2 = 10.5$ μs (42 %), while the $\text{MV}^\bullet$ time-evolved absorbance decays on a much slower timescale with bi-exponential time constants, $\tau_1 = 19$ μs (54 %) and $\tau_2 \sim 160$ μs (46 %).

8.3 Discussion

The results of Figure 8.2 show that the excited states of both BPA and Anq are capable of turning over $\alpha_2$ directly, without the participation of “Y356” on the peptide. We therefore propose that the BPA and Anq excited states oxidize Y731 directly in the $\alpha_2$; experiments with the Y731F-$\alpha_2$ variant will be needed to confirm this hypothesis. The $^3\text{BPA}$ excited state persists into the microsecond time regime, as shown by Figure 8.1, thus allowing this excited state plenty of time to oxidize Y731. For the Anq-F-R2C19 peptide, however, the $\text{•Anq}^-$ semiquinone radical anion is formed within the nanosecond laser pulse via oxidation of another, unidentified, residue on the peptide (Chapter VII). Therefore, oxidation of Y731 in $\alpha_2$ by $^3\text{Anq}$ must compete with this intra-peptide photochemistry (and reaction of $^3\text{Anq}$ with solvent) in order for this peptide to turnover $\alpha_2$. These competitive rates may account for the time-dependent turnover observed for this system (see Figure 8.2). The crystal structure $\alpha_2$ was obtained with the Y-R2C20 peptide bound, and shows significant ordering of the peptide for residues 360-375; however, the region containing Y356 is not located in the structure and is proposed to be thermally labile. From these observations we propose that the C-terminus of the (Ac-BPA/Anq)-F-R2C19 peptides bound to $\alpha_2$ are also thermally labile, and that intimate interaction of the photooxidant with Y731 may occur, at least transiently. Thus these excited states should be able to accept both a proton and an electron in forming $\text{•Y731}$. However, this process is not as efficient as oxidation of the proximate “Y356” in the (Ac-BPA/Anq)-Y-R2C19 peptides, as these systems generated higher percent turnover (Figure 7.5) than the F-containing constructs.
In contrast to Anq and BPA, the pyrene cation radical, $\bullet$Py$^+$-R2C19, can only accept an electron upon oxidation of amino acid residues. Nonetheless, Figure 8.5 shows that this oxidant is competent for initiating nucleotide reduction. These data are consistent with the conclusion based on work with $F_nY356$-$\beta_2$s that a proton is not required for radical transport at position 356 in $\beta_2$. The TA studies in Figure 8.6 (left) show that $\bullet$Py$^+$-R2C19 is formed within the nanosecond laser flash in the presence of 25 mM MV$^{2+}$ and persists into the tens of microseconds timescale, allowing this species plenty of time to oxidize residues in $\alpha_2$. The results of Figure 8.2 (right) confirm that the $\bullet$Py$^+$-R2C19 species is formed and decays much more rapidly when bound to $\alpha_2$, as compared to the species in free solution. We attribute this faster decay to oxidation of some residue in $\alpha_2$, however we cannot distinguish which one. Unfortunately, the absorption of reduced MV$^+$ radical overlaps the region where Y$^+$ would absorb (390-415 nm), and we do not observe buildup of any protein-based radicals. This complication, combined with the relatively low turnover for this construct, motivated us to search for other excited state oxidants that could turnover $\alpha_2$ at higher percent yields and would allow us to directly observe protein based radicals upon flash photolysis. The triplet MLCT excited states of Re(bpy)(CO)$_3$CN, when combined with the fluorotyrosine unnatural amino acids (Chapter VI), seemed to provide a reasonable construct for achieving these goals.

8.4 Experimental Section

**Materials.** Pyrene-1-carboxylic acid (Py-COOH), methyl viologen dichloride hydrate (MV$^{2+}$), trifluoroacetic acid (TFA), adenosine 5’-triphosphate (disodium salt from bacteria, ATP), cytidine 5’-diphosphate (sodium salt from yeast, CDP), piperidine, methyl viologen dichloride (MV$^{2+}$) and diisopropylethylamine (DIPEA), were purchased from Sigma-Aldrich. O-(7-Azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and O-(1,1-dimethylethyl)-N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-tyrosine (Fmoc-Y(tBu)-OH) were purchased from Applied Biosystems. [2-$^{14}$C]-CDP was purchased from Moravek Biochemicals and diluted to a specific activity of 5455 cpm/nmol. All chemicals were used as received, except for piperidine, which was freshly distilled from KOH under N$_2$ prior to use. Ac-BPA-F-R2C19 and Anq-F-R2C19 were prepared as described in Chapter VII. *E. coli* thioredoxin (TR, SA of 40 U/mg), *E. coli* thioredoxin reductase (TRR, SA of 1800 U/mg) and *E. coli* $\beta_2$ (SA of 6800 nmol/min mg) were isolated as previously described.
Synthesis of Pyrene-(Y)LVGQIDSEVDTDDLSNFQL [Py-(Y)-R2C19]. The pyrene-containing peptides were synthesized in a manner similar to the other R2C19 peptides described in Chapter VII. Briefly, solid phase peptide synthesis (SPPS) using Fmoc-protected amino acids was employed to extend the Fmoc-R2C19-PEG-PS resin bound peptide, which was available from a previous study. For the synthesis of the Py-Y-R2C19 peptide, the Fmoc-Y(\text{tBu})-OH was coupled to the resin-bound peptide as described in Chapter VII. The pyrene chromophore was incorporated into the peptide using pyrene-1-carboxylic acid. Py-COOH (25 mg, 4 eq) was combined with 125 mg of resin containing the free N-terminal, (Y(\text{tBu}))-R2C19 peptide, HATU (51 mg), DIPEA (53 \mu L), and 1.5 mL of DMF. The reaction mixture was shaken in a FisherScientific Vortex Genie 2 (VWR) for 80 min. The resin was rinsed with 2 mL of DMF (4 \times 1 min) and the coupling step was repeated. The resin was then rinsed with 2 mL DMF (4 \times 1 min) and 2 mL CH₂Cl₂ (3 \times 1 min) and dried in vacuo. The peptide was cleaved from the resin and worked up using the standard peptide cleavage cocktail and procedure described in Chapter VII.

Peptide Purification and Characterization. Both Py-R2C19 and Py-Y-R2C19 were purified and analyzed by reversed phase HPLC using methods similar to those described in Chapter VII. The molecular weight (MW) of the peptide was characterized by MALDI-TOF mass spectrometry as described in Chapter VII. The HPLC retention times (\(t_R\)) and MALDI-TOF m/z ratios for each peptide are listed in Table 8.1. Analytical HPLC traces for each peptide were recorded at 210 nm to confirm purity.

Isolation, Purification and Pre-reduction of \(\alpha_2\). \textit{E. coli} \(\alpha_2\) was isolated by standard procedures. To remove contaminating \(\beta_2\), it was further purified by the procedure described in Chapter VII. To pre-reduce \(\alpha_2\), ~30 mg were incubated with 30 mM DTT for 30 min at room temperature. Hydroxyurea, ATP and CDP were added to final concentrations of 30 mM, 3 mM and 1 mM, respectively, and the incubation continued for an additional 20 min. Another 10 mM DTT was added, and the mixture incubated for 10 min and desalted on a G-25 Sephadex column (~35 mL, 1.5 × 23 cm) pre-equilibrated in 50 mM Tris, 15 mM MgSO₄, pH 7.6.

\(\alpha_2\) Activity Assay and Competitive Inhibition Assay for Binding of Peptide to \(\alpha_2\). \(\alpha_2\) (0.1 \mu M, specific activity = 1900 nmol min⁻¹ mg⁻¹), \(\beta_2\) (0.2 \mu M, specific activity = 6800 nmol min⁻¹ mg⁻¹), TR (30 \mu M), TRR (0.5 \mu M), NADPH (0.2 mM), CDP (1 mM), and ATP (1.6
mM) were combined in 50 mM HEPES, 15 mM MgSO₄, 1 mM EDTA buffer at pH 7.6. Enzyme activity was measured by the consumption of NADPH, which was monitored by the decrease in absorbance at 340 nm. This was then repeated with peptide concentrations ranging from 1 - 40 μM.

**Single Turnover Assays for Photoinitiated Nucleotide Reduction in Peptide-α₂ Complexes.** The assays were performed by the method described in Chapter VII, except that samples were irradiated at room temperature with the focused light from a 1000 W Xe arc lamp equipped with a 299 nm (BPA and Anq peptides) or 338 nm (pyrene peptides) long pass and IR filters. dC was then separated from C and quantitated as previously described.¹¹,¹²

**Physical Measurements.** MALDI-TOF mass spectrometry was performed with a Brüker Omniflex instrument in the DCIF using α-cyano-4-hydroxycinnamic acid as the matrix. The instrument was calibrated in positive ion mode with a quadratic polynomial using a mixture of angiotensin II (1046.5423), P14R synthetic peptide (1533.8582), and ACTH fragment 18-39 (2465.1989) (Sigma). All peptides synthesized were analyzed in negative ion mode due to the large number of carboxylate containing residues.

UV-vis absorption spectra were recorded on a Spectral Instruments 440 spectrophotometer. TA measurements were made with pump light provided by the third harmonic (355 nm) of an Infinity Nd:YAG laser (Coherent) running at 20 Hz as previously described.¹³ In the case of the BPA, 300 nm pump light was used as previously described owing to the lack of absorption at 355 nm for this chromophore. Error! Bookmark not defined. Transient absorption experiments for all compounds were performed in a 2 mm cuvette in 20 mM Tris buffer at pH 7.5. Sample volumes of 200 µL were employed for all the peptide spectroscopy experiments reported herein. All experiments were performed under ambient conditions.
8.5 References

Chapter IX

The Search For Intermediates in Photochemical RNRs
with Re(bpy)CO$_3$CN as the Radical Initiator

Parts of this chapter have been submitted for publication:

9.1 Motivation and Background

Re(bpy)(CO)₃CN exhibits an excited state that is a competent oxidant of Y⁻ (Chapter IV). Tyrosinate oxidation occurs within the nanosecond laser pulse, the Re(bpy••)(CO)₃CN and Y• intermediates could be distinguished by transient absorption spectroscopy, and this charge separated state persisted into the hundreds of nanoseconds timescale. This photochemistry was extended in Chapter VI to fluorotyrosine unnatural amino acids; these tyrosine analogues exhibit significantly lower pKas allowing us to generate FnY• from FnY⁻ in the pH range where RNR is active (6-8.5). The confluence of these results led us to synthesize [Re]-FnY-R2C19 peptides ([Re] = Re(bpy)(CO)₃CN) for application to the photochemical RNR system ([Re]-3,5-F₂Y-R2C19 peptide is shown in Figure 9.1).

The absorption spectra of FnY• are significantly shifted from that of Y• to allow for visualization of the •FnY₃56→Y₇31 radical hop from the peptide into α2. Furthermore, the ³[Re]* excited should be selective for FnY⁻ oxidation; thus allowing us to avoid protein decomposition via spurious photochemical reactions. We now describe the synthesis of these peptides, a thorough characterization of the peptide based photochemistry via time-resolved emission and transient absorption (TA) spectroscopies, photoinitiated deoxynucleotide production in the photochemical RNR, and investigation of the radical generation and transport process in the peptide:α2 complex via nanosecond TA.

9.2 Results

Synthesis. The [Re]-(F/Y/3,5-F₂Y)-R2C19 peptides were made by extending the Fmoc-R2C19-PEG-PS resin bound peptide, first with Fmoc-(F/Y/3,5-F₂Y)-OH, followed by the carboxylic acid containing Re complex, Re(bpy–COOH)(CO)₃CN using Fmoc solid-phase peptide synthesis conditions similar to those previously described.¹ New HPLC methods were required to purify the [Re]-3,5-F₂Y-R2C19 peptide: the crude reaction product was separated by preparative reversed-phase HPLC using 10 mM KPi buffer at pH 6 with a 10→ 65% acetonitrile gradient over 45 minutes. The purified peptides were characterized by analytical HPLC and mass spectrometry. MALDI-TOF MS was the most convenient MS technique for peptide characterization, however, as is commonly observed for Re(bpy)(CO)₃X complexes,
the [Re] chromophore loses its cyanide ligand during laser ablation and positive ion analysis. The full length peptide with CN$^-$ ligand intact can be characterized by ESI-FT MS. Table 9.1 lists the analytical HPLC retention times and mass spectral data for each of the peptides synthesized.

Photophysics and Photochemistry. Steady state emission spectroscopy was used to investigate quenching of the $^3$[Re]$^*$ excited state in the [Re]-3,5-F$_2$Y-R2C19 peptide. Figure 9.2 plots the emission spectra obtained as the pH of the solution is titrated from 4.5 to 11.5. Between pH 4.5-5.0 the emission red-shifts and decreases in intensity. We attribute this spectral shift to a change in the peptide conformation as the carboxylic side groups of Asp and Glu residues are deprotonated. Between pH 5 and 11.5, the emission energy and band shape does not change, however we observe a decrease in overall intensity. This emission quenching is consistent with deprotonation of the 3,5-F$_2$Y phenolic proton (pKa = 7.2 for the Ac-3,5-F$_2$Y-NH$_2$ model compound)\textsuperscript{13}, which induces electron transfer from 3,5-F$_2$Y$^-$ to the proximate $^3$[Re]$^*$ excited state (see Chapter VI). By titrating the $^3$[Re]$^*$ emission intensity with pH, we obtain a pKa value of 3,5-F$_2$Y on the peptide of 7.3 ± 0.1.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$t_R$ (min)$^a$</th>
<th>MW Calcd (m/z)</th>
<th>MW Found (m/z)</th>
<th>$IC_{50}^d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Re]-F-R2C19</td>
<td>25.5</td>
<td>2748</td>
<td>2721$^b$</td>
<td>-</td>
</tr>
<tr>
<td>[Re]-Y-R2C19</td>
<td>23.8</td>
<td>2764</td>
<td>2737$^b$</td>
<td>8</td>
</tr>
<tr>
<td>[Re]-3,5-F$_2$Y-R2C19</td>
<td>19.5</td>
<td>2800</td>
<td>1399$^c$, 2772$^b$</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$Retention time ($t_R$) from analytical HPLC trace. $^b$[M-CN]$^+$ ion from MALDI-TOF MS. $^c$[M-2H]$^{2-}$ ion from ESI-FT MS. $^d$Concentration of peptide at 50% RNR inhibition.

Figure 9.2. Emission spectra of [Re]-3,5-F$_2$Y-R2C19 as a function of pH, which was titrated from 4.5 to 11.5. Inset: pH Titration curve of integrated emission intensity.
We next used time-resolved emission and transient absorption spectroscopy to characterize the excited states produced in the \([\text{Re}]-\text{(F/Y/3,5-F}_2\text{Y)}\)-R2C19 peptides. The emission decay of \([\text{Re}]-3,5-\text{F}_2\text{Y-R2C19}\) at pH 10 could be fit to a bi-exponential decay function with \(\tau_1 = 1.9\) ns (66%) and \(\tau_2 = 10.7\) ns (34%). Similarly, the \([\text{Re}]-\text{Y-R2C19}\) emission decay at pH 12 exhibited bi-exponential behavior (\(\tau_1 = 0.5\) ns (86%) and \(\tau_2 = 3.0\) ns (14%). Both lifetime components for both peptides are quenched compared to the emission lifetime of \([\text{Re}]-\text{F-R2C19}\) at all pHs or \([\text{Re}]-\text{Y-R2C19}\) at pH < 10 (\(\tau = 50 \pm 2\) ns or 62 \(\pm 2\) ns in 20% glycerol). As with the Py-Y-R2C19 peptide (Chapter VIII), we attribute the bi-exponential emission decay to two separate conformations of the R2C19 peptide in solution.

Figure 9.3. TA spectra of 100 \(\mu\)M solutions of \([\text{Re}]-\text{F-R2C19}\) (left) and \([\text{Re}]-\text{Y-R2C19}\) (right) in 50 mM Tris buffer (pH 7.6), 20% glycerol, 1 mM CDP, 3 mM ATP, and 15 mM MgSO\(_4\) recorded at 65, 115, and 215 ns following a 355 nm laser pulse. Insets: Single wavelength kinetics traces (○) with single exponential fit (▬) recorded at 480 nm.

We next used time-resolved emission and transient absorption spectroscopy to characterize the excited states produced in the \([\text{Re}]-\text{(F/Y/3,5-F}_2\text{Y)}\)-R2C19 peptides. The emission decay of \([\text{Re}]-3,5-\text{F}_2\text{Y-R2C19}\) at pH 10 could be fit to a bi-exponential decay function with \(\tau_1 = 1.9\) ns (66%) and \(\tau_2 = 10.7\) ns (34%). Similarly, the \([\text{Re}]-\text{Y-R2C19}\) emission decay at pH 12 exhibited bi-exponential behavior (\(\tau_1 = 0.5\) ns (86%) and \(\tau_2 = 3.0\) ns (14%). Both lifetime components for both peptides are quenched compared to the emission lifetime of \([\text{Re}]-\text{F-R2C19}\) at all pHs or \([\text{Re}]-\text{Y-R2C19}\) at pH < 10 (\(\tau = 50 \pm 2\) ns or 62 \(\pm 2\) ns in 20% glycerol). As with the Py-Y-R2C19 peptide (Chapter VIII), we attribute the bi-exponential emission decay to two separate conformations of the R2C19 peptide in solution.

Nanosecond TA spectroscopy was used to characterize the products of the emission quenching. Figure 9.3 plots the TA spectra recorded 65, 115, and 215 ns following excitation of 100 \(\mu\)M solutions of \([\text{Re}]-\text{F-R2C19}\) (left) and \([\text{Re}]-\text{Y-R2C19}\) (right). The spectra contain features at 380 and 480 nm, which decay with a time constant (64 \(\pm 1\) ns) identical to that of the excited state emission decay. Moreover, the spectra resemble that previously assigned for the \(^3[\text{Re}]^*\) MLCT excited state in model complexes with Y and F amino acids.\(^{14}\) We therefore attribute these spectra to the \(^3[\text{Re}]^*\) MLCT excited state on the peptide. Figure 9.4 presents the TA spectra of 100 \(\mu\)M solutions of \([\text{Re}]-3,5-\text{F}_2\text{Y-R2C19}\). The left panel shows the spectrum obtained for a pH 8.5 solution (deprotonated 3,5-\text{F}_2\text{Y}^-), and exhibits features at 395 and \(~525\) nm following the 355 nm laser pulse. This spectrum resembles that of the \(\bullet[\text{Re}]-3,5-\text{F}_2\text{Y}\bullet\)
The left inset plots the single wavelength absorbance kinetics obtained at 525 nm; the spectrum decays with a time constant of 70 ns, corresponding to a rate of $1.7 \times 10^7$ s$^{-1}$ for charge recombination on the full length peptide. The 3,5-F$_2$Y$^•$ radical can be trapped by oxidizing the •[Re]–-3,5-F$_2$Y$^•$ charge separated state in a bimolecular fashion with a reagent in solution. The right panel of Figure 9.4 plots the TA spectrum following 355 nm excitation of a solution of [Re]-3,5-F$_2$Y-R$_2$C$_{19}$ at pH 8.5 in the presence of 10 mM Ru(III)(NH$_3$)$_6$$^{3+}$ quencher. A clear “double-hump” feature is present with $\lambda_{max} = 395$ nm, which is characteristic of the absorption spectrum of 3,5-F$_2$Y$^•$. The spectrum decays with a time constant of 9.6 μs, as determined by the single wavelength absorbance kinetics at 395 nm (Figure 9.4, right inset). Thus by using the Ru(III) quencher, we can extend the lifetime of the 3,5-F$_2$Y$^•$ on the peptide well into the microsecond time regime.

Binding to the α$_2$ subunit of RNR. Binding of the [Re]-Y-R$_2$C$_{19}$ peptide to α$_2$ was first probed by the standard competitive inhibition assay. Figure 9.5 plots the relative RNR activity vs. the concentration of peptide competitive inhibitor in solution. From these data, we estimated that the peptide binds to α$_2$ with an approximate $IC_{50}$ of 8 μM. Under these conditions the $IC_{50}$ can be used to approximate the dissociation equilibrium constant, $K_D$. Thus, the larger [Re] complex does not inhibit binding of the peptide to α$_2$; in fact, the binding is of comparable strength to other hydrophobic pyrene- and anthraquinone-terminated
peptides used in this thesis (Chapters VII and VIII) and is stronger than the Y-R2C19 peptide alone.

Since the [Re] chromophore is emissive and $^3$MLCT excited states are sensitive to solvent and environment polarity, we can use the emission from the $^3[\text{Re}]^*$ excited state as a probe of peptide binding to $\alpha_2$. Figure 9.6 plots the emission spectra of a 5 $\mu$M solution of the [Re]-Y-R2C19 peptide as the $\alpha_2$ concentration is increased from $0\rightarrow50$ $\mu$M. Upon binding to $\alpha_2$, the emission band blue shifts and increases in intensity. This is consistent with the exclusion of water from the coordination sphere of the [Re] chromophore upon binding to a hydrophobic region of the protein.$^6,^7$ The emission maxima were plotted as a function of $\alpha_2$ concentration (Figure 9.6, inset) and fit to the binding model in eq 2, yielding a dissociation constant, $K_D$, of 4 $\mu$M, which, given the error associated with the emission measurement, is comparable to the value obtained with the competitive inhibition binding assay. Time resolved emission was then used to further probe the homogeneity of the peptide:$\alpha_2$ binding interaction. For this experiment, 5 $\mu$M peptide and 20 $\mu$M $\alpha_2$ (with 3mM ATP and at pH 7.5) were used to ensure that most of the peptide was bound. Under all conditions investigated, the time resolved $^3[\text{Re}]^*$ emission recorded at 600 nm was best fit with a bi-exponential decay function. One emission decay component was set equal to that of the free peptide while the other emission component was allowed to vary. In 20% glycerol, we obtained decay components of 60 ns (75%) and ~200 ns (25%), which did not change upon addition of 1 mM CDP substrate. These data reveal that the conformation of the peptide bound to $\alpha_2$ is heterogeneous, with at least two major binding modes. For one binding mode, the [Re] chromophore is solvated similar to that in free solution, while the other binding mode partially desolvates the [Re] chromophore resulting in a blue-shifted emission energy with longer emission lifetime.

**Figure 9.6.** Emission spectra of [Re]-Y-R2C19 (5 $\mu$M) at pH 7.5 as the $\alpha_2$ concentration is increased from $0\rightarrow50$ $\mu$M. Inset: Emission maximum as a function of $\alpha_2$ concentration (○) with fit (▬) to binding model in eq 2.
We next used the [Re] emission as a probe of the pKa of the 3,5-F2Y on the peptide bound to α2. Figure 9.7 plots the emission intensity of a 5 μM solution of [Re]-3,5,-F2Y-R2C19 in the presence of 20 μM α2 as the pH is increased from 6.3 to 9.0. The red spectra in the figure correspond to the emission of 5 μM [Re]-3,5,-F2Y-R2C19 in the absence of α2 at pH 6.3 (lower spectrum) and 9.0 (upper spectrum). These spectra illustrate that binding of 5 μM peptide to 20 μM α2 causes as blue shift and an increase in the \(^3\text{[Re]}^*\) emission intensity, both at pH 6.3 and 9.0. As the pH is titrated from 9.0 to 6.3 for the solution containing the bound peptide, the \(^3\text{[Re]}^*\) emission intensity decreases with an apparent pKa of 7.3 ± 0.1.

The same pKa was obtained whether the integrated emission or emission maximum (at 600 or 650 nm) was plotted vs. pH. As was observed in Figure 9.2, deprotonation of the phenol of 3,5-F2Y initiates electron transfer between 3,5-F2Y– and \(^3\text{[Re]}^*\) resulting in a decrease in emission intensity. We thus attribute the emission quenching in Figure 9.7 to electron transfer between \(^3\text{[Re]}^*\) and 3,5-F2Y– on the peptide bound to α2. Thus the pKa of the bound 3,5-F2Y does not shift significantly from that of the peptide in free solution. These data show that we should be able to make 3,5-F2Y• radicals bound to α2 to photochemically initiate deoxynucleotide production.

![Figure 9.7. Emission spectra of [Re]-3,5-F2Y-R2C19 (5 μM) and α2 (20 μM) as the pH is titrated from 9.0 to 6.3. The red spectra were recorded at pH 6.3 (lower spectrum) and pH 9.0 (upper spectrum) in the absence of α2. Inset: Integrated emission intensity as a function of pH (○) with fit (▬) to monoprotic titration curve.]

Stability of α2 under photolysis conditions and Inactivation of α2 with Ru(III)\((\text{NH}_3)_6^{3+}\). We developed new photolysis conditions for the photochemical RNRs reported in this chapter, therefore we wanted to test the stability of α2 during the experiment. We also considered using

![Figure 9.8. Amount of dCDP produced per α2 under single turnover conditions after incubation with the labeled conditions. Photolysis was performed with \(\lambda>299\) nm in a temperature controlled (25°C) bath.]

197
Ru(III)(NH₃)₆³⁺ as a bimolecular oxidant of the •[Re]-3,5-F₂Y• charge separated state on the peptide bound to α₂, and therefore we investigated the stability of α₂ to this compound. Figure 9.8 plots the number of equivalents of dCDP produced after incubation of α₂ with 3 mM ATP and 0.75 mM [2-¹⁴C]-CDP under the labeled conditions, followed by incubation with β₂ for 10 minutes. The bars represent an average of two data points, one obtained at 2 and one at 5 minutes. α₂ incubated in the dark produces 2.0 equivalents of dCDP per dimer, which decreases slightly under photolysis conditions (1.8 eq per α₂). Incubation of α₂ with 25 mM Ru(III)(NH₃)₆³⁺ results in further decrease in α₂ turnover (1.5 eq) which is enhanced under photolysis (1.0 eq). The ammonia ligands of Ru(III)(NH₃)₆³⁺ are somewhat labile⁸ and may exchange for thiolates of cysteine residues in α₂. Binding of active site thiolates in α₂ to Ru(III) would prevent nucleotide reduction and effectively inactivate α₂.

**Photochemical RNR turnover.** Figure 9.9 plots the turnover achieved upon photolysis of 200 μM [Re]-(F/Y/3,5-F₂Y)-R2C₁₉ peptide with 20 μM α₂ at pH 7.5 and 8.2 in 20% glycerol in the presence of 0.75 mM [5-³H]-CDP substrate and 3 mM ATP effector under single turnover conditions. Data were collected after 2, 5, and 10 minutes using light of λ > 348 nm and are an average of two separate experiments. The data were also corrected for background counts from radiochemical impurities, which were assessed by [Re]-F-R2C₁₉ + α₂ dark.

![Figure 9.9. Single turnover assays for α₂ with [Re]-(F/Y/3,5-F₂Y)-R2C₁₉ peptides in 20% glycerol. Data was collected after 2, 5, and 10 minutes of photolysis with light of λ > 348 nm. Red and blue bars are at pH 8.2 and 7.5, respectively.](image-url)
controls. From the plot in Figure 9.8, we see that the [Re]-F-R2C19:α2 system is essentially inactive towards photochemical nucleotide reduction. The [Re]-Y-R2C19:α2 and [Re]-3,5-F₂Y-R2C19:α2 systems, however, exhibit a time-dependent photochemical RNR activity. Higher percent turnover was measured at pH 7.5 compared to pH 8.2 for both systems. The [Re]-3,5-F₂Y-R2C19:α2 construct approaches 30% turnover at 10 minutes, which is the highest turnover recorded for any photochemical RNR to date. Adding glycerol to the solution increased the overall turnover yield, along with the reproducibility of the experiment.

Transient Spectroscopy of the Peptide:α2 Complex. Since the [Re]-Y-R2C19 peptide is active for photochemical deoxynucleotide reduction, we wondered if we could observe the [Re]₀-Y• charge separated state upon binding this peptide to α2, instead of the ³[Re]* excited state observed for the peptide free in solution (Figure 9.3). Figure 9.10 plots the TA spectra recorded for 100 μM solutions of [Re]-F-R2C19 (left) and [Re]-Y-R2C19 (right) in the presence of 135 μM α2, 1 mM CDP, and 3 mM ATP in 20% glycerol at pH 7.5. The spectra in both panels resemble those of the ³[Re]* excited state (see Figure 9.3). For both peptides, the absorbance decay at 480 nm could be fit to a bi-exponential decay function with τ₁ ≡ 62 ns (55%) and τ₂ = 150 ns (45%) for the [Re]-F-R2C19:α2 system and τ₁ ≡ 62 ns (62%) and τ₂ = 185 ns (38%). These data are consistent with previous results discussed above that suggest the peptide binds to α2 in a heterogeneous fashion. In search for a signals corresponding to Y• and bpy•⁻ that would be formed in the event of radical formation on the [Re]-Y-R2C19
peptide, the spectra for both peptides at a common time point were normalized and the signal for [Re]-F-R2C19 subtracted from that of [Re]-Y-R2C19. In no case could a clear absorption feature be distinguished between 380 and 420 nm (Y•) or at 525 nm (bpy•−).

We next investigated the [Re]-3,5-F2Y-R2C19 peptide bound to variants of α2 by TA spectroscopy. The Y731F-α2 variant provides a control with which we can isolate the photochemistry to the peptide (radical transport from the peptide into α2 should not occur with F at position 731). Figure 9.11 plots the TA spectra recorded for a 100 μM solution of [Re]-3,5-F2Y-R2C19 in the presence of 140 μM Y731F-α2, 1 mM CDP, and 3 mM ATP in 20% glycerol at pH 8.2. Spectra were recorded at 65, 115, and 215 ns. Inset: Single wavelength absorbance kinetics recorded at 395 nm.

By relieving the Y731F mutation, we wondered if we would see a disappearance of the 395 nm signal (3,5-F2Y•) and an increase in signal corresponding to Y• (~410 nm), as would be the result for radical injection into α2. Figure 9.12 plots the TA spectra obtained upon excitation of a 100 μM solution of [Re]-3,5-F2Y-R2C19 in the presence of 216 μM α2.
1 mM CDP, and 3 mM ATP. As with the Y731F-α2 variant, the spectrum at the earliest time point (65 ns) resembles an admixture of the $^{3}[\text{Re}]^*$ excited state and the $[\text{Re}]^{0-3,5}\text{F}_2\text{Y}^•$ charge separated state, except that the peaks at 380 and 480 nm ($^{3}[\text{Re}]^*$) are more pronounced, the 525 nm (bpy$^•^-$) and 395 nm (3,5-F$_2\text{Y}^•$) features are decreased in intensity. These data are consistent with inefficient quenching of the $^{3}[\text{Re}]^*$ excited state by the 3,5-F$_2\text{Y}$ residue upon binding of the peptide to α2. This effect is more pronounced for wt-α2 than for Y731F-α2.

To simplify the spectra and maximize our ability to visualize 3,5-F$_2\text{Y}^•\rightarrow$$\text{Y731}$ radical transport, we decided to use a bimolecular quencher to oxidize both the unquenched $^{3}[\text{Re}]^*$ excited state and the $[\text{Re}]^{0}$ species in the $[\text{Re}]^{0-3,5}\text{F}_2\text{Y}^•$ charge separated state. Oxidation of $^{3}[\text{Re}]^*$ would produce $[\text{Re}]^{II)^+}$, which is a strong oxidant$^{14}$ and should rapidly oxidize 3,5-F$_2\text{Y}$. Also, by mutating Y730 to F and C439 to S, we wondered if the 3,5-F$_2\text{Y}^•$ produced on the

![Figure 9.13](image)

**Figure 9.13** TA spectra of a 100 μM solution of [Re]-3,5-F$_2\text{Y}$-R2C19 with 10 mM Ru(III)(NH$_3$)$_6^{3+}$ (upper left) and 200 μM α2 (upper right), 175 μM Y730F-α2 (lower left), or 175 μM C439S-α2 (lower right). Conditions: 1 mM CDP, 3 mM ATP, 15 mM MgSO$_4$ and 50 mM Tris buffer at pH 8.3. Spectra were recorded at (▬) 415 ns, (▬) 1, (▬) 2, and (▬) 10 μs.
peptide would oxidize Y731 and become trapped in α2. Figure 9.13 displays the spectra produced upon excitation of a 100 μM solution of [Re]-3,5-F2Y-R2C19 with 10 mM Ru(III)(NH3)63+ (upper left) and 200 μM α2 (upper right), 175 μM Y730F-α2 (lower left), or 175 μM C439S-α2 (lower right). In all cases, the spectra resemble that of the 3,5-F2Y• radical displayed in Figure 9.4 (right) with a peak at 395 nm. We next compared the spectra obtained at the earliest time point (415 ns) for each of the systems. Figure 9.14 presents an overlay of these spectra normalized to the peak at 395 nm. The band shape of the peak is the same for every peptide:α2 system, and in no case do we observe a shoulder to the red, as would be expected for observation of a tyrosyl radical in α2.

9.3 Discussion

The results described above show that the [Re]-Y-R2C19 peptide binds to α2 with micromolar affinity, but that the peptide:α2 complex is heterogeneous in solution with at least two different binding modes of the peptide to the protein. Binding of the peptide inhibits RNR activity (Figure 9.5) and affects the steady state emission of the 3[Re]* excited state (Figure 9.6), however, analysis of the 3[Re]* time-resolved emission reveals at least two environments of the chromophore with different solvation/polarity. The X-ray structure of α2 (Figure 9.15) was obtained with the Y-R2C19 peptide bound, however the C-terminal residues (356-360) were not located in the
structure. Together, these data support a model in which the C-terminus of the bound peptide is thermally labile. However, there must be transient interactions between the “Y356” residue on the peptide with α2 that result in the stronger binding of Y-R2C19 peptides compared to F-R2C19 (see Chapter VII).

The photochemical RNR assays with the [Re]-F/Y/3,5-F2Y-R2C19 peptides reveal that radical generation on the peptide is required for turnover in systems based on the [Re] chromophore. The [Re]-F-R2C19 peptide is inactive towards deoxynucleotide production, which is in contrast to the Ac-BPA-, Anq-, and Py•+-based peptides described in Chapter VIII. Both the Py•+ and 3[Re]* oxidants can only accept an electron in oxidation of Y, however the 3[Re]* lifetime only persists into the hundreds of nanoseconds timescale when bound to α2, compared to Py•+ which lasts into the microsecond timescale. We therefore surmise that the relatively short lifetime of the 3[Re]*, in combination with its ability to serve as only an ET oxidant, prevent the excited 3[Re]*-F-R2C19 peptide from oxidizing Y731 directly and turning over α2.

The fact that the [Re]-Y-R2C19 peptide is competent for photochemical RNR turnover is somewhat surprising, given that we see no evidence for [Re]0-Y• formation in the TA studies of the peptide free in solution (Figure 9.3) or bound to α2 (Figure 9.10). Either a small amount of [Re]0-Y• is formed upon excitation of [Re]-Y-R2C19 in solution, or a proton acceptor exists in one of the binding modes of the peptide:α2 complex that is hydrogen bonded to Y356, thereby facilitating its oxidation as observed in Chapter V with buffers bound to Y in solution. In either case, the concentration of this species is too small to be detected by the spectroscopic methods employed above.

Excitation of the [Re]-3,5-F2Y-R2C19:α2 complex results in ~30% deoxynucleotide formation per α. The active RNR complex has been proposed to be an asymmetric dimer10,11 and hence the activity of the peptide:α2 complex may be limited to 50% owing to half-site reactivity in α2. If this is indeed the case, then we are approaching the single turnover limits with the [Re]-3,5-F2Y-R2C19 peptide. This chromophore-fluorotyrosine combination also allows us to visualize, for the first time, a tyrosyl radical on the peptide bound to α2 with TA spectroscopy (Figures 9.11 and 9.13). To visualize radical injection into α2, we would expect to see a decrease in the TA signal at 395 nm (3,5-F2Y•), concomitant with an increase in the signal at ~410 nm (•Y731 or •Y730). The Y730F and C439S mutants were designed to
prevent further radical transport and deoxynucleotide production, therein trapping the radical at Y731. Unfortunately, as evidenced by Figures 9.13 and 9.14, we see no evidence for a shoulder at 410 nm on the 395 nm, 3,5-F₂Y• peak. Even at longer times (10 μs), after decay of most of the 3,5-F₂Y• on the peptide in Figure 9.13, a clear feature at 410 nm for trapped •Y731 in the Y730F and C439S mutants cannot be resolved. From these data, we conclude that the conformation of the peptide:α2 complex that supports photochemical RNR activity is in equilibrium with other, non-productive peptide:α2 conformations. Under steady state photolysis, this conformation may be sampled many times for RNR turnover, but in the single laser-pulse TA experiment, the concentration of the productive conformation is too low for visualization of radical transport using our current TA methods.

9.4 Experimental Section

Materials. Trifluoroacetic acid (TFA), adenosine 5’-triphosphate (disodium salt from bacteria, ATP), cytidine 5’-diphosphate (sodium salt from yeast, CDP), piperidine, and diisopropylethylamine (DIPEA), were purchased from Sigma-Aldrich. O-(7-Azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), N-[(9H-fluoren-9-ylmethoxy)-carbonyl]-L-phenylalanine (Fmoc-F-OH) and O-(1,1-dimethylethyl)-N-[(9H-fluoren-9-ylmethoxy)carbonyl]-L-tyrosine (Fmoc-Y(tBu)-OH) were purchased from Applied Biosystems. 1-Hydroxybenzotriazole (HOBt) was obtained from NovaBiochem. [5-³H]-Cytidine diphosphate was purchased from Amersham and purified as described below. Calf-intestine alkaline phosphatase (10 U/μL) was purchased from New England Biolabs. [2-¹⁴C]-CDP was purchased from Moravek Biochemicals and diluted to a specific activity of 6330 cpm/nmol. Piperidine was freshly distilled from KOH under N₂ prior to use. Fmoc-3,5-F₂Y was made in two steps from 2,6-difluorophenol, pyruvate, and ammonia using pyruvate formate lyase as previously described.¹²,¹³ Re(bpy-COOH)(CO)₃CN was prepared as described in Chapter IV.¹⁴

Purification of [5-³H]-CDP. 250 μCi of [5-³H]-CDP (Amersham, 24 μCi/nmol, 97.9% radiochemical purity) was diluted to a specific activity of ~0.018 μCi/nmol with cold cytidine diphosphate (Sigma). The diluted stock solution was then purified over a diethylaminoethyl (DEAE)-Sephadex A25 column. The resin was purchased in the chloride form from Sigma-Aldrich, swelled in 10 mM triethylammonium bicarbonate (TEAB) solution, and poured into a 60 mL column. The resin was washed with 5 column volumes of 1M TEAB solution,
followed by five column volumes of deionized water. The stock [5-\textsuperscript{3}H]-CDP solution was then slowly loaded onto the column and eluted with a 0→600 mM TEAB gradient (450 mL × 450 mL). Fractions (400 drops) were collected automatically by a fraction collector and every other fraction analyzed by a 96 well plate UV-vis absorption spectrometer at 260 and 280 nm. Figure 9.16 plots the absorption trace for this column separation; fractions were pooled between the two horizontal lines (49-56). The solvent and TEAB were removed \textit{in vacuo}, and the resulting product dissolved in deionized water. The entire column separation procedure was repeated, yielding a 20 mM stock solution with 11,100 Cpm/nmol (99.95 \% radiochemical purity as assayed by the C/dC ion exchange separation procedure\textsuperscript{15,16}).

**Synthesis of [Re]-(F/Y/3,5-F\textsubscript{2}Y)LVGQIDSEVDTSDDLNSFQL [[Re]-(F/Y/3,5-F\textsubscript{2}Y)-R2C19].** Solid phase peptide synthesis (SPPS) using Fmoc-protected amino acids was employed to extend the Fmoc-R2C19-PEG-PS resin bound peptide, which was available from a previous study.\textsuperscript{17} For the syntheses of the [Re]-(F/Y/3,5-F\textsubscript{2}Y)-R2C19 peptides, typically 250 mg of the Fmoc-R2C19-PEG-PS resin bound peptide (0.2 mmol/g) was loaded into a 10 mL Bio-Rad Poly-Prep column containing a porous 30 μm polyethylene bed in the bottom to hold the resin. The N-terminal Fmoc protecting group was cleaved by shaking the resin in a solution of 3.6 mL 0.1 M HOBt in 20% piperidine/DMF for 3 × 8 min using a FisherScientific Vortex Genie 2 (VWR). The resin was then washed with 3 × 4 mL of DMF and CH\textsubscript{2}Cl\textsubscript{2}. Fmoc-F-OH/ Fmoc-Y(tBu)-OH/ Fmoc-3,5-F\textsubscript{2}Y was then coupled to the free N-terminus by shaking the resin for 2 × 70 - 80 min in a solution of 0.5 M amino acid, 0.45 M HATU and 1 M DIPEA; the volume was adjusted accordingly such that the amino acid was in 6 - 8 fold excess. The Re(bpy-COOH)(CO)\textsubscript{3}CN chromophore was then coupled in a similar manner with some modifications: the coupling solution consisted of 27 mg of Re(bpy-COOH)(CO)\textsubscript{3}CN in 1.5 mL DMF with 77 mg of HATU and 100 μL of DIPEA due to limited solubility of the chromophore. The coupling reaction was repeated to ensure high yields of the desired product. Cleavage of the peptide from the resin was carried out by shaking in 5 mL of
95% TFA, 2.5% triisopropylsilane, 2.5% water for 4 h. The resin was then washed for 2 × 1 min with 2 mL of TFA. The cleavage cocktail and washings were combined, evaporated under a stream of N₂, and taken up in 15 mL of ether to precipitate the crude peptide, which was pelleted in a centrifuge and the ether decanted. The precipitate was dissolved in 0.1 M ammonium bicarbonate HPLC buffer via sonication over several minutes.

**Peptide Purification and Characterization.** Peptides were purified by reversed phase HPLC using the system described in Chapter VII. Samples were manually injected onto a preparative Waters XTerra MS C-8 column (30 × 100 mm), which had been previously equilibrated with 10% acetonitrile/10 mM potassium phosphate buffer (pH 6). A linear gradient of 10 → 50 % acetonitrile vs. phosphate buffer over 50 min at a flow rate of 10 mL/min was used to elute the peptides. The eluant absorbance was monitored at 355 and 210 nm, where the [Re] chromophore and peptide amide bond absorb, respectively. Fractions containing the desired product were collected by hand, pooled, and reinjected onto the C-8 prep column which had been equilibrated with 0.1 M ammonium bicarbonate. The loaded column was rinsed with 5 column volumes of ammonium bicarbonate buffer, and the peptide eluted with 1:1 acetonitrile/ 0.1 M ammonium bicarbonate. The eluant was collected by hand, lyophilized, taken up in 50 mM Tris buffer at pH 7.5, combined, and stored at −80 °C. Analytical HPLC was employed to confirm the purity of the peptides. The samples were manually injected onto an analytical Waters XTerra MS C-8 (4.6 × 100 mm) column and eluted with a 10 → 65 % acetonitrile vs. 10 mM phosphate buffer (pH 6) over 45 minutes at a flow rate of 1 mL/min. The molecular weight (MW) of the peptide was characterized by MALDI-TOF mass spectrometry as described below. The HPLC retention times (t_R) and MALDI-TOF m/z ratios for each peptide are listed in Table 8.1. Analytical HPLC traces for each peptide were recorded at 210 nm to confirm purity. The concentration of each peptide stock solution was estimated by UV-vis absorbance using the known ε_{355 nm} = 5300 M⁻¹ cm⁻¹ for the [Re]-3,5-F₂Y-OMe dipeptide.

**Isolation, Purification and Pre-reduction of \(\alpha_2\).** *E. coli* \(\alpha_2\),¹⁸ \(\text{Y730F/Y731F-}\alpha_2\),¹⁹ and C439S-\(\alpha_2\)²⁰ were isolated by standard procedures. To remove contaminating \(\beta_2\), these proteins were further purified using a POROS HQ/20 anion exchange column (Applied Biosystems) on a BIOCAD Sprint Perfusion Chromatography System (Applied Biosystems). The column was loaded with \(\alpha_2\) (10 - 15 mg) and washed with Tris buffer (50 mM, pH 7.6)
for 5 min. α2 was eluted with a linear gradient of 0 - 700 mM NaCl over 30 min at a flow rate of 4 mL/min. Fractions were collected by hand and concentrated on a YM-30 membrane (Millipore). This procedure reduced background turnover of CDP 7 - 8 fold. To pre-reduce α2, ~30 mg were incubated with 30 mM DTT for 30 min at room temperature. Hydroxyurea, ATP and CDP were added to final concentrations of 30 mM, 3 mM and 1 mM, respectively, and the incubation continued for an additional 20 min. Another 10 mM DTT was added, and the mixture incubated for 10 min and desalted on a G-25 Sephadex column (~35 mL, 1.5 × 23 cm) pre-equilibrated in 50 mM Tris, 15 mM MgSO₄, pH 7.6.

**α2 Activity Assay and Competitive Inhibition Assay for Binding of Peptide to α2.** α2 (0.1 μM, specific activity = 1900 nmol min⁻¹ mg⁻¹), β2 (0.2 μM, specific activity = 6800 nmol min⁻¹ mg⁻¹), TR (30 μM), TRR (0.5 μM), NADPH (0.2 mM), CDP (1 mM), and ATP (1.6 mM) were combined in 50 mM HEPES, 15 mM MgSO₄ buffer at pH 7.6. Enzyme activity was measured by the consumption of NADPH, which was monitored by the decrease in absorbance at 340 nm. This was then repeated with peptide concentrations ranging from 1 - 150 μM.

**Single Turnover Assays for Photoinitiated Nucleotide Reduction in Peptide-α2 Complexes.** The assays were performed by a method modified from that described in Chapter VII. For the data in Figure 9.8 and 9.9, [2-¹⁴C]-CDP (0.75 mM, 6330 cpm nmol⁻¹) or [5-³H]-CDP (0.75 mM, 11,100 Cpm/nmol) substrate was used, respectively. FPLC purified, pre-reduced α2 (20 μM, specific activity = 1800 nmol min⁻¹ mg⁻¹), peptide (200 μM), ATP (3.0 mM), and labeled-CDP in 50 mM Tris, 15 mM MgSO₄ buffer (pH 7.5 unless otherwise indicated) were added to a 1 cm quartz micro-cuvette with total solution volume of 200 μL. The data in Figure 9.8 and 9.9 were also collected with samples containing 25 mM Ru(III)(NH₃)₆³⁺ or 20% glycerol, respectively. The reaction was initiated by the addition of CDP, quickly mixed, spun down in a minicentrifuge (< 10 s, 2000 g) to consolidate the liquid, and pipetted into the cuvette. Samples were irradiated at room temperature with the focused light from a 1000 W Xe arc lamp equipped with a 299 (Figure 9.8) or 348 nm (Figure 9.9) long pass filters. The cuvette was contained in a pyrex beaker filled with water at 25 °C to regulate the temperature during photolysis. Fractions (60 μL) were removed at 2, 5 and 10 min intervals and immediately quenched by heating in a boiling water bath for 2 min. The precipitated protein was then spun down for 10 min in a minicentrifuge at 20,000 g.
supernatant (50 of the 60 μL) was transferred to a new Eppendorf tube, diluted with 14 units of alkaline phosphatase and 120 nmol of carrier dC to a final volume of 170 μL, and incubated at 37 °C for 2 h in a sealed Eppendorf vial. dC was then separated from C and quantitated as previously described.\textsuperscript{15,21}

**Analysis of [Re]-Y-R2C19 binding to α2.** Figure 9.6 plots the binding of the [Re]-Y-R2C19 peptide to α2 as monitored by steady state emission spectroscopy. Solutions of 5 μM peptide were titrated with α2 from concentrations of 0→65 μM. Emission from [Re] did not significantly change at higher concentrations of α2; thus at 65 μM we concluded that each molecule of α2 was bound to two [Re]-Y-R2C19 peptide. The fraction of bound peptide as a function of α2 concentration, $\chi_{\text{bound}}(\alpha_2)$, was then calculated from the equation:

$$
\chi_{\text{bound}}(\alpha_2) = 2 \cdot \frac{I_{600\text{nm}}(\alpha_2) - I_{600\text{nm}}(0)}{I_{600\text{nm}}(65\mu M) - I_{600\text{nm}}(0)}
$$

where $I_{600\text{nm}}$ is the emission intensity at 600 nm. The inset of Figure 9.6 plots $\chi_{\text{bound}}(\alpha_2)$ vs. [α2]. The data were fit to the following kinetic model:

$$
[\alpha_2:P] \xrightleftharpoons{K_D} [\alpha_2] + [P] \\
[\alpha_2:P_2] \xrightleftharpoons{K_D} [\alpha_2:P] + [P]
$$

where P = [Re]-Y-R2C19. This treatment does not imply that two peptides bind to α2 simultaneously. From this model we can derive an expression for $\chi_{\text{bound}}$:\textsuperscript{22}

$$
\chi_{\text{bound}} = \frac{2[\alpha_2]^2}{K_D^2 + [\alpha_2]} + \frac{[\alpha_2]}{K_D} + 1
$$

assuming that $K_D$ is the same for binding of the first and second peptide, as previously discussed.\textsuperscript{3} Thus by plotting $\chi_{\text{bound}}$ vs. [α2] and fitting the data to eq. 2, we can derive $K_D$.

**Physical Measurements.** ESI-FT mass spectrometry was performed with a Brüker Daltonics APEXII instrument housed in the DCIF. MALDI-TOF mass spectrometry was performed with a Brüker Omniflex instrument in the DCIF using α-cyano-4-hydroxycinnamic acid as the matrix. The instrument was calibrated in positive ion mode with a quadratic polynomial using a mixture of angiotensin II (1046.5423), P14R synthetic peptide
(1533.8582), and ACTH fragment 18-39 (2465.1989) (Sigma). All peptides synthesized were analyzed in positive ion mode as the [M-CN]$^+$ ion.

UV-vis absorption spectra were recorded on a Spectral Instruments 440 spectrophotometer. Steady state emission spectra were recorded on an automated Photon Technology International (PTI) QM 4 fluorimeter equipped with a 150-W Xe arc lamp and a Hamamatsu R928 photomultiplier tube. Samples were excited at 330 nm in a 2 mm × 1 cm cuvette with four polished windows. Time resolved emission measurements on the <20 ns timescale were made with the frequency doubled (400 nm) pump light provided by a Ti:sapphire laser system (100 fs pulsewidth) and collected on a Hamamatsu C4334 Streak Scope streak camera as previously described. Time resolved emission measurements on the >20 ns timescale were made with pump light provided by the third harmonic (355 nm) of an Infinity Nd:YAG laser (Coherent) running at 20 Hz.

Nanosecond transient absorption (TA) measurements were made with the same laser running at 10 Hz. Thus system was extensively modified from that previously described. Probe light was provided by a pulsed 75 W Xe-arc lamp (Photon Technologies Incorporated). The lamp was pulsed with 5 A current (1 ms pulse width) and triggered externally at 10 Hz. The signal light passed through a Triax 320 spectrometer, where it was dispersed by a 300 × 500 blazed grating and collected with either an intensified gated CCD camera (ICCD, CCD 30-11, Andor Technology, 1024 × 256 pixels, 26 μm$^2$) for TA spectra or a photomultiplier tube (PMT) for TA kinetics at a single wavelength. PMT outputs were collected and averaged with a 1 GHz oscilloscope (LeCroy 9384CM)A TTL pulse synchronized with the Q-switch of Infinity laser was delayed 99 ms before triggering the pulser for the probe light. All electronic delays were created with SRS DG535 delay generators (Stanford Research Systems). Electronic shutters (Uniblitz) were used to create the pulse sequence illustrated in Figure 9.17.

To produce a TA spectrum, the series of four spectra were taken: $I_P$ (pump on/probe off), $I$

![Figure 9.17 Timing of the nanosecond TA instrument running at 10 Hz.](image)
(pump on/probe on), $I_B$ (pump off/probe off), and $I_0$ (pump off/probe on). Transient spectra were corrected for fluorescence and background light using these spectra by the calculation: $\Delta OD = \log([I_0 - I_B]/[I - I_F])$. For experiments involving $\alpha_2$, the spectra reported are an average of 125 of the four-spectra sequences. Sample sizes were typically 200 $\mu$L in a $2 \times 10$ mm cuvette containing a Teflon-coated mini-stirbar. Both the white light and pump beams were focused and overlapped to pass through the 2mm-wide window of the cuvette, providing a total pathlength of 1 cm. To provide optimal beam overlap, the pump beam was reflected off a small mirror in front of the collimating lens for the probe beam after the sample, as illustrated in Figure 9.18.

![Figure 9.18](image)

**Figure 9.18** Diagram of the nanosecond TA instrument running at 10 Hz.
9.5 References


Chapter X

Towards Photoactive β2 Subunits
10.1 Motivation

The results of Chapter IX suggest that observation of radical transport from •Y356 on the R2C19 peptide into α2 may be precluded by the conformational flexibility of the C-terminus of the peptide bound to α2. Multiple conformations of the peptide bound to α2 are evident from the data, and we postulate that the productive conformation supporting radical transport is only present at concentrations that are too low for observation with our transient spectroscopic methods.

In the natural system in vitro, RNR activity is regulated by conformational triggers in the α2:β2 complex.1 By replacing β2 with the Y-R2C19 peptide, we may be missing some of the conformational triggers that enable radical transport between Y356 and C439. The assumption here is that the conformational trigger in the α2:β2 complex in some way affects the radical transport through Y356. Several lines of evidence support this assumption. First, the pKa of 3-NO2Y356 in the 3-NO2Y356-β2 subunit was determined to shift by almost +1 pKa unit upon binding to α2, but only in the presence of RNR substrate and effector.2 Also, in the DOPA356-β2 subunit, •Y122→DOPA356 radical transport is only observed upon binding of DOPA356-β2 with α2 in the presence of RNR substrate and effector.3 The shift in pKa of Y356 upon binding to α2 predict a change in the thermodynamics of oxidation of this residue, while the result with DOPA356-β2 shows that the kinetics of •Y356 formation change upon subunit interaction.

Thus, in order to observe radical transport in a photochemical RNR, replacement of the Y-R2C19 peptide with the entire β2 subunit that is functionalized for photochemical •Y356 generation may be a fruitful endeavor. These constructs may capture RNR in a conformation appropriate for radical transport, which can be initiated by a laser pulse. Binding of met-β2 (β2 with •Y122 reduced) with reduced α2 should shift the enzyme conformation to favor product formation (also favoring forward radical propagation). In such cases, phototriggering •Y356 formation halfway along the radical transport pathway in RNR may allow for visualization of the •Y356→Y731 radical transport step. This chapter presents the first attempts towards preparing chromophore-functionalized β2 subunits for application to photochemical RNRs based on the α2:β2 complex.
10.2 [Re]-β2

10.2.1 Background

One potential method of generating a photoactive β2 subunit involves synthesis of a photoactive Y-R2C19 peptide that can be attached to the truncated β2 subunit by well established protein intein ligation methods.\textsuperscript{2} This semisynthetic method relies on the generation of the C-terminal peptide tail of R2 via solid-phase peptide synthesis (SPPS). The peptide must contain an N-terminal cysteine residue that can then be ligated to a truncated, thioester-terminated recombinant R2 subunit via thiotransesterification. By directly incorporating the photo-oxidant into the C-terminal peptide during SPPS, we have synthetic control over the location of the chromophore with respect to Y356. The modified protein can then be used to study reverse radical transport in β2, as well as forward radical transport in the α2:β2 complex. Based on the work presented in the foregoing chapters, [Re] is the excited state radical initiator of choice with 3,5-F\textsubscript{2}Y at position 356.

Figure 10.1 depicts the design plan that was implemented for studying the reverse radical transport:

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10.1.png}
\caption{Proposed photochemistry for [Re]-3,5-F\textsubscript{2}Y356-β2.}
\end{figure}

The basic design can be summarized as: (1) Excitation of the [Re] chromophore covalently attached to 3,5-F\textsubscript{2}Y356-β2 at position 355 will generate the [Re\textsuperscript{0}]-3,5-F\textsubscript{2}Y• charge separated state. (2) A bimolecular quencher can be used at this point to oxidize [Re\textsuperscript{0}] and prevent back electron transfer with the 3,5-F\textsubscript{2}Y• radical. (3) Once formed at the surface of met-β2, the 3,5-F\textsubscript{2}Y• radical can then initiate reverse radical transport along the 3,5-F\textsubscript{2}Y356•→W48→Y122 pathway, reforming the stable •Y122. This experiment seeks to provide kinetic resolution of
reverse radical transport in β2 and afford a method to test the hypothesis that D237 controls the thermodynamics W48 oxidation (see Chapter II). The met-[Re]-3,5-F2Y356-β2 (with ·Y122 reduced) can also be bound to α2 in the presence of RNR substrate and effector so that the experiment is applicable to the study of forward radical transport in RNR.

10.2.2 Results and Discussion

A synthetic method utilizing solid-phase peptide synthesis (SPPS) to generate a N-terminal Cys-peptide containing the [Re] chromophore and Y for ligation to the β2 subunit was developed. For this preliminary work, we focused on smaller, 4-residue peptides, rather than the full length R2C19. The strategy focused on utilizing an amino acid with two, orthogonally protected amine groups, such that the Re(bpy–COOH)(CO)3CN chromophore could be coupled to one amine group and the peptide extended off of the other. Scheme 10.1 shows the synthetic scheme for the Cys-Pro([Re])-Tyr-Leu-NH2 peptide.

MBHA resin was chosen, as it is stable to both trifluoroacetic acid (TFA) and piperidine. Fmoc-Y(tBu)-OH was coupled to Fmoc-Leu-MBHA resin using standard Fmoc-peptide coupling conditions. N-Boc-cis-4-N-Fmoc-amino-L-proline was chosen as the amino acid containing two orthogonal protecting groups. After coupling this residue to Y, the Fmoc group was cleaved with piperidine and Re(bpy–COOH)(CO)3CN chromophore coupled to the free amine (Step 1 in
Scheme 10.1). The Boc-protecting group was then cleaved with TFA and peptide synthesis continued. Cleavage of the final product from the resin could be afforded with triflic acid. The resulting tetra-peptide was purified by HPLC under basic conditions (see below) and analyzed by MALDI-TOF mass spectrometry. The tetra-peptide was sparingly soluble under the conditions necessary for ligation to truncated β2.

10.3 Y356C-BP β2

10.3.1 Background

The second method for developing photochemical RNRs involves site-specific labeling of β2 at or near position 356 in the C-terminal region of the protein with photochemical Y• generators. If the label is incorporated at position 356, then •Y731 may be generated directly if the photooxidant is in proximity to this residue in the α2:β2 complex. We chose benzophenone as the photooxidant for our first attempt owing to its relatively small size (minimal perturbation of the α2:β2 interaction) and its known ability to generate Y• upon excitation (see Chapter III). Furthermore, 4-acetamidobenzophenone (4-Ac-BP) has a major advantage for kinetics studies: the chromophore has an exceptionally large absorption cross-section outside of the protein absorption envelope with $\varepsilon (300 \text{ nm}) = 23,000 \text{ M}^{-1}\text{cm}^{-1}$, ten times that of BPA at this wavelength. This chromophore thus has ample absorption cross-section outside the protein envelope, thus allowing us to work at relatively low (~50 μM) concentrations of RNR.

Labeling was accomplished by introducing a cysteine residue via site directed mutagenesis into the C-terminus of β2. For the labeling to be specific to the cysteine of choice, the two surface accessible Cys residues of R2, C268 and C305, must also be mutated to Ser. The Stubbe lab has constructed the triple mutant: C268S, C305S, Y356C-R2 for these studies. Commercially available 4-(iodoacetamido)-benzophenone (BPIA, Scheme 10.2) was used to label the Y356C residue, as previously described for other protein derived Cys residues.6

[Diagram showing the labeling of β2 with benzophenone derivative.]
10.3.2 Results and Discussion

4-Ac-BP was used as a control compound for comparison to the benzophenone-labeled β2 subunit. Figure 10.2 plots the TA spectra recorded upon photolysis of solutions of 4-Ac-BP (20 mM Tris, pH 7.5) with 320 nm nanosecond-pulsed laser light in the absence (left panel) and presence (right panel) of 10 mM L-tyrosine methyl ester. The spectra in the left panel are broad with a peak at ~490 nm and decay on the microsecond timescale. Photolysis of the same sample in the presence of L-tyrosine methyl ester (right panel), produces spectra with the characteristic double hump feature ($\lambda_{\text{max}} = 410$ nm) corresponding to Y• (see Chapter III) along with a sharp peak at 348 nm and a broad peak with $\lambda_{\text{max}} \sim 570$ nm. The latter two features resemble that of the ketyl radical of BPA, reported in Chapter III, red-shifted 15-20 nm. We therefore attribute these features to that of the ketyl radical of 4-acetamidobenzophenone (4-Ac-BP•). From these data, it is clear that the excited state of 4-Ac-BP is a competent photooxidant of tyrosine.
Figure 10.3 TA spectra recorded after 320 nm photolysis of 50 μM solutions of met-Y356C(BP)-β2 in the absence (A) and presence (B) of 70 μM α2, and 50 μM Y356C(BP)-β2 with 70 μM α2 (C). Spectra were recorded 150, 300 ns, 1 and 5 μs after the laser flash (top to bottom). Conditions: 50 mM Tris (pH 7.5), 15 mM MgSO4, 1 mM CDP, and 3 mM ATP.

Figure 10.3 plots the TA spectra recorded upon photolysis of solutions containing Y356C(BP)-β2 with 320 nm laser light. Note that photolysis of wt-β2 or α2 subunits alone at this wavelength produces no observable transient signals on the nanosecond timescale. Panel A of Figure 10.5 plots the spectra observed following excitation of a 50 μM solution of met-Y356C(BP)-β2 (∗Y122 reduced) with 15 mM MgSO4, 1 mM CDP, and 3 mM ATP (pH 7.5). The spectra contain a sharp peak at 360 nm and a broad feature with λmax ~ 455 nm that decay on the microsecond timescale. In the presence of 70 μM α2 (panel B) and for 50 μM Y356C(BP) with 70 μM α2 (panel C), the peak at 360 nm is maintained, but the broad feature at 455 nm has decreased in intensity. The feature at 360 nm is reminiscent of the spectrum of 4-Ac-BP• which exhibits a sharp peak at 348 nm (see above). We would expect the thioether
modification of the 4-acetamido group in Y356C(BP) to perturb the spectrum of the 4-Ac-BP• radical. We therefore tentatively assign the 360 nm feature to the ketyl radical of 4-acetamidobenzophenone attached to β2 Y356C(BP•). Based on this assignment, we can conclude that the excited state of the BP chromophore has oxidized some residue in either the β2 or α2 subunit. The spectrum recorded at 150 ns in panel B contains a weak feature at 410 nm, which may correspond to a tyrosyl radical.

10.4 Conclusions

We have developed a synthetic strategy in Section 10.2 to generate β2 C-terminal peptides containing the [Re] chromophore for ligation to the truncated β2 subunit. The solubility problems of the tetra peptide should be resolved upon synthesis of the full length peptide, which contains multiple Asp and Glu residues (the [Re]-Y-R2C19 peptide is soluble at neutral pH). Using the synthetic methodology described here and the previously established peptide-protein ligation methods for the β2 subunit, we may be able to generate semi-synthetic β2 subunits containing covalently attached [Re] photooxidants for study of the PCET of radical transport in RNR. Time resolved emission and absorption spectroscopies should be used to verify Y• formation on the full length peptide. If the proline linkage does not afford the appropriate photochemistry, other orthogonally protected, bis-amine containing amino acids can be used, such as Boc-3-(Fmoc-amino)-L-alanine.

The results reported in Section 10.3 represent initial attempts to characterize the excited state chemistry of the 4-Ac-BP chromophore, both free in solution and covalently attached to the β2 subunit via a thioether linkage. The data show that this chromophore is a competent photooxidant of tyrosine, and TA spectra corresponding to 4-Ac-BP• and Y• in the charge separated state have been characterized. The spectra obtained for the “control” experiment in left panel of Figure 10.2 do not resemble the spectra for the control experiment in panel A of Figure 10.3. Perhaps the thioether linked 4-Ac-BP chromophore would provide a better control. Synthesis of this compound could be accomplished by allowing benzophenone-4-iodoacetamide to react with cysteine.

The peak at 410 nm in the spectrum recorded at 150 ns in panel B may indeed be due to a tyrosine radical in α2. To confirm this interpretation, flash photolysis of met-Y356C(BP)-β2 and Y356C(BP)-β2 should also be carried out with the Y730F-α2 and C439S-α2 variants to trap any tyrosyl radicals generated along the transport pathway in α2. Similar experiments
with the Y731F-α2 should result in no Y• production if •Y731 is indeed the observed radical. Photochemical assays should also be carried out with the Y356C(BP)-β2 subunit and α2 to confirm light initiated turnover.

10.5 Experimental Section

Materials. Boc-Leu-MBHA resin, 1-hydroxybenzotriazole (HOBT), N-α-Fmoc-S-trityl-L-cysteine (Fmoc-Cys(Trt)-OH), Benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) (Novabiochem), O-(1,1-dimethylethyl)-N-[[(9H-fluoren-9-ylmethoxy)carbonyl]-L-tyrosine (Fmoc-Y(tBu)-OH), O-(7-Azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (Applied Biosystems), (2S,4S)-N-tert-butoxycarbonyl-4-N-(9-fluorenylmethoxycarbonyl)aminopyrrolidine-2-carboxylic acid (N-Boc-cis-4-N-Fmoc-amino-L-proline), triflic acid, trifluoroacetic acid (TFA), piperidine, diisopropylamine (DIPEA), thioanisole, N-methylmorpholine (NMM), 1,2-ethanedithiol and dimethylformamide (DMF) (Sigma Aldrich) were used as received. Re(bpy-COOH)(CO) 3CN7 was prepared as previously described. Y356C(BP)-β2 was prepared by Lars Plate in the Stubbe Lab by labeling the Y356C–β2 mutant with benzophenone-4-iodoacetamide as previously described for myosin. 8 4-Acetamidobenzophenone (4-Ac-BP) was synthesized as previously described. 9

Synthesis of Cys-Pro([Re])-Tyr-Leu-NH2. The peptide was synthesized using a combination of Boc and Fmoc solid-phase peptide synthesis (SPPS) strategies. Boc-Leu-MBHA resin (250 mg, 0.54 mmol/g) was loaded into a 10 mL Bio-Rad Poly-Prep column containing a porous 30 μm polyethylene bed in the bottom to hold the resin. All reaction/washing steps were performed by shaking the resin with a FisherScientific Vortex Genie 2 (VWR).

Boc-protecting groups were cleaved by shaking the resin in 2 mL of 1:1 TFA:CH2Cl2 (1 × 1 min, 1 × 20 min) followed by neutralization of the resin with 2 mL of 10% NMM in CH2Cl2 (2 × 5 min). Fmoc-protecting groups were cleaved with 2 mL of 20 % Piperidine/DMF (3 × 7 min). Trt-protecting groups were cleaved with 94:1:5 CH2Cl2:TFA:TIS. Amino acid (Fmoc-Y(tBu)-OH, N-Boc-cis-4-N-Fmoc-amino-L-proline, and Fmoc-Cys(Trt)-OH) coupling steps were achieved by combining 4 eq of the N-protected amino acid to be coupled with HATU (185 mg, 3.6 eq and DIPEA (188 μL) in 1.1 mL of DMF and the reaction shaken for 80...
minutes. In between coupling and Fmoc- (Boc-) protecting group cleavage steps, the resin was rinsed with 4 × 2 mL of DMF (CH₂Cl₂).

Coupling of Re(bpy-COOH)(CO)₃CN was achieved by combining 2 eq of the Re(I) complex with BOP (239 mg, 4 eq), DIPEA (188 μL) in 4 mL of DMF and the reaction mixture shaken overnight. Note that higher yields can be achieved with the BOP coupling reagent, however it should only be used if a tBu-protected form of the tyrosine phenol is used on the peptide. If the free phenol is employed (as with Fmoc-3,5-F₂Y)¹⁰, then HATU should be used as the coupling reagent and the reaction run for 2 × 80 min.

The peptide was cleaved from the resin following cleavage of the S-Trt and N-Boc protecting groups of the Cys residue and washing of the resin. The resin (50 mg) was placed in a conical vial and thioanisole (100 μL) and 1,2-ethanedithiol (50 μL) were added. The vial was chilled in an ice bath, TFA (1 mL) was added, the mixture stirred for 5 min. Triflic acid (100 μL) was then added and the vial sealed and stirred at room temperature for 3 hours. The reaction mixture was filtered through the Bio-Rad Poly-Prep column and the filtrate concentrated under a stream of nitrogen gas. The peptide was isolated by precipitation with 30 mL of ether, followed by consolidation of the precipitate via centrifugation and canting off of the ether. The resulting pellet was dissolved in 10% MeCN, 0.1 M (NH₄HCO₃) (pH 10) for purification by HPLC.

**Purification and Characerization of Cys-Pro[(Re)]-Tyr-Leu-NH₂.** The title peptide was purified by reversed phase HPLC using the system described in Chapter VII. Samples were manually injected onto a preparative Waters XTerra MS C-8 column (30 × 100 mm), which had been previously equilibrated with 10% acetonitrile/ 0.1 M ammonium bicarbonate buffer (pH 8). A linear gradient of 10 → 55 % acetonitrile vs. ammonium bicarbonate buffer over 50 min at a flow rate of 10 mL/min was used to elute the peptide. The eluant absorbance was monitored at 355 and 210 nm, where the [Re] chromophore and peptide amide bond absorb, respectively. Fractions containing the desired product were collected by hand, pooled, and lyophilized to dryness. MALDI-TOF mass spectrometry was used to characterize the peptide as described below [(M–CN)⁺ Calc. (Obs.) 975.25 (974.2)].

**Physical Measurements.** MALDI-TOF mass spectrometry was performed with a Brüker Omiflex instrument in the DCIF using α-cyano-4-hydroxycinnamic acid as the matrix. The instrument was calibrated in positive ion mode with a quadratic polynomial using a mixture of
angiotensin II (1046.5423), P14R synthetic peptide (1533.8582), and ACTH fragment 18-39 (2465.1989) (Sigma). Transient absorption spectroscopy was carried out as described in Chapter IX, except that the excitation laser light was tuned to 320 nm.
10.6 References


Acknowledgements

The work in this thesis would not have come to fruition without the support and energy of many people who I hold near and dear to my heart. First and foremost, I need to thank my parents for raising me and having the courage and insight to send me away when I was in high school. Thank you for challenging me, believing in me, for being a constant source of support, and for being my friends. Also thanks to my brothers, Shaun and Todd, for being such good friends over the years. Shaun, you have been and will always be my role model and Todd, you can be one of the funniest people I know.

Thanks to my friends from Davidson who have kept in touch with me and kept me sane over the years. Graham Gravley, Diego Marquez, and Adam Kennedy, I have no doubt that we will be friends for life and things always pick up right where they left off. Prof. Durwin Striplin was my first mentor in chemistry and has now become one of my best friends. Thanks for giving me a chance to explore my curiosity in chemistry and helping me fall in love with the science. I hope I can always call you up to listen to your philosophy on life and on science, as they always provide a source of inspiration.

Since joining the ranks here at MIT, many people have provided words of wisdom, friendship, awkward moments, or forms of entertainment all of which need recognition. Thanks to the laser lab crew of Niels Damrauer, Justin Hodkgiss, Zhi-Heng Loh, and Liz Young for teaching me how to think about my experiments, providing New Zealand puppet shows while tuning the regen, teaching me how to use and abuse the Infinity, and for keeping us all in line, respectively. Now, can someone tell me how to buy a lense? Special recognition go to Bart “B-Money” Bartlett and David “Mank-the-Tank” Manke, the original members of perhaps the first chemistry fraternity at MIT founded at 68 Beacon St. Aetna Wun provided the plushy interior. Adam Veige is the most destructive person I know, so thanks for helping me keep things in perspective, teaching me how to hail a cab properly, and for always getting us started on the right foot, even if you “had a bad feeling” about that night. Thanks to Matthew F. Shores for being so conversational and Julien Bachmann for being so Swiss. Becky Somers always has a smile on her face and somehow manages to deal with the rest of the Nocera group. Jenny Yang was a great bay-mate for so many years and always provides a good listening ear. Joel Rosenthal does the best Nocera impressions and keeps him from getting out of control.
Currently Dan has assembled a crack team of postdocs that keep the lab running. Special thanks go to Dino Villagrán for being a real Mexican. Thanks to Matt Kanan for introducing the lab to Pinnocchio’s in Harvard Square, for nights at Grafton St with the X-factor, and for challenging me to reevaluate everything I thought I knew. Ted Betley has been the recipient of far too many streece melt-downs over the last year and for that I am eternally grateful. Without him I may not be here today. I wish him all the best at Harvard and beyond. Up for another game of Guitar Hero?

“Good luck” to the next wave of Nocera students. “Little” Emily McLaurin is a force to be reckoned with who can sense oxygen with one hand and spin records with the other, all while riding a skateboard. The photochemical hydrogen project is in the best of hands with Tim “the Hobbit” Cook who adds depth to the lab with his incredible artistic and Photoshop talents. May the force be with you, Montana, in ripping apart water to make oxygen. To Marshak, Yogi, and Jay, thanks for your fresh perspective on things that once seemed old to me.

Having Arthur Esswein as a fellow classmate to share in the trials and tribulations of MIT has been a blessing. Arthur is a man of multiple personalities and has had many nicknames over the years: Little Krodel, Alfred, Artie, the Corrector, JR, Artie Bukes, little Buddy… Seriously though, thanks for being a good friend and for putting up with me.

Several others outside the Nocera lab need mentioning. Leslie Murray helped me survive first year and has been there for me ever since. Lindsay McQuade is a great running partner and was always there to listen when I was freaking out. Josh Figueroa helped me through third year orals and always provides a unique perspective on things with his own dialect. Thanks to Leif Hammarström for his interest in me and his enthusiasm for my science. He has been a wonderful collaborator and I have learned much from our interactions. Tania Irebo always makes you feel welcome, whether in the lab, outside in the cold, dark winters of Sweden, or in the hot tub in Ventura. Much of the work in this thesis couldn’t have been possible without the help of Mohammad Seyedsayamdost. I have been extremely fortunate that he was willing to dedicate time and energy to my project. Thanks for always making me feel welcome in the Stubbe lab, for teaching me biochemistry, and for being a good friend.

Thanks to Joseph Sadighi for being a wonderful thesis chair, always interested to hear about what I have been up to. Steve Lippard made my thesis defense a very memorable and rewarding experience. I feel that JoAnne Stubbe has invested enough of her time and energy
in me to qualify as my co-advisor. Thank you for your commitment and enthusiasm to science and for being interested in the details of every experiment. I am always inspired by your passion for learning and determination to get the science right.

I find it hard to express in words the appreciation I have for my advisor, Daniel G. Nocera. Thank you for taking a “dumb kid from a small school in the South” and molding him into a scientist. As a man with Italian heritage, you can’t help but be emotional, but you wear your heart on your sleeve and face life every day with an honesty and enthusiasm that I find inspiring. Thank you for teaching me to think about the importance of my science, to always think of a better way to explain myself, and to be fearless in following your passions. Also thanks for introducing me to fine dining in Boston, beers at the Muddy, the car ride back from the ET Gordon Conference at Salve Regina, guys night out in the North End, being my father from Australia at the Phoenix, taking me in after the break up, Leng Leng’s last day (the best day ever), ladies lunch, and all the stories from your rock star exploits. Thank you.
Biographical Note

Steven Young Reece was born on February 26, 1980 in Kinston, NC and raised there by his parents Sonny and Susan. He grew up the middle child with two brothers, Shaun and Todd, who proved to be the best friends a boy could have. Being the oldest, Shaun was the role model and introduced Steven to such things as music, science, and swimming. Shaun and Steven had the same high school chemistry teacher, Mr. David Sawyer, now a meteorologist in Alabama, who was the first to spark Steven’s interest in chemistry.

Growing up, weekdays were spent at school in K-town and swimming laps with the local swim club, but the weekends provided a good escape to the beautiful beaches of North Carolina. Sneads Ferry, NC provided a quality education on combing the beach for sand dollars, waterskiing, pulling in flounder nets at sunrise, and trawling for shrimp at sunset. Always an introspective and quiet kid, he found the physical connection with the ocean a serene experience.

At the age of sixteen, Steve was “shipped off” to boarding school at Phillips Exeter Academy in Exeter, NH. Thus began his “formal” education on life that opened his eyes to the complexity of the world around him. At Exeter, Steve developed a love for the hypnotizing music of the Great Highland bagpipe through lessons with the wonderfully skilled and insightful Brian Yates, a native of Winchester, MA. In 1998, Steven migrated south to continue education at Davidson College in Davidson, NC where he learned the value of persistence and the true meaning of friendship as a four-year member of the varsity swim team. At the end of his freshman year, he joined the lab of Prof. Durwin Striplin to try his hand at chemical research. Without this single event this thesis probably wouldn’t be written. Dr. Striplin proved to be a wonderful mentor; Steve fell in love with inorganic synthesis and photochemistry, while gaining an appreciation for how chemistry can contribute to the search for renewable sources of energy.

In 2002, Steve moved back north to continue studying chemistry at MIT in Cambridge, MA. He was persistent and fortunate enough to join the lab of Prof. Daniel Nocera. Through work in Dan’s labs and those of his collaborator, Prof. JoAnne Stubbe, Steve has been molded from someone with a naïve love and passion for science to a more careful, thoughtful chemist who appreciates the complexity of his chosen discipline. His interests have broadened to include bioinorganic and biophysical chemistry. In early fall 2007, Steve will leave MIT to continue his scientific journey as a postdoc in the labs of Prof. Michael Marletta at the University of California, Berkeley.
Curriculum Vitae of Steven Y. Reece

Work Address
Department of Chemistry, 2-301
Massachusetts Institute of Technology
77 Massachusetts Ave.
Cambridge, MA 02139-4307

Contact Information
phone (work): 617•258•7268
phone (cell): 617•448•1227
FAX: 617•253•7670
e-mail: streece@mit.edu

Education
2002-2007 MASSACHUSETTS INSTITUTE OF TECHNOLOGY
Ph.D. in Inorganic Chemistry
Thesis: “Proton-coupled electron transfer and amino acid radical hopping in class I E. coli ribonucleotide reductase”

1998-2002 DAVIDSON COLLEGE
B.S. with High Honors in Chemistry • Minor in Mathematics • Cum Laude

Experience
2007- Postdoctoral Research Fellow, Univ. of Cal., Berkeley • Michael A. Marletta, Advisor
2002-2007 Graduate Research Assistant, MIT • Daniel G. Nocera, Advisor
2002-2003 Teaching Assistant, MIT
Lecture course: Principles of Chemical Science
Lab course: Introduction to Experimental Chemistry
1998-2002 Undergraduate Research Assistant • Durwin R. Striplin, Advisor
2001-2002 Varsity Swim Team Captain
Team member 1998-2002

Awards and Affiliations
2006 Morse Travel Grant (MIT)
2005 Travel Award, Div. of Inorg. Chem., Fall 2005 ACS National Meeting
2002 Student Awardee of the American Institute of Chemists Foundation (AICF)
2001 Samuel Bell Merit Scholarship (Davidson College)
2000 Porter P. Vinson Chemistry Award (Davidson College)
1998 Cum Laude graduate of Phillips Exeter Academy
2002- American Chemical Society member

Publications


