Photoinitiated Proton-Coupled Electron Transfer and Radical Transport Kinetics in Class Ia Ribonucleotide Reductase

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Photoinitiated Proton-Coupled Electron Transfer and Radical Transport Kinetics in Ribonucleotide Reductase

by

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ABSTRACT

Proton-coupled electron transfer (PCET) is a critical mechanism in biology, underpinning key processes such as radical transport, energy transduction, and enzymatic substrate activation. Ribonucleotide reductases (RNRs) rely on PCET to mediate the rate-limiting step in the synthesis of DNA precursors. E. coli class Ia RNR consists of two dimeric subunits: $\alpha_2$ contains the active site, while $\beta_2$ contains a stable diferric-tyrosyl radical cofactor. During turnover, transport occurs over 35 Å, from Y122 in $\beta_2$ to C439 in $\alpha_2$, where an active-site thyl radical mediates turnover. Radical transport is proposed to occur over a series of highly conserved redox-active amino acids, including Y356 in $\beta_2$, and Y731 and Y730 in $\alpha_2$. This thesis examines three subject areas of PCET that pertain to RNR:

Small-molecule model systems provide insights into tyrosine oxidation and radical generation. Under physiological conditions, tyrosine oxidation is accompanied by deprotonation and occurs by PCET. A critical factor in PCET reactions is the nature of the proton acceptor and the presence of hydrogen bonding. In a modular model system, pyridyl-amino acid-methyl esters are appended to rhenium(I) tricarbonyl phenanthroline to yield rhenium-amino acid complexes. In dichloromethane solution, bases coordinate to tyrosine by hydrogen bonding. Emission kinetics reveal base-dependent oxidation by PCET.

A photopeptide composed of the 19 C-terminal residues of $\beta_2$, fluorinated tyrosine in place of Y356, and a rhenium(I) bipyridine photooxidant enables photoinitated radical transport into $\alpha_2$. Transient absorption kinetics show rapid radical transport (10$^4$ s$^{-1}$) that is only observed when both Y731 and Y730 are present, suggesting a critical role for the Y731-Y730 dyad for radical transport in RNR.

An intact, photochemical $\beta_2$ enables studies of an $\alpha_2\beta_2$ complex. A bromomethylpyridine rhenium(I) phenanthroline photooxidant labels a single surface-cysteine mutant of $\beta_2$ at position 355 to yield [Re$^\circ$]–$\beta_2$. Under flash-quench conditions, transient absorption reveals a tyrosine radical. [Re$^\circ$]–$\beta_2$ binds $\alpha_2$ and is capable of light-initiated substrate turnover. Transient emission quenching experiments reveal Y356 oxidation that is dependent on the presence of Y731, in $\alpha_2$. This result suggests that a Y356–Y731–Y730 triad mediates radical transport across the subunit interface and into $\alpha_2$.

Thesis Supervisor: Daniel G. Nocera
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To my parents,

for their constant support and encouragement.
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Figure 3.14. Graphical residual analysis of kinetic decays of [Re]-F₃,Y-βC19:C₄₃S-α₂ and Re-F₃,Y-βC19:wt-α₂. (a) Monoexponential (left) and biexponential (right) residuals for [Re]-F₃,Y-βC19:C₄₃S-α₂. (b) Monoexponential (left) and biexponential (right) residuals for [Re]-F₃,Y-βC19:wt-α₂. The trend lines are a 20-point adjacent-averaged smoothing that are included as a guide for the eye. The region that deviates from linearity is in the early time points of each monoexponential trace, corresponding to a short lifetime component.

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Figure 3.16. Connectivity and timing of the ns TA system. The t = 0 is set by the Q-switch (Q-SW) output of the laser. This signal is used to set the delay and gate of the CCD through delay box #1 with by software. The Q-SW signal is also passed to delay box #2, which controls both pump and probe shutters. Because the shutters have a mechanical delay of milliseconds, timings are set so opening and closing occurs as far as possible from the laser pulse. The full shutter sequence is 400 ms, synced every 100 ms to the Q-SW signal. Delay box 2 is triggered by every second signal from the Q-SW in order to allow the full pump/probe open/closed sequence to function. The CCD receives each Q-SW signal to record the four states listed in Equation 3.1. Pump and probe shutter “open” and “closed” states are shown in green and red, respectively. Delay and gate timings are not to scale and vary by experiment.

Figure 4.1. X-ray crystal structure of [Re]-Br with a PF₆ anion. Thermal ellipsoids are reported at a 50% probability level. Hydrogen atoms, solvent molecules, and a second molecule of [Re]-Br are omitted for clarity.

Figure 4.2. Spectroscopic comparison of [Re]-Br and [Re]-S₃₅₅C-β. The UV-vis absorption (gold solid line) and emission spectra (λₑₓ = 355 nm) (gold dotted line) of [Re]-Br (MeCN solution) and the UV-vis absorption (blue solid line) emission (blue dotted line) spectra of [Re]-S₃₅₅C-β₁ (50 mM HEPES, 1 mM EDTA, pH 8.0) are shown. The absorption spectrum of S₃₅₅C-β₁ (grey solid line) (50 mM HEPES, 1 mM EDTA, pH 8.0) is also included for reference. The simulated absorption spectrum (red solid line) of [Re]-S₃₅₅C-β₁ by summing the absorption spectrum of twice the absorption spectrum of [Re]-Br and S₃₅₅C-β₁. The similarity of the actual and simulated [Re]-S₃₅₅C-β₁ UV-vis absorption traces suggests that binding has no significant impact on the spectroscopic properties of [Re].

Figure 4.3. ESI-MS spectrum for [Re]-S₃₅₅C-β₂. The mass spectrum of [Re]-S₃₅₅C-β₂ was reconstructed from ESI-MS data. The major peaks shown correspond to only expected labeled products, which include both [Re]-S₃₅₅C-β₁ (44922.6 expected) and the N-formylmethionyl variant, fMet-[Re]-S₃₅₅C-β₂ (45081.6 expected) as summarized in the following table (masses are reported in Da):

Figure 4.4. Nanosecond laser flash photolysis of [Re]-S₃₅₅C-β₂. Time-resolved spectroscopic data are recorded after excitation (λₑₓ = 355 nm) of [Re]-S₃₅₅C-β₂ (10 μM in 50 mM HEPES, 1 mM EDTA, pH 8.0). Top: Transient absorption spectra recorded 50 ns (black), 150 ns (brown), 500 ns (orange) and 1000 ns (grey) after excitation. Spectra are collected over 50 ns, beginning at the indicated time. The observed growth features are consistent with a [Re]* MLCT excited state. Asterisk indicates signal due to laser scatter. Middle: TA kinetics data observed at 450 nm and a monoexponential fit (solid line) (τ = 328 ns). Bottom: Time resolved emission data observed at 550 nm and a monoexponential fit (solid line) (τ = 311 ns).
Figure 4.5. Nanosecond laser flash photolysis of [Re(phen)(CO)₃(PyCH₃)]PF₆. Time-resolved spectroscopic data are recorded after excitation (λₑₓ = 355 nm) of [Re(phen)(CO)₃(PyCH₃)]PF₆ (20 μM in MeCN). Top: Transient absorption spectra recorded 50 ns (black), 150 ns (brown), 500 ns (orange) and 1000 ns (grey) after excitation. Spectra are collected over 50 ns, beginning at the indicated time. The observed growth features are consistent with a 3[Re]* MLCT excited state. Asterisk indicates signal due to laser scatter. Middle: TA kinetics data observed at 450 nm and a monoexponential fit (solid line) (τ = 237 ns). Bottom: Time resolved emission data observed at 550 nm and a monoexponential fit (solid line) (τ = 228 ns).

Figure 4.6. Strategy for the construction of a photoRNR-β. The attachment of the PO, [Re] = [Re(phen)(CO)₃(PyCH₂Br)] via the methylene group of the pyridyl ligand of the rhenium center is shown schematically. Radical initiation at Y₃₅₆: (1) a 355 nm laser pulse generates the [Re]* excited state, which (2) is quenched to produce (3) the [ReII] species, which oxidizes Y₃₅₆ to regenerate the [ReI] ground state and the radical. (4) The photogenerated Y₃₅₆ radical is observed and monitored by transient absorption spectroscopy. Graphics were generated from PDB entry 1MXR1* (P2).

Figure 4.7. Photochemically generated Y₃₅₆*. Time-resolved spectroscopic data are recorded after excitation (λₑₓ = 355 nm) of met-[Re]-S₃₅₅C-β₂. Top: Transient absorption spectrum of [Re]-S₃₅₅C-β₂ collected 1 μs after excitation (65 μM in 50 mM HEPES, 32.5 mM Ru⁹⁺(NH₃)₆Cl₃, 1 mM EDTA, pH 8.0). The spectrum shown is obtained from 2500 four-spectrum sequences taken on two samples (1250 four-spectrum sequences each), averaged, and smoothed using a low-pass filter on the basis of a fast Fourier transform (FFT). Bottom: Transient absorption kinetics for transient Y₃₅₆* (λₛₒₓ = 408 nm) and a biexponential fit (solid line) (50 μM in 50 mM HEPES, 25 mM Ru⁹⁺(NH₃)₆Cl₃, 1 mM EDTA, pH 8.0) (τ₁ = 8.1 ± 1.1 μs, τ₂ = 2.0 ± 0.8 μs). The trace shown is obtained from 5000 averages taken on a single sample.

Figure 4.8. As obtained transient absorption spectrum of Y*. Raw TA data (°) are shown in addition to the smoothed data (blue solid line), as shown above (Figure 4.7) for reference. Experimental parameters are identical to those given in the main text. Transient absorption spectrum of [Re]-S₃₅₅C-β₂ collected 1 μs after 355 nm excitation (65 μM in 50 mM HEPES, 32.5 mM Ru⁹⁺(NH₃)₆Cl₃, 1 mM EDTA, pH 8.0). The spectrum shown is obtained from 2500 four-spectrum sequences taken on two independent samples (1250 four-spectrum sequences each), averaged, and smoothed using a low-pass filter on the basis of a fast Fourier transform (FFT).

Figure 5.1. An intact, photochemical RNR where a radical is generated directly at Y₃₅₆(β) and is poised to follow the proposed radical transport pathway through αₙ to the active site. Figure prepared from PDB codes 4R1R1* (αₙ) and 1MXR1* (β₂).
The activity of wild-type β₂ (open circles) and S₃₅C–β₂ (closed circles). The activity of S₃₅C–β₂ is nonlinear and diminished with respect to wt–β₂. Each reaction contained, in a volume of 300 μL: 5 μM of the β₂ variant, 1 μM α₂, 30 μM TR, 0.5 μM TRR, 1 mM [³H]-CDP (10750 cpm/nmol), 3 mM ATP, and 1 mM NADPH in assay buffer (pH 7.6).

Figure 5.3. \( K_D \) for [Re]–β₂:wt–α₂ was determined by adding varying amounts of [Re]–β₂ to a solution of wt–RNR; the specific activity was measured for each reaction. Each reaction contained, in a volume of 300 μL: 0.2 μM wt–β₂, 0.1 μM wt–α₂, [Re]–β₂ (0–5 μM), 30 μM TR, 0.5 μM TRR, 1 mM CDP, 3 mM ATP, 0.2 mM NADPH in assay buffer (pH 7.6). Data were analyzed as previously reported, \( K_D = 0.71(8) \) μM. 157

Figure 5.4. Photochemical substrate turnover in the α₂:[Re]–β₂ complex formed from 50 μM met–[Re]–β₂ or met–[Re]–Y₃₅F–β₂ and 20 μM pre-reduced wt–α₂ or Y₇₃F–α₂ (as indicated) in 50 mM borate, 15 mM MgSO₄, 0.2 mM [³H]-CDP (10750 cpm/nmol), 3 mM ATP, 5% glycerol, pH 8.3, \( \lambda _{ex} = 325 \) nm. 157

Figure 5.5. Product formed in a photochemical single-turnover experiment using [Re]–β₂ and wt–α₂ was analyzed by HPLC. The product mixture was supplemented with equimolar dC and cytosine as carriers. Fractions were pooled according to peaks as shown, corresponding to cytosine (red) and dC (blue), and radioactivity measured for each. The relative amount of radioactivity, indicated as a percentage adjacent to each peak, confirms that dCDP is the product of phototurnover. Radioactivity observed in the cytosine fraction corresponds to a radioactive impurity present in all samples, as previously described. 3

Figure 5.6. Comparison of photochemical single-turnover experiments at pH 7.6 and 8.3. At pH 7.6 each experiment contained, in a volume of 300 μL: 30 μM [Re]–β₂, 10 μM wt–α₂, 0.2 mM [³H]-CDP (22713 cpm/nmol), and 3 mM ATP in assay buffer. At pH 8.3 each experiment contained, in a volume of 330 μL: 50 μM [Re]–β₂, 20 μM wt–α₂, 0.2 mM [³H]-CDP (10750 cpm/nmol), and 3 mM ATP in spectroscopy buffer. Error bars indicate 1σ deviations for independently prepared triplicate measurements.

Figure 5.7. Transient absorption spectra of [Re]–β₂ and [Re]–Y₃₅F–β₂ measured immediately after excitation on samples of 10 μM [Re]–β₂ (blue circles) or [Re]–Y₃₅F–β₂ (red circles), 1 mM CDP, and 3 mM ATP in spectroscopy buffer (pH 8.3), \( \lambda _{ex} = 355 \) nm.
Representative data of transient emission kinetics of [Re]−β₂ variants used for Table 5.1. Emission kinetics were measured on samples of 10 μM [Re]−β₂ or [Re]−Y₃₅₆F−β₂ and 25 μM wt−α₂ or Y₇₃₁F−α₂ (as indicated), 1 mM CDP, and 3 mM ATP in assay buffer (pH 7.6), λₑₓcₜ = 355 nm, λₒbs = 600 nm. Data and residuals are given as gray circles, solid lines represent monoexponential decay functions used to calculate lifetimes as shown above. Data reported in Table 5.1 are derived from triplicate data sets for each set of conditions.

Representative data of transient emission kinetics of [Re]−β₂ variants used for Table 5.1. Emission kinetics were measured on samples of 10 μM [Re]−β₂ or [Re]−Y₃₅₆F−β₂ and 25 μM wt−α₂ or Y₇₃₁F−α₂ (as indicated), 1 mM CDP, and 3 mM ATP in spectroscopy buffer (pH 8.3), λₑₓcₜ = 355 nm, λₒbs = 600 nm. Data and residuals are given as gray circles, solid lines represent monoexponential decay functions used to calculate lifetimes as shown above. Data reported in Table 5.1 are derived from triplicate data sets for each set of conditions.

Optimization of flash-quench conditions for [Re]−β₂. (a) transient absorption amplitudes and (b) lifetimes as a function of cumulative laser shots for varying concentrations of Ru³⁺(NH₃)₆Cl₃: 5 mM (brown square), 2 mM (blue circle), 1 mM (green triangle), and 0.5 mM (orange diamond). Transient absorption kinetics (λₒbs = 410 nm) were collected sequentially on samples of 50 μM [Re]−β₂ and Ru³⁺(NH₃)₆Cl₃ (as indicated) in spectroscopy buffer.

Inhibition of coupled RNR activity assay by Ru³⁺(NH₃)₆Cl₃. Incubation of NADPH (1 mM) in the presence of Ru(NH₃)₆Cl₃ (5 mM), TR (30 μM), and TRR (0.5 μM) (as indicated) in assay buffer results in consumption of NADPH as shown, indicating that TRR mediates the oxidation of NADPH by Ru³⁺(NH₃)₆Cl₃.

Measurement of Kᵢ for wt−α₂:[Re]−β₂ under flash-quench conditions. Varying amounts of wt−α₂ were added to solutions of [Re]−β₂ and transient emission kinetics were measured for each solution. Individual traces were fit to monoexponential decay functions and the resulting decay rates (kₒbs) analyzed according to Equation 5.8, from which the Kᵢ was determined to be 0.68(21) μM. Each reaction contained, in a volume of 750 μL: 1 μM [Re]−β₂, wt−α₂ (as indicated), 10 mM Ru³⁺(NH₃)₆Cl₃, 1 mM CDP, and 3 mM ATP in spectroscopy buffer (pH 8.3).

Transient absorption spectra (left) of photogenerated Y₃₅₆−5 μs after excitation and transient absorption kinetics (right) (λₒbs = 410 nm) of 50 μM [Re]−β₂ or [Re]−Y₃₅₆F−β₂ (as indicated), 75 μM wt−α₂ or Y₇₃₁F−α₂, 10 mM Ru³⁺(NH₃)₆Cl₃, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λₑₓ = 355 nm. Open circles show raw spectra and kinetic traces as collected; solid lines show monoexponential fits for kinetics traces and smoothed spectra.
Figure 5.14. Summary data for photogenerated Y\textsubscript{356}\textsuperscript{+}. Smoothed spectra (left) and summary data for transient absorption kinetics (right) taken from raw data shown in Figure 5.13. Colors indicate combinations of [Re]–β\textsubscript{2} and α\textsubscript{2} used for each experiment.

Figure 5.15. Transient absorption spectra of photogenerated Y\textsubscript{356}\textsuperscript{+} 5 μs after excitation of 50 μM [Re]–β\textsubscript{2} or [Re]–Y\textsubscript{356}F–β\textsubscript{2} (as indicated), 10 mM Ru\textsuperscript{iii}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λ\textsubscript{ex} = 355 nm. Open circles show raw spectra and kinetic traces as collected; solid lines show smoothed spectra.

Figure 5.16. Transient absorption spectrum of photogenerated Y\textsuperscript{+} and W\textsuperscript{+} 5 μs after excitation of 50 μM [Re]–β\textsubscript{2}, 75 μM wt–α\textsubscript{2}, 10 mM Ru\textsuperscript{iii}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λ\textsubscript{ex} = 355 nm. Open circles show raw spectra and kinetic traces as collected; solid lines show monoexponential fits for kinetics traces and smoothed spectra.

Figure 5.17. Kinetics of transient tryptophan radical signal formation and decay (λ\textsubscript{obs} = 525 nm) after excitation of 50 μM [Re]–β\textsubscript{2}, 75 μM wt–α\textsubscript{2}, 10 mM Ru\textsuperscript{iii}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λ\textsubscript{ex} = 355 nm. Long (left) and short (right) timescales are shown. The full data are shown in the top panels, the lower panels show the region of tryptophan radical decay (left) and growth (right) in greater detail.

Figure 5.18. Summary of TA spectra for photogenerated W\textsuperscript{+}. Smoothed spectra of photogenerated Y\textsuperscript{+} and W\textsuperscript{+} 5 μs after excitation of 50 μM [Re]–β\textsubscript{2} or [Re]–Y\textsubscript{356}F–β\textsubscript{2}, 75 μM wt–α\textsubscript{2} or Y\textsubscript{356}1 F–α\textsubscript{2} (as indicated), 10 mM Ru\textsuperscript{iii}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λ\textsubscript{ex} = 355 nm. Smoothed spectra from data as shown in Figure 5.16. Colors indicate combinations of [Re]–β\textsubscript{2} and α\textsubscript{2} used for each experiment.

Figure 5.19. Transient absorption spectra for photogenerated W\textsuperscript{+} in the absence of α\textsubscript{2}. As-collected (grey circles) and smoothed spectra (solid lines) of photogenerated W\textsuperscript{+} 5 μs after excitation of 50 μM [Re]–β\textsubscript{2} or [Re]–Y\textsubscript{356}F–β\textsubscript{2}, 10 mM Ru\textsuperscript{iii}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λ\textsubscript{ex} = 355 nm.

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Table 1.1. Thermodynamic parameters for cysteine, tyrosine, and tryptophan as previously reported or extrapolated from known values. Values in black were directly measured, those in grey were calculated as indicated. Reduction are potentials reported in V vs. NHE. Values in black were directly measured, those in grey were calculated as indicated. Reduction are potentials reported in V vs. NHE. Reds are potentials reported in V vs. NHE. As reported. As reported. As calculated from $E^0(C-SH+/C-SH)$, $E^0(C-S/C-S^-)$, and $pK_a(C-SH)$ using Equation 1.A. As reported. As reported. As reported. As reported. As reported. As reported. As reported. As reported. As calculated from $E^0(W-NH+/W-NH)$, $pK_a(W-NH^+)$, and $pK_a(W-NH)$ using Equation 1.A. As reported. As reported. As calculated from $E^0(Y-O+/Y-O^-)$, $pK_a(Y-OH^+)$, and $pK_a(Y-OH)$ using Equation 1.A. As reported. As reported. As reported.

Table 1.2. Reduction potential and $pK_a$ data for unnatural tyrosines used to study radical transport in RNR as previously compiled from previous studies.

Table 2.1. Values for rate constants shown in Figure 2.5 for excited-state dynamics of [Re]-F and [Re]-Y-OH associated to base.

Table 3.1. Time constants (μs) and amplitudes (%) of $F_3 Y$-TA kinetic decays. Monoexponential or biexponential weighted least-squares regression was used. The signals presented are either [Re]-F$_3$ Y-βC19 alone or in the presence of each α$_2$ subunit used in this study.

Table 4.1. Selected metric parameters for [Re]-Br. Distances are reported in Å; angles are reported in degrees.

Table 4.2. Selected Crystal Data and Structure Refinement Parameters for [Re]-Br.

Table 5.1. Tyrosine Dependent Excited-State Quenching at the α$_2$β$_2$ Interface.
Chapter 1  Introduction
1.1. Proton-Coupled Electron Transfer

Electron transfer (ET) and proton transfer (PT) are ubiquitous processes seen across a broad range of chemistry and biology. In reactions where both a proton (H\textsuperscript{+}) and electron (e\textsuperscript{−}) are transferred, frequently the two processes are coupled. These reactions, known collectively as proton-coupled electron transfer (PCET) reactions, are essential to many chemical transformations and represent a fundamental reaction mechanism.\textsuperscript{1-3}

1.1.1. Types of PCET Mechanisms

The simplicity of its definition belies the complexity and variety of PCET processes.\textsuperscript{4} The proton and electron can be transferred simultaneously in a concerted mechanism, or sequentially in a stepwise mechanism. Figure 1.1 illustrates this principle, where the edges represent stepwise mechanisms with discrete electron transfer (ET) and proton transfer (PT) steps (ETPT or PTET), while any trajectory through the interior represents a concerted mechanism, where both a proton (H\textsuperscript{+}) and electron (e\textsuperscript{−}) are transferred in a single microscopic step without the formation of intermediates.

![Diagram of PCET mechanisms](image)

**Figure 1.1.** PCET reactions can be represented schematically in a square scheme, where pathways along the perimeter represent individual proton and electron transfer steps. In this case, the electron and proton donor (D−H) can be transferred stepwise to the acceptor (A), along the edges of the scheme, or in a concerted reaction, represented by the shaded area. Driving force for the given step is proportional to the differences in pK\textsubscript{a} (ΔpK\textsubscript{a}) and reduction potentials (ΔE\textsubscript{p}) as written, with ΔG\textsuperscript{0} = −F×ΔE\textsuperscript{p} for single electron transfer steps and ΔG\textsuperscript{0} = −ln10×RTΔpK\textsubscript{a} for single proton transfer steps.
Similarly, the proton and electron donors and acceptors can occur in a wide variety of configurations, and they need not be located on the same atom or even molecule. The precise arrangement of donors and acceptors can affect the mechanism of PCET. For example, unidirectional PCET reactions, where both proton and electron follow the same path, are fundamentally limited to short distance scales, while in bidirectional PCET, where electron and proton acceptors are distinct, proton movement remains limited to short distances, but can be coupled to a long-range electron transfer.\textsuperscript{1,5}

1.1.2. \textit{pH} Dependence of PCET Reactions in Aqueous Solution

As a result of the coupling between PT and ET, the apparent reduction potential \(E^0\) for a generic species \(X-H\) is a function of pH in aqueous solution for pH regime between the p\(K_a\) of reduced, p\(K_a(XH)\), and oxidized, p\(K_a(XH\textsuperscript{*})\), species. Above p\(K_a(XH)\), the driving force is proton independent and has the reduction potential of \(E^0(X\textsuperscript{*}/X^-)\). For \(pH < pK_a(XH\textsuperscript{*})\), the reaction is proton independent and has a driving force corresponding to \(E^0(XH\textsuperscript{*}/XH)\). The variation of \(E^0\) with pH is given below, as a function of both p\(K_a\) values and the standard reduction potential for the reaction \(E^0_{PCET}\), which is \(E^0_{PH0}(X\textsuperscript{*}/XH)\).

\[
E^0 = E^0_{PCET} + \ln 10 \frac{RT}{F} \log \left[ \frac{10^{-pH} \frac{1 + 10^{(pH-pK_a(XH))}}{1 + 10^{-pH+pK_a(XH\textsuperscript{*})}}} \right]
\]

This equation furnishes a Pourbaix diagram for the coupled 1 electron, 1 proton PCET of a generic species, X–H, shown below with the relevant p\(K_a\) and proton-independent reduction potentials indicated. For p\(K_a(XH\textsuperscript{*}) < pH < pK_a(XH)\), the \(E^0\) varies linearly with pH with a slope of \(\ln10 \times RT/F\) per pH unit, corresponding to \(-59\) mV per pH unit. Notably, the four key thermodynamic parameters for PCET reactions of XH and X—p\(K_a(XH)\), p\(K_a(XH\textsuperscript{*})\), \(E^0(X\textsuperscript{*}/X^-)\), \(E^0(XH\textsuperscript{*}/XH)\)—are not mutually independent. Using the thermodynamic square scheme shown in Figure 1.1, knowledge of any three of the four parameters defines the fourth.
1.1.3. **PCET in Biology**

PCET reactions are critical to a wide variety of biological reactions, including energy conversion, enzymatic substrate activation, and charge transport.\textsuperscript{6-10} The coupling of proton and electron transfers enables difficult oxidations to be achieved while avoiding thermodynamically inaccessible, high energy intermediates.

PCET is critical in the enzymatic activation and synthesis of O\textsubscript{2}. In cytochrome c oxidase, O\textsubscript{2} is reduced to water; concomitantly, four protons are pumped across a membrane to generate a gradient used to drive various uphill reactions, such as ATP synthesis.\textsuperscript{11} PCET is critical to both O\textsubscript{2} reduction and proton pumping processes.\textsuperscript{12,13} The reverse reaction, the photosynthetic oxidation of water to O\textsubscript{2}, occurs at the oxygen evolving complex (OEC) of photosystem II, where PCET plays a critical role in charge transport to the OEC and in water oxidation.\textsuperscript{14,15} In cytochrome P450s,\textsuperscript{16} PCET drives O\textsubscript{2} activation to generate oxidizing heme species,\textsuperscript{17} as well as in the subsequent substrate activation.\textsuperscript{18} In addition to these specific examples, PCET is implicated generally in broad classes of reactions, including O\textsubscript{2} activation at metal cofactors, hydrogen-atom abstraction, and the formation and reactions of amino-acid radicals, making PCET a broadly applicable and important mechanism in biology.
1.2. Ribonucleotide Reductases

Ribonucleotide reductases (RNRs) are a ubiquitous class of enzymes that catalyze the synthesis of DNA precursors, deoxyribonucleoside diphosphates (dNDPs). Nucleoside diphosphates (NDPs) are reduced at the active site by a highly conserved radical mechanism that is mediated by an active-site cysteine residue.19

![Mechanism of NDP reduction at RNR](image)

**Figure 1.3.** Mechanism of NDP reduction at RNR. Numbering shown is for the *E. coli* class Ia enzyme.

1.2.1. *E. coli* Class Ia Ribonucleotide Reductase

The *E. coli* class Ia RNR consists of two homodimeric subunits, α₂ and β₂. The active complex of RNR is an α₂:β₂, the structure of which is shown below. Although individual crystal structures of the α₂ and β₂ subunits exist,20,21 a crystal structure of the active complex is not known. The α₂:β₂ complex shown below is based on a docking model proposed on the basis of electrostatic and shape complementarity21 and more recently confirmed by pulsed electron double resonance (PELDOR)22 and small-angle X-ray scattering (SAXS).23
The large subunit, \( \alpha_2 \), contains the active site of nucleotide reduction, as well as two allosteric regulation sites. The active site binds four distinct NDP substrates (\( N = A, U, C, G \)) and contains the site of the key cysteine thyl radical (C\(_{439}\)) as well as two cysteine residues (C\(_{225}\), C\(_{402}\)) which provide the reducing equivalents for NDP reduction. One allosteric site, termed the activity site, regulates the overall rate of catalysis where binding of ATP promotes activity while dATP is an inhibitor. The second allosteric site, known as the specificity site, modulates the relative reduction of the four substrates by binding deoxynucleoside triphosphates (dNTPs, \( N = A, T, G \)) or ATP. Recent work has shown a dATP-induced change in quaternary structure to give an inactive \( \alpha_4:\beta_4 \) complex.\(^{23}\) The smaller subunit, \( \beta_2 \), contains the site of radical initiation, a diferric-tyrosyl radical cofactor (Y\(_{122}\)) where an unusually stable (\( t_s = 4 \) days at 4 °C for \( E. coli \)) radical resides. Interaction between subunits is dominated by the highly flexible C-terminal tail of \( \beta \) including residues 360–375.\(^{26,27}\)

### 1.2.2. Long-Range Radical Transport in Class Ia RNR

During turnover, the radical is transferred between Y\(_{122}\) in \( \beta_2 \) to C\(_{439}\) in \( \alpha_2 \). These residues are separated by over 35 Å, as determined on the basis of the docking model and PELDOR.\(^{22}\) Single step
tunneling over this distance (predicted $k_{ET} \sim 10^{-4} - 10^{-9}$ s$^{-1}$ for $\beta \sim 1.1 - 1.4$ Å$^{-1}$) is incongruent with the observed rate of turnover (10 s$^{-1}$). As such, a model for radical transport that includes hopping through a series of highly-conserved redox-active aromatic amino acids has been proposed. This model consists of $Y_{122}$ (the site of radical initiation) and $Y_{356}$ in $\beta_2$, as well as $Y_{731}$, $Y_{730}$, and $C_{439}$ (the catalytic active-site thiyl radical) in $\alpha_2$. The criticality of these residues for radical transport has been confirmed on the basis of site-directed mutagenesis studies. Additionally, $W_{46}$ is proposed to play a role in transport; although direct evidence for a $W_{46}^\cdot$ or $W_{46}^\ast$ during turnover has not been observed; direct interrogation by site-directed mutagenesis is not possible as $W_{46}$ is essential for generation of $Y_{122}^\ast$.

**Figure 1.5.** Conserved, redox-active amino acids responsible for long-range radical transport in Class Ia RNR. Figure prepared from PDB entries 1RLR ($\alpha_2$) and 1RIB ($\beta_2$).

In order to account for the long distance between $Y_{122}$ or $W_{46}$ and $Y_{356}$, bidirectional PCET has been proposed for radical transport through $\beta_2$, while the shorter distance transport through $\alpha_2$ can occur by a unidirectional mechanism. Accordingly, orthogonal proton acceptors for $W_{46}$ and $Y_{356}$ have been proposed to be $D_{237}$ and $E_{350}$, respectively. Furthermore, recent work has identified the proton acceptor for $Y_{122}$ during turnover to be a hydroxo ligand to $Fe_1$ of the diferric cluster.
1.2.3. Amino-Acid Radical Generation and Transport Occur by PCET Under Physiological Conditions

Based on the proposed mechanism for long-range radical transport in Class Ia RNR, reversible oxidation of cysteine, tyrosine, and tryptophan occur during turnover. Given the acidity of the corresponding radical cations, deprotonation is thermodynamically favored at neutral pH, making amino-acid radical generation and transport a PCET process.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>$E^0(XH-*/XH)$</th>
<th>$E^0(X-*/X^-)$</th>
<th>pK$_a$(XH)</th>
<th>pK$_a$(XH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>1.35$^c$</td>
<td>0.73$^d$</td>
<td>-2$^f$</td>
<td>8.5$^f$</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.22$^f$</td>
<td>0.50$^b$</td>
<td>4.3$^f$</td>
<td>17$^f$</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.44$^b$</td>
<td>0.72$^f$</td>
<td>-2$^f$</td>
<td>10.1$^f$</td>
</tr>
</tbody>
</table>

**Table 1.1.** Thermodynamic parameters for cysteine, tyrosine, and tryptophan as previously reported or extrapolated from known values. Values in black were directly measured, those in grey were calculated as indicated. Reduction are potentials reported in V vs. NHE. As reported.\(^{33}\) As reported.\(^{34,35}\) \(^c\)Calculated from $E^0(C-SH^*/C-SH)$, $E^0(C-S^*/C-S^-)$, and pK$_a$(C-SH) using Equation (1.A). As reported.\(^{36}\) As reported.\(^{37,38}\) \(^h\)Calculated from $E^0(W-NH^*/W-NH)$, pK$_a$(W-NH$^+$), and pK$_a$(W-NH) using Equation (1.A). As reported.\(^{39}\) As reported.\(^{40}\) \(^i\)Calculated from $E^0(Y-OY^-*/Y-OY^{-})$, pK$_a$(Y-OH$^+$), and pK$_a$(Y-OH) using Equation (1.A).\(^{41}\) As reported.\(^{42}\) \(^m\)As reported.\(^{43}\) As reported.\(^{44}\)

The corresponding Pourbaix diagrams illustrate the pH dependence of the reduction potentials of these amino acids.

**Figure 1.7.** Pourbaix diagrams for cysteine, tryptophan, and tyrosine based on values above and calculated from Equation (1.A).

The data above correspond to amino acids in the radical transport pathway of Class Ia RNR. The values shown above are for the amino acid in aqueous solution. In a protein environment, these parameters can deviate significantly from aqueous solution. Variations in polarity and hydrogen bonding can significantly alter the reduction potentials and pK$_a$ of these amino acids. Thus, the protein environment can tune the redox properties of amino acids to promote stability or specific reactivity.
1.2.4. Interrogating the Radical Transport Pathway with Unnatural Amino Acids

Because turnover in Class Ia RNR is conformationally gated, direct interrogation of the radical transport pathway requires making modifications to the natural system. Site-directed mutagenesis to redox-inactive residues, tyrosine to phenylalanine or cysteine to serine, precludes radical transport. For tyrosine, the use of unnatural amino acids enables systematic variation of reduction potential and \( pK_a \), the key thermodynamic parameters for PCET. Using a battery of unnatural amino acids—including 2,3-dihydroxyphenylalanine (DOPA), 3-aminotyrosine (NH$_2$Y), a variety of fluorotyrosines (F$_n$Y), and 3-nitrotyrosine (NO$_2$Y)—enables tuning of the reduction potential over a 450 mV range and $pK_a$ over a 4 units. These parameters are summarized in the table below.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>$E^o_{\text{aqz}}$ / V vs. NHE</th>
<th>$pK_a$</th>
<th>$E^o_{\text{aqz}}$ / mV vs. YO-/YOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>0.83</td>
<td>9.9</td>
<td>0</td>
</tr>
<tr>
<td>DOPA</td>
<td>0.57</td>
<td>9.7</td>
<td>-260</td>
</tr>
<tr>
<td>3-NH$_2$Y</td>
<td>0.64</td>
<td>10</td>
<td>-190</td>
</tr>
<tr>
<td>3-FY</td>
<td>0.79</td>
<td>8.4</td>
<td>-40</td>
</tr>
<tr>
<td>3,5-F$_3$Y</td>
<td>0.77</td>
<td>7.2</td>
<td>-60</td>
</tr>
<tr>
<td>2,3-F$_3$Y</td>
<td>0.86</td>
<td>7.8</td>
<td>+30</td>
</tr>
<tr>
<td>2,3,5-F$_3$Y</td>
<td>0.86</td>
<td>6.4</td>
<td>+30</td>
</tr>
<tr>
<td>2,3,6-F$_3$Y</td>
<td>0.93</td>
<td>7.0</td>
<td>+100</td>
</tr>
<tr>
<td>2,3,5,6-F$_4$Y</td>
<td>0.97</td>
<td>5.6</td>
<td>+160</td>
</tr>
<tr>
<td>3-NO$_2$Y</td>
<td>1.02</td>
<td>7.2</td>
<td>+190</td>
</tr>
</tbody>
</table>

Table 1.2. Reduction potential and $pK_a$ data for unnatural tyrosines used to study radical transport in RNR as previously compiled from previous studies.

The pH dependence of the apparent reduction potential ($E^o$) makes the relative driving force between residues vary by pH. The pH dependence of $E^o$ can be calculated based on the driving force at any particular pH, such as that given above at pH 7, and the $pK_a$. 

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Initial studies focused on incorporation of Y356 as an attractive target given (i) the inability to locate Y356 crystallographically, (ii) the importance of Y356 for intersubunit radical transport, and (iii) the amenability of position 356 for substitution using expressed protein ligation (EPL), given its position on the conformationally flexible, solution accessible C-terminal tail of β. Using EPL, a variety of unnatural tyrosines have been incorporated at Y356 including 4-aminophenylalanine, DOPA, NO2Y, and five different FY derivatives. Unnatural amino acid substitution at additional pathway positions in β2 and α2 is not possible given by EPL given their positions within the subunits, as EPL requires ligation of a synthetic peptide to the C-terminus of an abbreviated subunit, not possible for positions in the interior of the protein.

The goal of site-specific incorporation of unnatural tyrosines at any position in RNR was achieved using evolved, orthogonal tRNA/tRNA synthetase pairs. This approach has been applied for incorporation of NO2Y, NH2Y, and several FY derivatives into positions along the radical transport pathway throughout α2 and enabling modulation of reduction potentials and pKₐ at each pathway position, from thermodynamic radical sinks (NH2Y, DOPA) to thermoneutral variants (FY, F₂Y) and stronger oxidants (NO2Y, F₃Y, F₄Y) that either result in reactive radicals or block radical transport. Using these...
variants, the radical transport pathway has been interrogated in detail, and a number of important and unique features of long-range radical transport in RNR have been unveiled.

1.2.5. **Unnatural Amino Acid Studies Have Illuminated Radical Transport Through the Pathway**

Beginning at Y₁₂₂, studies of NO₂⁻Y₁₂₂⁻β₂ have shown that the pKₐ at that position is significantly elevated to >9.6, compared to 7.1 for nitrotyrosine in solution, consistent with a very hydrophobic environment and the unusual stability of Y₁₂₂⁻. Furthermore, in the presence of α₂, substrate, and effector, NO₂⁻Y₁₂₂⁻ is reduced to form the nitrotyrosinate, rather than nitrotyrosine, an unusual uncoupling of PT and ET, and cannot be reoxidized by pathway radicals returning from the active site. The observation of NO₂⁻Y₁₂₂⁻ reduction, a step not observed for natural Y₁₂₂⁻, indicates that radical transport is uncoupled from conformational change using the more reactive NO₂⁻Y₁₂₂⁻; the rate of NO₂⁻Y₁₂₂⁻ reduction is significantly faster than turnover in the wild-type enzyme, suggesting that the rate-limiting conformational change is related to radical transport from Y₁₂₂⁻.

The role of Y₃₅₆ in radical transport has been investigated in detail, as mentioned above. Y₃₅₆ is a particularly important target given the lack of structural information about the C-terminal region of β₂ and the role of Y₃₅₆ in mediating intersubunit PCET. Studies with DOPA₃₅₆⁻β₂ (E°ₚH=7.0 (DOPA⁻/DOPA) = -260 mV vs Y⁻/Y) enabled observation of a kinetically competent trapped radical, demonstrating the redox-activity of Y₃₅₆, and further, showed that substrate, allosteric effector, and α₂ are required for radical transport. In F⁻Y₃₅₆⁻β₂, RNR activity is sensitive to the apparent F⁻Y₃₅₆ reduction potential, but not protonation state. This result indicated that Y₃₅₆ was neither a proton acceptor or donor to subsequent radical transport steps, consistent with a bidirectional PCET mechanism for reactions of Y₃₅₆⁻.

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Figure 1.9. Relative activity of F\textsubscript{Y}\textsubscript{356} as a function of apparent fluorotyrosine reduction potential. Data shown with open circles are where pH < pK\textsubscript{a} and closed circles where pH > pK\textsubscript{a}, colors indicate identity of F\textsubscript{Y}: 3,5-F\textsubscript{2}Y (blue), 3,5-F\textsubscript{2}Y (red), 2,3,5-F\textsubscript{3}Y (purple), 2,3,6-F\textsubscript{3}Y (green), 2,3,5,6-F\textsubscript{4}Y (orange).

Critically, the pH rate profiles of F\textsubscript{Y}\textsubscript{356} revealed three distinct regimes. For variants where $E^0(F_\text{Y}/F_\text{Y}) < 80$ mV vs. Y*/Y, wild-type activity was observed, while for $80$ mV < $E^0(F_\text{Y}/F_\text{Y})$ < $200$ mV vs. Y*/Y, activity was dependent upon $E^0(F_\text{Y}/F_\text{Y})$. For the most oxidizing fluorotyrosines, where $E^0(F_\text{Y}/F_\text{Y}) > 200$ mV vs. Y*/Y, activity was greatly diminished and showed little potential dependence. The potential dependence of the second regime suggested that under these conditions, the rate-limiting step was PCET, not conformational change, and further, provided a quantitative estimate for range of potentials over which radical transport is maintained. Such a shift in rate-limiting step might enable observation of radical intermediates along the pathway. Subsequent work using 2,3,5-F\textsubscript{3}Y\textsubscript{356} which falls into the predicted PCET-limited regime, confirmed the change in rate-limiting step, but no intermediates were observed.

To directly study radicals generated in a\textsubscript{2}, the less oxidizing NH\textsubscript{2}Y has been used ($E^0_{pH 7}$(NH\textsubscript{2}Y*/NH\textsubscript{2}Y)=−190 mV vs Y*/Y) as a thermodynamic sink. Substitution of NH\textsubscript{2}Y at positions for Y\textsubscript{730} and Y\textsubscript{731} enabled spectroscopic observation of NH\textsubscript{2}Y*, which was shown to be kinetically competent for deoxynucleotide formation. Subsequent work confirmed that the observed radicals and turnover were indeed pathway dependent, and not the result of NH\textsubscript{2}Y acting as a general radical trap, and analysis of NH\textsubscript{2}Y* and product formation kinetics resulted in a detailed model for radical transport through a\textsubscript{2}. The ability to trap on-pathway NH\textsubscript{2}Y* at Y\textsubscript{731} and Y\textsubscript{730} in a\textsubscript{2}, combined with trapping of DOPA* at Y\textsubscript{356}−β\textsubscript{2}, was
also exploited in a PELDOR study to determine distances between $Y_{122}$ (in the unreacted half of $\beta_2$) and trapped pathway radicals.\textsuperscript{22} The distances observed for $\text{NH}_2Y*$ at $Y_{731}$ and $Y_{730}$ were in agreement with those predicted for the active complex of $\alpha_2$ and $\beta_2$, thereby providing experimental confirmation for the docking model.

\textbf{Figure 1.10.} Distances between unreacted $Y_{122}*$ and trapped pathway radicals ($\text{DOPA}_{356}$, $\text{NH}_2Y_{731}$, $\text{NH}_2Y_{730}$, and an active-site radical from the inhibitor $N_3\text{UDP}$) as determined by PELDOR.\textsuperscript{22,60} These distances provide validation for the docking model. Figure reproduced from a previous report.\textsuperscript{44}

Trapping of $\text{NH}_2Y*$ on the pathway has enabled a number of studies which highlight unique aspects of long-range radical transport in RNR. Subsequent studies of trapped $\text{NH}_2Y_{730}$* have shown that subunit binding is 25-fold stronger when radical is on pathway.\textsuperscript{61} The increased stability of the $\alpha_2;\beta_2$ complex has enabled characterization by small-angle X-ray scattering (SAXS) and electron microscopy, which reveal a compact $\alpha_2;\beta_2$ complex, further confirmation of the docking model.\textsuperscript{21} The transient increase in $\alpha_2;\beta_2$ complex stability suggests an adaptation to protect the on-pathway radical. Further study of $\text{NH}_2Y_{730}$* by electron-nuclear double resonance (ENDOR) and DFT calculations has shown a network of hydrogen bonds including $Y_{731}$, $\text{NH}_2Y_{730}$*, $C_{439}$, and suggest H-bonding with a conserved water molecule.\textsuperscript{62}

The combination of numerous studies has enabled the construction of a thermodynamic landscape across the pathway, including $Y_{122}$ and $Y_{356}$ in $\beta_2$, and $Y_{731}$, $Y_{730}$, and $C_{439}$ in $\alpha_2$.\textsuperscript{44} Based on experiments with
strong oxidants at $Y_{122}$ ($N\text{O}_2 Y^\cdot$, $2,3,5-F_3 Y^\cdot$) and a redox-inactive block ($Y_{356}^\cdot$), $W_{48}$ appears not to be a discrete intermediate in radical transport, however, $W_{48}$ may be mediating PCET between $Y_{122}$ and $Y_{356}^\cdot$ or may be involved in conformational gating during turnover.\textsuperscript{64,65,66} $Y_{122}$ is proposed to be less oxidizing than $Y_{356}^\cdot$ as evidenced by the fact in the reaction of $F_n Y_{122} - \beta_2$ with $Y_{731} F - \alpha_2$ in the presence of substrate and effector, $Y_{356}^\cdot$ is only observed in the case where $F_n Y$ is more oxidizing than natural tyrosine.\textsuperscript{63} Based on the equilibrated populations of $Y_{356}^\cdot$, $Y_{731}^\cdot$, and $Y_{730}^\cdot$, this part of the pathway is proposed to be, in order of increasing reduction potential, $Y_{356}^\cdot < Y_{731}^\cdot \approx Y_{730}^\cdot$.\textsuperscript{64} $C_{439}$ is proposed to be comparable in reduction potential to $Y_{730}^\cdot$ and $Y_{731}^\cdot$ on the basis of solution potentials for thiols, and work that shows intramolecular radical transfer between tyrosine and cysteine.\textsuperscript{65} The overall model is therefore $Y_{122} < Y_{356}^\cdot < Y_{731}^\cdot \approx Y_{730}^\cdot < C_{439}$.\textsuperscript{29} As such, the overall process of PCET along the radical transport pathway is uphill, and chemistry is driven by irreversible chemistry of NDP reduction at the active site.

1.3. Photochemical RNR

A key feature of radical transport in the native RNR system is that radical transport is masked by conformational changes. The above approaches modify the system by using unnatural tyrosines to the properties of individual pathway residues. By rapidly generating an on-pathway radical photochemically, previously inaccessible intermediates—masked by a prior slow conformational step—can be generated and their subsequent reactivity studied.

1.3.1. C-terminal Tail of $\beta$ Enables Access to $Y_{356}^\cdot$ in and Abbreviated Construct

The photochemical RNR (photoRNR) approach has utilized the properties of the conformationally flexible C-terminus of the $\beta$ protein, which mediates both the association of $\alpha_2$ and $\beta_2$ and intersubunit radical transport by virtue of containing $Y_{356}^\cdot$. A peptide consisting of the 19 C-terminal residues of $\beta$ ($\beta C 19$) binds to $\alpha_2$ with $K_D \approx 20 \mu M$.\textsuperscript{26} The 20th amino acid from the C-terminus is $Y_{356}^\cdot$. Appending this tyrosine and a photooxidant (PO) yields a PO-$Y-\beta C 19$ photopeptide. The efficacy of this system was first demonstrated using tryptophan as a photooxidant.\textsuperscript{66} The W-$Y-\beta C 19$ system exhibited light-dependent formation of dCDP in the presence of $\alpha_2$, CDP, and ATP. Product formation was shown to be pathway dependent—no turnover was observed in the presence of $Y_{731} F - \alpha_2$—and dependent on the presence of
the appropriate allosteric effector.

1.3.2. **Photooxidant Choice is Critical for Efficient Radical Generation**

Subsequent variations of the photoRNR system established design criteria for photooxidants. In the case of tryptophan, which requires excitation within the protein envelope, inner filter effects from the $\alpha_2$ protein preclude efficient excitation of the photooxidant. Studies using organic photooxidants showed that both benzophenone and anthroquinone were competent photooxidants for phototurnover. The absorbance profiles of these photooxidants enable excitation at longer wavelengths, up to 300 nm with benzophenone and 375 nm with anthroquinone. Furthermore, light-dependent turnover was pathway dependent, and the oxidizing triplet excited states for both photooxidants, and the corresponding $\text{PO}^+ - \text{Y}^\bullet$ charge separated states are spectroscopically observed. Benzophenone and anthroquinone photoRNRs had the advantages of sufficiently red absorption features for selective excitation outside the protein envelope and enabling the observation of key transient species. Nevertheless, these highly oxidizing species resulted in protein degradation. This observation motivated the development of a less-reactive photooxidant less susceptible to deleterious side reactions with $\alpha_2$.

The family of rhenium tricarbonyl bisimmine complexes are useful photooxidants for tyrosine radical generation. In particular, the complex $\text{Re}^{1}(\text{CO})_3(\text{bpy})(\text{CN})$ (bpy = 4′-methyl-2,2′-bipyridine-4-carboxylic acid) has a long-lived, oxidizing triplet metal-to-ligand charge transfer (3MLCT) excited state, which was capable of reversible tyrosine oxidation. Using this photooxidant in a photoRNR construct offered numerous promising results, including competence for substrate turnover as well as direct observation of the competent radical in a $[\text{Re}^{3+}-3,5$-F$_2$Y$^\bullet$] charge-separated state. Here, fluorotyrosine was employed as its $pK_a$ allows deprotonation under physiological conditions, such that the 3,5-difluorotyrosine residue was deprotonated, making the initial oxidation reaction a rapid electron transfer, rather than a PCET, reaction. Further study defined the $\alpha_2$-peptide binding equilibrium and investigated the kinetics of peptide binding, identifying multiple conformations for $[\text{Re}]$ in the bound peptide.

1.4. **Thesis Scope**

Despite the great value of model systems for PCET and tyrosine oxidation in general, installation
of well-defined proton acceptors adds synthetic complexity and frequently requires derivatization of the tyrosine phenol, perturbing its electronic structure. In apolar media, the tyrosine phenol group can form hydrogen-bonded complexes with bases in solution. Using this strategy, we developed a modular model system for the study of tyrosine oxidation. In this system the base is chosen post-synthetically at the point of sample preparation, enabling facile variation of the PT driving force and proton acceptor. The ET driving force is tunable based on the identity of the 1,10-phenanthroline ligand, which enables changes in the excited-state reduction potential \( E^0[Re^{+/-}] \). In this model system, we have characterized base association, measured a rate of PCET, and determined the relevant equilibrium and kinetic parameters for excited-state quenching. The rates of PCET depend on the identity of the proton acceptor, indicating a concerted PCET mechanism.

![Figure 1.11](image.png)

**Figure 1.11.** A highly modular model system with a self-assembled, hydrogen-bonded proton acceptor enables the study of PCET in tyrosine oxidation.

The remaining chapters of this thesis focus on the further development of photoRNR systems and their use to photochemically investigate the mechanism of long-range radical transport in class Ia RNR. Given the promising results obtained with the \( \text{Re}^+(\text{CO})_3(\text{bpy})(\text{CN}), \, 3,5-\text{F}_2\text{Y} \) based photoRNR system, we pursued detailed investigations of the closely related 2,3,6-trifluorotyrosine (2,3,6-\( \text{F}_3\text{Y} \)) photopeptide, contained in Chapter 3. In this work, we thoroughly characterize the photochemistry of the [Re] photopeptide in the presence of redox-inactive radical pathway mutants of the \( \alpha_2 \) subunit. By directly observing the flash-quench generated 2,3,6-\( \text{F}_3\text{Y}^{*} \), we find the unexpected requirement that \( Y_{730} \) must be
present for oxidation of Y\textsubscript{731} to be observed within the timescale of 2,3,6-F\textsubscript{3}Y\textsuperscript{*} decay.

\[ \beta_2 \text{ surrogate} \quad \text{C-terminus} \quad \text{photopeptide} \quad \text{radical kinetics} \quad \text{monitored by TA} \quad \alpha_2 \text{ oxidation requires} \quad \text{both Y\textsubscript{731} and Y\textsubscript{730}} \]

Figure 1.12. Detailed investigations of the photoRNR system enable the direct observation of radical injection into the \( \alpha_2 \) subunit, reveal a PCET rate for this reaction, and identify the requirement for Y\textsubscript{730} to observe PCET, suggesting a Y\textsubscript{731}Y\textsubscript{730} dyad that is key for radical transport.

Despite the insights gained from the photopeptide system, detrimental conformational flexibility in the photopeptide, fidelity to the natural system, and the potential to extend our studies into \( \beta_2 \) motivated the construction of a full-length photochemical \( \beta_2 \). In Chapter 4, we developed such a system using cysteine alkylation to attach a novel rhenium phenanthroline photooxidant via a bromomethylpyridine ligand.\textsuperscript{73} Initial photochemical studies reveal 3[Re\textsuperscript{I}]*, as expected, and signals for Y\textsuperscript{*} under flash-quench conditions.

Figure 1.13. Alkylation of a cysteine residue installed at position 355 in a single-surface cysteine mutant results in a [Re]–\( \beta_2 \) which furnishes a [Re\textsuperscript{I}]* excited-state capable of Y oxidation.

Having developed an intact, photochemically active [Re]–\( \beta_2 \) subunit, detailed studies of this construct and its complex with \( \alpha_2 \) were performed.\textsuperscript{74} This work reveals formation of a stable \( \alpha_2:[\text{Re}–\beta_2 \) complex and photoinitiated substrate turnover. Detailed nanosecond flash photolysis experiments
reveal Y_{356} oxidation by [Re']* and spectroscopically observed radicals under flash-quench conditions. Most importantly, Y_{356} oxidation is sensitive to the presence of Y_{731}, suggesting a further extension of our previous model for a Y_{731}-Y_{730} dyad for radical transport in α₂ to a tyrosine triad, Y_{356}-Y_{731}-Y_{730} that is operant for transport through the α₂-β₂ interface as well as the α₂ subunit.

![Diagram](image)

**Figure 1.14.** [Re]-β₂ provides a platform for the study of photoinitiated PCET in assembled α₂β₂ complexes of RNR. Photochemical substrate turnover results in product formation in α₂ while time-resolved studies reveal details of tyrosine oxidation at the subunit interface.
1.5. References


(43) CRC Handbook of Chemistry and Physics, 94th Edition.


Chapter 2  Photochemical Tyrosine Oxidation with a Hydrogen-Bonded Proton Acceptor by Bidirectional Proton-Coupled Electron Transfer
Portions of this chapter have been published:

2.1. Introduction

Tyrosyl radicals are key intermediates in a wide variety of energy conversion processes. Tyrosine oxidation has been a subject of significant focus in the literature. Specifically, the proton transfer (PT) component of tyrosine oxidation has been of particular interest both in natural and model systems. Tyrosyl radicals are essential to a biology as diverse as that derived from photosystem II, cytochrome c oxidase and ribonucleotide reductase (RNR). We have developed biophysical tools that permit tyrosine radicals (Y*) to be photogenerated in biological systems with emphasis on RNR. These photoRNRs have been invaluable for deciphering the PCET mechanism by which radicals are generated and transported in this enzyme. The value of Y* photogeneration in the development of new biophysical tools for radical enzymology provides a further imperative for a mechanistic understanding of tyrosine photooxidation.

Formation of tyrosyl radical from tyrosine involves both deprotonation and one-electron oxidation of the native amino acid. Stepwise mechanisms are thermodynamically demanding and result in high-energy intermediates, implicating a PCET mechanism for this reaction. In natural systems, PCET processes occur with exquisite sensitivity and remarkable selectivity by separating the PT coordinate from the ET coordinate, i.e. by installing a bidirectional PCET pathway. This requirement is due to the relatively large mass of a proton in comparison to an electron. Hence, whereas the electron can tunnel over long distances, the proton cannot; the control of the PT coordinate over a different length scale to that of the electron is essential for the kinetic feasibility of a PCET reaction and the attendant opportunity to exercise kinetic control over the reaction.

Well-defined, pre-organized PT pathways and the incorporation of a well-defined proton acceptor are essential for the generation of Y*. A variety of studies have demonstrated the importance of hydrogen bonding on the kinetics of phenol oxidation by PCET. Herein, we report the use of a bidirectional scaffold for the study of tyrosine oxidation that enables the independent variation of the driving force for both proton transfer (PT) and electron transfer (ET), which are critical determinants of the PCET governing tyrosine photooxidation. Pyridyl-amino acid-methyl esters (Py-AA) have been appended to a rhenium(I) tricarbonyl 1,10-phenanthroline core [Re] to yield rhenium–amino acid complexes with tyrosine ([Re]–Y–OH) and phenylalanine ([Re]–F). A PCET network is self-assembled by association.
of [Re]-Y-OH to a base, which is both well-defined and easily varied to permit the PT to be examined in concert with ET to the photoexcited [Re] core. We find that the efficacy of radical generation depends intimately on the strength of the associated base.

2.2. Results

2.2.1. Model System Synthesis and Assembly

The [Re]-AA compounds shown in Scheme 2.1 were developed as a modular platform where relevant driving forces could be varied for the study of PCET. A similar method of their synthesis has recently been reported.\(^4\)

![Scheme 2.1. Synthesis of [Re]-AA](image)

The PCET network is self-assembled by addition of base (pyridine or imidazole) to a solution of [Re]-Y-OH in dichloromethane, as shown in Figure 2.1. This approach for forming the bidirectional PCET network provides a modular and versatile platform for studying tyrosine oxidation.

![Figure 2.1. Self-assembly of a network for the photooxidation of tyrosine by bidirectional PCET with a hydrogen-bonded proton acceptor. For this work, \(N_{\text{base}}\) corresponds to either pyridine or imidazole.](image)
of Y•. Moreover, because F is redox inactive, any disparity in excited-state lifetimes between [Re]-F and [Re]-Y-OH is directly attributable to reactivity at the tyrosine phenol.

2.2.2. *Equilibrium Determination by Emission Quenching*

Ground state absorption and steady state emission spectra of [Re]-Y-OH and [Re]-F (Figure 2.2) are nearly identical to one another and dominated by the electronic properties of the [Re] center.

![Figure 2.2. Electronic absorption and emission spectra of [Re]-Y-OH (blue) and [Re]-F (gold). Spectra shown collected at 50 μM [Re]-AA in dichloromethane.](image)

Hydrogen-bonded association between the tyrosine phenol proton of [Re]-Y-OH and base is evident from emission spectra. In dichloromethane solution, [Re]-Y-OH is highly emissive from a 3MLCT excited state (ν[Re']*). As shown in Figure 2.3, emission from [Re]-Y-OH is quenched significantly upon addition of base (pyridine or imidazole). The equilibrium constants, $K_A$, for association between [Re]-Y-OH and the added base may be measured from the concentration dependence of this quenching. Equilibrium constants were measured for both pyridine ($K_A = 16 ± 2 \text{ M}^{-1}$) and imidazole ($K_A = 157 ± 13 \text{ M}^{-1}$) by fitting the concentration dependence of emission intensity as previously reported.25 The stronger binding to imidazole than to pyridine is in accordance with the relative aqueous pKₐ values. These equilibrium rate constants correspond to reaction free energies of $\Delta G^o = -6.9 \text{ kJ mol}^{-1}$ and $\Delta G^o = -12.5 \text{ kJ mol}^{-1}$ for pyridine and imidazole, respectively.
Figure 2.3. Excited-state quenching titrations monitored by steady-state emission of [Re]-Y-OH. Addition of pyridine (top) or imidazole (bottom) to solutions of [Re]-Y-OH (50 μM in dichloromethane) results in significant quenching of steady-state emission ($\lambda_{em} = 400$ nm). Spectra are shown in the absence of base (blue) and the presence of increasing concentrations of base (grey). Association constants are calculated using integrated emission intensity as previously reported (insets). 25

2.2.3. Nanosecond Flash Photolysis

Transient absorption spectra indicate that the $3[Re']^*$ excited state reacts with base by electron transfer. The transient absorption spectra and single-wavelength kinetic data for solutions of [Re]-Y-OH and [Re]-F in dichloromethane are shown in Figure 2.4 (top) in the absence of base. Transient spectra exhibit absorption features as expected for compounds of this type. 26 Significant growth features are observed at 300 and 450 nm. The transient signals decay monoexponentially; $3[Re']^*$ excited state in [Re]-Y-OH is slightly shorter than [Re]-F. This is consistent with oxidation of tyrosine by the [Re]-Y-OH. The $3[Re']^*$ excited state is highly oxidizing and is of sufficient potential to oxidize base ($E^0(Re^{7+/0}) = 1.7$ V vs. NHE) 27. This reduction potential was measured in acetonitrile and provides only an estimate for the present report where experiments are conducted in dichloromethane. When pyridine or imidazole is added to solutions of [Re]-F and [Re]-Y-OH, the TA signal of the $3[Re']^*$ excited state is considerably
shortened, more so for imidazole than pyridine (Figure 2.4, bottom). Concentrations of base were chosen to ensure that at least 95% of \([\text{Re}]\cdot \text{Y-OH}\) was bound. We note that a transient signal for photooxidized tyrosine is not observed, indicating that the intermediate does not accumulate owing to back electron transfer rate that is faster than the forward PCET rate constant for quenching.

![Figure 2.4](image)

**Figure 2.4.** Transient absorption spectra (\(\lambda_{ex} = 355\) nm) of \([\text{Re}]\cdot \text{F}\) (gold) and \([\text{Re}]\cdot \text{Y-OH}\) (blue) (50 \(\mu\)M in dichloromethane) in the absence of base (top), and in the presence of 0.20 M imidazole (bottom). Spectra are collected immediately after the laser pulse and every 1 \(\mu\)s (in the absence of base) or 150 ns (in the presence of base) thereafter. Single-wavelength emission decay kinetics monitored at 550 nm are shown in the insets. Decays are monoexponential. The lifetimes derived from the monoeponential fit, shown by the solid line, are given.

The quenching paths of \(^5\text{[Re]}^+\) for \([\text{Re}]\cdot \text{F}\) and \([\text{Re}]\cdot \text{Y-OH}\) are shown in Figure 2.5. In this model, we assume that the intrinsic decay processes of and bimolecular processes of \([\text{Re}]\cdot \text{Y-OH}\) and \([\text{Re}]\cdot \text{Y-OH---N}_{\text{base}}\) cannot be distinguished in excited state decay profiles.

The excited-state reactivity of \([\text{Re}]\cdot \text{F}\) with base is straightforward; the emission will decay with intrinsic radiative and nonradiative process defined by \(k_0\) (\(= 1/\tau_0\)) or react with base by electron transfer, defined by the bimolecular quenching rate constant \(k_q\).
Figure 2.5. Excited-state quenching pathways for [Re]-F (top) and [Re]-Y-OH---NBase (bottom). The rate constants $k_0$ and $k_q$ are assumed to be the same for both paths, and are defined by the natural lifetime of [Re]-F and the bimolecular quenching of [Re]-F by base, respectively.

In a Stern-Volmer process, the overall observed emission rate constant for reaction ($= 1/\tau_p$) should follow the kinetics,

$$k_{obs} = \frac{1}{\tau_p} = k_0 + k_q \text{[base]}$$

As predicted by Equation 2.1, Stern-Volmer plots are linear with increasing concentration of base. As determined from the data in Figure 2.6, the bimolecular quenching rate constants are $k_q = 3.2(6) \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ and $3.1(6) \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ for pyridine and imidazole, respectively. These data are summarized in Table 2.1.

The excited-state decay processes of $^3\text{[Re]}^*$ in the [Re]-Y-OH---N_{Base} assembly are richer. In addition to the natural decay of the $^3\text{[Re]}^*$ excited state and its bimolecular quenching by base, two unique unimolecular processes arise from electron transfer from Y-OH to $^3\text{[Re]}^*$ and PCET. The excited state dynamics of the rhenium center should not be affected by the remote amino acid inasmuch as it is not conjugated to the ligands of the rhenium center. Hence $k_0$ and $k_q$ to a first approximation, should be the same for both systems. One possible exception to this approximation may be the difference in reduction potential of the bound base relative to base in solution; however, the large excess of free base is expected...
to dominate the kinetics of base oxidation by $^3[\text{Re}]^\ast$. Moreover, the Re–F center provides a reference for the ET process between the tyrosine not associated to base and the excited rhenium center. The $k_{\text{ET}}$ rate constant is given by the following equation, where $\tau_y$ and $\tau_x$ are the excited-state lifetimes for [Re]–F or [Re]–Y–OH, respectively, in the absence of base.

$$k_{\text{ET}} = \frac{1}{\tau_{\text{obs}}} - \frac{1}{\tau_0} = \frac{1}{\tau_y} - \frac{1}{\tau_F}$$

From the data in Figure 2.4 and rate constants summarized in Table 2.1, a $k_{\text{ET}} = 6.1 \times 10^4 \text{ s}^{-1}$ is determined. Whereas the kinetics associated with ET are straightforward, the PCET process is more complicated. The emission decay dynamics are modulated by the equilibrium between [Re]–Y–OH and $N_{\text{base}}$. For this reason, a linear Stern-Volmer relation is not expected. The base concentration dependence of the emission lifetime is shown in Figure 2.6.

Figure 2.6. Excited-state quenching titrations monitored by emission lifetime. Re–F and Re–Y (50 µM in dichloromethane) are excited ($\lambda_{\text{ex}} = 355$ nm) and time resolved emission ($\lambda_{\text{obs}} = 550$ nm) kinetics are recorded on the ns to µs timescale. The excited-state quenching rate-constants ($k_{\text{obs}}$) are calculated from the emission lifetimes for Re–AA, measured as a function of base concentration. Data for Re–F (bottom) are fit to Equation (2.2) (gold); data for Re–Y (top) are fit to Equation (2.3) (blue).
The rate of excited-state decay for [Re]–Y–OH varies as a function of base concentration according to the following equation,

\[
(2.3) \quad k_{\text{obs}} = \frac{1}{\tau_Y} = k_0 + k_q[\text{base}] + k_{\text{ET}} + k_{\text{PCET}} \frac{K_A[\text{base}]}{1 + K_A[\text{base}]}
\]

Detailed derivations of these rate laws (Equations (2.1) and (2.3)) are available below. The last term of the above equation accounts for the equilibrium between [Re]–Y–OH and base, and the PCET process enabled by that equilibrium. The only unknown variable in the above equation is \( k_{\text{PCET}} \). Fitting the emission lifetime, \( k_{\text{obs}} \), to the concentration of base furnishes \( k_{\text{PCET}} = 4.1(6) \times 10^5 \text{ s}^{-1} \) and \( k_{\text{PCET}} = 4.8(8) \times 10^6 \text{ s}^{-1} \) and for pyridine and imidazole, respectively. These values, and all relevant equilibrium and rate-constants are summarized in Table 2.1.

<table>
<thead>
<tr>
<th>Base-Independent Parameters</th>
<th>[Re]–F</th>
<th>[Re]–Y–OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_0 / \text{s}^{-1} )</td>
<td>( 7.1 \times 10^5 )</td>
<td>( 7.1 \times 10^6 )</td>
</tr>
<tr>
<td>( k_T / \text{s}^{-1} )</td>
<td>n/a</td>
<td>( 6.1 \times 10^4 )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Base-Dependent Parameters</th>
<th>pyridine</th>
<th>imidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_A / \text{M}^{-1} )</td>
<td>16(2)</td>
<td>157(13)</td>
</tr>
<tr>
<td>( k_q / \text{M}^{-1} \text{s}^{-1} )</td>
<td>( 3.2(6) \times 10^5 )</td>
<td>( 3.1(6) \times 10^7 )</td>
</tr>
<tr>
<td>( k_{\text{PCET}} / \text{s}^{-1} )</td>
<td>( 4.1(6) \times 10^5 )</td>
<td>( 4.8(8) \times 10^6 )</td>
</tr>
</tbody>
</table>

\( ^a \) Determined from natural lifetime of [Re]–F in dichloromethane at room temperature. 
\( ^b \) Calculated from equation (2.2) from data in Figure 2.4.
\( ^c \) Calculated from data in Figure 2.2 according to ref. 25.
\( ^d \) Calculated from equation (2.1) from data in Figure 2.4.
\( ^e \) Calculated from equation (2.3) from data in Figure 2.6.

**Table 2.1.** Values for rate constants shown in Figure 2.5 for excited-state dynamics of [Re]–F and [Re]–Y–OH associated to base.

### 2.3. Discussion

These PCET rates are comparable to those previously observed in unimolecular systems incorporating hydrogen bonding to phenols within bimolecular\(^7\) and unimolecular (tethered)\(^8\) frameworks. In these cases, the photoacceptor is tris(bipyridine)ruthenium(II), which is less oxidizing than...
the Re(I) polypyrrolidyl excited state used in this work. Hydrogen bonding facilitates PCET by substantially decreasing the inner-sphere contribution to reorganization energy from the phenol and by introducing 'promoting' vibrational modes; the thermodynamic strength of the hydrogen bond has been observed to have a lesser impact on PCET rate enhancement.\textsuperscript{19} This is not the case here. The rate-enhancement for phenol oxidation in [Re]-Y-OH---N\textsubscript{base} varies with the strength of the hydrogen bond as has also recently been observed in protein maquettes.\textsuperscript{28} The correlation may be more apparent for [Re]-Y-OH---N\textsubscript{base} owing to the similarity of the hydrogen bond type formed by imidazole and pyridine. Previous studies on phenol photooxidations have emphasized hydrogen-bonding networks that are formed from carboxylates, where differences in the hydrogen-bonded adduct (e.g., six- or seven-membered rings formed in the two cases) likely introduce important vibrational effects and the greater sensitivity to the vibrational modes of the hydrogen bonding network. When this complexity is removed, it appears that the PCET rate for photooxidation follows a more straightforward thermodynamic trend with the strength of the hydrogen bonds within the PCET network.

With respect to the observed variation of $k_{\text{PCET}}$ with respect to the proton acceptor pK$\text{a}$, previous work in a bimolecular system has shown that as the strength of the base (proton acceptor) increases, faster rates of PCET are observed for phenol oxidation.\textsuperscript{29,30} In recent related work, the importance of hydrogen-bond distance (rather than strength) on the rate of PCET reactions has been investigated.\textsuperscript{31,32} Although the present scaffold does not offer a way to directly control the proton transfer distance, the facile variation of hydrogen-bond strength that can be achieved with this scaffold may yield valuable insights of interest to this discussion.

2.4. Conclusion

The assembly of a rhenium-tyrosine complex associated to base establishes a network for the photogeneration of tyrosyl radical by a PCET mechanism. An equilibrium interaction between the tyrosine phenol proton and bases in solution provides a well-defined proton acceptor, simplifying analysis of PCET kinetics. The observed rates of tyrosine oxidation associated to base are consistent with those reported in the literature for intramolecular model systems. The approach affords for the self-assembly of
a modular scaffold for the study of bidirectional PCET by simply varying the added base. To this end, the approach reduces the complexity associated with the synthesis of tethered networks.

2.5. Experimental Information

Syntheses of Py-Y, Py-F, and [Re]-Y-OH, and characterization of the binding between [Re]-Y-OH and base were performed by Jay L. Yang.

2.5.1. General Considerations

The [Re]-AA compounds are synthesized via the route shown in Scheme 2.1. Syntheses were accomplished by methodologies described below and products were spectroscopically and analytically characterized in detail to ensure compound identification and purity.

Steady-state spectroscopy was performed using dilute solutions of [Re]-AA (AA = Y or F) complexes. Emission titrations were performed by adding sequential volumes of pyridine (neat) or imidazole (1.0 M in dichloromethane) to a sample of [Re]-Y-OH (50 μM in dichloromethane). Equilibrium constants were determined for the association of [Re]-Y-OH and pyridine or imidazole by monitoring emission quenching as a function of base added. Fitting to determine the equilibrium association constants for [Re]-Y-OH binding to bases was performed according to published methods.25

Time-resolved absorption and emission experiments were completed by nanosecond flash photolysis, which were performed using a system which was significantly modified from one previously described.11,33 Emission lifetimes were measured for both [Re]-Y-OH and [Re]-F as a function of pyridine and imidazole concentrations. Errors reported for rate constants and equilibrium constants measured are two standard deviations, as calculated from the standard error of the corresponding fit.

1H NMR spectra were obtained using a Varian Inova-500 NMR spectrometer at the MIT Department of Chemistry Instrumentation Facility (DCIF) and internally referenced using the proteo impurity for the relevant deuterated solvent (d6-acetone or d2-dichloromethane). Chemical shifts are reported relative to tetramethylsilane (TMS). Elemental analysis data were obtained from Midwest Microlab, LLC (Indianapolis, IN). UV-vis absorption spectra were collected on a Varian Cary 5000 UV-vis-NIR spectrometer; steady-state emission data were collected using a PTI QM 4 Fluorometer equipped
with a 150 W Xe-arc lamp for excitation and a photomultiplier tube (Hamamatsu R928) cooled to -78°C for detection. Samples for both absorption and emission experiments were dilute solutions of the complexes as indicated (10 – 50 μM) in quartz spectroscopy cells.

2.5.2. Materials

L-tyrosine methyl ester (98%, Aldrich), isonicotinic acid (Aldrich), 1-hydroxybenztriazole hydrate (HOBT) (Advanced ChemTech), N-methylmorpholine (NMM) (Alfa-Aesar), dichloromethane (DCM) (99.5%, Sigma-Aldrich), L-phenylalanine methyl ester hydrochloride (98%, Aldrich), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC·HCl) (98%, Aldrich), dimethylformamide (DMF) (Anhydrous, 99.8%, Sigma-Aldrich), citric acid monohydrate (Mallinckrodt Chemicals), sodium bicarbonate (NaHCO₃) (Sigma-Aldrich), magnesium sulfate (MgSO₄) (anhydrous, Sigma-Aldrich), ethyl acetate (EtOAc) (99.9%, Sigma-Aldrich) pentacarbonylchlororhenium(I) (Re(CO)₅Cl) (98%, Strem), 1,10-phenanthroline (Phen) (99%, Aldrich), thallium(I) hexafluorophosphate (TlPF₆) (97%, Strem), acetonitrile (MeCN) (99.9%, Sigma-Aldrich), toluene (99.5%, Sigma-Aldrich), acetone (99.9%, Sigma-Aldrich), diethyl ether (Et₂O) (99.0%, Sigma-Aldrich), d⁶-acetone ((CD₃)₂CO) (Cambridge Isotope Laboratories), d²-dichloromethane (CD₂Cl₂) (Cambridge Isotope Laboratories), pyridine (Anhydrous, 99.8%, Sigma-Aldrich), and imidazole (99.5%, Sigma-Aldrich) were used as received. Tricarbonyl(1,10-phenanthroline)(acetonitrile)rhenium(I) hexafluorophosphate ([Re(Phen)(CO)₃(NCMe)]PF₆) was prepared as previously reported.³⁴

2.5.3. Synthesis

2.5.3.1. 4-Isonicotinoyl-L-tyrosine methyl ester (Py-Y)

Isonicotinic acid (837 mg, 6.8 mmol, 1.0 eq.), L-tyrosine methyl ester (1361 mg, 7.0 mmol, 1.0 eq.), HOBT (994. mg, 7.4 mmol, 1.1 eq.), EDC·HCl (1438 mg, 7.5 mmol, 1.1 eq.), and NMM (3.4 mL, 31 mmol, 4.1 eq.) were dissolved in 250 mL DMF/DCM (1:10 v/v) and stirred at room temperature for 3 days. The reaction mixture was washed sequentially with 1% citric acid (w/v in H₂O, 100 mL), 1% NaHCO₃ (w/v in H₂O, 100 mL), and H₂O (4 × 100 mL). The organic layer was dried over MgSO₄ and the solvent removed by rotary evaporation. Crude product was separated by flash chromatography (silica,
EtOAc) to yield pure Py-Y as a white solid (594 mg, 29%). $^1$H NMR (500 MHz, CD$_2$Cl$_2, 20$°C): $\delta = 8.71$ (m, 2H, Py-H), 7.56 (m, 2H, Py-H), 6.98 (m, 2H, Ar-H), 6.75 (m, 2H, Ar-H), 6.65 (d, 1H, N-H), 4.98 (m, 1H, C$_o$-H), 3.77 (s, 3H, OCH$_3$), 3.18 (m, 2H, C$_\beta$-H); Anal. calcd. for C$_{16}$H$_{16}$N$_2$O$_4$: C, 63.99; H, 5.37; N, 9.33; found: C, 64.08; H, 5.42; N, 9.32.

2.5.3.2. 4-Isonicotinoyl-L-phenylalanine methyl ester (Py-F)

Isonicotinic acid (423 mg, 3.4 mmol, 1.0 eq.), L-phenylalanine methyl ester hydrochloride (746 mg, 3.5 mmol, 1.0 eq.), HOBt (520 mg, 3.9 mmol, 1.1 eq.), EDC-HCl (724 mg, 3.8 mmol, 1.1 eq.), and NMM (1.5 mL, 14 mmol, 4.1 eq.) were dissolved in 250 mL DMF/DCM (1:10 v/v) and stirred at room temperature for 3 days. The reaction mixture was washed sequentially with 1% citric acid (w/v in H$_2$O, 100 mL), 1% NaHCO$_3$ (w/v in H$_2$O, 100 mL), and H$_2$O (4 x 100 mL). The organic layer was dried over MgSO$_4$ and the solvent removed by rotary evaporation. Crude product was separated by flash chromatography (silica, EtOAc) to yield pure Py-F as a waxy white solid (656.3 mg, 67%). $^1$H NMR (500 MHz, CD$_2$Cl$_2, 20$°C): $\delta = 8.71$ (m, 2H, Py-H), 7.56 (m, 2H, Py-H), 7.28 (m, 3H, Ar-H), 7.14 (m, 2H, Ar-H), 6.71 (d, 1H, N-H), 5.03 (m, 1H, C$_o$-H), 3.77 (s, 3H, OCH$_3$), 3.25 (m, 2H, C$_\beta$-H); Anal. calcd. for C$_{16}$H$_{16}$N$_2$O$_3$: C, 67.59; H, 5.67; N, 9.85; found: C, 67.98; H, 5.73; N, 9.89.

2.5.3.3. Tricarbonyl (1,10-phenanthroline)(Py-Y)rhenium(I) hexafluorophosphate (Re-Y)

[Re(Phen)(CO)$_3$(NCMe)]PF$_6$ (213 mg, 0.34 mmol, 1.0 eq.) and Py-Y (131 mg, 0.44 mmol, 1.3 eq.) were dissolved in 20 mL acetone and heated to reflux with stirring overnight. The solvent was removed by rotary evaporation and the resulting yellow oil was redissolved in 10 mL DCM, to which 200 mL Et$_2$O was added to precipitate the desired product. The resulting solid was isolated by vacuum filtration to yield Re-Y as a pale yellow powder (264 mg, 88%). $^1$H NMR (500 MHz, CD$_2$Cl$_2, 20$°C): $\delta = 9.57$ (m, 2H, Ar-H), 8.76 (s, 2H, Ar-H), 8.34 (m, 2H, Ar-H), 8.13 (m, 4H, Ar-H), 7.40 (m, 2H, Ar-H), 6.83 (m, 2H, Ar-H), 6.63 (d, 1H, N-H), 6.56 (m, 2H, Ar-H), 4.76 (m, 1H, C$_o$-H), 3.69 (s, 3H, OCH$_3$), 3.01 (m, 2H, C$_\beta$-H). Anal. calcd. for C$_{31}$H$_{24}$F$_6$N$_2$O$_4$PF$_6$: C, 41.57; H, 2.70; N, 6.25; found: C, 41.36; H, 2.80; N, 6.17.

2.5.3.4. Tricarbonyl (1,10-phenanthroline)(Py-F)rhenium(I) hexafluorophosphate (Re-F)

[Re(Phen)(CO)$_3$(NCMe)]PF$_6$ (234 mg, 0.37 mmol, 1.0 eq.) and Py-F (134 mg, 0.47 mmol, 1.3 eq.) were dissolved in 20 mL acetone and heated to reflux with stirring overnight. The solvent was
removed by rotary evaporation and the resulting yellow oil was redissolved in 10 mL DCM, to which 200 mL Et₂O was added to precipitate the desired product. The resulting solid was isolated by vacuum filtration to yield Re-F as a pale yellow powder (280 mg, 87%). ¹H NMR (500 MHz, CD₂Cl₂, 20 °C): δ = 9.56 (m, 2H, Ar-H), 8.78 (s, 2H, Ar-H), 8.30 (m, 2H, Ar-H), 8.14 (m, 4H, Ar-H), 7.45 (m, 2H, Ar-H), 7.20 (m, 3H, Ar-H), 7.09 (m, 2H, Ar-H), 6.82 (d, 1H, N-H), 4.79 (m, 1H, Ce-H), 3.66 (s, 3H, OCH₃), 3.04 (m, 2H, C₆-H).

2.5.4. Emission Quenching Titrations

Emission titrations were performed by adding sequential volumes of pyridine (neat) or imidazole (1.0 M in DCM) to a sample of Re-Y (50 μM in DCM). Emission spectra were collected using a fluorometer and rescaled to account for changes in Re-Y concentration using the following equation.

\[
I_r = I_c \left( \frac{V_0}{V_0 + \Delta V} \right)
\]

Here, \(I_r\) is the rescaled emission spectrum, \(I_c\) is the corrected emission spectrum obtained from the fluorometer directly, \(V_o\) is the initial volume of the sample, and \(\Delta V\) is the total additional volume added to the sample to reach the indicated concentration. The rescaled emission spectra were integrated; the integrated emission intensity, I, was used in subsequent analysis.

2.5.5. Nanosecond Laser Flash Photolysis

Nanosecond timescale laser flash photolysis experiments utilized a system that has previously been reported, with a number of modifications. For this report, samples were flowed without recirculation to prevent interference from decomposition products. Within the system previously described and referenced above, one of two diffraction gratings was used for each of these experiments. All reported transient absorption experiments (full spectra and single wavelength kinetics) were performed using the 250 nm blaze grating (300 grooves/mm); emission kinetics studies employed the 500 nm blaze grating (300 grooves/mm). Transient absorption spectra reported are an average of 1000 four-spectrum sequences; for time-resolved emission experiments, individual traces are an average of 200 sweeps.
2.5.6. **Equilibrium Association Constant Determination**

Equilibrium constants were determined for the association of [Re]-Y-OH and pyridine or imidazole by monitoring emission quenching as a function of base added. The titration was performed in accordance with the procedure described above in the experimental section to yield corrected emission spectra as shown in the main text (Figure 3). For each concentration, the integrated emission intensity was computed by summing the individual intensity values over the range of wavelengths for the emission spectrum. Fitting to determine the equilibrium association constants for [Re]-Y-OH binding to bases was performed according to published methods.\(^{25}\)

2.5.7. **Base-Dependent Quenching Kinetics**

Emission lifetimes were measured for both [Re]-F and [Re]-Y-OH as a function of pyridine and imidazole concentrations. For each of the four possible combinations, six different concentrations were measured. For each independent sample, five transient emission kinetics traces were collected.

In order to extract excited state lifetimes, each trace is fit to an exponential decay function in OriginPro (v. 8.0, OriginLab, Northampton, MA). Each fit yields an exponential decay constant, taken to be the measured excited-state lifetime for the given trace. Emission kinetics were monitored at 550 nm.

2.5.8. **Detailed Analysis of Excited-State Decay Pathways**

The observed lifetimes, \(\tau_{\text{obs}}\), for both [Re]-F and [Re]-Y-OH correspond to the excited-state lifetime and rate constant for excited-state decay, \(k_{\text{obs}}\), according to Equation (2.4).

\[
\frac{d[\text{Re}^*-\text{AA}]}{dt} = -k_{\text{obs}}[\text{Re}^*-\text{AA}] = -\frac{1}{\tau_{\text{obs}}}[\text{Re}^*-\text{AA}]
\]

Figure 2.5 illustrates the proposed pathways for [Re]-Y-OH excited-state decay. There are both static and dynamic quenching components, which correspond to reactions of bound and free [Re]-Y-OH, respectively. To construct a rate law, each of these decay pathways must be incorporated. Each of the terms below, and their associated rate constants, is associated with a particular excited-state decay pathway. The overall rate law for [Re]-Y-OH excited-state decay is therefore composed of four terms.

The first term is an intrinsic unimolecular rate of excited state decay which depends on [Re*-Y],

\[76\]
the total concentration of $[^1\text{Re}^* \text{MLCT}]$ excited-state and $k_o$ is the intrinsic rate constant for excited state decay in the absence of any charge-transfer processes. The second decay pathway involves intramolecular oxidation of tyrosine and depends upon $k_{ET}$, the rate constant for intramolecular oxidation of the tyrosine phenol without proton transfer to base.

The bimolecular terms include a rate for intermolecular base oxidation, where $k_q$ is the rate constant for bimolecular oxidation of base in solution by the [Re] excited state and [B] is the concentration of base in solution. The final term shown corresponds to intramolecular tyrosine oxidation with associated proton transfer to a hydrogen-bonded base, where $k_{PCET}$ is the rate constant for tyrosine oxidation with concomitant proton transfer to base, and $[^1\text{Re}^*-\text{Y}]_b$ is the concentration of [Re]-Y-OH molecules that are bound to base.

$$\frac{d[^1\text{Re}^* - \text{Y}]}{dt} = -k_o[^1\text{Re}^* - \text{Y}] + k_{ET}[^1\text{Re}^* - \text{Y}] - k_q[^1\text{Re}^* - \text{Y}][\text{B}] - k_{PCET}[^1\text{Re}^* - \text{Y}]_B$$

(2.6)

In order to define a raw law only in terms of measured parameters and experimental variables, it would be useful to replace the term for bound [Re]-Y-OH molecules only, $[^1\text{Re}^* - \text{Y}]_b$, in terms of the total concentration, $[^1\text{Re}^* - \text{Y}]$. The concentration of bound [Re]-Y-OH is related to the concentration of base in solution, the total concentration of [Re]-Y-OH in solution, the equilibrium constant for this association reaction, $K_A$, which can be measured by steady-state emission quenching. $[^1\text{Re}^* - \text{Y}]_u$ is the concentration of [Re]-Y-OH molecules that are not bound to base.

$$K_A = \frac{[^1\text{Re}^* - \text{Y}]_B}{[\text{B}][^1\text{Re}^* - \text{Y}]_U}$$

(2.7)

By definition, $[^1\text{Re}^* - \text{Y}]_b$ and $[^1\text{Re}^* - \text{Y}]$ are related by $\chi_B$.

$$[^1\text{Re}^* - \text{Y}]_B = \chi_B[^1\text{Re}^* - \text{Y}]$$

(2.8)

Similarly, $[^1\text{Re}^* - \text{Y}]_u$ and $[^1\text{Re}^* - \text{Y}]$ are related by $\chi_u$.

$$[^1\text{Re}^* - \text{Y}]_U = (1 - \chi_B)[^1\text{Re}^* - \text{Y}]$$

(2.9)
Substituting for \([\text{Re}^*-\text{Y}]_0\) and \([\text{Re}^*-\text{Y}]_a\) in terms of \(\chi_y\), the fraction of bound \([\text{Re}]-\text{Y-OH}\).

\[
K_A = \frac{\chi_B}{[B](1 - \chi_B)}
\]

Rearranging, \(\chi_B\) is as follows.

\[
\chi_B = \frac{K_A[B]}{1 + K_A[B]}
\]

Substituting:

\[
[\text{Re}^*-\text{Y}]_B = \frac{K_A[B]}{1 + K_A[B]}[\text{Re}^*-\text{Y}]
\]

Therefore, the overall rate law is:

\[
\frac{d[\text{Re}^*-\text{Y}]}{dt} = -(k_0 + k_{ET} + k_q[B] + k_{PCET} \frac{K_A[B]}{1 + K_A[B]})[\text{Re}^*-\text{Y}]
\]

Recalling Equation (2.5):

\[
\frac{1}{\tau_Y} = k_0 + k_{ET} + k_q[B] + k_{PCET} \frac{K_A[B]}{1 + K_A[B]}
\]

In order to calculate the desired rate constant, \(k_{\text{PCET}}\), the other four parameters \((k_0, k_{ET}, k_q, K_A)\) must be determined. Fitting the data to Equation (2.14) should theoretically yield values for \(k_{\text{PCET}}, k_q, K_A\), and the sum \(k_0 + k_{ET}\); however, the relatively small number of data points makes accurately determining all of these constants infeasible. By simplifying the system to systematically exclude individual effects, each rate constant can be determined with greater accuracy.

Returning to the basic rate law and recalling the inability of \([\text{Re}]^-\text{F}\) to undergo phenol oxidation reactions or participate in association with base, we obtain a much-simplified expression.

\[
\frac{d[\text{Re}^*-\text{F}]}{dt} = -(k_0 + k_q[B])[\text{Re}^*-\text{F}]
\]

Again substituting with Equation (2.5), we obtain a similar relationship for lifetimes of the \([\text{Re}]^-\text{F}\), which as expected, is equivalent to the Stern-Volmer relationship.

\[
\frac{1}{\tau_F} = k_0 + k_q[B]
\]
Using a typical Stern-Volmer analysis, the base dependence of [Re]-F lifetimes yields values for $k_0$ and $k_\phi$. As described above, analysis of a steady-state emission titration yields values for $K_A$. With these values in hand, values for $k_{ET}$ and $k_{PCET}$ can be extracted from a fit where previously determined values for $k_0$, $k_\phi$, and $K_A$ are used.

An alternative approach is to directly use the difference in excited-state lifetimes between [Re]-F and [Re]-Y-OH, a quenching rate constant, to determine the overall rate of tyrosine oxidation. This approach relies on the fact all processes except tyrosine oxidation that occur for [Re]-Y-OH must necessarily occur for [Re]-F as well. The quenching rate constant, $k_{obs}$, is therefore, in terms of the observed rate constants, defined by Equation (2.17).

\[
(2.17) \quad k_{obs} = \frac{1}{\tau_Y} - \frac{1}{\tau_F}
\]

At a given base concentration, subtracting Equation (2.16) from (2.14),

\[
(2.18) \quad \frac{1}{\tau_Y} - \frac{1}{\tau_F} = k_{ET} + k_{PCET} \frac{K_A[B]}{1 + K_A[B]}
\]

Finally, substituting into (2.17):

\[
(2.19) \quad k_{obs} = k_{ET} + k_{PCET} \frac{K_A[B]}{1 + K_A[B]}
\]

This data can be fit to Equation (2.19) leaving all three parameters ($K_A$, $k_{ET}$, and $k_{PCET}$) free; however, having previously determined the equilibrium association constant in a steady-state titration experiment, $K_A$ can be taken as known, leaving only $k_{PCET}$ and $k_{ET}$ free.
2.6. References


Chapter 3  Deciphering Radical Transport in the Large Subunit of Class I Ribonucleotide Reductase
Portions of this chapter have been published:

3.1. Introduction

The most recent iteration of photoRNR employed a photopeptide composed of a rhenium-phenanthroline photooxidant [Re], an unnatural 3,5-difluorotyrosine (3,5-F\textsubscript{2}Y), and the 19 C-terminal residues of the β protein ([Re]-F\textsubscript{2}Y-βC\textsubscript{19}).\textsuperscript{1} Exploiting the favorable properties of the \textsuperscript{3}MLCT excited state of [Re] and the rapid radical initiation kinetics of deprotonated fluorotyrosines,\textsuperscript{2,3} this previously reported photoRNR system resulted in a number of highly promising observations, including the highest-to-date photochemical activity yields, and the direct spectroscopic detection of a charge-separated state competent for turnover.\textsuperscript{1}

A major motivation for photoRNR is the measurement of photoinitiated radical transport kinetics, which had not been achieved to date. In order to make this measurement, we used a closely related system, replacing 3,5-F\textsubscript{2}Y with 2,3,6-trifluorotyrosine (F\textsubscript{3}Y) to yield ([Re]-F\textsubscript{3}Y-βC\textsubscript{19}). Using this construct, we performed a comparative study of F\textsubscript{3}Y\textsuperscript{•} decay kinetics in the presence of four α\textsubscript{2} variants (Figure 3.1), which allowed us to observe radical injection into α\textsubscript{2} and measure the rate and pathway dependence of this PCET reaction.

3.2. Results

3.2.1. Synthesis, Purification and Characterization of Materials

The synthesis of [Re]-F\textsubscript{3}Y-βC\textsubscript{19} was accomplished on solid phase using established methods. The identity of the modified peptide was confirmed by high-resolution MALDI-TOF MS, its purity by HPLC, and the pK\textsubscript{a} of the F\textsubscript{3}Y phenol when incorporated within the peptide was found to be 7.1 ± 0.1; these data are shown in Figure 3.2. The observed pK\textsubscript{a} of F\textsubscript{3}Y in [Re]-F\textsubscript{3}Y-βC\textsubscript{19} is in accordance with previous measurements of model dipeptides of the F\textsubscript{n}Y residue.\textsuperscript{3}
Figure 3.1. A photoactive peptide [Re]-2,3,6-F$_3$Y--βC19 displays a fluorotyrosyl radical at the equivalent position to Y$_{356}$ in β$_3$. The method of radical transport into α$_2$ from 2,3,6-F$_3$Y--Y$_{353}$--Y$_{730}$--C$_{439}$ is deciphered by monitoring the radical in the presence of the stop mutants Y$_{731}$F--α$_2$, Y$_{730}$F--α$_2$, C$_{439}$S--α$_2$ as well as wt--α$_2$. 
Figure 3.2. Purification, identification, and pKₐ of [Re]-F₃Y-βC19. (a) HPLC of purified peptide on C-18 resin with a gradient of 5–15% MeCN in 0.1% aqueous Et₂N. (b) UV–vis absorption spectrum of the peak at 9.5 min in (a). (c) HRMS of the peptide in (a). (d) Determination of the pKₐ of the phenolic proton by fluorometric titration. Each data point is the intensity at 600 nm for 5 μM [Re]-F₃Y-βC19.

We determined the Kᵦ between the a₂ subunit and the [Re]-F₃Y-βC19—under the conditions of spectroscopy—to be 9 ± 1 μM using a competitive inhibition assay (Figure 3.3), which agrees with measurements on our previous photoRNR systems.¹⁴
Due to the modest affinity of the peptide for the subunit, the spectroscopic measurements require large amounts of $\alpha_2$, thus the expression and purification of each mutant (Figure 3.4) was accomplished on gram scale with hexahistidine affinity chromatography.

**Figure 3.3.** Competitive inhibition assay between $[\text{Re}]_3Y-\beta\text{C19}$ and wt-RNR. $[\text{Re}]_3Y-\beta\text{C19}$ was titrated against a solution of intact wt-RNR including substrate (CDP) and effector (ATP). Activity was measured using a spectrophotometric readout of NADPH consumption. For each measurement, the solution contained 100 nM wt-$\alpha_2$, 200 nM wt-$\beta_2$, 30 $\mu$M TR, 0.5 $\mu$M TRR, 200 $\mu$M NADPH, 1 mM CDP, 3 mM ATP, 15 mM MgSO$_4$, 5% v/v glycerol and the specified amount of $[\text{Re}]_3Y-\beta\text{C19}$ in 50 mM borate buffer (pH 8.3).

**Figure 3.4.** Purification and activity of $\alpha_2$ mutants. (a) SDS-PAGE of each mutant after purification indicates a monomer molecular weight at the expected retention (85 kD). (b) Quantification of the gel lanes in (a) by integrating the band density indicates that $>98\%$ of the protein in solution is $\alpha_2$ for each mutant prepared. (c) Activity of each enzyme quantified by counting turnover of [H]-labeled CDP.
3.2.2. [Re]-F₃Y-βC19 Charge-Separated State

Spectroscopic observation of F₃Y radical injection into α₂ requires a detailed understanding of all transient spectroscopic features present. The generation of the F₃Y radical was confirmed by comparing the excitation of a peptide that cannot be oxidized, [Re]-F-βC19, to the peptide containing the fluorotyrosine. Photolysis of [Re]-F-βC19 at pH 8.3 generates a 3MLCT excited state, [Re]+*, which cannot oxidize the adjacent phenylalanine. [Re]+* can be monitored by recording emission kinetics at λ_max = 610 nm, and decays with a monoexponential lifetime of 59 ns. In contrast to [Re]-F-βC19, excitation of [Re]-F₃Y-βC19 at pH 8.3 generates [Re]+* that decays with biexponential kinetics (Figure 3.5).

![Figure 3.5. Time-resolved decay of emission of [Re]-F₃Y-βC19. Data were recorded at 610 nm after 7 ns 355 nm excitation over a 500 ns window (a) and fit with a monoexponential decay (b). The prominent structure in the residuals lead us to perform biexponential fitting (c), revealing τ₁ = 58 ± 2 ns and τ₂ = 22 ± 2 ns. The solution contained 10 μM [Re]-F₃Y-βC19, 1 mM CDP, 3 mM ATP, 15 mM MgSO₄, and 5% v/v glycerol in 50 mM borate buffer (pH 8.3).](image-url)
The long component of the decay in Figure 3.5 (58 ns, 19%) corresponds to $[\text{Re}^+]^*$ that is unchanged by the proximal $\text{F}_3\text{Y}$, while the short component of the decay (22 ns, 81%), represents the quenching of the $[\text{Re}^+]^*$ by the deprotonated $\text{F}_3\text{Y}^-$. The decay is biexponential as a result of measuring two chemical species in solution—protonated and deprotonated tyrosine. Whereas the emission decay of the $[\text{Re}^+]^*$ can be used to reveal the quenching of the $^3\text{MLCT}$, the photoproducts of the quenching reaction may be captured by transient absorption (TA) spectroscopy. Figure 3.6 shows the time-resolved spectral features upon excitation of $[\text{Re}]^{-}\text{F}_3\text{Y}^{-}\beta\text{C19}$ at three different time points. The $[\text{Re}^+]^*$ appears as broad bands at 380 and 480 nm; the decay of the transient signal at these two TA wavelengths yields decay time constants of $60 \pm 2$ and $61 \pm 2$ ns, respectively. In addition, new spectral features corresponding to the bpy$^-$ and the $^{*}\text{F}_3\text{Y}$ appear in the spectrum at $\lambda_{\text{max}} = 525$ and 425 nm, respectively. The $\text{F}_3\text{Y}^*$ signal decays monoexponentially with a time constant of 68 ns. In contrast, the signal for the bpy$^-$ is best fit to a biexponential as a combination of growth and decay.

![Figure 3.6. TA spectra of the charge-separated state $[\text{Re(bpy}^-\text{)}]^{-}\text{F}_3\text{Y}^{-}\beta\text{C19}$ collected 25 ns (top), 75 ns (middle) and 125 ns (bottom) after a 7 ns excitation pulse of laser light, $\lambda_{\text{exc}} = 355$ nm. Lifetime decays of the transient spectrum were measured at the four wavelengths indicated by the dashed lines: $\Delta \lambda_{\text{det}}$ at 380 and 480 nm correspond to the $^3\text{MLCT}$ of $[\text{Re}]^*$; $\lambda_{\text{em}} = 525$ nm is that of bpy$^-$; and, $\lambda_{\text{em}} = 425$ nm is that of $\text{F}_3\text{Y}^*$. The solution contained 50 pM $[\text{Re}]^{-}\text{F}_3\text{Y}^{-}\beta\text{C19}$ with 1 mM CDP, 3 mM ATP, 15 mM MgSO$_4$, and 5% glycerol in 50 mM sodium borate (pH 8.3).](image)

This complication in the measured TA kinetics, which are not compensated for emission signals, arises from the spectral congestion between the emission of $[\text{Re}^+]^*$ excited state ($\tau = 22$ ns) and the decay of bpy$^-$ anion over 76 ns. The appearance of signals for both the $\text{F}_3\text{Y}^*$ and the bpy$^-$ anion with
the concomitant disappearance of [Re']* establishes that the excited state is quenched by deprotonated tyrosine in an intramolecular charge transfer event resulting in a charge-separated [Re(bpy*)]−F₃Y•−βC₁₉ intermediate. The duration of this charge-separated state agrees well with previously reported lifetimes for model compounds.

3.2.3. *Generation of a long-lived -F₃Y by the flash-quench method*

The rapid decay of the charge-separated state (discussed above) on a sub 100 ns timescale precludes faithful measurements of radical injection rates slower than 10⁷ s⁻¹. The likelihood for oxidative injection from the F₃Y• radical into α₂ increases as the lifetime of the radical increases. The F₃Y• lifetime can be increased by employing the flash-quench method to irreversibly remove the electron from the quenched [Re']* excited state by electron transfer to a flash quencher (FQ), Ru(NH₃)₆Cl₃. By removing the electron from the system with the FQ, back electron transfer is averted and the flash-quenched Re¹¹ is able to oxidize F₃Y. Spectra of Y• and the F₃Y• were collected 50 ns after irradiating a solution of either [Re]−Y−βC₁₉ (at pH 12) or [Re]−F₃Y−βC₁₉ (at pH 8.3) in borate buffer and in the presence of 200 mol equivalents of FQ. The spectra, which are shown in Figure 3.7, exhibit the expected isolated peaks for the F₃Y• radical at λ_max = 425 nm and the Y• radical at λ_max = 412 nm. We also observe that the F₃Y• radical in Tris buffer shifts to λ_max = 418 nm.

![Figure 3.7](image)

**Figure 3.7.** Normalized TA spectra of flash/quenched peptides. Data were gated over 25 ns after a 50 ns delay from the 7 ns 355 nm excitation pulse. Each measurement consists of 500 four-spectrum sequences. Each final spectrum plotted here is an average three identically prepared samples. Individual solutions contained 100 μM [Re]−X−βC₁₉, 200 eq. (20 mM) Ru(NH₃)₆Cl₃, 1 mM CDP, 3 mM ATP, 15 mM MgSO₄, and 5% v/v glycerol in 50 mM pH 8.3 Tris or borate buffer, as shown.
The optimal FQ concentration was determined by titrating solutions of 50 μM [Re]-F₃Y-βC19 with 5–1000 eq. of FQ. As Figure 3.8 shows, the best concentration of FQ was determined to be 20 mol eq., which was the concentration used to perform all subsequent kinetic analysis involving the F₃Y* radical. At 20 mol eq. of FQ, not all of the [Re]⁺ is quenched, and thus the flash quench TA spectrum of [Re]−•F₃Y−βC19 taken 25 ns after laser excitation (λ_{exc} = 355 nm) appears as a sum of the F₃Y* and the remaining charge-separated state.

![Figure 3.8](image-url)

**Figure 3.8.** Optimization of flash/quench conditions for maximum F₃Y* signal intensity and lifetime. Decay of the [Re]−F₃Y−βC19 was collected at λ_{det} = 425 nm following a 7 ns, 355 nm excitation. Each decay is an average of the data collected for three samples of identically prepared solution. Each lifetime (a) was determined by monoexponential weighted non-linear least squares regression of the averaged data. Each solution consisted of 50 μM [Re]−F₃Y−βC19 with varying concentrations of Ru(NH₃)₆Cl₃ in the presence of 15 mM MgSO₄ and 5% glycerol in 50 mM borate buffer (pH 8.3). Signal to noise ratios (b) were calculated from the decays by dividing the amplitude at t = 1 μs (signal) by the amplitude between the highest and lowest data points recorded in the last 5 μs of data (noise).
Figure 3.9 shows the decay profile of the TA spectrum at $\lambda_{det} = 425$ nm. A fast time component of 100 ns corresponding to the recombination of the charge separated state is followed by a slower time component corresponding to the decay of the $F_3Y$ radical produced by flash quenching. The TA spectral profile of the flash-quenched radical may be spectrally isolated by delaying its detection (typically by 0.5–1 $\mu$s) until after the decay of the charge-separated state.

![Graph showing decay profile](image)

**Figure 3.9.** Decay profile at 425 nm for photoexcited [Re]$\cdot F_3Y$--$\beta$C19 under optimized flash/quench conditions. Data were recorded after 7 ns 355 nm excitation of a solution containing 50 $\mu$M [Re]$\cdot [F_3Y]$--$\beta$C19, 20 eq. (1 mM) Ru(NH$_3$)$_6$Cl$_3$, 1 mM CDP, 3 mM ATP, 15 mM MgSO$_4$, and 5% v/v glycerol in 50 mM borate buffer (pH 8.3). The rapid decay corresponds to [Re(bpy$^-$)$_2$]$\cdot F_3Y$--$\beta$C19, which has not been flash-quenched; the long decay corresponds to [Re]$\cdot F_3Y$--$\beta$C19, in which the [Re] photoproduct has been flash quenched.

Figure 3.10a shows the TA spectrum of a flash-quenched peptide 500 ns after laser excitation; the $\lambda_{max} = 425$ nm of $F_3Y$ is clearly observed. The radical, monitored at 395, 410, and 425 nm, decays monoexponentially with lifetimes of $\tau_{395} = 14.7 \pm 0.2$ $\mu$s, $\tau_{410} = 13.5 \pm 0.2$ $\mu$s and $\tau_{425} = 14.5 \pm 0.4$ $\mu$s, Figure 3.10b. In this optimized system, the photochemical yield of $F_3Y$ formation is 4.9%, as calculated in Section 3.5.

3.2.4. **Two conformations of [Re]$\cdot F_3Y$--$\beta$C19 bound to $\alpha_2$**

The relatively weak binding between [Re]$\cdot X$--$\beta$C20 and $\alpha_2$ manifests in the dynamics of the peptide N-terminus, as previously shown. Both the [Re] and Y$_{356}$ are located in this flexible region of the peptide, and when [Re]$\cdot X$--$\beta$C19 is in the presence of $\alpha_2$, the lifetime of the [Re]$^*$ reports on the local
environment of both the [Re] complex as well as the proximal fluorotyrosine. The decay signal from the [Re']*—in the presence of an amino acid that it cannot oxidize—is biexponential when the peptide is bound to the protein. The fast time decay, at 60 ns, is similar to [Re']* in solution (59 ns), and was assigned to an “off” state where the chromophore is largely solvated. A longer time decay, at 155 ns, was ascribed to the N-terminus binding closely to the surface of α₂ in an “on” state, owing to occluding solvent from [Re].

Figure 3.10. (a) TA spectra and of [Re]-FY-βC19 collected 500 ns (top), 5 μs (middle) and 30 μs (bottom) after a 7 ns 355 nm excitation pulse of laser light. (b) Lifetime decay of TA signal at 395 nm (blue), 410 nm (green), and 425 nm (brown). The monoexponential fit is shown by the solid lines. The solution contained 50 μM [Re]-FY-βC19 with 20 eq. (1 mM) [Ru(NH₃)₆]Cl₂, 1 mM CDP, and 3 mM ATP, 15 mM MgSO₄, and 5% glycerol in 50 mM borate buffer (pH 8.3). Colored vertical bands (a) indicate the portion of the spectrum polled for recording the kinetic decays in (b).
The \([\text{Re}^+]\) lifetime measurements were repeated for the \([\text{Re}]\)-X-\(\beta\)C19 peptide with non-oxidizable (X = F) and oxidizable (X = F\(_3\)Y) amino acids associated to each of the \(\alpha_2\) mutants prepared here. All decays were biexponential; the lifetimes obtained from a fit of the lifetime decay of \([\text{Re}]\)-F-\(\beta\)C19 are shown in Figure 3.11a. The “off” lifetime increases marginally from 52 to 60 ns, and the “on” lifetime of 180 ns is the same for all four mutant proteins. A biphasic decay for the \([\text{Re}]\)-F\(_3\)Y-\(\beta\)C19 peptide bound to \(\alpha_2\) is also obtained (Figure 3.11b), but with significantly attenuated lifetimes (“on” lifetime of 29 ns, “off” lifetime of 100 ns) owing to the quenching of \([\text{Re}^+]\) by F\(_3\)Y as described above. The similarity of the percentage of the short and long lifetime components to the overall decay suggests similar binding conformations for both peptides. It is likely that all four states of the \([\text{Re}^+]\) (on/quenched, off/quenched, on/unquenched, off/unquenched) are present in solution.

![Graph showing lifetime measurements](image)

**Figure 3.11.** Lifetime of \([\text{Re}^+]\) excited state of 10 \(\mu\)M \([\text{Re}]\)-X-\(\beta\)C19 for (a) X = F and (b) X = F\(_3\)Y peptide in the presence of \(\alpha_2\) mutants at 20 \(\mu\)M, in a solution of 1 mM CDP, 3 mM ATP, 15 mM MgSO\(_4\), and 5% glycerol, in 50 mM borate (pH 8.3). For biexponential decays, the percentage contribution of short and long components to the decay is listed.

### 3.2.5. \([\text{Re}]\)-F\(_3\)Y-\(\beta\)C19 lifetimes in the presence of \(\alpha_2\) mutants

The emission lifetimes permit the formation of the F\(_3\)Y\(^-\) to be monitored. Radical injection, however, requires that the F\(_3\)Y\(^-\) on the photopeptide in the presence of each \(\alpha_2\) mutant be directly observed by TA spectroscopy. Figure 3.12a shows the spectra recorded at 1 \(\mu\)s for the peptide alone, and in the
presence of each protein. For the free peptide and for the Y731F mutant, for which radical injection is blocked, the typical spectrum of the $F_3Y^*$ with $\lambda_{\text{max}} = 425$ nm is observed, with a small shoulder at 410 nm; this spectral profile is typical of fluorotyrosyl radicals. Figure 3.12b displays the TA spectrum of $F_3Y^*$ in the presence of mutants that are theoretically capable of radical injection.

![Spectra of photoexcited [Re]-F$Y^*$-βC19 alone, and in the presence of α2 variants. Data were gated over 25 ns after a 1 μs delay from the 7 ns 355 nm excitation pulse. Each measurement consists of 500 four-spectrum sequences averaged to create a single spectrum. Each final spectrum plotted here is an average of data from three samples of identically prepared solution. Each solution contained 50 μM [Re]-F$Y^*$-βC19, 20 mol eq. (1 mM) [Ru(NH$_3$)$_6$]Cl$_2$, 1 mM CDP, 3 mM ATP, 15 mM MgSO$_4$, and 5% v/v glycerol in 50 mM borate buffer (pH 8.3).](image)

The radical profile changes significantly. The $\lambda_{\text{max}} = 425$ nm broadens and a pronounced shoulder at $\lambda_{\text{max}} = 400$ nm appears; the spectrum of the C439S mutant shown in 3.13a is representative. We chose to monitor kinetics at three wavelengths, 425, 410 and 395 nm, as these correspond to the $\lambda_{\text{max}}$ of $F_3Y^*$,
its typically observed shoulder, and the new, shifted shoulder that appears in the presence of the protein. For $Y_{731}F$ and $Y_{730}F$, the TA signal decays monoexponentially at all wavelengths. In contrast, the signals for $[\text{Re}]^{-}F_{3}Y^{-}\beta C19$ in the presence of $C_{439}S$ and wt mutants decay biexponentially with a significantly shorter lifetime component. Figure 3.13b shows the decay of $F_{3}Y$ for the $C_{439}S$ mutant.

![Figure 3.13](image)

**Figure 3.13.** (a) TA spectra of $[\text{Re}]^{-}F_{3}Y^{-}\beta C19$ in the presence of $C_{439}S$--$\alpha_{2}$ collected 1 $\mu$s (top), 15 $\mu$s (middle) and 30 $\mu$s (bottom) after a 7 ns 355 nm excitation pulse of laser light. (b) Lifetime decay of TA signal at 395 (blue), 410 (green), and 425 (brown) nm. The biexponential fit is shown by the solid lines. The solution contained 50 $\mu$M $[\text{Re}]^{-}F_{3}Y^{-}\beta C19$, 100 $\mu$M $C_{439}S$--$\alpha_{2}$, 20 eq. (1 mM) $\text{Ru(NH}_{3})_{6}\text{Cl}_{3}$, 1 mM CDP, and 3 mM ATP, 15 mM MgSO$_{4}$ and 5% glycerol in 50 mM borate buffer (pH 8.3). Colored vertical bands (a) indicate the portion of the spectrum polled for recording the kinetic decays in (b).
Figure 3.14. Graphical residual analysis of kinetic decays of [Re–F3Y–βC19:C439S–α2] and [Re–F3Y–βC19:wt–α2]. (a) Monoexponential (left) and biexponential (right) residuals for [Re–F3Y–βC19:C439S–α2]. (b) Monoexponential (left) and biexponential (right) residuals for [Re–F3Y–βC19:wt–α2]. The trend lines are a 20-point adjacent-averaged smoothing that are included as a guide for the eye. The region that deviates from linearity is in the early time points of each monoeponential trace, corresponding to a short lifetime component.
The residuals for the mono- and biexponential fits are shown in Figure 3.14. A summary of the lifetimes of the TA signal of F₃Yₚ at the three different wavelengths for the free peptide and the peptide in the presence of Y₇₃F₋α₂, Y₇₃0F₋α₂, C₄₃₉S₋α₂ as well as the wt₋α₂ is plotted in 3.15. These lifetimes and their amplitude components for the four systems are given in Table 3.1.

![Figure 3.15. Lifetime of [Re]-F₃Y₋βC19 peptide, and it in the presence of the four α₂ variants. Decay lifetimes of TA signal of F₃Yₚ were observed at 395 (blue), 410 (green) and 425 (brown) nm.](image)

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<th>410 nm</th>
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<td>19 ± 4</td>
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</table>

* Biexponential decay kinetics
* Normalized amplitudes of biexponential components

Table 3.1. Time constants (μs) and amplitudes (%) of F₃Yₚ TA kinetic decays. Monoexponential or biexponential weighted least-squares regression was used. The signals presented are either [Re]-F₃Y₋βC19 alone or in the presence of each α₂ subunit used in this study.
For the peptide in solution, in the presence of Y$_{731}$F--α$_2$ or Y$_{730}$F--α$_2$, there is no significant change in radical lifetime. With C$_{499}$S--α$_2$ and the wt--α$_2$ subunit, the TA signal of F$_3$Y$^\bullet$ exhibits a biexponential decay owing to the contribution of a significantly shorter lifetime component, which we assign to radical injection into the protein.

3.3. Discussion

The unnatural tyrosine—2,3,6-trifluorotyrosine (F$_3$Y)—permits a photoRNR to be constructed by binding [Re$^-$]-F$_3$Y-PC19 to α$_2$. This particular photoRNR construct provides three important features to enable the kinetics of radical initiation and propagation to be uncovered. First, Figure 3.2d establishes the pK$_a$ of F$_3$Y, when incorporated into the full-length peptide, to be 7.1 ± 0.1. In order to generate proton-independent oxidation of the F$_3$Y by the [Re$^+$]$^*$ (electron transfer only), the F$_3$Y must be in its deprotonated state. Thus the proton-independent oxidation of F$_3$Y by [Re$^+$]$^*$ may occur at a mild pH; we chose to perform spectroscopic experiments at pH 8.3 where F$_3$Y is ~94% deprotonated. Second, the reduction potential of F$_3$Y is ~180 mV above tyrosine at pH 8.3,$^1$ thus providing a driving force for oxidation of the tyrosines of α$_2$. Third, the absorption maximum of the F$_3$Y$^\bullet$ is sensitive to its local environment, shifting 7 nm by changing buffer identity in solution (Figure 3.7). This spectral shift allows F$_3$Y$^\bullet$ to be spectrally isolated and the radical can be time resolved at multiple wavelengths.

3.3.1. F$_3$Y radical photoinitiation

The F$_3$Y$^\bullet$ is generated efficiently by laser flash photolysis of [Re$^-$] incorporated within the [Re$^-$]-F$_3$Y-βC19 peptide. The TA spectrum of the photolyzed peptide in the presence of substrate, effector, and buffer establishes that the $^3$MLCT excited state of [Re$^-$], [Re$^+$]$^*$, is readily quenched by F$_3$Y$^-$ to generate the intramolecular charge-separated state [Re(bpy$^\bullet$)]-F$_3$Y$^\bullet$-βC19. The absorption features characteristic of the charge transfer photoproducts, F$_3$Y$^\bullet$ and bpy$^\bullet^\bullet$, are observed in the TA spectrum of Figure 3.6. These features decay concomitantly with one another, and they persist for a time longer than the [Re$^+$]$^*$ excited state. Identical lifetimes for the charge-separated state were observed for each of the protein mutants prepared here.

In order to extend the lifetime of F$_3$Y$^\bullet$ and isolate its spectral signatures, radical photoinitiation
was performed in the presence of the reversible flash-quench reagent \([\text{Ru(NH}_3)_6\text{Cl}_3\] The Ru\textsuperscript{III} complex oxidatively quenches \([\text{Re}^+]^*\) within the laser pulse to furnish the \([\text{Re}^\text{II}]\) ground state. \([\text{Re}^\text{II}]\) is also thermodynamically competent for tyrosinate oxidation, subsequently regenerating the \([\text{Re}^+]\) ground state and a free \(\text{F}_3\text{Y}^*\). Since neither the \([\text{Re}^+]^*\) nor the intramolecular charge-separated state are present upon flash quenching, the resulting TA features are solely representative of the radical. Spectra of \(\text{F}_3\text{Y}^*\) and \(\text{Y}^*\) under flash quench conditions clearly show disparate absorption maxima (Figure 3.7), which concur with those observed for tyrosyl radicals in the \(\beta\text{C19}\) construct that were generated with the same photooxidant in \([\text{Re]}-\text{F}_3\text{Y}\) and \([\text{Re]}-\text{Y}\) dipeptides. In addition, we found that the \(\lambda_{\max}\) of the \(\text{F}_3\text{Y}^*\) changes as a function of buffer composition, highlighting the sensitivity of the radical to its environment (Figure 3.7). There is an optimum concentration of the FQ reagent. High concentrations of FQ decrease the observed yield of radical because contaminating Ru\textsuperscript{II} is present at higher concentration in solution, which is able to reduce the photogenerated radical. Figure 3.8 shows that 20 mol eq. generates the highest yield of radical with the longest observable lifetime.

3.3.2. Spectroscopy of \([\text{Re]}-\text{F}_3\text{Y}-\beta\text{C19} \) bound to \(\alpha_2\) mutants

The equilibrium binding of \([\text{Re}]-labeled\) peptides with the \(\alpha_2\) subunit can be measured by fluorometric titration. However, in this case, the quenching of \([\text{Re}]\) by the deprotonated \(\text{F}_3\text{Y}\) was so efficient that the emission intensity of \([\text{Re}^+]^*\) was too low to provide a reliable measurement of the binding between the peptide and \(\alpha_2\). Thus we reverted to competitive inhibition assay measurements to reveal that \(K_{D}\) is \(9 \pm 1 \mu\text{M}\) (Figure 3.3). With this \(K_{D}\), spectroscopic measurements employed a mixture of 50 \(\mu\text{M}\) peptide and 100 \(\mu\text{M}\) \(\alpha_2\) to ensure that 86% of the peptide was bound to \(\alpha_2\).

Even in this bound state, the N-terminus of the photopeptide resides in two states, one in which it is closely associated with the \(\alpha_2\) subunit ("on") and one in which it is largely solvent exposed ("off"), consistent with previous observation. This on/off dynamic was measured by monitoring the emission lifetime of the \([\text{Re}^+]^*\) for a control \([\text{Re]}-\text{F}-\beta\text{C19}\) peptide, Figure 3.11a. The lifetime of the solvent-exposed excited state is much shorter than when it is adsorbed to the protein surface. For the experiments reported here, we also wished to define this two-state dynamic for the fluorotyrosine-containing peptide bound to each of the \(\alpha_2\) mutants. All constructs displayed the two-state behavior, as shown by Figure 3.11b. We
note that the \([\text{Re}^+]^*\) lifetime of the \(F_3Y-\beta C19:Y_{731}F-\alpha_2\) construct corresponding to the “on” state \((\tau = 29\) ns) is unquenched relative to its lifetime in buffered solution \((\tau = 22\) ns). This observation confirms that the highly oxidizing \([\text{Re}^+]^*\) is unable to extract electrons from protein sidechains via direct oxidation of tyrosine within the protein. Consistent with this observation, the “on” lifetime of the \([\text{Re}^+]^*\) is the same for all constructs (Figure 3.11). Thus the primary quenching pathway of the \([\text{Re}^+]^*\) is electron transfer solely from the adjacent \(F_3Y\) on the peptide.

The decay of \([\text{Re}^+]^*\) is accompanied by the appearance of absorption features characteristic of \(F_3Y^*\) at \(\lambda_{\text{max}} = 425\) nm in borate buffer and \(\lambda_{\text{max}} = 418\) nm in an otherwise identical solution of the mildly more hydrophobic Tris buffer (Figure 3.7). The lifetime of the photogenerated \(\cdot F_3Y\) is provided, independent of protein oxidation, by the \([\text{Re}^-]_3F_3Y-\beta C19:Y_{731}F-\alpha_2\) construct (Figure 3.1a). As summarized in Figure 3.15, the lifetime of the \(\cdot F_3Y\) marginally depends upon the wavelength of observation \((\tau_{395} = 13.3 \pm 0.2\) µs, \(\tau_{410} = 14.2 \pm 0.2\) µs, and \(\tau_{425} = 16.5 \pm 0.4\) µs). At \(\lambda_{\text{det}} = 425\) nm, this lifetime is slightly shorter than that observed for the free peptide in solution. The lifetime at \(\lambda_{\text{det}} = 410\) nm matches the solution lifetime, and the lifetime at \(\lambda_{\text{det}} = 395\) nm is longer than the lifetime when protein is not present. These lifetimes correspond to \(k_0\) denoted in Figure 3.1.

This heterogeneity suggests that the radical, the spectral features of which are sensitive to local environment, may experience multiple conformations when in the presence of the unnatural phenylalanine on the surface of the protein. The local environment at \(Y_{356}\) does depend on the residue at 731 in the intact \(\alpha_2;\beta_2\) system. The \(Y_{356}^*\) radical may be trapped when \(\cdot \text{NO}_2Y_{122}\) is introduced into \(\beta_2\). The \(g_{\alpha}\) value for trapped \(Y_{356}^*\) in \(Y_{731}F-\alpha_2\) \((2.0073)\) is shifted relative to wt-\(\alpha_2\) \((2.0063)\). The \(g_{\alpha}\) value for \(Y^*\) in proteins is known to vary between 2.006 and 2.009 as a function of local environment, with the lower values corresponding to tyrosyl radicals involved in hydrogen bonding. This result implies that \(Y_{356}^*\) is involved in hydrogen-bonded stabilization in the wt enzyme, but not \(Y_{731}F-\alpha_2\). Together, these results suggest that the radical in the presence of \(Y_{731}F\) experiences multiple solution/surface conformations. Of these different conformations, the amplitude of the monoexponential lifetime of \(F_3Y^*\) conveys that a significant portion of the peptide N-terminus \((20–30\%)\) is in the conformer of the “on” state. However, even in this conformation, \(F_3Y^*\) cannot inject the radical into \(\alpha_2\) owing to the phenylalanine block.
3.3.3. Radical propagation into $\alpha_2$

With the ability to isolate the $F_3Y^\ast$ radical and temporally profile its lifetime in the absence of protein oxidation, the kinetics for photoinitiated radical transfer along the RNR pathway are revealed for the first time. The F point mutation was moved from position 731 to position 730 (Figure 3.1b), which would allow for the oxidation of $Y_{731}$ but block any further transport of the radical into the $\alpha_2$ subunit. Photogeneration of $[\text{Re}] - F_3Y^\ast - \beta\text{C19}$ in the presence of $Y_{730} F - \alpha_2$ again reveals monoexponential decays at the three wavelengths: 425 nm, $14.0 \pm 0.2 \mu$s; 410 nm, $13.7 \pm 0.2 \mu$s; 395 nm, $14.4 \pm 0.2 \mu$s. The lifetimes are all within error of one another, suggesting that the radical is in a homogenous environment in the presence of the native $Y_{731}^\ast$, to which it can hydrogen bond. A new spectral feature at 400 nm is a signature of this hydrogen-bonded environment. This result suggests that the radical residing in the "on" conformations has a spectral signature that is shifted to higher energy relative to the free peptide or the dynamic $Y_{731} F$ radical. Though both "on" and "off" conformations are present, the observed lifetime of $F_3Y^\ast$ should be monoexponential as it corresponds to the sum of $k_0$ and $k_1$ (Figure 3.1b). Inasmuch as $(k_0 + k_1)$ of $[\text{Re}] - F_3Y^\ast - \beta\text{C19} : Y_{730} F - \alpha_2$ is similar to $k_0$ of $[\text{Re}] - F_3Y^\ast - \beta\text{C19} : Y_{731} F - \alpha_2$, we conclude that radical injection into the surface of the protein is blocked when the radical cannot further propagate along the pathway.

Consistent with this interpretation, dramatic changes occur when $Y_{730}$ is present to propagate the radical into the subunit. As shown by the data in Figure 3.15, the rate of $[\text{Re}] - F_3Y^\ast - \beta\text{C19}$ decay increases significantly for both the $C_{439} S - \alpha_2$ mutant and the wt enzyme. In each case, both $Y_{730}$ and $Y_{731}$ are present, and in each case the lifetimes at all wavelengths become biexponential. For $C_{439} S$, in which a serine substitutes for the active site cysteine (Figure 3.1c) the TA signal at 395 nm consists of a long phase ($17.3 \pm 0.9 \mu$s, 81%) and a short phase ($2.2 \pm 1 \mu$s, 19%) (Figure 3.13b). Similar pairs of lifetimes were observed for 410 and 425 nm, as shown in Table 3.1. The amplitudes of each signal can be used to assign each phase of decay to a conformation on the protein surface. As in the measurement of emission decay of the $[\text{Re}]^\ast$, the large amplitude component (80%) of the radical decay is attributed to the portion of the peptide that is largely solvated, in the "off" state. The small amplitude (20%) matches the "on" state. In this conformation, the $F_3Y^\ast$ lifetime is much shorter, indicating that it is able to oxidize $Y_{731}^\ast$. Previous analysis of the crystal structure of $\text{wt-} \alpha_2$ concluded that there is hydrogen-bonding between $Y_{731}^\ast$ and $Y_{730}^\ast$. 103
Because the installation of $Y_{730}^F$—a change of only one hydroxyl group from $Y_{730}$—prevents oxidation of $Y_{731}$ from occurring, we postulate that the enzyme has evolved to employ hydrogen-bonding between $Y_{730}$ and $Y_{731}$ (a $Y-Y$ dyad) in proton-coupled tyrosine oxidation.

The "off" lifetime then serves as a baseline ($k_o$, Figure 3.1c) for the rate of oxidation of $Y-Y$ by $F_3^Y$. Substituting the averages across the three wavelengths for the lifetime of the radical in the "on" (2.5 ± 1.0 μs) and "off" (16.9 ± 0.7 μs) states into Equation (3.2) yields a rate constant for radical injection of $C_{439}^S-a_2$ to be $(4 ± 2) \times 10^5$ s⁻¹. This injection event is rate limited by the slower of $k_1$ and $k_2$, Figure 3.1c. Because oxidation of $Y_{731}$ is not possible without pre-arrangement with $Y_{730}$, as shown in the $Y_{730}^F-a_2$ mutant, we assign $k_1$ as the limiting step. A similar oxidation event is observed for the wt enzyme, which contains the active site cysteine. The averaged lifetimes for the long phase (15.4 ± 0.5 μs) and the short phase (3.0 ± 1.4 μs) of $^\bullet F_3^Y$ in [Re]$^+$-$F_3^Y$-$\beta$C19:wt-$a_2$ correspond to an injection rate of $(3 ± 2) \times 10^5$ s⁻¹. As in the $C_{439}^S-a_2$ experiment, this rate-constant reflects the rate-limiting step among $k_1$, $k_2$, and $k_3$, Figure 3.1d, and it is within error limit of the injection kinetics observed in [Re]$^+$-$F_3^Y$-$\beta$C19:$C_{439}^S-a_2$. The requirement of hydrogen-bonding between $Y_{731}$ and $Y_{730}$ again lead us to conclude that the rate-limiting oxidation for [Re]$^+$-$F_3^Y$-$\beta$C19:wt-$a_2$ is $k_1$, Figure 3.1d.

3.3.4. Comparison to known rates for tyrosine PCET

The need for the $Y-Y$ dyad to promote radical injection into $a_2$ is consistent with the PCET reactivity of tyrosine in model compounds. It is known that the rate constant for oxidation of tyrosine depends dramatically on the proximity of a hydrogen-bonding partner to the phenol. Specifically, in a series of phototriggered Ru(II)-(bpy)$_3$-$Y$ model dyads, the addition of a carboxylate at the ortho position of the tyrosine phenol induces a hydrogen bond and increases the rate for PCET at pH 8 from $10^4$ s⁻¹ to $10^5$–$10^6$ s⁻¹. The critical need to couple oxidation to a hydrogen bond is further demonstrated by its ability to drive less favored reactions. In this experiment, a pair of Ru(II)-(bpy)$_3$-$Y$ models containing benzimidazole hydrogen-bond partners were synthesized in which one variant contained a 0.2 eV lower driving force but a 0.2 Å shorter hydrogen-bond distance. The shorter hydrogen bond drives the rate of oxidation by nearly one order of magnitude greater than for the variant with the greater thermodynamic driving force but longer hydrogen bond. These observations for model tyrosyl radical systems are in line with the
similarity of the observed radical kinetics of $[{\text{Re}}]_{3}Y_{31}C19:Y_{30}F-a_{2}$ and $[{\text{Re}}]_{3}Y_{31}C19:Y_{30}F-a_{2}$. In $[{\text{Re}}]_{3}Y_{31}C19:Y_{730}F-a_{2}$, a hydrogen bond to $Y_{730}$ is absent thus preventing PCET.

3.4. Conclusion

These results show that radical injection and propagation in RNR with any appreciable rate requires that both $Y_{731}$ and $Y_{730}$ be in place to establish the hydrogen-bonded network needed for PCET.

With the Y-Y dyad present, we are able to transiently monitor a photogenerated radical on the RNR pathway for the first time. The rate for radical injection and transport in $a_{2}$ is fast, on the order of $3 \times 10^{5}$ s$^{-1}$. The ability to shut down this efficient PCET pathway for radical propagation by modifying the hydrogen-bonding network of the amino acids composing a colinear PCET pathway in $a_{2}$ suggests a finely tuned evolutionary adaptation of RNR to control radical transport in this enzyme.

3.5. Experimental Information

Synthesis and characterization of $[{\text{Re}}]_{X}C19$ photopeptides; construction, expression, purification, and characterization of $a_{2}$ subunits; and steady-state experiments were performed by Dr. Patrick G. Holder. Instrument control software was designed and developed by Bryce L. Anderson. Transient experiments were performed in collaboration with Dr. Patrick G. Holder.

3.5.1. Materials

The $C19$ peptide was synthesized on resin by Pi Proteomics (Huntsville, AL; piproteomics.com) by starting with Fmoc-Leu-PEG-PS resin (Applied Biosystems, 180 μmol/g), and using our previously described protocol. 4-methyl-4′-carboxyl-2,2′-bipyridine, RNR subunit $β_2$ (1.2 $Y_{125}/β_{2′}$ 5,400 nmol/min/mg), E. coli thioredoxin (TR, 40 U/mg), E. coli thioredoxin reductase (TRR, 1400 U/mg), $[{\text{Re}}]_{Y}C19$, and hydroxybenzotriazole (HOBT) were available from previous studies. Protected Fmoc-2,3,6-trifluorotyrosine was prepared as previously reported. $[^{3}\text{H}]$-CDP was purchased from ViTrax (Placentia, CA).

Other chemicals were of reagent grade or higher, sourced commercially and used as received. Acros: 2,3,5-trifluorophenol; Strem: rheniumpentacarbonyl chloride $[\text{Re(CO)}_{5}\text{Cl}]$ and
hexaammineruthenium(III) chloride \([\text{Ru(NH}_3\text{)}_6\text{]}\text{Cl}_3\); NovaBioChem: Fmoc-succinimide and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylenaminium hexafluorophosphatate (HCTU); Sigma: Lactate dehydrogenase (LDH) and adenosine-5'-triphosphate (ATP); Aldrich: sodium borate (\(\text{Na}_2\text{B}_4\text{O}_7\)), DL-dithiothreitol (DTT), \(\beta\)-mercaptoethanol (\(\beta\)-ME), ampicillin sodium salt (Amp), pyridoxal-5'-phosphate (PLP), phenylmethylsulfonyl fluoride (PMSF), streptomycin sulfate (strep), kanamycin sulfate (Km), ethyl acetate (EtOAc), dichloromethane (DCM), diethyl ether (Et\(_2\)O), trifluoroacetic acid (TFA), \(\text{NaN}_2\text{dmethylformamide (DMF), ethanol (EtOH), acetonitrile (MeCN), diisopropylethylamine (DIPEA), triethylamine (Et}_3\text{N), triisopropyl-silane (TIPS). Invitrogen: SOC Media; Mallinckrodt: ethylenediaminetetraacetic acid (EDTA), potassium phosphate (KPi); EMD: magnesium sulfate (\(\text{MgSO}_4\)), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane (Tris), ammonium sulfate [(\(\text{NH}_4\))\(_2\text{SO}_4\)], VWR (BDH): glycerol; BD Biosciences: Luria-Bertani media.

3.5.2. Cell Stocks, Plasmids and Primers

*E. coli* BL21 (DE3) cells were purchased from Novagen. *E. coli* XL-10 Gold cells were purchased from Agilent (formerly Stratagene). The pET-*nrdA*(wt) plasmid encoding for N-terminally (His)_6-tagged wt-\(\alpha_2\) was available from a previous study.\(^9\) Primers used in site-directed mutagenesis were purchased in purified form as a custom synthesis from Invitrogen.

3.5.3. Tools, Instrumentation and Methods

All protein chromatography was performed under gravity flow at an ambient temperature of 4 °C, with flow rates listed for each column prepared. Fractions were collected with a GE Healthcare Frac-920 collector as described for each column prepared.

High performance liquid chromatography (HPLC) was performed on an Agilent 1200 series instrument fitted with an inline Diode Array UV Absorption Detector and an inline Fluorescence detector. Analytical chromatography was accomplished on a Phenomenex Kinetix XB-C18 4.6 \(\times\) 100 mm reversed-phase column fitted with a guard cartridge. Separation gradients are listed with results. Semi-preparative scale purifications were accomplished on a Waters 19 \(\times\) 150 mm C18 reversed-phase column fitted with a guard cartridge. For these runs, the instrument was set to collect fractions for designated time periods at 106
0.5 min intervals.

$^1$H and $^{13}$C NMR spectra were obtained using a Bruker AVQ-400 NMR spectrometer at the MIT Department of Chemistry Instrumentation Facility (DCIF) and internally referenced using the proteo impurity for the relevant deuterated solvent (d-chloroform).

High resolution Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) was performed on a Bruker OmniFlex system in positive reflector detection mode. α-Cyanohydroxycinnamic acid was used as a matrix. Solutions of matrix were prepared day-of as a saturated solution in 1:1 MeCN:H$_2$O + 0.5% v/v TFA. The matrix and sample solutions were mixed at a ratio of 9:1 matrix:sample, by volume, and spotted on a stainless steel target and allowed to dry before analysis. Spectra were collected as an average of 500 shots. Peaks were calibrated externally each day using the proteomass peptide MALDI-MS calibration kit (Sigma) by following the manufacturer’s instructions. For each spectrum, four peaks were used to generate the reference file.

For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus from Bio-Rad, following the general protocol of Laemmli. Gels were pre-cast 7.5% TGX (Bio-Rad). All protein electrophoresis samples were heated for 2 min at 200 °C in the presence of DTT to ensure reduction of any disulfide bonds. Commercially available markers (Dual Color Protein Plus, Bio-Rad) were applied to at least one lane of each gel for assignment of apparent molecular masses. Gels were stained with Coomassie Brilliant Blue R-250 before imaging. Gel images were collected with a Bio-Rad Gel Doc 2000, saved using Quantity One version 4.4.0 software, and exported without modification as 16-bit .tif images.

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 (typically 75–100 µL) were collected with 1 s integration time and 1 nm step size. UV–vis spectra were recorded on a Varian Cary 5000 UV/VIS/NIR spectrometer. Samples (typically 75–100 µL) were measured as a baseline-subtracted spectrum with 0.1 s integration and 1 nm observation slit width at each data point.

Radioactivity scintillation counting for measurement of $[^3]$H decay counts was performed using
a Beckman LS 6500 Scintillation counter. Each counted solution was a mixture of 9 mL of scintillation fluid and 1 mL of sample solution, which was vigorously vortexed before counting for 10 min per sample.

3.5.4. \textit{pET-nrdA(wt) site-directed mutagenesis and transformation}

Site-directed mutagenesis (SDM) was carried out with the Quickchange Kit from Stratagene. Each mutant was generated by amplifying the template, pET-nrdA(wt), with Pfu Ultra II polymerase in the presence of the forward and reverse primers whose sequences are below. Amplification of pET-nrdA mutant plasmids was accomplished by transformation of SDM reaction mixtures into XL-10 gold cells by following the manufacturer’s instructions. Plasmids were isolated using a Miniprep kit from Qiagen, eluting the final plasmid with ddH$_2$O. DNA sequencing was performed by the MIT Biopolymers Lab. pET-nrdA transformation into BL21(DE3) cells was completed by following the manufacturer’s instructions.

3.5.5. \textit{Sequence of Mutagenesis Primers}

\textbf{pET-nrdA(Y$_{731}$ F)}
\begin{itemize}
  \item Forward: S'-G GTC AAA ACA CTG TAT TTT CAG AAC ACC CG-3'
  \item Reverse: S'-CG GGT GTE CTG AAA ATA CAG TGT TTT GAC C-3'
\end{itemize}

\textbf{pET-nrdA(Y$_{730}$ F)}
\begin{itemize}
  \item Forward: S'-C GGG GTC AAA ACG CTG TTT TAT CAG ACG ACC CGT G-3'
  \item Reverse: S'-C ACG GGT GTT CTG ATA AAA CAG CGT TTT GAC CCC G-3'
\end{itemize}

\textbf{pET-nrdA(C$_{439}$ S)}
\begin{itemize}
  \item Forward: S'-G CGT CAG TCT AAC CTG TCC CTG GAG ATA GCC C-3'
  \item Reverse: S'-G GGC TAT CTC CAG GGA CAG GTT AGA CTG ACG C-3'
\end{itemize}

3.5.6. \textit{NrdA Mutant Expression}

A solution of 75 \textmu L of the SOC media BL21(DE3) transformant was spread aseptically onto an LB-agar plate containing kanamycin (Km) at 50 \textmu g/mL. The plate was then incubated overnight at 37 °C. After 14 h of growth, the plate showed well-dispersed individual colonies. One colony from the plate was picked, which was incubated at 37 °C in 5 mL of LB media containing 50 \textmu g/mL Km on a rotating tumbler until saturated (10–20 h). One mL of this culture was then diluted into 100 mL of LB-Km media in a 500
mL Erlenmeyer flask and incubated at 37 °C while shaking at 220 rpm for 12 h. 50 mL of this saturated culture was then diluted into 10 L of LB-Km media in a Beckman Scientific fermentor. The temperature was set to 37 °C with air sparging at 10 L/h and stirring at 500 RPM. After 2.5 h of growth (OD₆₀₀ = 0.67), protein production was induced by adding 10 mL of 1 M IPTG, giving 1 mM in solution. Growth continued for 4 h, at which point the cells were harvested by centrifugation (10 min, 7,000 × g), flash frozen at 77 K, and stored at −80 °C. Typical yield was 3–5 g/L wet cell paste.

3.5.7. (His)_₆-α₁ purifications

Lysis buffer consisted of 50 mM Tris (pH 7.6 at 4 °C) containing 5% glycerol, 1 mM PMSF, 10 mM imidazole and 10 mM DTT. The solid DTT and PMSF (0.2 M in EtOH) were added just before use. The details for a single purification of the Y₇₃₀F mutant follow, and the other mutants were purified in the same manner. Frozen cell pellet (7.4 g) was thawed on ice with 5 mL of lysis buffer per gram of pellet (35 mL total). The suspension was then prepared for lysis with several passes through a Teflon/glass homogenizer. The cells were then lysed with a single pass through a SLM-Aminco French pressure cell (with the cell and its fittings pre-cooled on ice) at between 16,000 and 18,000 psi. The resulting suspension was then centrifuged (25,000 × g, 30 min). The pellet was discarded and the supernatant was poured into a stirring solution of 0.2 vol eq. (16 mL) of 6% w/v streptomycin sulfate at 4 °C. The solution was stirred for 30 min at 4 °C and centrifuged (25,000 × g, 30 min). The supernatant was then loaded onto a Ni-NTA superflo (Qiagen) column (25 mL), which had been equilibrated with lysis buffer containing 500 mM NaCl. The column was then washed with 10 CV (250 mL) of lysis buffer to remove cellular proteins, and collected in five 50 mL fractions. The protein was then eluted with a 500 mL linear gradient of 10–300 mM imidazole in lysis buffer and collected in 1.5 min (12 mL) fractions. The fractions containing only a protein (by SDS-PAGE) were pooled and concentrated to 30 mL while stirring in an Amicon pressure concentrator fitted with a 30 kD MWCO membrane at 50 psi N₂. The solution was then loaded onto a 200 mL G-25 column and eluted with spectroscopy buffer (50 mM sodium borate, pH 8.3 at 23 °C), containing 15 mM MgSO₄ and 5% v/v glycerol). The eluent was collected in 3 min (10 mL) fractions. Those fractions containing protein, as judged by the Bradford assay, were pooled and concentrated in 30 kD MWCO centrifugal concentrators. Final purity was determined by SDS-PAGE (Figure 3.4a,b).
concentration was determined with the known $\varepsilon_{278}$ of 0.189 $\mu$M$^{-1}$ cm$^{-1}$. Activity measurements were performed as previously described (Figure 3.4c).

3.5.8. Tyrosine Phenol Lyase (TPL) Expression and Purification

TPL expression and purification was adapted from existing procedures$^{21,22}$ with minimal changes. *E. coli* SVS pTZTPL was available as a glycerol stock solution from previous expression in our group. A small amount (~0.5 mL) from a frozen glycerol stock was used to inoculate 2 L of LB medium containing amp (100 $\mu$g/mL), and the culture was grown at 37 °C for 20 h in an incubator at 220 RPM. Cells were harvested by centrifugation (10 min, 3,000 x g). The resulting cell pellet (8 g) was suspended in 35 mL of standard buffer: 100 mM KPi (pH 7.0) with 0.1 mM PLP, 1 mM EDTA, and 5 mM β-mercaptoethanol. The cells were lysed by passage through a SLM-Aminco French pressure cell between 16,000 and 18,000 psi. The resulting solution was centrifuged (30 min, 25,000 x g) at 4 °C, and the supernatant was isolated and treated with 0.2 vol (4 mL) of 6% w/v protamine sulfate. The solution clouded immediately, and it was stirred 20 minutes more at 4 °C to ensure complete DNA precipitation. It was then centrifuged (30 min, 25,000 x g) at 4 °C. The supernatant was isolated. To the clear yellow solution was added solid (NH$_4$)$_2$SO$_4$ to reach 66% of saturation (39 g / 100 mL). The solution clouded immediately. It was stirred an additional 30 min at 4 °C to dissolve the salts, and it was then centrifuged (30 min, 25,000 x g) to pellet the protein. The supernatant was discarded, and the pellet was gently redissolved with 20 mL of standard buffer. The solution was then dialyzed in a 10 kD MWCO membrane (Pierce Biotechnology) for 18 h against 2 L of standard buffer that was 25% saturated with (NH$_4$)$_2$SO$_4$. The dialyzed protein solution was then loaded onto an octyl sepharose column, which was equilibrated with standard buffer that was 25% saturated with (NH$_4$)$_2$SO$_4$. The protein was then eluted with the same buffer and collected in 2 min fractions. The protein eluted within the first 40 fractions, as determined by TPL activity assays. The fractions containing enzyme activity were pooled and concentrated to 120 $\mu$M with a specific activity of 0.6 U/mg, flash frozen at 77 K, and stored at −80 °C. Yield 4 mL of 72 U/mL.
3.5.9. *Synthesis*

3.5.9.1. (N-Fmoc)-2,3,6-trifluorotyrosine

The Fmoc protection of 2,3,6-trifluorotyrosine has been previously described.\(^5\) It is detailed here due to minor changes in the purification that were found to increase material recovery yields. A 25 mL round bottom flask was charged with a magnetic stir bar and the fluorotyrosine (1 eq.). To this was added water (to make 0.67 M fluorotyrosine) and \(K_2CO_3\) (10% w/v). The solution was stirred magnetically, and it became tan-brown and homogenous. The flask was then cooled with stirring in an ice bath for 10 min. During this time, Fmoc-Succinimide (1.2 eq.) was dissolved in 1,4-dioxane (0.8 M Fmoc-Suc). The dioxane solution was then added to the cold tyrosine solution dropwise over 4-5 min, giving final solution concentrations of 0.33 M fluorotyrosine and 0.4 M Fmoc-Suc. The solution was stirred while the ice melted to rt (4 h). The reaction progress was monitored by TLC in 20% v/v ethyl acetate/dichloromethane containing 0.1% v/v TFA. After the reaction finished progressing, it was transferred to a separatory funnel and washed once with ethyl acetate. The solution was then acidified with ~5 mL of 1 M HCl, during which the solution became cloudy. The material was then extracted with three fractions of ethyl acetate, dried over sodium sulfate, vacuum filtered through a 1 cm pad of celite, and condensed under reduced pressure. The product was purified by flash column chromatography on silica gel. The yellow oil was dissolved in 5% v/v ethyl acetate/dichloromethane containing 0.05% v/v TFA, which was loaded onto the silica column. The product was then eluted with 20% EtOAc/DCM + 0.05% TFA, and collected in 10 mL fractions. The solution was condensed under reduced pressure, and the yellow oil was triturated with DCM, which caused the off-white product to precipitate. The suspension was then reconcentrated under reduced pressure. The trituration/evaporation procedure was repeated four additional times to ensure removal of free TFA, and the final material was dried at room temperature for 12 h at 50 mTorr. Yield 270 mg (28%), off-white solid. All characterization of the compound matched that previously reported.\(^5\)

3.5.9.2. Re(I)(CO)₃(CN)(Mebpy-COO-PFP) ([Re]-OPFP)

The synthesis of Re(I)(CO)₃(CN)(Mebpy-COOH) ([Re]-COOH) has been previously described.\(^1\) It was determined that the coupling yields of the rhenium carboxylate to the N-terminus of the peptide were low under previously reported HATU coupling conditions. The [Re]-COOH was pre-
activated as a pentafluorophenyl ester. To do so, [Re]-COOH (235 mg, 0.46 mmol, 1 eq.) was diluted with 2.6 mL DCM and 75 µL DIPEA. The solution was transferred to a 25 mL round-bottom flask under a nitrogen atmosphere, and the vial used to dissolve the carboxylate was washed with an additional 2 mL DCM and 50 µL DIPEA and transferred to the reaction flask. The flask headspace was purged with N₂ and then PFP-TFA (258 mg, 0.92 mmol, 158 µL, 2 eq.) was added dropwise via syringe. The solution was stirred magnetically for 2 h, at which point an additional 1 eq. of PFP-TFA was added and the reaction stirred 90 min more. The reaction was monitored by TLC viewed in 4:1 DCM:EtOAc. Upon consumption of the baseline starting material determined by TLC, the reaction solution was condensed under reduced pressure. The product was purified by flash chromatography on silica gel (2 x 15 cm). The product was loaded onto the column in DCM, and washed with DCM to elute a pale yellow band (R_f = 0.9) corresponding to hydrolyzed pentafluorophenol. This band was discarded. The bright orange product band was then eluted with 3:1 DCM:EtOAc and collected in 10 mL fractions. Those showing product by UV illumination of TLC spots were pooled, condensed under reduced pressure, and dried in vacuo. Yield 221 mg (71%, bright red-orange solid). ¹H NMR (400 MHz, CDCl₃): δ = 2.65 (s, 3H, bpy-CH₃), 7.43 (dd, 1H, J = 5.6, 0.8, bpy-H), 8.17 (dd, 1H, J = 1.6, 6, bpy-H), 8.23 (s, 1H, bpy-H), 8.85 (d, 1H, J = 0.8, bpy-H), 8.89 (d, 1H, J = 5.6, bpy-H), 9.31 (d, 1H, J = 6, bpy-H). ¹³C NMR (400 MHz, CDCl₃): 21.6 (bpy-CH₃), 122.99 (bpy), 125.00 (bpy), 126.50 (bpy), 129.09 (bpy), 136.51 (bpy), 143.61 (bpy), 151.98 (bpy), 153.08 (bpy), 154.21 (bpy), 154.72 (PFP C-F), 157.73 (PFP C-F), 159.59 (PFP C-F), 190.07 (-O-(C=O)-Ar, ester), 194.59 (Re-CO), 194.92 (Re-CN). HPLC: 99%, 6.6 min (isocratic 1:1 MeCN:H₂O w/0.1% TFA).

3.5.9.3. Synthesis of (N-Fmoc)-(2,3,6-trifluorotyrosyl)-LVGQIDSEVDTLDSNFQL (F₃Y-βC19)

The as-received βC19 resin (126 µmol/g for the protected peptide, 1 eq., 992 mg resin) was added to a 20 mL econopac column (Bio-Rad). To this was added 10 mL of deblocking solution (20% v/v piperidine/DMF containing 0.1 M HOBT). The column was capped and vortexed for 10 min at room temperature. The solution was drained and the deblocking was repeated twice more. The resin was then washed with DMF (5 x 10 mL for 60 s each) and DCM (3 x 10 mL for 60 s each). To the column was then added the reaction solution, including (N-Fmoc)-2,3,6-trifluorotyrosine (343 mg, 75 mM, 6 eq.),
DIPEA (250 μL, 194 mg, 0.15 M, 12 eq.), and DMF (9.75 mL). The reaction was initiated by the addition of HCTU (280 mg, 67.5 mM, 5.4 eq.). The column was vortexed for 2 h at room temperature. The solution was then drained, and the resin was again washed five times with DMF and three times with DCM, as described above. The resin was then dried in the econopac column by pulling on the outlet with vacuum for 2 h. The product was characterized by HPLC and MALDI-MS of a test cleavage of 10 mg of the resin, which confirmed the conjugation. The dry material was stored at 4 °C.

3.5.9.4. Synthesis of [Re]-F₃Y-βC19

The Fmoc-F₃Y-βC19 resin (122 μmol/g for the protected peptide, 1 eq., 1.02 g resin) was added to a 20 mL econopac column (Bio-Rad). To this was added 10 mL of deblocking solution (20% v/v piperidine/DMF containing 0.1 M HOBT). The column was capped and vortexed for 10 min at room temperature. The solution was drained and the deblocking was repeated twice more. The resin was then washed with DMF (5 × 10 mL for 60 s each) and DCM (3 × 10 mL for 60 s each). The reaction solution was then added to the column, including [Re]-OPFP (224 mg, 33 mM, 2.6 eq.), HOBT (110 mg, 80 mM, 6.5 eq.), and DMF. The column was vortexed for 90 min at room temperature. The solution was then drained, and the resin was again washed five times with DMF and three times with DCM, as described above. To isolate the peptide from the resin, the econopac was then filled with 10 mL of 95/2.5/2.5 TFA/TIPS/H₂O, capped, and vortexed for 4 h at room temperature. After, the solution was bright yellow and it was drained into a 20 mL scintillation vial and condensed by hand under a stream of N₂ to ~2 mL. We note that TFA vapors are noxious and toxic and proper personal protective equipment is imperative. Condensation of TFA by rotary evaporation was avoided. After condensation, the solution was dripped into 45 mL of Et₂O stirring in a 50 mL falcon tube, which caused immediate precipitation of a bright yellow flocculent solid. The falcon tube was then capped and incubated at 4 °C for 12 h to encourage further precipitation. The solution was then centrifuged (8,000 x g, 30 min) and the supernatant discarded. The yellow pellet was then resuspended in 25 mL of Et₂O with vigorous vortexing, and recentrifuged, as before. This wash step was necessary to remove residual TFA, which makes the final peptide difficult to dissolve in dilute basic solutions. The final product was then dried for 12 h under a stream of N₂. The solid was stored at 4 °C.
3.5.9.5. Purification of [Re]-F₃Y-βC₁₉

HPLC analysis of received βC₁₉ peptide established that further purification was necessary. As such, the final peptide was purified by RP-HPLC. A separation method was first developed on analytical scale. It was found that a 2–17.5% gradient of MeCN in water with 0.1% v/v Et₃N gave adequate separation in 20 min. For semi-preparative scale, this same gradient was applied to a 40 min separation time. For each semi-preparative run, fractions were collected in 0.5 min increments across the peak of interest. Injections consisted of a 500 μL solution of ~10 μmol of crude material dissolved in 5% v/v Et₃N. Fractions containing the desired product, as determined by inline UV and fluorescence, were pooled and condensed in vacuo (50 mTorr). From 16 serial semi-preparative runs 30 mg of purified peptide was recovered (8% isolated yield) (Figure 3.2). HPLC: 98%, 9.5 min (5–15% MeCN in H₂O w/ 0.1% Et₃N over 15 min). MALDI-MS: [M-CN]⁺ e.m. expected 2791.03; found 2790.81.

3.5.10. pKₐ of [Re]-F₃Y-βC₁₉

The pKₐ of the peptide was determined by fluorometric titration (Figure 3.2d). To prepare samples at 5 μM for spectroscopy, 13 μL of a 73 μM stock solution of peptide was diluted into 187 μL of buffer at the following pH values: 5.99, 6.19, 6.41, 6.62, 6.80, 7.00, 7.20, 7.38, 7.55, 7.79, 8.00, 8.20, 8.40 and 8.58. From 5.99 to 7.00 the buffer was 100 mM potassium phosphate. From 7.20 to 8.58 the buffer was 100 mM Tris. Each spectrum was recorded by exciting at 315 nm and monitoring from 475–800 nm, in 1 nm increments with 1s integration per nm. The intensity at 600 nm was then plotted versus pH and fit using Origin software according to a method previously outlined,²³ with the assumption that protonation and deprotonation does not occur in the excited state.

3.5.11. Kᵦ of [Re]-F₃Y-βC₁₉ and wt-α₂

To measure the dissociation of the photopeptide from α₂, we used a previously developed competitive inhibition assay.²⁴ The inhibitor was titrated against a solution of wt–RNR. In a total volume of 150 μL, each solution contained 100 nM wt–α₂, 200 nM wt–β₂, 200 μM NADPH, 30 μM TR 500 nM TRR, 1 mM CDP, 3 mM ATP, inhibitor peptide, and buffer. The volume of inhibitor peptide (250 μM stock) and buffer (50 mM borate, pH 8.3 with 15 mM MgSO₄ and 5% glycerol) were adjusted so that the
peptide was present in the total volume at 5, 15, 25, 35, and 50 μM.

3.5.12. Nanosecond Laser Flash Photolysis

3.5.12.1. Instrumentation

Transient measurements were recorded on a home-built system the timing schematic of which is shown in Figure 3.16. Pump light was provided by the third harmonic (355 nm) of a Quanta-Ray Nd:YAG laser (Spectra-Physics) running at 10 Hz with a power output set to 2 mJ/pulse using a combination of the instrument’s power knob and a neutral density filters. Laser power was measured using a Coherent J-25MB-HE head coupled to a Coherent Labmax TOP meter. Probe light was the output of a 75 W Xe-arc lamp (A1010B arc lamp, LPS-220B power supply, Photon Technologies Incorporated). A TTL pulse synchronized with the Q-switch of Infinity laser was used to generate the 0 time point of the system.

Figure 3.16. Connectivity and timing of the ns TA system. The t = 0 is set by the Q-switch (Q-SW) output of the laser. This signal is used to set the delay and gate of the CCD through delay box #1 with software. The Q-SW signal is also passed to delay box #2, which controls both pump and probe shutters. Because the shutters have a mechanical delay of milliseconds, timings are set so opening and closing occurs as far as possible from the laser pulse. The full shutter sequence is 400 ms, synced every 100 ms to the Q-SW signal. Delay box 2 is triggered by every second signal from the Q-SW in order to allow the full pump/probe open/closed sequence to function. The CCD receives each Q-SW signal to record the four states listed in Equation 3.1. Pump and probe shutter “open” and “closed” states are shown in green and red, respectively. Delay and gate timings are not to scale and vary by experiment.
All timed delays were created with SRS DG535 delay generators (Stanford Research Systems) controlled by software via a GPIB interface. Uniblitz electronic shutters model VS14S2Z0R3 (Vincent Associates, Rochester, NY) were used to create the pulse sequence illustrated in Figure 3.16. Both the white light and pump beams were focused and overlapped at a 15° angle to pass through the cylindrical bore of a flow cuvette (585.3/Q/10/Z15/AR, Starna Inc., Atascadero, CA), providing a total pathlength of 1 cm. The sample was flowed from a 1.5 mL eppendorf tube, through the flow cell, then the pump (Cole-Parmer L/S 7518-00), the inline filter (see below) and back to the eppendorf tube, through a combination of pump tubing (Masterflex L/S 13) and the included fittings for the flow cell. The resulting signal light was passed into a Triax 320 spectrometer with a slit width of 0.4 mm (correlating to 5 nm).

3.5.12.2. Transient absorption (TA) spectroscopy

Signal light entering the spectrometer was first dispersed by a 300 × 250 blazed grating. The dispersed light was recorded on an intensified gated CCD camera (ICCD, CCD 30-11, Andor Technology, 1024 × 256 pixels, 26 μm²), whose delay and exposure were timed from the output of the SRS DG535 delay generator. A series of four spectra were taken using the on/off state of the pump and probe shutters: \( I_p \) (pump on/probe off), \( I \) (pump on/probe on), \( I_s \) (pump off/probe off), and \( I_o \) (pump off/probe on). Spectra were corrected for fluorescence and background light using these data by the equation:

\[
\Delta OD = -\log \left( \frac{I - I_F}{I_o - I_B} \right)
\]

3.5.12.3. Transient absorption kinetics

For TA kinetics, signal light entering the spectrometer was first dispersed by a 300 × 500 blazed grating, and selected wavelengths were passed through an output with a slit width of 0.4 mm (5 nm). The resulting transient pulses were recorded with a photomultiplier tube (Hammamatsu 928A) poised at 1.00 kV. PMT outputs were collected and averaged with a 1 GHz oscilloscope (LeCroy 9384CM), controlled by software, which also collects averaged data. The oscilloscope was triggered by the output of a photodiode that was aligned to respond to the backscattered light from the flow cuvette. Unlike in collection of TA spectra, both the pump and probe light shutters were open for the duration of the measurement. To
perform a specific number of scans, we first opened the probe shutter and the spectrometer slits to admit light to the detection. The software was then set to record a desired interval, the oscilloscope memory was cleared, and finally the pump shutter was opened, which triggered the photodiode to begin recording. After accumulating the desired number of scans, the software recorded averaged data from the oscilloscope.

3.5.12.4. Time-resolved emission kinetics

Time-resolved emission data were generated with the same system as the transient absorption kinetics above, but without the use of probe light.

3.5.12.5. Instrument Control and Data Acquisition Software

Instrument control and data acquisition for the nanosecond laser system were achieved using custom software written in python (python.org) using assorted libraries packaged together by the Python(x,y) project (http://code.google.com/p/pythonxy/, v. 2.6.5.6).

The software has two main programs, the kinetics mode and full spectrum mode. These differ in the method of detection and the information obtained. The full spectrum program, ccd_main_program.py, initializes the HORIBA Jobin Yvon Triax 320 spectrometer for use with the Andor iCCD camera. The timing of the camera is controlled by a Stanford Research Systems delay, controlled by GPIB serial interface. Data are read from the iCCD using the supplied dynamic link library after the desired number of exposures and displayed on screen. It can then be stored to disk.

The kinetics mode program, kinetics_main_program.py, initializes the spectrometer for use with a Hamamatsu PMT. The signal channel from the PMT is connected to the LeCroy oscilloscope equipped with GPIB interface. After the signal is found manually on the scope, the software can be used to automate the acquisition of a series of averages. The data are then displayed on screen, and can be saved to disk.

3.5.12.6. Sample Information for Flash Photolysis

Solutions for time-resolved spectroscopy were prepared in 1.5 mL eppendorf tubes at room temperature. Reagents were added to the vial in the following order: 1st half of buffer, flash quencher (if used), ATP, CDP, peptide, 2nd half of buffer (to encourage mixing), and then protein (if used). Care was taken to avoid using pipette tips for more than one draw of a protein solution. It was found that the second draw of these very concentrated protein solutions in the same pipette tip can lead to small amounts
of precipitation on the plastic sidewall. All samples were equilibrated in a water bath at 23 °C for 3 min before analysis. Unless otherwise noted, "buffer" for all transient spectroscopy samples consisted of 50 mM sodium borate (pH 8.3 at 23 °C) with 15 mM MgSO₄ and 5% v/v glycerol.

Each solution was analyzed while flowing at 10 mL/min through the cuvette. Great care was taken to ensure that no bubbles were in the path length of the cuvette during measurement. In addition, we found that in alkaline solutions, the flash quencher decomposed over time into RuO₂. At low concentration this side product can slowly deposit on the cell glass, and at high concentrations it rapidly clouds the solution. To alleviate these problems, during the experiment the samples were run continuously through a 13 mm 0.2 μm Supor inline filter (Pall Corp., Port Washington, NY) to remove the byproduct as it is generated. We found that 650 μL was the minimum volume required to flow a sample through the cuvette, pump lines, and inline filter.

Each sample for time-resolved emission measurements of [Re]-F₃Y-βC19 contained in final concentration: 10 μM [Re]-F₃Y-βC19, 200 μM Ru(NH₃)₆Cl₃, 1 mM CDP, and 3 mM ATP. Each 650 μL buffered solution used for time-resolved emission measurements of [Re]-F₃Y-βC19 with α₂ variants contained in final concentration: 10 μM [Re]-F₃Y-βC19, 20 μM α₂ mutant, 200 μM Ru(NH₃)₆Cl₃, 1 mM CDP, 3 mM ATP. Each buffered solution used for transient absorption measurements of [Re]-F₃Y-βC19 contained in final concentration: 50 μM [Re]-F₃Y-βC19, 1 mM Ru(NH₃)₆Cl₃, 1 mM CDP, 3 mM ATP. Each 650 μL buffered solution used for transient absorption measurements of [Re]-F₃Y-βC19 with α₂ variants contained in final concentration: 50 μM [Re]-F₃Y-βC19, 100 μM α₂ mutant, 1 mM Ru(NH₃)₆Cl₃, 1 mM CDP, 3 mM ATP.

3.5.13. Data Analysis for Transient Spectroscopy

The calculation of rate constants for the oxidation of Y by •F₃Y was performed using the equation,

\[
(3.2) \quad k_{\text{ox}} = k_{\text{on}} - k_{\text{off}} = \frac{1}{\tau_{\text{on}}} - \frac{1}{\tau_{\text{off}}}
\]

Here, \( k_{\text{on}} (\tau_{\text{on}}) \) is the rate constant (time constant) for \( F₃Y^- \) decay when the tyrosyl is in the "on" conformation, bound to the protein, and \( k_{\text{off}} (\tau_{\text{off}}) \) is the rate constant (lifetime) for \( F₃Y^- \) decay when the tyrosyl is in the "off" solvated, conformation. The error of each measurement was propagated using
standard methods. Transient absorption spectra were an average of three independently collected data sets, corrected for noise inherent to the instrument by fast Fourier transform (FFT) filtering of high frequency noise across the data set. To determine the filter level, a FFT was first performed on the raw data to determine the frequency of signal/noise cutoff. That cutoff filter was then applied to the data set using OriginPro. Kinetic decay traces were collected from the PMT as intensity values corresponding to a change in voltage. Transient optical density was calculated using:

\[
\Delta OD = -\log \left( \frac{V}{V_0} \right)
\]

\( V_0 \) was determined by averaging the first 40 data points collected before the 0 time point.

Each decay trace from which a lifetime was calculated was an average of 3–6 individually collected data sets. The standard deviation at each \( x,y \) pair was used as the weight term in the fitting. All rate constants were calculated using weighted least-squares regression analysis of Cartesian data pairs in Origin by modulation of variables until the reduced \( \chi^2 \) ceased changing. The goodness-of-fit parameter \( (R^2) \) was used as a starting point for determining the accuracy of fit; all fits reported are 0.98 or greater \( R^2 \). Subsequently, graphical residual analysis was employed. Those residuals demonstrating significant asymmetry or periodicity with respect to the independent variable were fit again with an additional phase. Error bars were calculated as 67% confidence intervals (one standard deviation). Decays of short (ns) lifetimes were fit using all data points appearing after the time point with maximum amplitude. Decays of radical lifetimes were fit by excluding those data points corresponding to the residual charge-separated state of [Re] by starting at the 1 \( \mu s \) timepoint.

3.5.13.1. Calculations and propagation of uncertainty

Equation 3.2 describes the calculation of the rate constant for radical-induced oxidation of the protein subunits. For clarity, the standard equations\(^{25} \) used to propagate the instrumental error associated with measurement are provided below.

The error for a single rate constant measurement was calculated as a relative error, as follows:

\[
E_x = \sqrt{E_{on}^2 + E_{off}^2}
\]
where $E_x$ denotes the error for each rate constant (either $E_{on}$ or $E_{off}$), $f$ is the error fraction, $E_{\tau}$ is the error in the lifetime measurement, and $\tau$ is the measured lifetime ($\tau_{on}$ or $\tau_{off}$). The error for the calculated oxidation rate constant (given by Equation (3.2)), is then calculated as follows:

\[(3.5)\quad E_x = f_x k_x\]

Where,

\[(3.6)\quad f_x = \frac{E_{\tau}}{\tau}\]

and $E_{\tau}$ is the total error of the measurement, and $E_{on}$ and $E_{off}$ are the error of each kinetic measurement, respectively. Similarly, the error for a single lifetime measurement was propagated to an averaged lifetime measurement.

3.5.13.2. Calculation of Radical Yield

The photochemical yield of trifluorotyrosyl radical ($\Phi_\gamma$), measured in molecules of radical generated per photon, was calculated using the equation:

\[(3.7)\quad \Phi_\gamma = \frac{N_A V_{\text{Bore}} \left( \frac{\Delta OD}{\epsilon_{\gamma,\lambda}} \right)}{(E_p \lambda / hc) \left( 1 - 10^{-\epsilon_{355,\text{Re}} [\text{Re}] l} \right)}\]

Here, $N_A$ is Avogadro's number, $V_{\text{Bore}}$ is the volume of the cuvette in the path length (70 $\mu$L), $\Delta OD$ is the transient absorption of the radical ($5.2 \times 10^{-3}$), $\epsilon_{\gamma}$ is the approximate extinction coefficient of the radical itself (2,750 M$^{-1}$ cm$^{-1}$ for phenoxy radical), $l$ is the sample path length (1 cm), $E_p$ is the energy of each laser pulse (2.0 mJ), $\lambda$ is the wavelength of observation (355 nm), $h$ is Planck's constant, $c$ is the speed of light, $\epsilon_{355,\text{Re}}$ is the extinction coefficient of the rhenium complex (5,300 M$^{-1}$ cm$^{-1}$), and [Re] is the concentration of the complex (50 $\mu$M). For the trifluorotyrosyl radical measured in the presence of $Y_{731}$, $\Phi_\gamma = 0.049$, or 4.9%.

3.5.14. Software

Graphs were generated from Cartesian data pairs in Origin (v. 8.0; OriginLab Corp, Northhampton,
MA, www.originlab.com). 16-bit.tif gel images were first processed with Adobe Photoshop (v. CSS, Adobe Systems Inc., San Jose, CA, www.adobe.com) by setting the image levels automatically, then cropping the pixels and saving. ImageJ (version 1.43u, NIH, http://rsb.info.nih.gov/ij/) was then used to subtract the background using a 50-pixel rolling-ball, and protein purity was calculated from integration of the area under vertical slices of the optical density plots of the gel bands. Mass spectra were transformed from the collected raw data with the Bruker CompassXport tool (v. 3.0; Bruker Daltonics, Inc., Billerica, MA, www.bdal.com) by mmass software27 (v. 3.9, www.mmass.org), which was further used for mass calibration and export. NMR analysis was performed with SpinWorks (v. 3.17; University of Manitoba, www.umanitoba.ca/chemistry/nmr/spinworks). Final plots of all graphical data were prepared with Adobe Illustrator (v. CSS).
3.6. References


Chapter 4  Construction of an Intact Photochemical $\beta_2$
Subunit
Portions of this chapter have been published:

4.1. Introduction

Peptide based photoRNR systems have enabled a number of interesting and important advances,1-5 including the direct measurement of radical transport into the \( \alpha_2 \) subunit,\(^5\) as shown in Chapter 3. Nevertheless, these studies were complicated significantly by weak photopeptide binding and conformational flexibility, where up to 80% of bound peptide was present in a conformation unable to effect radical injection.

Direct covalent attachment of a PO to the \( \beta_2 \) subunit can significantly advance the study of radical transport in RNRs. Full-length \( \beta_2 \) subunits for photoRNR would: \( (i) \) enhance binding between the site of radical generation, \( Y_{356} \), and the \( \alpha_2 \) subunit; \( (ii) \) permit PCET to be examined in the \( \beta_2 \) subunit; \( (iii) \) provide a platform for the first photoinitiated measurement of PCET in the \( \alpha_2 \beta_2 \) holoenzyme; and in doing so \( (iv) \) minimize complications arising from conformational flexibility.

We now describe the creation of such a photoRNR by developing a PO bearing an electrophilic carbon capable of alkylating surface-accessible protein thiolates. The native \( \beta_2 \) subunit has two solvent-accessible cysteine residues \( C_{268} \) and \( C_{305} \), both of which can be mutated to serine without loss of activity.\(^6\) In the present report, we mutated these two residues to serine while also changing the serine at position 355 in the native protein to cysteine \( (S_{355}C-\beta_2) \). This permits a PO to be placed adjacent to \( Y_{356} \). A new PO, a rhenium(I) tricarbonyl phenanthroline complex \((\text{[Re]-Br}=\text{[Re(phen)(CO)}_3\text{(PyCH}_2\text{Br)]}^+\))\(^6\), has been synthesized and characterized. By using the bromobenzyl derivative, the single surface cysteine variant of \( \beta_2 \) can be labeled selectively to yield \( \text{[Re]}-S_{355}C-\beta_2 \). By using the flash-quench technique and transient absorption spectroscopy, we show that the production of \( Y_{356}^* \) may be phototriggered in \( \beta_2 \) and the radical is long lived, thus constituting a \( \beta_2 \) photoRNR.

4.2. Results

4.2.1. \([\text{Re]}-\text{Br Photooxidant Synthesis}\)

The selection of an appropriate photooxidant (PO) is essential to the construction of a photoRNR. The PO must \( (i) \) exhibit spectral separation from the protein envelope for its excitation, \( (ii) \) possess an excited-state lifetime and reduction potential sufficient to effect amino acid oxidation,
and (iii) be photostable. We have found that rhenium polypyridyl complexes, such as \( \text{Re}^I(NN)(CO)_3L \) (\( NN = 1,10\)-phenanthroline (phen); 2,2'-bipyridine (bpy) or derivatives; \( L = \text{Cl, SCN, CN}; \) or phosphine and pyridine derivatives), are superior phototriggers of amino acid radicals, especially tyrosine. They absorb well outside the absorption envelope of the protein and are strong photooxidants. For this study, the rhenium polypyridyl complex needs to be conjugated with the protein. To accomplish this, we prepared \( \text{[Re(phen)(CO)₃(PyCH₂Br)]PF₆} \) according to the reaction sequence in Scheme 4.1.

![Scheme 4.1. Synthesis of [Re]-Br](image)

The presence of the primary bromide on the pyridyl ring provides a site for facile bioconjugation of the complex to the protein. \( \text{[Re(phen)(CO)₃(MeCN)]PF₆} \) is prepared as previously published.\(^8\) 4-pyridylcarbinol displaces the bound MeCN ligand of \( \text{[Re(phen)(CO)₃(MeCN)]PF₆} \) to yield the alcohol \( \text{[Re(phen)(CO)₃(PyCH₂OH)]PF₆} \), which is subsequently brominated with \( \text{PBr₃} \) to yield the protein-reactive \( \text{[Re(phen)(CO)₃(PyCH₂Br)]PF₆} \). The initial reaction yields a mixture of bromide and hexafluorophosphosphate salts, which is otherwise highly pure and suitable for use in protein labeling experiments. Treatment of the initial bromination product with TIPF₆ allowed us to isolate the pure PF₆⁻ salt. The final product was obtained in analytically pure form and 46% overall yield. Crystallization of this material allowed structural characterization of \( \text{[Re(phen)(CO)₃(PyCH₂Br)]PF₆} \) Figure 4.1.
The structural parameters (Table 4.1) are consistent with an approximately octahedral geometry about rhenium with an attenuated N-Re-N angle as is typical of a phenanthroline complex.

Table 4.1. Selected metric parameters for [Re]-Br. Distances are reported in Å; angles are reported in degrees.

Spectroscopic studies of ultraviolet-visible absorption and steady-state emission (Figure 4.2) show the characteristic features of an electronic structure derived from metal-to-ligand charge transfer.⁹
Figure 4.2. Spectroscopic comparison of [Re]-Br and [Re]-S\textsubscript{355}C-β\textsubscript{2}. The UV-vis absorption (gold solid line) and emission spectra (λ\textsubscript{ex} = 355 nm) (gold dotted line) of [Re]-Br (MeCN solution) and the UV-vis absorption (blue solid line) emission (blue dotted line) spectra of [Re]-S\textsubscript{355}C-β\textsubscript{2} (50 mM HEPES, 1 mM EDTA, pH 8.0) are shown. The absorption spectrum of S\textsubscript{355}C-β\textsubscript{2} (grey solid line) (50 mM HEPES, 1 mM EDTA, pH 8.0) is also included for reference. The simulated absorption spectrum (red solid line) of [Re]-S\textsubscript{355}C-β\textsubscript{2} by summing the absorption spectrum of twice the absorption spectrum of [Re]-Br and S\textsubscript{355}C-β\textsubscript{2}. The similarity of the actual and simulated [Re]-S\textsubscript{355}C-β\textsubscript{2} UV-vis absorption traces suggests that binding has no significant impact on the spectroscopic properties of [Re].

4.2.2. S\textsubscript{355}C-β\textsubscript{2} Labeling with [Re] Photooxidant

The choice of site, residue 355, for labeling β\textsubscript{2} was guided by our previous work using PO-Y\textsubscript{356}-βC19 peptides in place of β\textsubscript{2}. This peptide, representing the C-terminus (355–375 of β\textsubscript{2}) contains the elements in large part responsible not only for subunit interactions, but also activity, as Y\textsubscript{356} mediates radical transport between α\textsubscript{2} and β\textsubscript{2}.\textsuperscript{110} The S\textsubscript{355} residue was thus targeted as the site of labeling since it is directly adjacent to Y\textsubscript{356} and it occupies a position analogous to that of the photooxidant in PO-Y-βC19, a site that has been shown to allow Y\textsubscript{356} generation, radical injection into α\textsubscript{2}, and subsequent substrate turnover.\textsuperscript{1-3}

To attach the PO to a single site, a β\textsubscript{2} needed to be prepared with a single, surface-accessible cysteine at residue 355 (S\textsubscript{355}C-β\textsubscript{2}). Previous studies had shown that wt-β\textsubscript{2} contained two such cysteines, C\textsubscript{268} and C\textsubscript{305}, and that their mutation to serine gave C\textsubscript{268S}C\textsubscript{305S}-β\textsubscript{2} with activity identical to wt-β\textsubscript{2}.\textsuperscript{6} For
rapid affinity purification, all $\beta_2$ constructs were constructed with an N-terminal (His)$_6$-tag. Site-directed mutagenesis was performed with (His)$_6$-C$_{268}$S$_{305}$S-$\beta_2$ to give (His)$_6$-C$_{268}$S$_{305}$S$_{355}$C-$\beta_2$. The protein was expressed in media containing 1,10-phenanthroline, a chelating agent, in order to express (His)$_6$-C$_{268}$S$_{305}$S$_{355}$C-$\beta_2$ in the absence of Fe$^{II}$ to directly obtain apo-S$_{355}$C-$\beta_2$ in large amounts. Alternative protocols, where expression occurs in the presence of Fe$^{II}$, requires a partial denaturing chelation step prior to cofactor reconstitution which is not conducive to large scale isolation. Final purification resulted in homogenous apo-S$_{355}$C-$\beta_2$ in a yield of 39 mg/g of cell paste. Reconstitution of the diferric tyrosyl cofactor gave protein with 0.96 tyrosyl radicals/$\beta_2$, demonstrating that the mutation does not disrupt cofactor assembly.

Prior to labeling with photooxidant, the concentration of solvent-accessible thiolate groups of S$_{355}$C-$\beta_2$, taken to indicate the number of surface-exposed cysteine residues, was established by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). As expected, the results of the assay revealed 2.0 reactive thiols per $\beta_2$. Sequential incubation of S$_{355}$C-$\beta_2$ with dithiothreitol (DTT) and [Re]-Br at pH 8 for 2 h resulted in covalent labeling of C$_{355}$ via the methylene linker of the pyridyl ligand (i.e., Re($\text{Phen}$)(CO)$_3$(PyCH$_2$-)). Complete labeling was confirmed by a second DTNB assay, which indicated less than 500 nM free thiolate, consistent with labeling of >98% of solution-accessible cysteine residues. Labeling was further confirmed by ESI-MS (Figure 4.3) and shows peaks corresponding only to labeled species.

![Figure 4.3. ESI-MS spectrum for [Re]-S$_{355}$C-$\beta_2$. The mass spectrum of [Re]-S$_{355}$C-$\beta_2$ was reconstructed from ESI-MS data. The major peaks shown correspond to only expected labeled products, which include both [Re]-S$_{355}$C-$\beta_2$ (44922.6 expected) and the N-formylmethionyl variant, fMet-[Re]-S$_{355}$C-$\beta_2$ (45081.6 expected) as summarized in the following table (masses are reported in Da):](image-url)
4.2.3. Time-Resolved Spectroscopy of Radical Initiation

Electronic absorption and emission spectra (Figure 4.2) of [Re]-S$_{355}$C-$\beta_2$ show the expected signatures of the [Re] complex. The MLCT transition is observed as a shoulder at $\lambda_{\text{abs,max}} = 330$ nm, and a yellow-green emission of the triplet excited state occurs at $\lambda_{\text{em,max}} = 560$ nm; the emission spectrum shows only a small blue shift relative to [Re]-Br in acetonitrile. The rising absorption envelope of the protein occurs well into the UV spectral region. The absorption spectrum for [Re]-S$_{355}$C-$\beta_2$ is modeled well as the sum of the unlabeled S$_{355}$C-$\beta_2$ spectrum and twice the spectrum of [Re]-Br. This similarity is as expected for a [Re]-S$_{355}$C-$\beta_2$ construct where each $\beta$ monomer has been labeled with [Re], yielding a final [Re]:$\beta_2$ ratio of 2:1, which is reflected in the absorption spectrum, indicating that the environment of [Re] when bound to protein is similar to that of free [Re] in solution.

Nanosecond timescale transient absorption (TA) and time-resolved emission experiments were performed on [Re]-S$_{355}$C-$\beta_2$ to probe the effect of labeling on the spectroscopic properties of the $^3$[Re]$^*$ excited-state. TA spectra (Figure 4.4) show two growth features at 300 and 450 nm; these growths are due to MLCT excited state absorptions. Time-resolved emission data (Figure 4.4) reveal an excited-state lifetime of $311$ ns, in agreement with the excited state lifetime derived from transient absorption kinetics measured at 450 nm, which reveal a lifetime of $328$ ns.

In comparison, time-resolved spectroscopic data for a small molecule analogue [Re(phen)(CO)$_3$(PyCH$_3$)]PF$_6$ (Figure 4.5) show similar transient absorption features, exhibit an emission lifetime of $228$ ns in acetonitrile (in the presence of ambient O$_2$ as present in protein experiments) and a corresponding lifetime of $237$ ns monitored by transient absorption measured at 450 nm. The longer lifetime observed for [Re] bound to $\beta_2$ in comparison to free [Re] in solution indicates that the modified cysteine thioether does not quench the [Re]$^*$ excited state, making it available for amino acid oxidation and radical initiation.
Figure 4.4. Nanosecond laser flash photolysis of [Re]-S\textsubscript{55}-C-β. Time-resolved spectroscopic data are recorded after excitation (λ\textsubscript{ex} = 355 nm) of [Re]-S\textsubscript{55}-C-β\textsubscript{2} (10 μM in 50 mM HEPES, 1 mM EDTA, pH 8.0).

Top: Transient absorption spectra recorded 50 ns (black), 150 ns (brown), 500 ns (orange) and 1000 ns (grey) after excitation. Spectra are collected over 50 ns, beginning at the indicated time. The observed growth features are consistent with a ^3[Re]\textsuperscript{*} MLCT excited state. Asterisk indicates signal due to laser scatter.

Middle: TA kinetics data observed at 450 nm and a monoexponential fit (solid line) (τ = 328 ns).

Bottom: Time resolved emission data observed at 550 nm and a monoexponential fit (solid line) (τ = 311 ns).
Figure 4.5. Nanosecond laser flash photolysis of [Re(phen)(CO)₃(PyCH₃)]PF₆. Time-resolved spectroscopic data are recorded after excitation (λₑₓ = 355 nm) of [Re(phen)(CO)₃(PyCH₃)]PF₆ (20 μM in MeCN). Top: Transient absorption spectra recorded 50 ns (black), 150 ns (brown), 500 ns (orange) and 1000 ns (grey) after excitation. Spectra are collected over 50 ns, beginning at the indicated time. The observed growth features are consistent with a [Re]* MLCT excited state. Asterisk indicates signal due to laser scatter. Middle: TA kinetics data observed at 450 nm and a monoexponential fit (solid line) (τ = 237 ns). Bottom: Time resolved emission data observed at 550 nm and a monoexponential fit (solid line) (τ = 228 ns).
Whereas the excited-state reduction potential for \([\text{Re(Phen)(CO)}_3\text{(Py)}]^+\) \(E^0(\text{Re}^{III/II}) = 1.7\) V vs. NHE), calculated from previously reported excited-state energies and reduction potentials,\(^{13}\) is sufficient to oxidize tyrosine under these conditions \(E^0 = 0.77\) V vs. NHE at pH 8.0),\(^{14}\) rapid back electron transfer or significant transient absorption signals due to residual the \([\text{Re}]^{I^*}\) excited state may preclude detection of a transient tyrosine radical. In order to circumvent this issue, we employed the flash-quench technique. In the presence of an oxidant, \((\text{Ru}^{III}(\text{NH}_3)_6\text{Cl}_3)\), the \([\text{Re}]^+\) is rapidly quenched to yield the oxidized ground state \([\text{Re}^{II}]\), which also has an adequate potential for tyrosine oxidation \(E^0(\text{Re}^{II/II}) = 2.0\) V vs. NHE),\(^{13}\) precludes any rapid back reaction, and lacks significant absorption in the spectral region of interest.

As summarized in Figure 4.6, the sequence of events is: (i) laser excitation generates \([\text{Re}]^+\) excited state, which is (ii) rapidly reduced to the oxidized \([\text{Re}]^{II}\) ground state; (iii) the \([\text{Re}]^{II}\) ground state oxidizes the adjacent tyrosine residue, \(Y_{356}\), to yield \(Y_{356}^\cdot\).

![Figure 4.6](image-url)  

**Figure 4.6.** Strategy for the construction of a photoRNR-\(\beta\). The attachment of the PO, \([\text{Re}] = [\text{Re(phen)} - (\text{CO})_3\text{(PyCH}_2\text{Br})]\) via the methylene group of the pyridyl ligand of the rhenium center is shown schematically. Radical initiation at \(Y_{356}\): (1) a 355 nm laser pulse generates the \([\text{Re}]^*\) excited state, which (2) is quenched to produce (3) the \([\text{Re}]^{II}\) species, which oxidizes \(Y_{356}\) to regenerate the \([\text{Re}]^+\) ground state and the radical. (4) The photogenerated \(Y_{356}^\cdot\) radical is observed and monitored by transient absorption spectroscopy. Graphics were generated from PDB entry 1MXR.\(^{15}\)
In the presence of Ru\textsuperscript{III}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3} (500 eq. / Re-\textbeta\textsubscript{2}), transient absorption features due to the \textsuperscript{3}[Re]\textsuperscript{*} MLCT are significantly quenched upon laser excitation. After 1 \mu s, the only apparent features in the transient absorption spectrum are due to tyrosyl radical, Y\textsubscript{356} (Figure 4.7). In order to maximize signal-to-noise, a total of 1250 averages (5000 component spectra) were collected on each of two independent samples and averaged to give a total of 2500 averages. The transient radical vanishes with biexponential kinetics and exhibits lifetimes of 8.1 ± 1.1 and 2.0 ± 0.8 \mu s, with the longer component having approximately twice the amplitude of the shorter component (Figure 4.7). The data presented are an average of 5000 kinetic traces taken on a single sample and averaged.

![Figure 4.7](image.jpg)

**Figure 4.7.** Photochemically generated Y\textsubscript{356}. Time-resolved spectroscopic data are recorded after excitation (\lambda = 355 nm) of met-[Re]-S\textsubscript{55}C-\textbeta\textsubscript{2}. Top: Transient absorption spectrum of [Re]-S\textsubscript{55}C-\textbeta\textsubscript{2} collected 1 \mu s after excitation (65 \mu M in 50 mM HEPES, 32.5 mM Ru\textsuperscript{III}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}, 1 mM EDTA, pH 8.0). The spectrum shown is obtained from 2500 four-spectrum sequences taken on two samples (1250 four-spectrum sequences each), averaged, and smoothed using a low-pass filter on the basis of a fast Fourier transform (FFT). Bottom: Transient absorption kinetics for transient Y\textsubscript{356} (\lambda\textsubscript{abs} = 408 nm) and a biexponential fit (solid line) (50 \mu M in 50 mM HEPES, 25 mM Ru\textsuperscript{III}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}, 1 mM EDTA, pH 8.0) (\tau\textsubscript{1} = 8.1 ± 1.1 \mu s, \tau\textsubscript{2} = 2.0 ± 0.8 \mu s). The trace shown is obtained from 5000 averages taken on a single sample.
The presence of high concentrations of Ru\textsuperscript{III}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3} results in shortened apparent lifetimes; the intrinsic chemical lifetime of this species is likely to be significantly longer than that observed here. Therefore, the given lifetimes are valid only for the specific conditions given. Additionally, in the context of previously reported lifetimes in the literature for photoinitiated tyrosine radicals, the observed lifetimes are short; UV photolysis of tyrosine in aqueous solution yields a tyrosyl radical that decays with a lifetime of 77 μs.\textsuperscript{16} This decrease in lifetime is attributed to reduction of Y\textsubscript{356} by Ru\textsuperscript{II}, generated stoichiometrically when Ru\textsuperscript{III}(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3} oxidatively quenches the 3[Re]\textsuperscript{*} excited state. While large concentrations of flash-quench reagent maximize the observed signal for tyrosyl radical, the corresponding increase in Ru\textsuperscript{II} increases the rate of Y\textsuperscript{*} reduction, thereby causing a decrease in the observed lifetime. In the absence of flash quencher, large transient absorption signatures due to 3[Re]\textsuperscript{*} show no significant features due to Y\textsuperscript{*} or a charge-separated state.

The maximum amplitude of the Y\textsuperscript{*} TA spectrum ($\lambda_{\text{max}} = 412$ nm, $\Delta\text{OD}_{412} = 3.9$ mA) and other experimental parameters can be used to calculate an estimated quantum yield of tyrosyl radical formation ($\Phi_{Y} \approx 0.02$). Details of the approximation are given in the Supporting Information. The low observed quantum yield can be attributed to a number of factors. One significant consideration is that $\Phi_{Y}$, as given is inherently limited by the emission quantum yield ($\Phi_{\text{em}} = 0.262$).\textsuperscript{17} In addition to this limitation, incomplete quenching of the 3[Re]\textsuperscript{*} excited state, rapid charge recombination with Ru\textsuperscript{II}, and signal decay that occurs before the observed spectrum ($t = 1$ μs for the spectrum, $\tau_2 = 2.0 \pm 0.8$ μs) would lead to significant decreases in the apparent yield of Y\textsuperscript{*}.

The observed multieponential decay kinetics for Y\textsubscript{356}\textsuperscript{*} may indicate distinct conformations of the flexible C-terminal tail of Re-β\textsubscript{2}. In previous work, we have observed multiple peptide conformations for Re-Y-βC19 bound to the α\textsubscript{2} subunit, resulting in biexponential [Re] emission kinetics, where the PO can be closely associated with the subunit or may be largely exposed to solution. Similar dynamics of the C-terminal tail in the intact β\textsubscript{2} subunit may result in the observed multieponential kinetics for Re-β\textsubscript{2}.

4.3. Discussion

Presuming that Y\textsubscript{356} is largely solvated and therefore that the pK\textsubscript{a} of Y\textsubscript{356} and protonation state
at pH 8.0 are not largely perturbed from the aqueous value of 10, under the experimental conditions, tyrosine oxidation necessarily occurs by a PCET mechanism, as Y$_{356}$ is largely protonated at pH 8.0.

4.4. **Conclusion**

[Re(Phen)(CO)$_3$(PyCH$_2$Br)]PF$_6$, a photooxidant bearing an electrophilic group for the selective alkylation of solution-accessible cysteine residues on proteins, has been synthesized and characterized. Its steady-state emission and electronic absorption spectra are consistent with an emissive excited state competent for photochemical tyrosine oxidation. [Re(Phen)(CO)$_3$(PyCH$_2$Br)]PF$_6$ has been shown to selectively and effectively alkylate surface-exposed residues on the ribonucleotide reductase mutant holo-S$_{355}$C$\beta_2$ to yield a complex [Re]$-S_{355}$C$\beta_2$, as confirmed by mass spectrometry. Both steady-state and time-resolved spectroscopic properties for [Re]$-S_{355}$C$\beta_2$ are consistent with those reported for related compounds, indicating that the excited-state properties of [Re] are not significantly perturbed by conjugation to $\beta_2$.

The successful labeling of S$_{355}$C$\beta_2$ leads to the creation of a photoRNR-$\beta_2$. Nanosecond flash photolysis, employing the flash-quench technique, affords Y$_{356}^*$, which was spectroscopically observed. The photoRNR-$\beta_2$ construct is a powerful tool for the examination of PCET. Future studies will use the photoRNR-$\beta_2$ to trigger radical initiation and transport in RNR. Moreover, the generation of Y$_{356}^*$ in an intact $\alpha_2$$\beta_2$ construct provides a platform for time-resolved photochemical study of PCET in RNR. The expected enhancements in binding and minimization of detrimental conformational dynamics due to the use of an intact $\beta_2$ subunit, in addition to increased fidelity to the natural system will be a great asset to these studies, which are currently underway.

4.5. **Experimental Information**

Construction and initial expression of the S$_{355}$C$\beta_2$ mutant and extinction coefficient determination by quantitative Bradford assay were performed by Dr. Daniel A. Lutterman. X-ray crystal structure determination was performed by Dr. Thomas S. Teets.
4.5.1. Materials

Tetrahydrofuran (THF) (99.9%, Sigma-Aldrich), dichloromethane (DCM) (99.5%, Sigma-Aldrich), acetonitrile (MeCN) (99.9%, Sigma-Aldrich), toluene (99.5%, Sigma-Aldrich), diethyl ether (Et₂O) (99.0%, Sigma-Aldrich), methanol (MeOH) (99.9%, Sigma-Aldrich), phosphorous tribromide (PBr₃) (1.0 M in DCM, Aldrich), sodium sulfate (Na₂SO₄) (anhydrous, 99%, Sigma-Aldrich), pentacarbonylchlororhenium(I) (Re(CO)₅Cl) (98%, Strem), 1,10-phenanthroline (phen) (99%, Aldrich), thallium(I) hexafluorophosphate(V) (TlPF₆) (97%, Strem), 4-pyridylcarbinol (PyCH₂OH) (99%, Aldrich), BL21-CodonPlus(DE3)-RIL competent cells (Stratagene), kanamycin (kan) (Sigma), Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega), phenylmethanesulfonyl fluoride (PMSF) (Sigma), 1,4-dithiothreitol (DTT) (Promega), 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma), DNase I (Roche), Ni²⁺-NTA resin (Qiagen), and dimethyl formamide (DMF) (Sigma-Aldrich) were used as received. \((\text{1,10-phenanthroline})(\text{acetonitrile})\text{rhenium(I) hexafluorophosphate} ([\text{Re}(\text{phen})(\text{CO})₃(\text{NCMe})]\text{PF}_₆)\) and \((\text{1,10-phenanthroline})(\text{4-picoline})\text{rhenium(I) hexafluorophosphate} ([\text{Re}(\text{phen})(\text{CO})₃(\text{PyCH₃})]\text{PF}_₆)\) were prepared as previously reported.

4.5.2. Physical Methods

\(^1\text{H}\) NMR spectra were collected at the MIT Department of Chemistry Instrumentation Facility (DCIF) on a Varian Inova-500 NMR spectrometer and internally referenced to tetramethylsilane (TMS) using the proteo impurity for the deuterated solvent (d₆-acetone). UV-vis absorption spectra were obtained using a Varian Cary 5000 UV-vis-NIR spectrometer; data were collected on dilute solutions (10 – 50 µM) in quartz spectroscopy cells. Steady-state emission spectra were collected using a PTI QM 4 Fluorometer, which was equipped with a 150 W Xe-arc lamp for excitation and a photomultiplier tube (Hamamatsu R928) cooled to –78 °C for detection; samples were as described above for UV-vis absorption experiments. IR spectra of powder samples were recorded on a PerkinElmer Spectrum 400 FT-IR/FIR Spectrometer equipped with a Pike Technologies GladiATR attenuated total reflectance (ATR) accessory with a monolithic diamond crystal stage and a pressure clamp. Elemental analysis was performed by Midwest Microlab, LLC (Indianapolis, IN). ESI-MS measurements were performed by the Proteomics Core Facility at MIT. DNA sequencing was performed by the MIT Biopolymers Laboratory.
4.5.3. Synthesis

4.5.3.1. Tricarbonyl(1,10-phenanthroline)(4-hydroxymethylpyridyl)rhenium(I) Hexafluorophosphate, [Re(phen)(CO)₃(PyCH₂OH)]PF₆

[Re(phen)(CO)₃(NCMe)]PF₆ (640 mg, 1.01 mmol, 1.0 eq) and PyCH₂OH (219 mg, 2.01 mmol, 2.0 eq) were dissolved in 150 mL THF in a round bottom flask equipped with a reflux condenser, yielding a clear yellow solution. The reaction mixture was heated and maintained at reflux for 18 hours. After allowing the solution to cool, the solvent was removed by rotary evaporation and the resulting oil redissolved in a minimal amount of DCM. Slow addition of 200 mL diethyl ether resulted in the formation of a bright yellow precipitate, which was isolated by vacuum filtration on a fritted funnel and left to dry overnight under vacuum (672 mg, 94%). ¹H NMR (500 MHz, (CD₃)₂CO, 20 °C): δ = 9.92 (dd, 2H, phen-H), 9.10 (dd, 2H, phen-H), 8.52 (m, 2H, Py-H), 8.37 (s, 2H, phen-H), 8.35 (dd, 2H, phen-H), 7.31 (m, 2H, Py-H), 4.60-4.62 (m, 1H, OH), 4.56 (d, 2H, CH₂).

4.5.3.2. Tricarbonyl(1,10-phenanthroline)(4-bromomethylpyridyl)rhenium(I) Hexafluorophosphate, [Re(phen)(CO)₃(PyCH₂Br)]PF₆ ([Re]-Br)

[Re(phen)(CO)₃(PyCH₂OH)]PF₆ (706 mg, 1.00 mmol, 1.0 eq) was dissolved in 100 mL DCM. The solution was cooled in an ice bath to 0 °C and a solution of PBr₃ in DCM (1.0 M, 10 mL, 10 mmol, 10 eq) was slowly added. The mixture was allowed to return to room temperature and stirred overnight. The solution was cooled on an ice bath prior to slowly adding 10 mL MeOH to quench excess PBr₃. After returning to room temperature, solvent was removed under a stream of N₂. The resulting solids were resuspended in 100 mL DCM, washed with twice with 50 mL H₂O, dried over Na₂SO₄, which was removed by filtration. The remaining solvent was removed by rotary evaporation, and the resulting solids were redissolved in 200 mL THF. To this mixture, a solution of TlPF₆ (3.51 g, 10 mmol, 10 eq) in 50 mL THF was added. Precipitation began immediately; the reaction was stirred overnight at room temperature. Solvent was removed from the crude reaction mixture by rotary evaporation and the resulting solids were resuspended in a minimal volume of DCM, yielding a deep yellow solution and dense white precipitate. The solid material was removed by filtration and rinsed with additional DCM. The filtrates were combined and the solvent removed. The resulting oil was redissolved in a minimal volume of DCM to which Et₂O...
was slowly added in excess to yield a colorless solution and yellow precipitate. The product was isolated by vacuum filtration and dried under vacuum overnight to yield $[\text{Re(phen)(CO)}_3(\text{PyCH}_2\text{Br})]\text{PF}_6 ([\text{Re}]^\text{-Br})$ as a bright yellow powder (372 mg, 49%). $^1$H NMR (500 MHz, $(\text{CD}_3)_2\text{CO}, 20 \degree\text{C}$): $\delta = 9.94$ (dd, 2H, phen-H), 9.13 (dd, 2H, phen-H), 8.66 (m, 2H, Py-H), 8.39 (s, 2H, phen-H), 8.36 (dd, 2H, phen-H), 7.45 (m, 2H, Py-H), 4.51 (s, 2H, CH$_2$); IR (solid, cm$^{-1}$): 2024, 1925, 1895 (C=O stretch); Anal. calcd. for C$_{21}$H$_{14}$N$_3$O$_3$PF$_6$BrRe: C, 32.87; H, 1.84; N, 5.48; P, 4.04; Br, 10.41; found: C, 33.30; H, 2.08; N, 5.38; P, 4.23; Br, 10.27.

4.5.4. X-ray Crystal structure of $[\text{Re(phen)(CO)}_3(\text{PyCH}_2\text{Br})]\text{PF}_6$

Single crystals suitable for X-ray diffraction were obtained from vapor diffusion of Et$_2$O into a concentrated solution of $[\text{Re(phen)(CO)}_3(\text{PyCH}_2\text{Br})]\text{PF}_6$ in MeCN overnight. The crystals were mounted on a Bruker three circle goniometer platform equipped with an APEX detector. A graphite monochromator was employed for wavelength selection of the Mo Ka radiation ($\lambda = 0.71073$ Å). The data were processed and refined using the program SAINT supplied by Siemens Industrial Automation. Structures were solved by Patterson methods in SHELXS and refined by standard difference Fourier techniques in the SHELXTL program suite (6.10 v., Sheldrick G. M., and Siemens Industrial Automation, 2000). Hydrogen atoms bonded to carbon were placed in calculated positions using the standard riding model and refined isotropically.
Table 4.2. Selected Crystal Data and Structure Refinement Parameters for [Re]–Br.

4.5.5. Construction, expression, purification, and of S_{55}C–β_{2}

Site-directed mutagenesis was performed using a Quickchange kit from Stratagene. pET9d–C_{268}S_{50}S_{50}, encodes (His)_{6}–C_{268}S_{50}S_{50}–β_{2}^{18} and was used as the template to generate S_{55}C mutant using primers (5’–CAATCTGCGCAGCATACAAACTGACITCCACTTCC–3’ (reverse) and 5’–GGAAGTGGAAGTCAGTTGTTATCTGGTCGGGCAGATTG–3’ (forward), Invitrogen) to give S_{55}C–β_{2}. The mutation was confirmed by DNA sequencing at the MIT biopolymers lab. The resulting plasmid encoding S_{55}C–β_{2} was transformed into competent cells (BL21-CodonPlus(DE3)-RIL), which were grown on (LB) plates (50 μg/mL kanamycin) and incubated overnight (37 °C, 12 h). A single
colony was chosen and used to inoculate a small scale culture (5 mL LB, 50 μg/mL kanamycin), which was incubated with shaking (37 °C, 12 h); this subculture was subsequently used to inoculate a larger culture (100 mL LB, 50 μg/mL kanamycin, 1:100 dilution). The 100 mL culture was incubated (37 °C, 12 h) with shaking and used to inoculate cultures (2 × 2 L LB, 50 μg/mL kanamycin, 1:200 dilution), which were incubated (37 °C) with shaking. The growth of this culture was monitored by scattering at 600 nm. At an OD₆₀₀ of ~0.7, an aqueous solution of 1,10-phenanthroline in 0.1 M HCl (to aid in solubility) was added to a final concentration of 0.1 mM. After 15 min, overexpression was induced with IPTG (0.5 mM total concentration). After an additional 4 h, cells were harvested by centrifugation (14,000 g, 20 min) and stored at -80 °C prior to purification (5.9 g, 1.5 g/L). Successful expression was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the cell lysate before and after induction.¹⁹

A portion of the frozen cell pellet (11.7 g) was thawed at 0 °C and resuspended in 60 mL (5 mL/g) lysis buffer (50 mM tris(hydroxymethyl)aminomethane (Tris), 500 mM NaCl, 10 mM imidazole, pH 7.6). Phenylmethanesulfonyl Fluoride (PMSF) was prepared as a 20 mM stock in isopropyl alcohol (PrOH) and added to the cell suspension to a final concentration of 200 μM. The cells were homogenized and subsequently lysed by passing through a French press (14,000 psi) twice. DNAse I (600 U, 10 U/mL lysate) was added to the suspension, which was briefly stirred (10 min, 4 °C). The mixture was centrifuged (25,000 g, 25 min) to remove insoluble material. An additional aliquot of PMSF was added to the supernatant, yielding a final concentration of 400 μM.

The supernatant was slowly loaded onto a nickel affinity column (25 mL Ni²⁺-NTA resin, 1 mL/min). The flow through was reapplied to the column (7 mL/min) to ensure complete binding. The bound protein was washed with 750 mL (30 column volumes) lysis buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 7.6). The protein was then eluted with 50 mM Tris, 500 mM NaCl, 200 mM imidazole, pH 7.6 and the protein fractions combined. The imidazole was removed by Sephadex G-25 chromatography (650 mL) equilibrated with β₂ Buffer (50 mM Tris, 5% Glycerol, pH 7.6) and concentrated to 172 μM, using ε₂₈₀ = 0.131 μM⁻¹ cm⁻¹. The protein was judged to be >95% pure based on SDS-PAGE.¹⁹

The diferric-tyrosyl radical cofactor was reconstituted by established procedures.²⁰ Specifically,
apo-S\textsubscript{355} C-\(\beta\)\textsubscript{2} (6 mL, 172 \(\mu\)M in \(\beta\)\textsubscript{2} Buffer) was degassed at 0 \(^\circ\)C by alternating cycles of vacuum and argon backfill, ending with the sample under vacuum. In an glovebox under inert \(\text{N}_2\) atmosphere, \(\text{Fe}^{II}(\text{NH}_4)\text{SO}_4\) (160 \(\mu\)L, 33.6 mM, 5 equiv) was added to the degassed protein solution while stirring. The resulting solution was maintained at 4 \(^\circ\)C (15 min). After removing the sample from the glove box, \(\text{O}_2\) saturated \(\beta\)\textsubscript{2} buffer ([\(\text{O}_2\]) \(\sim\) 1.9 mM, 2.7 mL, 5 equiv) was added to the sample, which was subsequently loaded on a G-25 Sephadex column (75 mL) to remove excess \(\text{Fe}^{II}(\text{NH}_4)\text{SO}_4\). Fractions containing protein, as identified by Bradford assay, were combined and concentrated to 211 \(\mu\)M holo-S\textsubscript{355} C-\(\beta\)\textsubscript{2}. Radical content was measured using the drop line correction method\textsuperscript{20} and found to be 0.96 / \(\beta\)\textsubscript{2}.

4.5.6. \textit{C}_{355} surface accessibility assay

The solution accessibility of S\textsubscript{355} C-\(\beta\)\textsubscript{2} thiolates was assessed by reaction with \(5,5'-\text{dithiobis-(2-nitrobenzoic acid)}\) (DTNB). Immediately prior to assay, S\textsubscript{355} C-\(\beta\)\textsubscript{2} was incubated with dithiothreitol (DTT) (20 mM, 30 min). Excess DTT was then removed using a Sephadex G-25 column (15 mL) equilibrated in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM EDTA, pH 8.0. The pooled S\textsubscript{355} C-\(\beta\)\textsubscript{2} (10 \(\mu\)M) was titrated with DTNB (50 \(\mu\)M total concentration in the assay buffer) and the reaction was monitored by UV-vis spectroscopy (2 min intervals, 10 min total). The concentration of free 5-thio-2-nitrobenzoic acid (TNB) was calculated (\(e_{410,\text{TNB}} = 13,600 \text{ M}^{-1} \text{ cm}^{-1}\)) and corrected for background DTNB reaction by comparison with a sample prepared under identical conditions in the absence of protein. The assay was repeated in triplicate. The concentration of reactive thiolate groups was found to be 19.8 \(\mu\)M (1.98 per holo-S\textsubscript{355} C-\(\beta\)\textsubscript{2}), as expected.

4.5.7. \textit{Labeling of S}_{355} C-\(\beta\)\textsubscript{2} with [Re(phen)(CO)}\textsubscript{3}(PyCH\textsubscript{2}Br)]\textsuperscript{+}

For labeling with [Re]-Br, holo-S\textsubscript{355} C-\(\beta\)\textsubscript{2} (1 mL, 211 \(\mu\)M) was treated with 10 mM DTT for 30 min to reduce disulfide bonds and excess DTT was removed using Sephadex G-25 column (15 mL). Labeling was performed immediately after the reduction reaction and accomplished by incubating S\textsubscript{355} C-\(\beta\)\textsubscript{2} with 5 equiv of [Re(phen)(CO)}\textsubscript{3}(PyCH\textsubscript{2}Br)]PF\textsubscript{6} from a concentrated stock (50 mM in DMF) and gently shaking for 2 h at room temperature. Rhenium labeled protein ([Re]-S\textsubscript{355} C-\(\beta\)\textsubscript{2}) was isolated from remaining [Re(phen)(CO)}\textsubscript{3}(PyCH\textsubscript{2}Br)]PF\textsubscript{6} and DMF using another Sephadex G-25 column (15 mL).
The resulting product ([Re]-S_{355}C-\beta_2) was subsequently concentrated to 130 \mu M as determined by a Bradford assay and labeling verified by ESI-MS. Wild-type \beta_2 was used as a standard for the Bradford assay. UV-vis absorption and steady-state emission spectra were also recorded for this construct. For experiments using the reduced (met) form of [Re]-S_{355}C-\beta_2, reduction of the radical was performed prior to labeling by incubating with 30 mM hydroxyurea for 30 min, desalting on a Sephadex G-25 column, and labeling performed as described above.

4.5.8. Time-Resolved Methods

Nanosecond time-resolved emission and transient absorption (TA) experiments were performed using a previously reported system with a number of significant modifications. Excitation (pump) light was provided by the third harmonic (\lambda = 355 nm) of a Nd:YAG laser (Quanta-Ray Lab-190-10, Spectra-Physics) with a repetition rate of 10 Hz; white light continuum (probe) was provided by a 75 W Xe-arc lamp (Photon Technologies). In the TA experiments, the probe beam was aligned directly with the sample bore while the pump beam was positioned at 15° with respect to the probe. Both beams were focused on the sample. After passing through the sample, the probe light entered a monochromator (Triax 320, Jobin Yvon Horiba) and was dispersed by a blazed grating (250 nm, 300 grooves/mm). The entrance and exit slits for the monochromator were 0.4 mm in all experiments reported herein, corresponding to a spectral resolution of 5 nm, excepting emission kinetics experiments on small molecules, which utilized 0.2 mm slits, corresponding to a 2.5 nm spectral resolution. Laser excitation energy was adjusted to 2.0 mJ per pulse for all experiments using a combination of neutral density filters and built-in pulse energy control of the Nd:YAG laser.

For single wavelength kinetics (both TA and emission), the signal was amplified by a photomultiplier tube (R928, Hamamatsu) and collected on a 1 GHz digital oscilloscope (9384CM, LeCroy); acquisition was triggered by a photodiode positioned to collect scattered pump light at the sample. Time-resolved emission experiments were performed in the same configuration; however, probe light was not used.

Transient absorption spectra were collected using a gated intensified CCD camera (ICCD) (DH520–25F–01, Andor Technology). Acquisition delays and gate times for the CCD were set by a digital delay generator (DG535, Stanford Research Systems), which is synchronized to the Q-switch.
output of the Nd:YAG laser. The final data were calculated from a combination of four spectra: $I$ (pump on/probe on), $I_p$ (pump on/probe off), $I_o$ (pump off/probe on), and $I_b$ (pump off/probe off). Pump and probe beams were selectively admitted to the sample to produce these four conditions using electronically controlled fast shutters (Uniblitz T132, Vincent Associates), which were triggered by an additional digital delay generator (DG535, Stanford Research Systems), which was also synchronized to the Q-switch output of the Nd:YAG laser. The resulting TA spectrum was obtained from the calculation $\Delta OD = -\log[(I - I_p)/(I_o - I_b)]$ to corrects for sample emission and extraneous background light.

For all protein experiments, 750 μL samples were recirculated to address sample decomposition. For small molecule experiments, samples were flowed irreversibly. Samples were held in a quartz flow-cell (585.3-Q-10/Z1S, Starna) with a 1 cm path length and 3 mm diameter windows; the total sample bore was 70 μL. Fresh samples were used for all experiments as indicated. When observing the unquenched excited state of Re-βα, spectra reported are an average of 1250 four-spectrum sequences, transient absorption kinetics are an average of 5000 sweeps, and emission kinetics are an average of 1000 sweeps. For experiments using the flash-quench technique to observe tyrosyl radical, the reported spectrum is an average of 2500 four-spectrum sequences; the reported single wavelength kinetics are an average of two 5000 sweep series (10000 sweeps total), each of which was taken on fresh sample. The transient absorption spectrum shown has been smoothed using a low pass filter to remove high-frequency noise components due to instrumentation; the cutoff frequency was selected on the basis of a fast Fourier transform. Figure 4.8 shows the comparison between data as collected and after smoothing.
4.8. As obtained transient absorption spectrum of Y-. Raw TA data (○) are shown in addition to the smoothed data (blue solid line), as shown above (Figure 4.7) for reference. Experimental parameters are identical to those given in the main text. Transient absorption spectrum of [Re]–S₃₅₅C–β₂ collected 1 µs after 355 nm excitation (65 µM in 50 mM HEPES, 32.5 mM Ru³⁺(NH₃)₆Cl₄, 1 mM EDTA, pH 8.0). The spectrum shown is obtained from 2500 four-spectrum sequences taken on two independent samples (1250 four-spectrum sequences each), averaged, and smoothed using a low-pass filter on the basis of a fast Fourier transform (FFT).

The reported errors are the standard error of the fit, as obtained from least-squares analysis. Additionally, an inline syringe filter (0.2 µm Acrodisc, Supor membrane, 13 mm, Pall Corporation) was used for all flash-quench experiments to collect precipitate resulting from flash-quench by products. For small molecule experiments, spectra reported are an average of 2500 four-spectrum sequences.

All experiments were conducted in buffer containing 50 mM HEPES and 1 mM EDTA at pH 8.0. Experiments in the absence of oxidative quencher were done using 10 µM [Re]–S₃₅₅C–β₂ (Figure 4.4). Flash-quench experiments were performed in the presence of 500 eq. Ru³⁺(NH₃)₆Cl₄ per [Re]–S₃₅₅C–β₂. The reported spectrum of tyrosyl radical was collected 1 µs after excitation of a sample containing 65 µM met–[Re]–S₃₅₅C–β₂ and 32.5 mM Ru³⁺(NH₃)₆Cl₄ in buffer (Figure 4.7, top); single wavelength kinetics were recorded using two samples of 50 µM met–[Re]–S₃₅₅C–β₂ and 25 mM in buffer (Figure 4.7, bottom).

4.5.9. Estimated Quantum Yield of Y- Formation

The approximate quantum yield with which Y- is generated, Φᵢᵧ, in [Re]–S₃₅₅C–β₂ is given by
Equation (4-1).

$$\Phi_{Y^*} = \frac{\text{molecules of } Y^* \text{ generated}}{\text{number of photons absorbed}}$$

Under our experimental conditions, these parameters may be derived from a variety of known experimental parameters. Our estimate of $\Phi_y$ presumes that the entire region of the probe beam has been irradiated by the excitation laser pulse. The number of molecules of $Y^*$ generated for the observed experimental volume for a single laser flash can be calculated from Avogadro’s number ($N_A$), the sample bore volume ($V_{bore}$), and the observed concentration of $Y^*$ according to Equation 4-2.

$$\text{molecules of } Y^* \text{ generated} = N_A V_{bore} [Y^*]$$

$[Y^*]$ is determined from the intensity of the $Y^*$ signal in the transient absorption spectrum ($\Delta OD$) and molar extinction coefficient for $Y^*$ ($\varepsilon_{Y^*}$).

$$[Y^*] = \frac{\Delta OD}{\varepsilon_{Y^*} l}$$

The number of photons absorbed can be calculated from the sample transmittance and the number of photos per laser pulse, $N_p$.

$$\text{number of photons absorbed} = (1 - T) N_p$$

Combining Equations (4-1), (4-2), and (4-4) gives Equation (4-5).

$$\Phi_{Y^*} = \frac{N_A V_{bore} [Y^*]}{(1 - T) N_p}$$

$N_p$ is calculated from the laser pulse energy ($E_p$), the laser wavelength ($\lambda$), Planck’s constant ($h$), and the speed of light ($c$).

$$N_p = \frac{E_p \lambda}{hc}$$

Similarly, the transmittance can be calculated from the sample absorbance, which can be in turn be calculated using the Beer–Lambert law from the molar extinction coefficient for [Re]$\text{S}_{355}$ C–$\beta_2$ at the
excitation wavelength ($\varepsilon_{555,Re}$), the sample concentration ([$\text{Re}-\beta_2$]), and the sample path length ($l$).

$T = 10^{-A} = 10^{-\varepsilon_{555,Re}[\text{Re}-\beta_2]l}$

Combining Equations (4-5), (4-6), and (4-7) gives Equation (4-8).

\[
\Phi_Y = \frac{N_A V_{\text{Bore}} \left( \frac{\Delta OD}{\varepsilon_{Y*1}} \right)}{\left( \frac{E_p \lambda}{h c} \right) (1 - 10^{-\varepsilon_{555,Re}[\text{Re}]l})}
\]
4.6. References


Chapter 5  Photochemical $Y_{356}$ Oxidation is Modulated by $Y_{731}$ Across the $\alpha_2:\beta_2$ Interface of Class la $E. coli$ Ribonucleotide Reductase
5.1. Introduction

Having developed a photochemical $\beta_2$ subunit (Chapter 4), we sought to study its properties in the presence of $\alpha_2$ and study photoinitiated radical transport in an assembled $\alpha_2\beta_2$ complex. We now report the photochemical generation of $Y_{356}^*$ in an intact $\alpha_2\beta_2$ complex, which is competent for photoinitiated substrate turnover. In order to decipher the nature of tyrosine oxidation processes by $[\text{Re}]$ at the subunit interface, the $Y_{356}F$ variant ([Re]$-Y_{356}F$-$\beta_2$) was prepared. By performing a comparative emission kinetics study of [Re]$-\beta_2$ and [Re]$-Y_{356}F$-$\beta_2$ in the presence of wt-$\alpha_2$ and $Y_{731}F$-$\alpha_2$ constructs, a photooxidation process to furnish radicals is ascribed to interfacial tyrosine radical transport.

![Figure 5.1](image.png)

Figure 5.1. An intact, photochemical RNR where a radical is generated directly at $Y_{356}(\beta)$ and is poised to follow the proposed radical transport pathway through $\alpha_2$ to the active site. Figure prepared from PDB codes 4R1R (\(\alpha_2\)) and 1MXR (\(\beta_2\)).

5.2. Results

5.2.1. Characterization of [Re]$-\beta_2$

RNR activity assays were performed on the unlabeled $S_{355}C$-$\beta_2$ and labeled [Re]$-\beta_2$ to ascertain the effect of labeling on activity. The 1,100 U/mg (Figure 5.2) activity of unlabeled $S_{355}C$-$\beta_2$ is less than $\beta_2$ (7,600 U/mg), indicating that the subunit interface is perturbed by the modification.
Figure 5.2. The activity of wild-type β₂ (open circles) and S₃₅ C-β₂ (closed circles). The activity of S₃₅ C-β₂ is nonlinear and diminished with respect to wt-β₂. Each reaction contained, in a volume of 300 μL: 5 μM of the β₂ variant, 1 μM α₂, 30 μM TR, 0.5 μM TRR, 1 mM [³H]-CDP (10750 cpm/nmol), 3 mM ATP, and 1 mM NADPH in assay buffer (pH 7.6).

Further perturbation of β₂ occurs upon [Re] labeling as [Re]-β₂, in the dark, shows an additional decrease in activity to 142 U/mg. The activity of [Re]-β₂ is not due to trace amounts of unlabeled S₃₅ C-β₂, which is present at 2% by a DTNB assay. Accounting for an activity of 1100 U/mg in 2% of the sample (22 U/mg), the remaining fraction (authentic [Re]-β₂) has an activity of 122 U/mg. To avoid complications from steady-state turnover, [Re]-β₂ was treated with hydroxyurea (HU) to reduce Y₁₁₂⁺ of the native radical cofactor, yielding met-[Re]-β₂. The met form was used for all experiments except the above activity assays and is subsequently referred to as [Re]-β₂ for convenience.

The [Re] group does not preclude binding of [Re]-β₂ to α₂. A competitive inhibition assay⁴ (Figure 5.3) reveals that the observed dissociation constant (Kₐ₂([Re]-β₂) = 0.71(8) μM) is comparable to that observed for aminotyrosine (NH₂Y) substituted β₂ at position 356.⁵

5.2.2. Photochemical Substrate Turnover

The efficacy of photooxidant labeling is revealed by activity measurements of [Re]-β₂ under illumination (λ > 325 nm) and in the presence pre-reduced wt-α₂. The two cysteines in the active-site of wt-α₂ are reduced, and there is no additional reductant available. Figure 5.4 shows that dCDP product is formed at 0.11(2) dCDP/α₂ under photochemical conditions.
Figure 5.3. $K_D$ for [Re]–$\beta_2$:wt-$\alpha_2$ was determined by adding varying amounts of [Re]–$\beta_2$ to a solution of wt–RNR; the specific activity was measured for each reaction. Each reaction contained, in a volume of 300 μL: 0.2 μM wt–$\beta_\gamma$, 0.1 μM wt–$\alpha_\gamma$, [Re]–$\beta_2$ (0–5 μM), 30 μM TR, 0.5 μM TRR, 1 mM CDP, 3 mM ATP, 0.2 mM NADPH in assay buffer (pH 7.6). Data were analyzed as previously reported, $K_D = 0.71(8)$ μM.

Figure 5.4. Photochemical substrate turnover in the $\alpha_2$-[Re]–$\beta_2$ complex formed from 50 μM met-[Re]–$\beta_2$ or met-[Re]–$\text{Y}_{356}\text{F}$–$\beta_2$ and 20 μM pre-reduced wt–$\alpha_2$ or $\text{Y}_{356}\text{F}$–$\alpha_2$ (as indicated) in 50 mM borate, 15 mM MgSO$_4$, 0.2 mM [H]-CDP (10750 cpm/nmol), 3 mM ATP, 5% glycerol, pH 8.3, λ > 325 nm.
Previous studies have shown the possibility of slow cleavage of substrate to release base when active-site mutations or mechanism-based inhibitors are used.\textsuperscript{7-10} Under the protocol used to quantitate turnover (the radiolabeled base) cytosine in the case of dCDP substrate, is not separated from deoxycytidine (dC, the expected product of the reaction subsequent to dephosphorylation).\textsuperscript{11} Therefore, the identity of the product was confirmed by an HPLC assay (Figure 5.5) as previously reported.\textsuperscript{5}

![HPLC assay result](image)

**Figure 5.5.** Product formed in a photochemical single-turnover experiment using [Re]-3 and wt-\(\alpha_2\) was analyzed by HPLC. The product mixture was supplemented with equimolar dC and cytosine as carriers. Fractions were pooled according to peaks as shown, corresponding to cytosine (red) and dC (blue), and radioactivity measured for each. The relative amount of radioactivity, indicated as a percentage adjacent to each peak, confirms that dCDP is the product of phototurnover. Radioactivity observed in the cytosine fraction corresponds to a radioactive impurity present in all samples, as previously described.\textsuperscript{5}

The phototurnover yield of the \(\alpha_2:[\text{Re}]-\beta_2\) construct is significantly greater at pH 7.6 (0.44(4) dCDP/\(\alpha_2\)) (Figure 5.6).

5.2.3. *Photochemistry of [Re]*\textsuperscript{*} in [Re]-\(\beta_2\)

Having established that [Re]-\(\beta_2\) is photochemically active for substrate turnover, we sought to generate a radical within the \(\alpha_2:[\text{Re}]-\beta_2\) complex. Photoexcitation (\(\lambda = 355\) nm) of [Re]-\(\beta_2\) or [Re]-Y\textsubscript{556}F-\(\beta_2\) yields transient absorption features (Figure 5.7) consistent with the \(3\)MLCT excited state ([Re\textsuperscript{+*}], as previously reported,\textsuperscript{12} but no signatures corresponding to tyrosyl radical are observed.
Figure 5.6. Comparison of photochemical single-turnover experiments at pH 7.6 and 8.3. At pH 7.6 each experiment contained, in a volume of 300 µL: 30 µM [Re]-β, 10 µM wt-α, 0.2 mM [3H]-CDP (22713 cpm/nmol), and 3 mM ATP in assay buffer. At pH 8.3 each experiment contained, in a volume of 330 µL: 50 µM [Re]-β, 20 µM wt-α, 0.2 mM [3H]-CDP (10750 cpm/nmol), and 3 mM ATP in spectroscopy buffer. Error bars indicate 1σ deviations for independently prepared triplicate measurements.

Figure 5.7. Transient absorption spectra of [Re]-β and [Re]-Y_{35}F-β, measured immediately after excitation on samples of 10 µM [Re]-β (blue circles) or [Re]-Y_{35}F-β (red circles), 1 mM CDP, and 3 mM ATP in spectroscopy buffer (pH 8.3), λ_{exc} = 355 nm.

Scheme 5.1 shows a photophysical model for the system.
In the absence of tyrosine (left), the observed lifetime, $\tau_0$, is defined by nonradiative ($k_{NR}$) and radiative ($k_R$) decay rate constants as follows,

$$k_0 = \frac{1}{\tau_0} = k_R + k_{NR}$$ (5.1)

For the kinetics associated with the $\beta_2$ subunit, $\tau_0$ is given by $[\text{Re}] - Y_{356} - F - \beta_2$. For $[\text{Re}] - \beta_2$:wt-$\alpha_2$, $\tau_0$ is given by $[\text{Re}] - Y_{356} - F - \beta_2$:wt-$\alpha_2$, while for $[\text{Re}] - \beta_2$:Y$_{731}$-F-$\alpha_2$, $\tau_0$ is given by $[\text{Re}] - Y_{356} - F - \beta_2$:Y$_{731}$-F-$\alpha_2$. For each case, measurement of $\tau_0$ differs from measurement of the observed lifetime only by $Y_{356}$, in order to isolate effects only from this residue. The presence of $Y_{356}$ in $\beta_2$ adds an additional decay pathway via charge separation ($k_{CS}$), which may be determined from the observed lifetime ($\tau_{obs}$),

$$k_{CS} = k_{obs} - k_0 = \frac{1}{\tau_{obs}} - \frac{1}{\tau_0}$$ (5.2)

where $\tau_{obs}$ is inversely related to the observed rate constant ($k_{obs}$) (i.e., $k_{obs} = 1/\tau_{obs}$).

The lifetime of $[\text{Re}]^*$ in $[\text{Re}] - \beta_2$ ($\tau_{obs} = 344(25)$ ns, pH 8.3, Table 5.1) is significantly shorter than that for the construct with $Y_{356}$ replaced by F ($\tau_{obs} = 525(22)$ ns, for $[\text{Re}] - Y_{356} - F - \beta_2$, pH 8.3, Table 5.1). We ascribe the shortened lifetime of $[\text{Re}]^*$ in $[\text{Re}] - \beta_2$ to quenching of $[\text{Re}]^*$ by $Y_{356}$ (Scheme 5.1). These time-resolved emission studies of the $[\text{Re}] - \beta_2$ constructs suggest that the inability to detect tyrosine radical signal is due to rapid charge recombination of the $[\text{Re}]^0$-$Y_*$ charge separated state ($k_{CR} > k_{CS}$).

Using the lifetime of $[\text{Re}]^*$ in $[\text{Re}] - Y_{356} - F - \beta_2$ to provide $\tau_0$, the rate constant for charge separation, $k_{CS}$, is calculated from Equation (5.2) to be $k_{CS} = 10.0(8) \times 10^4 \text{s}^{-1}$. 

**Scheme 5.1.** Excited-State Deactivation Pathways for $[\text{Re}]^*$
Tyrosine-dependent Excited-state Quenching Pathway Residues

<table>
<thead>
<tr>
<th>Pathway Residues</th>
<th>$\tau_{500\text{nm}}$ / ns</th>
<th>$k_{cs} \times 10^{6}$ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 8.3</td>
<td>pH 7.6</td>
</tr>
<tr>
<td>Y</td>
<td>344(25)</td>
<td>403(31)</td>
</tr>
<tr>
<td>F</td>
<td>525(22)</td>
<td>607(7)</td>
</tr>
<tr>
<td>Y</td>
<td>433(5)</td>
<td>543(13)</td>
</tr>
<tr>
<td>F</td>
<td>574(4)</td>
<td>696(5)</td>
</tr>
<tr>
<td>Y</td>
<td>455(4)</td>
<td>590(17)</td>
</tr>
<tr>
<td>F</td>
<td>588(3)</td>
<td>732(11)</td>
</tr>
</tbody>
</table>

Enhancement in $Y_{431}$ Oxidation from $Y_{356} / 14(2)$% 23(6)%

*a* Emission lifetimes measured on samples of 10 μM met-[Re]-β, or met-[Re]-Y_{356}F-β, and 25 μM wt-α, or Y_{356}F-α, (as indicated), 1 mM CDP, and 3 mM ATP in buffer (as indicated), $\lambda_{em} = 355$ nm, $\lambda_{det} = 600$ nm.

*b* Errors shown in parentheses represent $2\sigma$ from triplicate measurements on independently prepared samples.

$\times 50$ mM borate, 15 mM MgSO₄, 5% glycerol, pH 8.3 (see Figure 5.8)

$\times 50$ mM HEPES, 15 mM MgSO₄, pH 7.6 (see Figure 5.9)

$\times k_{cs}$ is calculated according to Equation (5.2)

$\times$ Enhancement calculated from the ratio $k_{cs}(Y_{356})/k_{cs}(F_{356})$. The error reported is the error in the ratio, i.e., $k_{cs}(Y_{356})/k_{cs}(F_{356}) = 1.14(2)$, and is propagated from $2\sigma$ error in the lifetime measurements.

Table 5.1. Tyrosine Dependent Excited-State Quenching at the α₂:β₂ Interface

In the presence of wt-α, the lifetime of [Re⁺]* is also quenched within the intact α₂:[Re]-β₂ complex. Based on the measured $K_{Pr}$ under these experimental conditions, 96% of [Re]-β₂ in solution is bound to α₂. In this assembled α₂:[Re]-β₂ complex, the lifetime of the [Re⁺]* excited state increases for both [Re]-β₂ and [Re]-Y_{356}F-β₂ (Table 5.1) because the [Re] center resides in the hydrophobic environment of the protein. Notwithstanding, the lifetime of α₂:[Re]-β₂ is shorter than that of the [Re]-Y_{356}F-β₂ complex and a charge separation rate constant—again assigned to Y_{356} oxidation—is calculated to be $k_{cs} = 4.05(10) \times 10^{6}$ s⁻¹.

The charge separation reaction is affected by perturbations of the radical transport pathway in α₂. When Y_{731} (α) is replaced by phenylalanine, the rate constant for Y_{356} oxidation decreases to 3.28(11) $\times 10^{6}$ s⁻¹. This difference corresponds to a 23(6)% enhancement in Y_{356} (β) oxidation when Y_{731} (α) is present. At pH 8.3, a similar phenomenon is observed (Table 5.1, Figure 5.8), with faster charge separation but a smaller rate enhancement due to Y_{731} (α) (14(2)%).
Figure 5.8. Representative data of transient emission kinetics of [Re]-β<sub>c</sub> variants used for Table 5.1. Emission kinetics were measured on samples of 10 μM [Re]-β<sub>c</sub> or [Re]-γ<sub>356</sub>F-β<sub>c</sub> and 25 μM wt-α<sub>c</sub> or Y<sub>356</sub>F-α<sub>c</sub> (as indicated), 1 mM CDP, and 3 mM ATP in assay buffer (pH 7.6), λ<sub>exc</sub> = 355 nm, λ<sub>obs</sub> = 600 nm. Data and residuals are given as gray circles, solid lines represent monoexponential decay functions used to calculate lifetimes as shown above. Data reported in Table 5.1 are derived from triplicate data sets for each set of conditions.
Figure 5.9. Representative data of transient emission kinetics of [Re]-\(\beta_2\) variants used for Table 5.1. Emission kinetics were measured on samples of 10 \(\mu\)M [Re]-\(\beta_2\) or [Re]-\(\beta_2\), and 25 \(\mu\)M wt-\(\alpha_2\) or Y\(_{356}\)F-\(\alpha_2\) (as indicated), 1 mM CDP, and 3 mM ATP in spectroscopy buffer (pH 8.3), \(\lambda_{\text{exc}} = 355\) nm, \(\lambda_{\text{obs}} = 600\) nm. Data and residuals are given as gray circles, solid lines represent monoexponential decay functions used to calculate lifetimes as shown above. Data reported in Table 5.1 are derived from triplicate data sets for each set of conditions.
5.2.4. Flash-Quench Yields Long-Lived Radicals

With the knowledge that [Re]-β₂ is capable of photochemical Y₃⁶₆ oxidation to generate an on-pathway radical, we sought to circumvent the limitation imposed by \( k_{CR} > k_{CS} \) by employing the flash-quench methodology.¹⁴ When [Re]-β₂ is in the presence of Ru³⁺(NH₃)₆Cl₃, [Re]⁺ is rapidly quenched to reveal signals for a tyrosyl radical. As previously observed for the rhenium-bipyridine photopeptide system, increased concentrations of quencher result in larger observed signals but also in diminished Y• lifetimes;¹⁵ photostability measurements for [Re]-β₂ as a function of the Ru³⁺(NH₃)₆Cl₃ concentration reveal the maximum signal and longest Y• lifetime occur over the concentration range of 2–5 mM (Figure 5.10). In the presence of the a₂ subunit, less efficient quenching by Ru³⁺ is observed, requiring a concentration of 10 mM to obtain adequate radical signals.

![Figure 5.10](image)

**Figure 5.10.** Optimization of flash-quench conditions for [Re]-β₂. (a) transient absorption amplitudes and (b) lifetimes as a function of cumulative laser shots for varying concentrations of Ru³⁺(NH₃)₆Cl₃: 5 mM (brown square), 2 mM (blue circle), 1 mM (green triangle), and 0.5 mM (orange diamond). Transient absorption kinetics (\( \lambda_{abs} = 410 \text{ nm} \)) were collected sequentially on samples of 50 μM [Re]-β₂ and Ru³⁺(NH₃)₆Cl₃ (as indicated) in spectroscopy buffer.
The addition of 10 mM Ru\(^{III}\)(NH\(_3\)_\(_6\))Cl\(_3\) quenches [Re\(^+\)]\(^+\); in the absence of \(\alpha_2\), the [Re]–\(\beta_2\) lifetime is 65 ns. Addition of wt-\(\alpha_2\) to the solution results in a longer [Re\(^+\)]\(^+\) lifetime (137 ns) owing to the decreased solution exposure of the [Re] complex and an attendant decrease in bimolecular quenching by Ru\(^{III}\). To ensure that the concentrations of Ru\(^{III}\)(NH\(_3\)_\(_6\))Cl\(_3\) did not interfere with subunit binding, we measured \(K_D\) for [Re]–\(\beta_2\) in the presence of Ru\(^{III}\)(NH\(_3\)_\(_6\))Cl\(_3\). Although the typical coupled assay used to measure activity of RNR subunits cannot be performed when Ru\(^{III}\)(NH\(_3\)_\(_6\))Cl\(_3\) is present (Figure 5.11), the difference in [Re\(^+\)]\(^+\) lifetimes in the presence and absence of \(\alpha_2\) provides an alternative method to characterize subunit binding (Figure 5.12). Lifetime analysis of the binding shows that the presence of Ru\(^{III}\)(NH\(_3\)_\(_6\))Cl\(_3\) does not significantly perturb binding of [Re]–\(\beta_2\) (\(K_D([\text{Re}]-\beta_2) = 0.68(21) \mu\text{M}\).

Figure 5.11. Inhibition of coupled RNR activity assay by Ru\(^{III}\)(NH\(_3\)_\(_6\))Cl\(_3\). Incubation of NADPH (1 mM) in the presence of Ru(NH\(_3\)_\(_6\))Cl\(_3\) (5 mM), TR (30 \muM), and TRR (0.5 \muM) (as indicated) in assay buffer results in consumption of NADPH as shown, indicating that TRR mediates the oxidation of NADPH by Ru\(^{III}\)(NH\(_3\)_\(_6\))Cl\(_3\).
Figure 5.12. Measurement of $K_d$ for wt-α₂: [Re]-β₂ under flash-quench conditions. Varying amounts of wt-α₂ were added to solutions of [Re]-β₂ and transient emission kinetics were measured for each solution. Individual traces were fit to monoexponential decay functions and the resulting decay rates ($k_{obs}$) analyzed according to Equation 5.8, from which the $K_d$ was determined to be 0.68(21) μM. Each reaction contained, in a volume of 750 μL: 1 μM [Re]-β₂, wt-α₂ (as indicated), 10 mM Ru$^{III}$ (NH$_3$)$_6$Cl$_2$, 1 mM CDP, and 3 mM ATP in spectroscopy buffer (pH 8.3).

One potential issue when performing experiments at high concentrations of protein (up to 50 μM [Re]-β₂ and 75 μM α₂), is the formation of assemblies larger than α₂:β₂, as previously observed for the inhibited form of E. coli Class Ia RNR (formed in the presence of dATP). Under different conditions, such oligomers may not retain an intact subunit interface, precluding the generation of on-pathway radical or its subsequent transport. With 10 μM of each subunit, even in the absence of dATP, species larger than α₂:β₂ are observed by analytical ultracentrifugation. Nevertheless, PELDOR experiments performed at high concentration (100 μM each of α₂:β₂), including those previously discussed, show on-pathway radicals are generated under these conditions, confirming that radical transport is intact at high concentrations.

In the presence of both wt-α₂ and Ru$^{III}$ (NH$_3$)$_6$Cl$_2$, photoexcitation of [Re]-β₂ results in a typical Y• absorption feature at 410 nm (Figure 5.13). Under these experimental conditions, 97% of [Re]-β₂ in solution is bound to α₂. This observation confirms that [Re] is capable of generating Y• in an assembled α₂:β₂ complex.
Figure 5.13. Transient absorption spectra (left) of photogenerated $Y_{356}$ $\cdot$ 5 µs after excitation and transient absorption kinetics (right) ($\lambda_{ex} = 410$ nm) of 50 µM [Re]$_3$-β$_3$, or [Re]$_3$-Y$_{356}$-β$_3$ (as indicated), 75 µM wt-α, or Y$_{731}$-F-α, 10 mM Ru$_{10}$(NH$_3$)$_6$Cl$_3$, 1 mM CDP, and 3 mM ATP in spectroscopy buffer (pH 8.3), $\lambda_{ex} = 355$ nm. Open circles show raw spectra and kinetic traces as collected; solid lines show monoexponential fits for kinetics traces and smoothed spectra.
As in the unquenched experiments above, [Re]-Y_{356} F-β_{2} and Y_{731} F-α_{2} were employed in combination with [Re]-β_{2} and wt-α_{2} to determine the identity of the photogenerated radical. The presence of visible signals for Y• in the absence of Y_{356} (Figure 5.13) indicates that the observed signal is not entirely due to Y_{356}•. In fact, signals due to •Y radical persist even in the case where neither interface tyrosine is present, suggesting that oxidation of an off-pathway tyrosine is also occurring.

Despite these side-reactions under flash-quench conditions, the relative amplitudes of Y• indicate that a significant fraction (45(7)% based on single-wavelength kinetics, Figure 5.15) of the observed signal is due to Y_{356}•. In all cases where Y_{356}• is present, the observed amplitude of Y• is greater than the amplitudes observed in any case where Y_{356} is absent (Figure 5.14) though we note that the presence of the off-pathway radical precludes direct study of radical transport kinetics within the triad. Future experiments will target the installation of a fluorotyrosine at Y_{356}(β) in an effort to outcompete off-pathway oxidations by [Re], improve the yield of photogenerated radical and to provide a spectroscopically distinguishable radical signal.

**Figure 5.14.** Summary data for photogenerated Y_{356}•. Smoothed spectra (left) and summary data for transient absorption kinetics (right) taken from raw data shown in Figure 5.13. Colors indicate combinations of [Re]-β_{2} and α_{2} used for each experiment.

Upon photoexcitation of [Re]-β_{2} under flash-quench conditions in the absence of α_{2}, signals for transient tyrosine radicals (λ_{max} = 410 nm) are still observed (Figure 5.15). Notably, a lower intensity signal for Y• is still observed in the absence of Y_{356} suggesting off-pathway tyrosine oxidation.
Figure 5.15. Transient absorption spectra of photogenerated $Y_{355}$ 5 μs after excitation of 50 μM [Re]−$\beta_2$ or [Re]−$F_{355}$−$\beta_2$ (as indicated), 10 mM Ru(II)(NH$_3$)$_6$Cl$_3$, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), $\lambda _{ex} = 355$ nm. Open circles show raw spectra and kinetic traces as collected; solid lines show smoothed spectra.

5.2.5. Non-Specific Tryptophan Oxidation by [Re$^{II}$]

Under flash-quench conditions, transient absorption spectra reveal an absorption feature centered near 525 nm (Figure 5.16). This absorbance is assigned to tryptophan-based radical; however, the wavelength correlates directly to neither the deprotonated tryptophan radical ($W^-$) nor the protonated tryptophan radical cation ($WH^+$), which appear at 500 nm and 560 nm, respectively.$^{18}$

The kinetics of oxidized tryptophan formation and decay are shown below (Figure 5.17). The full kinetics (Figure 5.17, left) are fit to a triexponential decay, corresponding to residual emission at 525 nm, growth of the oxidized W signal, and decay of oxidized W. Measurement on early timescales (Figure 5.17, right) does not enable resolution of emission decay and radical growth kinetics, as evidenced by the unexpected large amplitude for tryptophan radical growth and the inaccurate lifetime for emission, which should be ~137 ns, in accordance with Figure 5.12.
Figure 5.16. Transient absorption spectrum of photogenerated Y- and W- 5 µs after excitation of 50 µM [Re]-β, 75 µM wt-α, 10 mM RuIII(NH)6Cl3, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λex = 355 nm. Open circles show raw spectra and kinetic traces as collected; solid lines show monoexponential fits for kinetics traces and smoothed spectra.

Figure 5.17. Kinetics of transient tryptophan radical signal formation and decay (λobs = 525 nm) after excitation of 50 µM [Re]-β, 75 µM wt-α, 10 mM RuIII(NH)6Cl3, 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), λex = 355 nm. Long (left) and short (right) timescales are shown. The full data are shown in the top panels, the lower panels show the region of tryptophan radical decay (left) and growth (right) in greater detail.

Notably, the radical is formed independently of tyrosine at positions Y356(β) or Y731(α) (Figure 5.18). In fact, slightly greater amplitudes for the W-centered species are observed when [Re]−Y356F−β2 is used than when Y356 is present.
**Figure 5.18.** Summary of TA spectra for photogenerated \( W \). Smoothed spectra of photogenerated \( Y \) and \( W \) 5 µs after excitation of 50 µM [Re]-β, or [Re]-Y\(_{556}\)F-β, 75 µM wt-α, or Y\(_{731}\)F-α (as indicated), 10 mM Ru\(_{III}(NH\_3)_6\)Cl\(_3\), 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), \( \lambda_{ex} = 355 \) nm. Smoothed spectra from data as shown in Figure 5.16. Colors indicate combinations of [Re]-β and α used for each experiment.

Additionally, \( W \) is still observed when α is absent (Figure 5.19). As in the presence of α, the signal for \( W \) has greater amplitude when Y\(_{556}\) is not present. The oscillations observed in the \( W \) region of the transient absorption spectra are an artifact of smoothing.

**Figure 5.19.** Transient absorption spectra for photogenerated \( W \) in the absence of α. As-collected (grey circles) and smoothed spectra (solid lines) of photogenerated \( W \) 5 µs after excitation of 50 µM [Re]-β, or [Re]-Y\(_{556}\)F-β, 10 mM Ru\(_{III}(NH\_3)_6\)Cl\(_3\), 1 mM CDP, and 3 mM ATP, in spectroscopy buffer (pH 8.3), \( \lambda_{ex} = 355 \) nm.
5.3. Discussion

5.3.1. [Re]–β₂ is Competent for Photoinitiated Substrate Turnover

[Re] modification of β₂ hinders catalytic substrate turnover. Photolysis of [Re]–β₂ under single-turnover conditions results in the product formation (Figures 5.4, 5.6), confirmed to be dCDP by HPLC (Figures 5.5). Product formation is only observed in the case where [Re]–β₂ and wt–α₂ were used. If either Y₃₅₆(β) or Y₇₃₁(α) are absent, no turnover is observed. Consistent with previous peptide-based systems, significantly more turnover is observed at pH 7.6 (0.44(4) dCDP/α₂) than at pH 8.3 (0.11(2) dCDP/α₂).

This product yield is less than that observed previously for photopeptide based photoRNR systems (~0.3 dCDP/α). However, we note that in the peptide surrogate, position 356 on the peptide was occupied by 3,5-difluorotyrosine (3,5-F₂Y), as opposed to tyrosine. Greater radical production yield is expected for 3,5-F₂Y because a significant proportion of the amino acid is deprotonated (pKa = 7.2) and tyrosine is not, and hence 3,5-F₂Y is more rapidly oxidized than tyrosine, despite having similar reduction potentials at this pH.

5.3.2. [Re]⁺ Oxidizes Y₃₅₆ Despite the Lack of Transient Absorption Features

A transient absorption spectrum of [Re]–β₂ shows no characteristic feature due to Y⁺ (λₘₐₓ = 410 nm), Figure 5.7. Furthermore, [Re]–Y₃₅₆F–β₂ shows identical excited-state features. Nevertheless, the difference in excited-state lifetimes between these two constructs can be attributed to Y₃₅₆ oxidation, and a rate constant for oxidation calculated using Equation (5.2). The fastest measured rate for Y₃₅₆ oxidation here is 1.0 × 10⁶ s⁻¹. Previously reported charge recombination rate constants for rhenium polypyridine tyrosine systems exceed 10⁷ s⁻¹. Accordingly, k₉CR appears to be much greater than k₉CS, thus consistent with our inability to observe Y⁺ in the transient absorption spectrum.

Y₃₅₆ photooxidation is observed within complexes of [Re]–β₂ with either wt–α₂ or Y₇₃₁F–α₂. The observed rates of charge separation are indeed faster at pH 8.3 than at pH 7.6, consistent with a PCET mechanism for Y₃₅₆ oxidation. Notably, for both pH 8.3 and pH 7.6, Y₃₅₆ oxidation is significantly faster for wt–α₂ than for Y₇₃₁F–α₂, Table 5.1.
5.3.3. *Y*<sub>731</sub> in *α*<sub>2</sub> Modulates *Y*<sub>356</sub> Photooxidation in *β*<sub>2</sub>

The observed decrease in the rate of *Y*<sub>356</sub> oxidation in the presence of *F*<sub>731</sub> provides evidence for direct interaction between *Y*<sub>356</sub>(*) and *Y*<sub>731</sub>(*) in the assembled interface. This result is consistent with our previous work showing that oxidation of *α*<sub>2</sub> by a photogenerated 2,3,6-trifluorotyrosine radical requires both *Y*<sub>731</sub> and *Y*<sub>730</sub>. This observation led to the proposal that the *Y*<sub>731</sub>(*)--*Y*<sub>730</sub>(*) dyad is an important element for radical transport. The current observation that *Y*<sub>356</sub> photooxidation is modulated by *Y*<sub>731</sub> suggests a significant interaction between *Y*<sub>356</sub> and *Y*<sub>731</sub> across the *α*<sub>2</sub>--*β*<sub>2</sub> interface. The present work therefore suggests further elaboration to our previous model of a coupled tyrosine dyad---*Y*<sub>356</sub>(*)--*Y*<sub>731</sub>(*)--*Y*<sub>730</sub>(*)---which is responsible for mediating intersubunit radical transport as well as radical transport through *α*<sub>2</sub> to *C*<sub>439</sub>(*) at the active site. This result is in line with the recent observation that a new radical, generated by using a highly oxidizing *NO*<sub>2</sub>*Y*<sub>122</sub>(*) equilibrates over *Y*<sub>356</sub>, *Y*<sub>730</sub>, and *Y*.<sup>17</sup>

Given the mechanistic hypothesis that unidirectional PCET operates in the *α*<sub>2</sub> subunit,<sup>21</sup> even a slight perturbation of the *Y*<sub>356</sub>(*)--*Y*<sub>731</sub>(*)--*Y*<sub>730</sub>(*) triad is likely to have an effect on radical transport. Several related studies have provided insight into the nature of interactions between adjacent pathway tyrosine residues. Hydrogen-bonding interactions involving the tyrosine phenol groups of these residues are crucial structural elements. Such a hydrogen-bonded network is consistent with our observations that removal of one hydroxyl group disrupts the oxidation of an adjacent residue.

In support of this model, rapid mixing studies of wt--*β*<sub>2</sub> and *NH*<sub>2</sub>*Y*<sub>730</sub>--*α*<sub>1</sub> (in the presence of CDP and ATP) results in generation of *NH*<sub>2</sub>*Y*<sub>730</sub>*Y*<sub>122</sub>(*) and concomitant loss of *Y*<sub>122</sub>(*)<sup>22</sup>. Examination of this trapped *NH*<sub>2</sub>*Y*<sub>730</sub> radical by electron-nuclear double resonance and density functional theory suggests a detailed model for the hydrogen bonding network in *α*<sub>2</sub> including *NH*<sub>2</sub>*Y*<sub>730</sub>, *C*<sub>439</sub>*Y*<sub>731</sub> and a key water molecule.<sup>23</sup> The importance of hydrogen bonding on the PCET oxidation of phenols has been well established in studies of small molecule models.<sup>24-31</sup>

Conformational changes may also occur upon substitution of nearby amino acids of the tyrosine triad. An effect of this type has been previously revealed by the *NH*<sub>2</sub>*Y*<sub>730</sub>--*α*<sub>2</sub> crystal structure; the observed electron density suggests a second conformation for *Y*<sub>731</sub>.<sup>3</sup> In the case of an F to Y mutation, a change in conformation may well result from the loss of a hydrogen bond to the substituted residue. A change in the
conformation of a tyrosine residue could significantly alter the distance between residues as well as the local environment of the redox-active phenol group.

5.3.4. Photogenerated [Re$^{III}$] is a Strong, Non-Specific Oxidant

Use of the flash-quench approach, using an oxidative quencher [Ru$^{III}$'(NH$_3$)$_6$]Cl$_3$, results in the generation of Y$^\cdot$ which can be directly observed in transient absorption experiments (Figure 5.13). Such signals are observed even in the case where neither interface tyrosine is present, in the photolysis of [Re−Y$_{356}$F−β$_2$ and Y$_{731}$F−α$_2$, indicating that oxidation of off-pathway tyrosines occurs. Furthermore, the lifetimes of these photogenerated tyrosine radicals are statistically indistinguishable for all four cases (Figure 5.14). In contrast, the amplitudes of these signals are distinguishable and indicates that the presence of Y$_{356}$ results in greater amplitudes than in the corresponding Y$_{356}$F mutation. We therefore assign a significant fraction of the observed signal to Y$_{356}$•, noting that significant amplitude remains that can be attributed to Y$_{731}$• and off-pathway oxidation.

Notably, a signal for oxidized tryptophan is also observed in these experiments (Figure 5.16); however, as in the case of Y$^\cdot$, W$^\cdot$/WH$^\cdot$ are formed independently of the presence of Y$_{356}$ and Y$_{731}$ (Figure 5.18). In fact, larger amplitude tryptophan radical signals are observed in cases where the Y$_{356}$F mutation is used, suggesting that tryptophan and tyrosine oxidation by [Re$^{III}$] are competitive. Furthermore, signals for oxidized tryptophan are still observed in the absence of α$_2$ (Figure 5.19), where a greater oxidized tryptophan amplitude is observed for [Re−Y$_{356}$F−β$_2$ than for [Re−β$_2$. Single-wavelength kinetics measurement of the tryptophan radical species (Figure 5.17) are convoluted by residual emission from unquenched [Re$^{III}$]*, but reveal growth of oxidized tryptophan as well as a radical species that lives for tens of microseconds (τ = 19.3 ± 0.8 μs).

A curious feature of this oxidized tryptophan signal is its appearance at 525 nm, rather than at the more characteristic wavelengths of 500 nm (W$^\cdot$) or 560 nm (WH$^\cdot$). This difference in wavelength can be attributed to a number of possible factors, such as a mixture of W$^\cdot$ and WH$^\cdot$ in the oxidation of a single tryptophan species, or consistent with the lack of specificity, the oxidation of multiple W residues resulting in a mixture of protonated and deprotonated products.
5.4. Conclusion

The generation of $Y_{360^{*}}$ within an assembled $\alpha_2\beta_2$ complex represents a key advance in the study of PCET in RNRs. The observation of a photogenerated, on-pathway radical enables examination of rapid radical transport along the $\alpha_1$ pathway, with the key advantages of added fidelity to the natural system and improved subunit binding. This result also opens the door for the study of photoinitiated PCET along the radical transport pathway in the $\beta_2$ subunit in the fully assembled protein complex, a longstanding goal of our studies in RNR.

5.5. Experimental Information

Photochemical turnover at pH 7.6, association constant determination under unquenched conditions, and protein preparation for emission quenching flash photoysis at pH 7.6 were performed by Lisa Olshansky.; the corresponding flash photolysis data were obtained collaboratively. Dr. Patrick G. Holder assisted in collection of flash photolysis and phototurnover data at pH 8.3.

5.5.1. Materials

Luria-Bertani medium (LB) (Sigma or BD Biosciences), agar (BD Biosciences), kanamycin disulfate salt (Km) (Sigma), phenylmethanesulfonyl fluoride (PMSF) (99%, Sigma), (+)-sodium L-ascorbate (99%, Sigma), tris(hydroxymethyl)aminomethane (Tris) (99%, Sigma), imidazole (Im) (99%, Sigma), Sephadex G-25 (Sigma), cytidine 5'-diphosphate sodium salt hydrate (CDP) (99%, Sigma), S-tritiated cytidine 5'-diphosphate sodium salt hydrate ([³H]-CDP) (ViTrax Radiochemicals), adenosine 5'-triphosphate disodium salt hydrate (ATP), (99%, Aldrich), β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) (97%, Sigma), sodium tetraborate (NaBi) (99%, Aldrich), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (99%, Aldrich), Ni,N-dimethylformamide (DMF) (99.9%, Sigma-Aldrich), streptomycin sulfate salt (Sigma-Aldrich), Ni-NTA Superflow (Qiagen), QIAprep Spin Miniprep Kit (Qiagen), DL-dithiothreitol (DTT) (99%, Promega), isopropyl-β-D-thiogalactopyranoside (IPTG) (99%, Promega), dNTP mix (mixture of 10 mM dATP, dCTP, dGTP, dTTP each in H₂O, pH 7.5) (Promega), hexaaammineruthenium(III) chloride (Ru°°(NH₃)₆Cl₃) (99%, Strem), nickel(II) sulfate hexahydrate (NiSO₄) (99.99%, Strem), E. coli XL10-Gold ultracompetent
cells (Agilent), *E. coli* BL21-CodonPlus(DE3)-RIPL (Agilent), PfuUltra II Fusion HotStart DNA Polymerase (Agilent), Mini-PROTEAN TGX precast 7.5% polyacrylamide gels (Bio-Rad), Precision Plus Protein Kaleidoscope Standards (Bio-Rad), glycerol (BDH/VWR), Amicon Ultra-15 centrifugal concentrators with Ultrace-30 membrane (Millipore), PLTK ultrafiltration discs, Ultrace-30 (Millipore), 4-(2-hydroxyethyl)piperazin-1-ylethanesulphonic acid (HEPES) (99%, EMD), anhydrous magnesium sulfate (MgSO₄) (99%, EMD), ethylenediaminetetraacetic acid disodium salt (EDTA) (99%, EMD), sodium hydroxide (NaOH) (EMD), concentrated hydrochloric acid (HCl) (Mallinckrodt), and sodium chloride (NaCl) (Mallinckrodt) were used as received.

Tricarbonyl(1,10-phenanthroline)(4-bromomethylpyridine)rhenium(I) hexafluorophosphate ([Re]-Br) was available from a previous study. Glycerol cell stock solutions for the expression of pET-28a-nrdA (encodes for wt-a₂) and pET-28a-Y₇₃₁F-nrdA (encodes for Y₇₃₁F-a₂) in *E. coli* BL21 (DE3) cells were available from previous a study. Plasmids encoding (His)₆-C₂₆₈S₃₀₅S-β₂ (pET-9d-C₂₆₈S₃₀₅S-nrdB) and (His)₆-C₂₆₈S₃₀₅S₃₅₅S-β₂ (pET-9d-C₂₆₈S₃₀₅S₃₅₅S-C-nrdB) were available from a previous study. Custom primers for site-directed mutagenesis were purchased from Invitrogen in desalted form and used as received. Thioredoxin (TR) and thioredoxin reductase (TRR) were available from previous work.

5.5.2. General Methods and Instrumentation

All aqueous solutions and other uses of water are of distilled deionized water (ddH₂O) (18 MΩ cm⁻¹). All buffers were adjusted to the noted pH at the appropriate temperature for use.

Glycerol cell stock solutions were prepared aseptically from 750 µL sterile 50% (v/v) aqueous glycerol and 750 µL saturated culture in LB, flash frozen at −78 °C, stored at −80 °C until use, and thawed at room temperature. Protein samples were stored in concentrated 1 mL aliquots, flash frozen at 77 K, stored at −80 °C until use, and thawed slowly on ice. DNA samples (plasmids and primers) were stored at −20 °C and thawed at room temperature.

Absorption spectra in the UV and visible region were collected on a Varian Cary 5000 UV-vis-NIR spectrophotometer on samples as indicated. High-performance liquid chromatography (HPLC) was performed using a Waters 717/2487 instrument with an Alltech Econosil C18 column (10
um, 250 mm x 4.6 mm). DNA sequencing was performed in the Massachusetts Institute of Technology Biopolymers Laboratory.

Spectroscopy buffer is composed of 50 mM borate, 15 mM MgSO₄ in 5% glycerol, pH 8.3. Assay buffer contains 50 mM HEPES, 15 mM MgSO₄, 1 mM EDTA, pH 7.6.

5.5.3. **RNR Subunit Construction, Expression, Purification, and Labeling**

In general, RNR subunit α₂ and β₂ mutants were obtained by a previously published method. Changes from this method and key experimental parameters are detailed below.

Construction of the plasmid for expression of (His)₆-C₂₆ S,C₃₀₅ C₅₅ Y₃₅₆ F-β₂ (pET-9d-C₂₆₈ S,C₃₀₅ S₃₅₅ C₅₅ Y₃₅₆ F-nrdB) was achieved by site-directed mutagenesis using pET-9d-C₂₆₈ S₃₅₅ S₅₅₅ F-nrdB as a template and the following forward (F) and reverse (R) primers. Relevant sections to affect the double mutation S₃₅₅ C/Y₃₅₆ F are underlined; specific nucleotides that are modified are indicated in bold.

F: 5′-AG GAA GTG GAA GTC AGT TGT TTT CTG GTC GGG CAG-3′
R: 5′-CTG CCC GAC CAG AAA ACA ACT GAC TJC TJC TJC CT-3′

For subunit purification of β₂, the cell lysate was supplemented with Fe²⁺ and ascorbate as previously reported. Reduction of the stable radical Y₁₂₂ in holo-β₂ to yield inactive met-β₂ and labeling of β₂ mutants was achieved by a method that has been previously reported. Concentrations of RNR subunits were determined from UV-absorption using the molar extinction coefficients (ε₂₈₀(α₂) = 0.189 μM⁻¹ cm⁻¹, ε₁₈₆(β₂) = 0.131 μM⁻¹ cm⁻¹, ε₂₈₀([Re]-β₂) = 0.189 μM⁻¹ cm⁻¹).

Purity (SDS-PAGE), radical content (determined by the dropline correction method), and activity (assessed by a radioactive activity assay), were measured by established methods as indicated.

5.5.4. **Dissociation constant (Kᵦ) determination**

The Kᵦ for [Re]-β₂ and wt-α₂ was determined by competitive inhibition as previously reported. These data are shown in Figure 5.3 and analyzed as previously reported.

In order to determine Kᵦ for modified β₂ subunits and wt-α₂ in the presence of Ru⁺⁺(NH₃)₆Cl₃, time resolved emission traces were recorded for [Re]-β₂ in the presence of varying concentrations of wt-α₂. The recorded emission traces were fit to monexponential decay functions and the resulting lifetimes were
taken as a measure of the solution exposure of [Re]. In a model where the second-order rate constant for collisional quenching by Ru(lll)(NH₃)₆³⁺ varies for [Re] in bound (kₐ) and free (kₕ) [Re]-β₂, the observed rate of excited-state deactivation (k₀bh) should vary linearly with the fraction of bound [Re]-β₂ (χₕ), as shown below.

\[
(5.3) \quad k_Q = k_B[Ru^{III}][Re - \beta_2]_B + k_F[Ru^{III}][Re - \beta_2]_F
\]

Scaling in terms of the total concentration of [Re]-β₂ and substituting in terms of \( \chi_h \):

\[
(5.4) \quad \frac{k_Q}{[R_u^{III}][Re - \beta_2]} = k_B \chi_B + k_F (1 - \chi_B)
\]

Rearranging in terms of an observed rate constant and measured emission lifetime (\( \tau \)):

\[
(5.5) \quad k_{obs} = \frac{1}{\tau_{obs}} = k_F + \chi_B (k_B - k_F)
\]

Taking the observed emission lifetime as a readout for \( \chi_h \), the following previously reported analysis can be applied. In this model, \([\alpha_2]_0\) and \([\beta_2]_0\) represent the total concentration of each subunit added to the experimental mixture.

\[
(5.6) \quad [\alpha_2 : \beta_2] = \frac{([\alpha_2]_0 + [\beta_2]_0 + K_D) - \sqrt{([\alpha_2]_0 + [\beta_2]_0 + K_D)^2 - 4[\alpha_2]_0[\beta_2]_0}}{2}
\]

Alternatively, where the data are better fit to the fraction of [β₂] bound (χ₀), the following previously reported equation is more suited:

\[
(5.7) \quad \chi_B = \frac{[\alpha_2 : \beta_2]}{[\beta_2]_0} = \frac{([\alpha_2]_0 + [\beta_2]_0 + K_D) - \sqrt{([\alpha_2]_0 + [\beta_2]_0 + K_D)^2 - 4[\alpha_2]_0[\beta_2]_0}}{2[\beta_2]_0}
\]

Combining these two approaches, the data were fit to the following equation:

\[
(5.8) \quad k_{obs} = k_F + (k_B - k_F) \frac{([\alpha_2]_0 + [\beta_2]_0 + K_D) - \sqrt{([\alpha_2]_0 + [\beta_2]_0 + K_D)^2 - 4[\alpha_2]_0[\beta_2]_0}}{2[\beta_2]_0}
\]
Data for the determination of the $K_D$ for [Re]-β$_2$-wt-α$_2$ in the presence of Ru$^{III}$(NH$_3$)$_6$Cl$_3$ are given in Figure 5.12.

5.5.5. **Nanosecond laser flash photolysis**

Laser experiments were performed using a system that has been previously described. Instrument control and data collection were performed by software designed by Bryce L. Anderson that has been previously reported. Samples for laser flash photolysis were prepared in 750 µL and recirculated to reduce sample decomposition. An inline filter (Acrodisc 13 mm 0.2 µM Supor Membrane, Pall Corporation) was used to collect solid photoproducots in all cases. Optical long-pass cutoff filters ($\lambda > 375$ nm) were used to filter probe light before detection to remove scattered 355 nm pump light.

Each experiment was repeated three times on independent samples to maximize signal to noise and obtain error limits for calculated values, except for emission kinetics experiments, which showed adequate signal to noise after only a single sample. Data were recorded for 1000 laser shots for each sample, resulting in either 1000 sweeps for single-wavelength kinetics or 500 four-spectrum sequences for transient absorption spectra, where only two of four conditions result in exposure to the pump beam.

5.5.6. **Error calculations for transient absorption and emission kinetics**

For transient emission kinetics, each sample provided sufficient signal to noise to be analyzed independently (Figure 5.8, Figure 5.9). Therefore, triplicate measurements were used to calculate a standard deviation directly.

For transient absorption kinetics of photogenerated tyrosine radicals (Figure 5.13), the individual traces obtained were of insufficient quality to obtain a reliable fit. In this case, a weighted least-squares fit was performed on the set of three traces; errors reported are derived from the error in this fit rather than from statistics on the individual samples.

5.5.7. **Optimization of flash-quench conditions**

In order to generate a detectable and long-lived radical, the flash-quench method was used with Ru(NH$_3$)$_6$Cl$_3$. Large concentrations of Ru(NH$_3$)$_6$Cl$_3$ in solution result in increased radical yield (larger intensity spectral features) but also lead to decreased radical lifetime. Figure 5.10 shows the effect of
sequential laser shots on the observed signal amplitudes and transient absorption lifetimes for a variety of Ru(NH$_3$)$_6$Cl$_3$ concentrations.

5.5.8. Single-turnover assays

Photochemically initiated RNR activity was assayed by photolysis under single-turnover conditions. Photochemical single turnover experiments were performed as previously reported.$^{16,38}$ Comparative data for phototurnover experiments at pH 7.6 and 8.3 are provided in Figure 5.6. In all cases, values reported are for light-dependent turnover corrected for small amounts of turnover observed in the dark. The identity of the observed product was confirmed by an HPLC assay, shown in Figure 5.5, as previously described.$^5$

5.5.9. Data analysis

All least squares fitting was performed using OriginPro 8.5 (OriginLab); acceptability of the fits was made on the basis of qualitative symmetry of residuals about zero amplitude and the $R^2$ factor. Smoothing was performed on transient absorption spectra under quenched conditions where indicated. Spectra were smoothed by applying a high-frequency cut-off filter on the basis of a fast Fourier transform (FFT) using the built-in smoothing functions of OriginPro 8.5.
5.6. References


Chapter 6  Future Work and New Directions
6.1. Introduction

The work detailed in the preceding chapters has opened a number of new avenues for photoinitiated PCET in RNR. Rhenium-phenanthroline labeled β₂ (([Re]−β₂) enables observation of tyrosine radicals (Chapter 4), studies of radical generation kinetics, and photochemical substrate turnover (Chapter 5).² These studies indicate that the radical transport pathway between Y₃₁₆ and the active site in α₂ is intact when rhenium-phenanthroline photooxidant ([Re]) is introduced and that [Re] is able to photogenerate on-pathway radicals. Variation of [Re]−β₂ enables a number of studies to further elucidate PCET along the radical transport pathway of RNR.

6.2. Radical Transport along the α₂ Pathway in an Intact α₂:β₂ Complex

Transient absorption of [Re]−β₂ reveals no signatures for tyrosine radical directly (Figures 4.4, 5.7), requiring a flash-quench approach to observe radicals (Figures 4.7, 5.13); however, low radical yield and signal-to-noise preclude resolution of radical transport kinetics in assembled α₂:β₂ (Figures 5.13, 5.14). Fluorotyrosine substitution results in incorporation of an amino acid that can be deprotonated at mild pH (Table 1.2),³,⁴ enabling rapid radical initiation by electron transfer rather than PCET. This strategy has been previously employed for photopeptide based studies (Chapter 3).⁶ By performing similar studies using the intact α₂:β₂, key properties for radical transport into and in α₂ can be elucidated.

The intact β₂ subunit offers significant advantages relative to the photopeptide, including tighter binding: Kₐ = 9 μM for the [Re]−F₃Y photopeptide, Kₐ = 0.7 μM for [Re]−β₂ (Chapters 3, 5). Furthermore, conformational flexibility leads to two distinct conformations of [Re] bound to α₂ only one of which is competent for radical injection into α₂ (Chapter 3). Installation of a fluorotyrosine at position 3₅₆ would generate an equivalent system to the photopeptide, enabling efficient radical generation while incorporating the advantages of the intact [Re]−β₂ construct. Furthermore, the more rapid kinetics of fluorotyrosine oxidation may help to out-compete off-pathway tyrosine and tryptophan oxidation (Figures 5.13, 5.16). Fluorotyrosine incorporation into β₂ can be accomplished by recently developed methods.⁸

Transient absorption studies of flash-quench photogenerated, long-lived F₃Y• kinetics in the presence of pathway-blocked mutants of α₂ will enable the study of radical transport kinetics, expected
to benefit from improved signal-to-noise and fidelity to the natural system, promising an opportunity to evaluate the importance of the previously proposed $Y_{721-726}$ dyad in the intact enzyme.

6.3. Probing Proton-Coupled Electron Transfer of $Y_{356}$

Generation of $[\text{Re}]^{-}Y_{356}^{-}\beta_{2}$ enables the systematic variation of the driving force for ET from $[\text{Re}]^{+}$. The driving force for photooxidation can also be modulated by variation of the phenanthroline ligand of $[\text{Re}]$, which tunes the $[\text{Re}^{+}]^{0}$ and $[\text{Re}^{2+}]^{1}$ reduction potentials. By doing so, the electron transfer properties of $Y_{356}$ can be studied, as previously done for the free fluorotyrosine amino acids in solution. A detailed analysis of these parameters will enable determination of key features of $Y_{356}$ such as the reorganization energy determination for oxidation of $F_{n}Y_{356}$ by $[\text{Re}]^{+}$. Variations in electron transfer kinetics and reorganization energy between fluorotyrosine in solution and at position in the pathway reveal the effect of the protein environment on $Y_{356}$.  

6.3.1. Probing the Role of $E_{350}$ in $Y_{356}$ Oxidation

By varying the protonation state of fluorotyrosine, the role of proton-coupling on the formation and the reactivity of $Y_{356}$ can be elucidated. In the case where tyrosine is protonated, or where unsubstituted tyrosine is used, the proton acceptor for $Y_{356}$ can be interrogated. A conserved glutamate residue on the C-terminal tail of $E_{350}$ is required for activity and is proposed to act as a base for proton-coupled oxidation of $Y_{356}$. Both $E_{350}A^{-}\beta_{2}$ and $E_{350}D^{-}\beta_{2}$ are inactive, consistent with a critical role for $E_{350}$ as a proton acceptor. In the case where $E_{350}$ directly accepts the proton from $Y_{356}$, deprotonation of $F_{n}Y_{356}$ should restore activity. Recent work has attempted to probe this hypothesis acceptor using $3,5-F_{2}Y_{356}^{-}E_{350}A^{-}\beta_{2}$ and $3,5-F_{2}Y_{356}^{-}E_{350}D^{-}\beta_{2}$ mutants and reveals no such effect where pH rate profiles for these mutants show extremely low activity (<1% relative to wt-$\beta_{2}$), suggesting a more complex role for $Y_{356}$. Photochemical studies of $[\text{Re}]^{-}E_{350}^{-}X^{-}\beta_{2}$ and the related $[\text{Re}]^{-}E_{350}^{-}X_{F}^{+}Y_{356}^{-}\beta_{2}$ can further elucidate the role of $E_{350}$ in $Y_{356}$ oxidation kinetics directly, rather than relying on the indirect measure of activity.

6.4. Photochemical Tryptophan Oxidation in $\beta_{2}$

In addition to studies in $\alpha_{2}$ is, $[\text{Re}]^{-}\beta_{2}$ has the potential to probe radical transport along the pathway.
in $\beta_2$, including PCET between $Y_{122}$ and $Y_{356}$, as well as probing the role of $W_{48}$ in radical transport. $W_{48}$ is redox-active and critical to radical initiation at $Y_{122}$, where $W_{48}\cdot$ is involved, but has yet to be confirmed to be a discrete intermediate in long-distance PCET during turnover. Under flash-quench conditions, photogenerated $[\text{Re}^{II}]$ generates off-pathway tyrosine radicals (Figure 5.13) and an unidentified tryptophan-based radical (Figure 5.16). Photochemical oxidation of a W residue is of potential relevance to $W_{48}\cdot$

The possible involvement of $W_{48}$ motivates the further investigation of this W-containing radical species in order to identify the potential involvement of $W_{48}\cdot$. Notably, the presence of $Y_{356}$ decreases the yield of photogenerated $W\cdot$, suggesting that either $Y_{356}$ oxidation is competitive with $W$, or that W radical formation occurs via $Y_{356}$. The role of $W_{48}$ in the observed W oxidation can be probed by mutation of $W_{48}$ with redox-inactive residues to generate $[\text{Re}]^{-}W_{48}\cdot A^{-}\beta_2$ and $[\text{Re}]^{-}W_{48}\cdot F^{-}\beta_2$; the $W_{48}\cdot F$ and $W_{48}\cdot A$ mutations have been previously studied. Changes in $W$ oxidation kinetics, yield, or $\lambda_{\text{max}}$ in such mutants would indicate the extent to which $W_{48}$ is involved in the observed $W\cdot$.

6.5. Photoinitiated Redox Reactions of the Diferric Tyrosyl Radical Cofactor

Having demonstrated the utility of photochemical $\beta_2$ by installing $[\text{Re}]$ at the subunit interface, the approach can be extended to other key aspects of $\beta_2$. Installation of $[\text{Re}]$ in the vicinity of $Y_{122}$ provides an opportunity to study redox reactions of the diferric tyrosyl radical cofactor, enabling a number of potential studies.

Photochemical reconstitution of the reduced (met) form of $\beta_2$ would elucidate aspects of $Y_{122}$ oxidation after turnover, while photoreduction studies of $Y_{122}\cdot$ in the active form of $\beta_2$ are related to the initial steps of long-range radical transport in turnover. Further, photochemical one-electron oxidation of the active $Y_{122}\cdot$ form are of relevance to the radical initiation process, where oxidized forms of the diiron cofactor ($\text{Fe}^{III}\text{Fe}^{IV}$) and a nearby tryptophan residue ($W_{48}\cdot$) are observed. As discussed in the proposed studies of $Y_{356}$ oxidation, the driving force for oxidation of $Y_{122}$ can be systematically varied by installation of fluorotyrosine, and more generally, the driving force for photooxidation can be varied by substitution of the phenanthroline ligand of $[\text{Re}]$. 189
One possible site for \([\text{Re}]\) labeling, a surface-accessible threonine residue, \(T_{243}\), is located two positions away from a histidine ligand, \(H_{241}\), to \(Fe_2\) of the diiron cofactor. The following figure illustrates this position, highlighting the through-bond path to \(Fe_2\) and relevant distances to the radical cofactor and \(W_{48}\).

**Figure 6.1.** A potential site for \([\text{Re}]\) labeling for photoinitiated studies at the diferric tyrosyl radical cofactor. Distances shown between \(T_{243} C_{\alpha}\) and the indicated atoms (\(W-NH, Y-OH, Fe\)).
6.6. References


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The work in this thesis would not have been possible without many people I have encountered over the past six years. They not only made many of the specific experiments presented herein possible, but also taught me lessons that have guided me throughout my entire graduate career. I am immensely thankful for each of these experiences and the knowledge I gained throughout my time here.

I am deeply grateful to Professor Daniel G. Nocera for allowing me to join his research group. I am continually amazed by his energy and ability to engage deeply on a broad range of subjects. The breadth his expertise and research program make his group an incredibly enriching place to be. In my time here, Dan has taught me to be a hunter (not a gatherer), ask the hard questions, always do the experiment, and most importantly, to figure it out. I am thankful to Dan for giving me the freedom to pursue my project independently, even when it meant lessons were learned the hard and slow way, and for helping me to learn how to ask the right questions.

As part of my research, I was fortunate to have the chance to collaborate with Professor JoAnne Stubbe. I am thankful to JoAnne for letting me work in her lab and for all her help and advice throughout my time here. JoAnne's enthusiasm and relentless pursuit of the right answer are inspiring. Thank you to Professor Stephen J. Lippard for chairing my thesis committee. I greatly enjoyed our annual meetings. Thank you to the inorganic faculty as a whole, who made my teaching, coursework, and oral exams educational and interesting experiences.

During my time in the Nocera group, I had the good luck of working with a number of great postdocs and graduate students on the RNR project. When I joined the lab, Danny Lutterman helped me get up to speed on the project, teaching me how to express and purify RNR and answering my day-to-day questions about everything. Thanks to Danny for all his guidance and help. Thank you to Jay Yang for his work establishing the rhenium-tyrosine small molecule model system. In the Stubbe lab, Ellen Minnihan and Ken Yokoyama were always willing to teach me new techniques and answer questions about all things RNR.

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of the project, from molecular biology to nanosecond spectroscopy, as well as PCET in general. Thank you to Pat for his help and advice, for introducing me to the wonders of PyMOL, Adobe Creative Suite, and appropriate dash use.

Bryce Anderson has been an outstanding and invaluable addition to the group. From his first few months in the lab working with Pat and me to optimize the nanosecond laser and then single-handedly rewriting the control software for both laser systems, Bryce Anderson was critical to obtaining the transient data in this thesis. Thank you to Bryce for sharing his expertise, I have greatly enjoyed our discussions about RNR, PCET, and the variety of other projects you have undertaken since joining our group. More recently, Lisa Olshansky and Dave Song have started working on photoRNR, bringing new ideas and excitement to the project. Thank you to Lisa for her work on the project. I am excited to see the project in such great hands and look forward to seeing where it goes in the future.

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I was lucky to join the Nocera lab together with Matt Chambers and Tom Teets our first year. Matt and Tom were not only the founding captains of Team Fun and Team Intensity, but also two of the best colleagues I know. Tom exemplifies unnecessary hustle both on the field and in the lab. Thanks to Tom for leading two championship Organometals softball teams, and for being an outstanding colleague, always willing to take time to answer questions or help with experiments, in particular for determining the X-ray structure presented in this thesis. Matt, our fearless leader in flag football and volleyball, is one of the most easygoing guys and best sports I know. Thanks to Matt for the many hours of productive discussions around his desk, and for bringing an occasional dose of calm when needed.

Many thanks to the senior graduate students and postdocs in the group for their help. As a younger student, they were experienced in navigating the graduate program and the group, yet were always approachable and willing to give advice. Many thanks to Emily McLaurin for teaching me how to
use the nanosecond laser and always being there to answer questions and give advice, and thank you for introducing me to the wonders of the Thirsty Ear Pub. Thank you to Tim Cook for being a source of sanity amidst the madness, including the truly big questions like how a charging Triceratops would fare against a rocket launcher. Thank you to Dino Villagrán for the many long discussions about virtually every subject imaginable, often running late into the night. Many students and postdocs who joined during my time here helped make my experience much more productive and enjoyable. Thank you to Casandra Cox for good times and introducing me to my cat, Chaz. Thank you to Bon Jun Koo for being a source of kindness, humor, and fun discussions of PCET. Special thanks to Dilek Dogutan for everything she does to keep everyone going in the same direction, but more so for the genuine kindness and interest with which she does it.

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Finally, everything in this thesis begins and ends with my family. My younger sister, Constanza, moved to Cambridge just before my fourth year began and has made all the difference. Whenever things have been frustrating or difficult, her sense of humor, kindness, and encouragement are always just around the corner. Words cannot describe the debt of gratitude I owe my parents. Since long before I came to MIT, their support and encouragement have given me the motivation to succeed and the courage to fail. Thank you for being there in every way imaginable and for always reminding me to be thankful, work hard, and have fun.
Biographical Note

Arturo Alejandro Pizano was born on December 6, 1985 in Los Angeles to Lourdes and Arturo Pizano. His younger sister, Maria Constanza, was born two years later. After living in Milpitas, CA from the age of four to seven, the family moved to New Jersey in the fall of 1993, eventually moving to Belle Mead, where Arturo and his sister grew up. He attended Montgomery High School, where his interest in chemistry grew with the encouragement of his chemistry teacher, Mrs. Georgia Muhs. He left MHS after his junior year to pursue his undergraduate degree at the California Institute of Technology, beginning in the fall of 2003.

While at Caltech, Arturo worked in the laboratories of Professor Jesse L. Beauchamp studying methods for generating droplets for field-induced droplet ionization mass spectrometry. As his interests in bioinorganic chemistry developed, he joined the group of Professor Harry B. Gray where he worked on protein folding studies of cytochrome c-b$_{562}$, an engineered four-helix bundle protein. Outside of the classroom and lab, Arturo worked in the governance of the Caltech honor code as Secretary of the Board of Control and later served as president of Ricketts House.

After graduating from Caltech with a B.S. in Chemistry, Arturo moved to Cambridge, MA in fall 2007 to begin doctoral studies in inorganic chemistry at the Massachusetts Institute of Technology. At MIT, he joined the group of Professor Daniel G. Nocera where he studied photoinitiated proton-coupled electron transfer and long-range radical transport in ribonucleotide reductase, in collaboration with Professor JoAnne Stubbe. In January 2013, he moved to Harvard University with Professor Nocera as a visiting student where he completed his graduate work in August 2013.
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