Chapter 2

PHOTO-TRIGGERED OXIDATION OF
RU-MODIFIED CYTOCHROME P450 BM3

Portions of this work are excerpted with permission from:


Copyright 2010 National Academy of Sciences
2.1. Background: Toward high-valent P450 intermediates

Cytochromes P450 (P450s) catalyze a dazzling array of regio- and stereospecific oxidation reactions, including the hydroxylation of aliphatic and aromatic hydrocarbons and the epoxidation of alkenes.\textsuperscript{1,2} This activity requires the controlled formation of extremely reactive species within the protein framework. In the native catalytic cycle, P450s activate dioxygen with the aid of two electrons from reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H); one oxygen atom is delivered to the organic substrate, and the other is released as water.

![Catalytic cycle for P450-catalyzed hydroxylation reactions.](image)

**Figure 2.1.** Catalytic cycle for P450-catalyzed hydroxylation reactions.

The consensus P450 catalytic cycle (Figure 2.1) implicates a ferryl porphyrin radical cation (compound I, CI, intermediate 7) as the active oxygenating agent.\textsuperscript{3} In the postulated mechanism, CI abstracts a hydrogen atom from the bound
substrate molecule to form a transient Fe$^{IV}$-hydroxide complex (compound II, CII, intermediate 6), and radical recombination (or rebound) with the substrate produces oxygenated product.$^{4,5}$ Substrate release from the active site and re-binding of a water molecule regenerates the ferric resting state of the enzyme.

As described in Chapter 1, the elusive high-valent species, CI and CII, have been the target of decades of time-resolved spectroscopic studies. One intrinsic challenge in their pursuit is that the native electron transfer (ET) events that activate dioxygen are slow,$^{6,7}$ and require the expensive redox cofactor, NADH. One method to circumvent these rate limiting reactions involves direct heme oxidation by oxygen atom donors such as peroxycacids (e.g., meta-chloroperoxybenzoic acid, mCPBA) or hydrogen peroxide. In 2010, rapid mixing with mCPBA was used to generate and trap CI in highly purified samples of the thermophilic P450 CYP119 from Sulfolobus acidocaldarius.$^{10}$ This reactive species was characterized by UV-visible absorption, electron paramagnetic resonance (EPR), and Møssbauer spectroscopies, and was found to be active for hydroxylation of model substrates such as lauric acid. Single-turnover hydroxylation studies yielded an observed second order rate constant of $k_{obs} = 1.1 \times 10^7$ M$^{-1}$s$^{-1}$.10

The events of H• abstraction to form CII, and subsequent radical-rebound to produce hydroxylated product, have yet to be directly observed. Mechanistic studies of P450 catalysis in cryogenic matrices suggest that the barrier to formation of compound I (Figure 2.1, 5→6) is higher than that for its reaction with substrate (6→7→1).11 In order to gain insights into these rapid, reactive processes, new methods must be developed to generate high-valent P450 species on a timescale that is comparable to the rate of reaction with substrate. Experiments in which enzyme oxidation is initiated by rapid mixing of enzyme with a chemical oxidant
or substrate have a dead time of 0.25 ms - 10 ms (e.g., for a Bio-Logic® SFM-400 stopped-flow). This timescale may be too slow to observe the fundamental bond-breaking and bond-making events of interest.

Photo-triggered methods provide a faster way to initiate reactions, with extremely high temporal resolution. Electronic transitions (including photo-induced excitation) are essentially instantaneous on the timescale of molecular motions. The dead time for monitoring a laser-triggered photochemical event is only limited by the temporal length of a laser pulse or the instrument response (~20 ns for the setup described herein, see Appendix B). The Gray laboratory has a long history of photo-initiating ET in proteins using inorganic photosensitizers, including ruthenium diimines and rhenium tricarbonyl complexes. Curious readers are directed toward a number of excellent reviews for further details.12–14

A flash-quench cycle elicits ET with protein redox cofactors. In this method, a laser pulse generates a reactive, electronic excited state (Figure 2.2). Interaction of excited state with exogenous small molecule quenchers results in electron transfer quenching to generate a more oxidized photosensitizer species. The charge separation accomplished by the bimolecular ET increases the lifetime of the reactive photosensitizer, and provides additional driving force. For example, ~500 mV of potential is gained by reversible, oxidative quenching of [Ru(bpy)₃]²⁺ (E° vs. NHE in water: Ru²⁺/²⁻ = 1.3 V; Ru*²⁺/²⁻ = 0.8 V), and the lifetime is increased from ~600 ns to nearly 100 μs.17,18

In earlier work, [Ru(bpy)₃]²⁺ (bpy = 2,2′-bipyridine) was employed in a bimolecular flash-quench photochemical oxidation procedure to generate CII and CI in the heme enzyme horseradish peroxidase (HRP) and in the “minienzyme” microperoxidase-8 (MP8), a heme-containing, 8-amino acid fragment from
cytochrome c.\textsuperscript{15,16} By oxidizing the active site directly, this route avoids the use of dioxygen or reactive oxygen species entirely. Instead, the high-valent ferryl species, which can incorporate an oxygen atom into substrate, is generated by oxidation of a water molecule that ligates the heme in the Fe\textsuperscript{III} resting state.

![Diagram of Ru and Fe oxidation](image)

**Figure 2.2.** [Ru\textsuperscript{II}(bpy)\textsubscript{3}]\textsuperscript{2+} flash-quench and oxidation of the a heme protein active site.

The approach of employing [Ru(bpy)\textsubscript{3}]\textsuperscript{2+} in solution was unsuccessful with P450, however, owing to the deep burial of the heme active site inside the polypeptide matrix of the enzyme. We circumvented this problem by covalently attaching the photosensitizer to the P450 surface. This has allowed us to successfully laser-trigger photochemical heme oxidation in a Ru-P450 system, monitor the kinetics of electron transfer using transient absorption (TA) spectroscopy, and extract kinetics parameters for the rate of heme oxidation. The details of photosensitizer-attachment, crystallographic characterization, laser-triggered heme oxidation, and kinetics modeling are discussed in this Chapter.
2.2. Motivation and selection of the photochemical system

Photosensitizer

As described in Chapter 1, the Gray laboratory has used a variety of photosensitizers for triggering ET in proteins, and selective tethering at histidine or cysteine can be achieved. The heme domain of P450 BM3 contains 13 native histidines, but only three native cysteines, one of which provides proximal ligation for the heme. We have selected cysteine-labeling, using a ruthenium(II) bis-bipyridine iodoacetamidophenanthroline \([\text{Ru(bpy)}_2(\text{IAphen})]^{2+}\) complex (Figure 2.3) that can be readily synthesized by published procedures.\(^{19}\) This photosensitizer is excited in the blue-green region of the visible spectrum, has a long excited state lifetime in deoxygenated water (900 ns),\(^{19}\) and, when oxidized to \(\text{Ru}^{III}\) by flash-quench, has a sufficiently high reduction potential to oxidize the enzymatic active site (\(\sim 1.3\) V vs. NHE).\(^{17}\)

![Diagram](https://example.com/diagram.png)

**Figure 2.3.** \([\text{Ru(bpy)}_2(\text{IAphen})]^{2+}\) tethering to cysteine, to form the conjugate Cys-Ru(bpy)$_2$(Aphen).
**Exogenous Oxidative Quencher**

We have selected ruthenium(III) hexaamine trichloride as a reversible, exogenous oxidative quencher. This complex is water soluble (> 50 mM, pH ≤ 8), has little or no absorbance in the regions of interest (390-500 nm), and undergoes rapid electron transfer with ruthenium diimine photosensitizers (vide infra). Some care must be taken to avoid decomposition: at high pH (> 8) and/or temperature (> 50 °C), [Ru(NH₃)₆]³⁺ decomposes and turns deep purple/black. In solutions of low ionic strength, this complex may also cause protein precipitation (observed for P450 and Nitric Oxide Synthase); this can be avoided by using protein solutions that contain 100-200 mM NaCl.

We also attempted use of methyl viologen (dichloride salt) and chlorocobalt(III)pentaamine (dichloride salt). Upon irradiation (e.g., laser flash-quench transient absorption studies), both of these quenchers caused Ru-P450 sample degradation as observed by permanent bleaching of the P450 Soret (see Appendix C). Therefore, all of the studies described in this Chapter use [Ru(NH₃)₆]Cl₃ as the oxidative quencher.

**P450 Mutants**

To achieve selective surface modification with the photosensitizer, we used site directed mutagenesis to remove two native cysteine residues (C62A, C156S) from the P450 BM3 heme domain. These two mutations had been made previously in order to achieve specific attachment at a non-native cysteine for photochemical and electrochemical methods. The resulting double-mutants are stable and active toward oxidation of fatty acids (e.g., palmitate and laurate) in the presence of oxygen donors (e.g., hydrogen peroxide or mCPBA). For our purposes, a single cysteine was introduced at residue 97 (K97C). This position was chosen by former
Gray group postdoctoral fellow, Lionel Cheruzel, for surface-exposure and proximity to the heme. The cysteine Cα to heme iron distance is 16.8 Å (Figure 2.4). Additionally, residue 97 is directly adjacent to Trp96, which lies within hydrogen-bonding contact of one of the heme propionates. The role of this intervening residue will be further explored in Chapters 3 and 4.

**Figure 2.4.** K97 labeling site. **Left:** Structure of the P450 BM3 heme domain from the proximal face (pdb 2IJ2) highlighting the heme (red), axial ligand C400 (yellow), W96 (purple), and K97 (orange). **Right:** Space-filling model illustrating surface exposure of K97 (orange). (Figures made with PyMol, 2009)
2.3. Results

2.3.1. Characterization

The Ru\textsuperscript{II}(bpy)_2(Aphen)–P450(BM3)C62A/C156S/K97C conjugate (abbreviated Ru\textsuperscript{II}K97C-P450\textsubscript{BM3}) has been characterized by ultraviolet-visible absorbance (UV-vis), X-ray diffraction, and steady state and time-resolved fluorometry. The mass of the conjugate (54,200 Da) corresponds to that of the apo (heme-free) unlabeled protein (53,520 Da) plus the ruthenium photosensitizer (777 Da), minus the mass of iodide.

\textit{UV-visible absorbance}

The triple-mutant P450 BM3 C62A/C156S/K97C has characteristic absorption features that are almost identical to wild-type: a Soret band at 418 nm, a near UV band at 360 nm, and Q-bands at 536 and 569 nm. The Ru\textsubscript{K97C}-P450\textsubscript{BM3} conjugate has an additional shoulder at 450 nm, due to the characteristic ruthenium diimine MLCT absorbance (\textbf{Figure 2.5}).
Figure 2.5. Absorption spectra of \([\text{Ru(bpy)}_2(\text{IAphen})]^{2+}\) (yellow), P450-BM3 C62A/C156S/K97C (blue), \(\text{Ru}_{K97C}\cdot\text{P450}_{BM3}\) (green), at approximately equal concentrations.

**Steady-state Luminescence**

The free photosensitizer \([\text{Ru(bpy)}_2(\text{Aphen})]^{2+}\) and \(\text{Ru}^{II}_{K97C}\cdot\text{Fe}^{III}_{P450}\) conjugate have been examined by steady-state luminescence spectroscopy. When excited with blue light (e.g., 480 nm), both samples show a broad luminescence band in the red region of the visible spectrum, with \(\lambda_{\text{max}} = 620\) nm. The steady-state spectra of \([\text{Ru(bpy)}_2(\text{Aphen})]^{2+}\) and \(\text{Ru}^{II}_{K97C}\cdot\text{Fe}^{III}_{P450}\) are nearly superimposable, and very closely resemble that of \([\text{Ru(bpy)}_3]^{2+}\) (\(\lambda_{\text{max}} = 626\) nm) (Figure 2.6). Un-labeled P450 is not luminescent in the visible region.
Figure 2.6. Steady-state luminescence spectra of Ru photosensitizers in deoxygenated water. \([\text{Ru}^{II}\text{(bpy)}_3]^{2+}\) (red), free photosensitizer \([\text{Ru}^{II}\text{(bpy)}_2\text{(Aphen)}]^{2+}\) (dark blue), and conjugate \([\text{Ru}^{II}\text{K97C-Fe}^{III}\text{P450}]^{2+}\) (light blue).

X-ray Crystal Structure Analysis

We have determined the X-ray crystal structure of \([\text{Ru}^{II}\text{K97C-Fe}^{III}\text{P450}]^{2+}\) to 2.4-Å resolution (Figure 2.7). Two monomers were found in the asymmetric unit; the root-mean-squared deviation (rmsd) between the C\(\alpha\) atom positions in the two monomers is 0.34 Å, confirming that the two polypeptides have nearly identical conformations. Interestingly, the substrate channel is occupied by two unidentified electron density peaks. Additionally, the structure of \([\text{Ru}^{II}\text{K97C-Fe}^{III}\text{P450}]^{2+}\) more closely resembles that of the substrate-bound (closed) P450-BM3 enzyme (rmsd of 0.44 Å for C\(\alpha\) with the structure 2UWH\(^{22}\)), in which the F and G helices (known as the
“lid domain”) contract inward toward the heme. In contrast, the substrate-free (open) form overlays with an rmsd of 0.66 Å (for the structure 2IJ2) (Figure 2.7).

**Table 2.1.** X-ray crystallographic data collection, refinement statistics, and validation.

<table>
<thead>
<tr>
<th>Data collection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength, Å</td>
<td>0.979</td>
</tr>
<tr>
<td>Unit cell, Å</td>
<td>117.08, 117.08, 273.85</td>
</tr>
<tr>
<td>Space group</td>
<td>P41212</td>
</tr>
<tr>
<td>Resolution range, Å</td>
<td>45.52–2.40 (2.53–2.40)*</td>
</tr>
<tr>
<td>No. of total reflections</td>
<td>793,591</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>74,965</td>
</tr>
<tr>
<td>Completeness, %</td>
<td>99.7 (99.6)</td>
</tr>
<tr>
<td>$R_{merge}$, %</td>
<td>12.6 (82.7)</td>
</tr>
<tr>
<td>$\langle l/\sigma(l)\rangle$</td>
<td>15.4 (4.2)</td>
</tr>
<tr>
<td>Wilson B-value, Å²</td>
<td>45.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement statistics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range, Å</td>
<td>10–2.4</td>
</tr>
<tr>
<td>No. of reflections used</td>
<td>69,343</td>
</tr>
<tr>
<td>Free R reflections, %</td>
<td>5.0</td>
</tr>
<tr>
<td>$R/R_{free}$</td>
<td>0.200/0.237</td>
</tr>
<tr>
<td>rmsd bond length, Å</td>
<td>0.0113</td>
</tr>
<tr>
<td>rmsd bond angle, deg</td>
<td>1.374</td>
</tr>
</tbody>
</table>

| Ramachandran analysis, %         |       |
| No. of residues in               |       |
| Favored regions                  | 97.4  |
| Allowed regions                   | 2.6   |
| Outlier regions                   | 0.0   |
| PDB entry                        | 3NPL  |

*Data for the outermost shell are given in parentheses.
Figure 2.7. The RuK97C-P450BM3 structure. (PDB: 3NPL) Ru$^{II}$(bpy)$_2$(Aphen) photosensitizer is colored blue, the heme is colored red and tryptophan96 is colored purple.

Figure 2.8. Overlay of RuK97C-P450BM3 with wild type substrate-free and substrate-bound forms. **Left:** Substrate-free (open) form (pink, PDB: 2IJ2), RMS 0.662. **Right:** Substrate-bound (closed) form (orange, PDB: 2UWH) RMS 0.440.
The Ru-photosensitizer is well defined in only one monomer of the crystal structure, owing to π-stacking of the bipyridine ligands with aromatic residues on adjacent crystal units (Figure 2.9). The distance between the Fe-heme and Ru-photosensitizer is 24 Å.

Figure 2.9. Stacking of Ru$_{97C}$-P450$_{BM3}$ with an adjacent crystal unit. P450: green, photosensitizer: blue. Adjacent crystal unit: pink. Trp90 and Tyr344 edge-to-edge distances of 3.5-4.5 Å with the two bipyridine ligands. Glu 244 stacks beneath one of the bipyridine ligands, within 3.5 Å of the plane of the phenanthroline ligand.

The Ru-photosensitizer in the second monomer, which lacks the π-stacking interactions with neighboring protein molecules, is highly disordered. This is probably due to flexibility of the cysteine-acetamide linkage. These observations suggest conformational freedom for the Ru-photosensitizer. The 24-Å Ru-Fe distance is likely near the maximum separation in the distribution of conformations sampled by the Ru complex in dilute Ru$^{II}_{97C}$-Fe$^{III}_{P450}$ solutions
since the ligands of the photosensitizer may form favorable hydrophobic with amino acid residues on the protein surface, decreasing the Fe-Ru separation.

### 2.3.2. Laser flash-quench experiments

In order to examine photo-triggered ET in a time-resolved manner, laser samples were prepared under inert atmosphere with either the Ru\textsuperscript{II}_{K97C}-Fe\textsuperscript{III}_{P450} conjugate or the free photosensitizer ([Ru(bpy)\textsubscript{2}(Aphen)]\textsuperscript{2+}) in the presence or absence of exogenous quencher. Formation of the ferryl species from the ferric aquo resting state requires loss of protons, and we anticipated that the photo-triggered ET reaction would be pH dependent. Samples were prepared in buffers: 20 mM sodium acetate (pH 6, 7), 50 mM tris or 50 mM sodium borate (pH 8).

**Time-resolved luminescence**

The excited state luminescence decays of both [Ru(bpy)\textsubscript{3}]\textsuperscript{2+} and the free photosensitizer [Ru(bpy)\textsubscript{2}(IAphen)]\textsuperscript{2+} are monoexponential; \(\tau([\text{Ru}(\text{bpy})\textsubscript{2}(\text{IAphen})]\textsuperscript{2+})\) is 720 ns. Somewhat unexpectedly, the time-resolved luminescence decay of Ru\textsuperscript{II}_{K97C}-Fe\textsuperscript{III}_{P450} (\(\lambda_{\text{obsd}} = 630\) nm) is biexponential, with components of \(\tau_1(*)\text{Ru}^{II}_{K97C}-\text{Fe}^{III}_{P450} = 670\) ns, \(\tau_2(*)\text{Ru}^{II}_{K97C}-\text{Fe}^{III}_{P450} = 140\) ns (Figure 2.10). This biexponential nature of the Ru\textsuperscript{II}_{K97C}-Fe\textsuperscript{III}_{P450} decay does not appear to be affected by protein concentration over a range of 1-20 \(\mu\)M (see Appendix C). This suggests that a monomer-dimer equilibrium is not the cause of this biexponential behavior. We attribute these two exponential components to two separate conformations of the tethered photosensitizer conformations that do not exchange on the timescale of the luminescence measurement. The disorder present in the crystal structure supports the possibility of multiple conformations, but we do not have sufficient information to speculate on their exact identities.
Figure 2.10. Time resolved 630 nm luminescence decays in the absence of quencher. **Top:** Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ (cyan), and **Bottom:** [Ru$^{II}$(bpy)$_2$(IАphen)]$^{2+}$ (orange). Monoexponential fits are in red, biexponential fit (Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ only) is in blue.
In the presence of the exogenous electron transfer quencher, \[ \text{Ru(NH}_3\text{)_6}\text{Cl}_3 \], luminescence lifetimes decrease. The lifetimes in the presence of quencher appear to be more monoexponential, and approximate rate constants are extracted using a monoexponential fit. Stern-Volmer analysis of \( \text{Ru}^{II}_{\text{K97C}}\text{-Fe}^{III}_{\text{P450}} \) luminescence decay in the presence of various \([\text{Ru(NH}_3\text{)_6}]^{3+}\) concentrations (Figure 2.11) produces a bimolecular quenching rate constant \( (k_q) \) of \( 1.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \) (pH 8); this is close to diffusion limited (Figure 2.12).

![Figure 2.11. Luminescence decays of Ru\text{K97C-P450BM3} (pH 8) at various concentrations of [Ru(NH}_3\text{)_6]^{3+} quencher.](image)
Figure 2.12. Stern-Volmer quenching of RuK97C-P450BM3 with [Ru(NH₃)₆]³⁺ at three pH values. Luminescence decay rates are determined from monoexponential fits. pH 8 borate buffer (blue circles), pH 7 sodium acetate buffer (green triangles), pH 6 sodium acetate buffer (red squares).

Transient absorption

Both the Ru photosensitizer and P450 heme have strong electronic absorbance in the 390-440 nm region: P450 Soret $\varepsilon(\lambda_{\text{max}}$: 418 nm) = 95,000 M⁻¹cm⁻¹;⁷ [Ru(bpy)₂(Aphen)]²⁺ $\varepsilon(\lambda_{\text{max}}$: 450 nm) = 16,600 M⁻¹cm⁻¹.¹⁹ The shapes and positions of these electronic transitions are sensitive to metal oxidation state and environment; each species has a distinct absorption profile. By monitoring multiple wavelengths (390-440 nm) over time, we can identify the formation and decay of ET intermediates following laser excitation. This process is greatly
facilitated by control studies (e.g., the absence of quencher, or the free photosensitizer), as well as comparison to absorption profiles in the literature.

In the absence of exogenous quencher, TA traces of [Ru(bpy)$_2$(Aphen)]$^{2+}$ and Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ following excitation ($\lambda_{ex} = 480$ nm) are essentially identical. Data at all wavelengths reveal bleaching ($\Delta$Abs < 0) from 390-440 nm, consistent with the well-characterized behavior of metal-to-ligand charge-transfer (MLCT) excited Ru-diimine complexes.$^{17}$ The transient signals return to baseline at the same rate as luminescence decays, and we find no evidence for the formation of additional transient species (Figure 2.13).

Figure 2.13. Single-wavelength transient absorption of Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ in the absence of quencher. 400 nm: blue; 420 nm: green; 440 nm: red, and a scaled overlay of the luminescence decay at 630 nm. The sample is in 50 mM borate buffer, pH 8. Laser excitation is $\lambda_{ex} = 480$ nm.
TA analysis of the free photosensitizer \([\text{Ru(bpy)}_2(\text{Aphen})]^{2+}\) in the presence of quencher allows us to identify transient features associated with Ru\(\text{III}\)-photosensitizer species. TA data at all wavelengths examined are characterized by bleaches with biphasic recovery; the first kinetics phase is attributed to \(\text{Ru}^{\text{II}}\rightarrow\text{Ru}^{\text{III}}\) ET quenching, and agrees with the luminescence decay rate (Figure 2.14). \([\text{Ru}^{\text{III}}(\text{bpy})_2(\text{IAphen})]^3+\) is characterized by a bleach at all wavelengths examined (390-440 nm); this feature decays to baseline within 100 µs, presumably through back-ET with reduced quencher ([Ru\(\text{II}(\text{NH}_3)_6\)]^{2+}) (see Appendix C for additional comments on this recombination rate).

![Figure 2.14](image.png)

**Figure 2.14.** Transient Absorption data for flash-quench of \([\text{Ru(bpy)}_2(\text{IAphen})]^{2+}\) with 17 mM \([\text{Ru(NH}_3)_6]^{3+}\). The scaled luminescence decay (630 nm) is overlayed (top, pink) for comparison.

Flash-quench of Ru\(\text{II}_{\text{K97C}}\)-Fe\(\text{III}_{\text{P450}}\) in the presence of quencher reveals substantially more complex kinetic behavior, indicating the presence of multiple intermediates
that form and disappear over the microsecond to second time range (Figure 2.15). The most prominent features are: a persistent bleach at 420 nm (which coincides with $\lambda_{\text{max}}$ of the ground state Fe$^{III}$ heme Soret), an increase in absorption at 390 nm on the microsecond timescale, and an increase in absorbance at 440 nm on the 10 ms – 1 s timescale. These features are distinct from $[\text{Ru}^{II}(\text{bpy})_2(\text{IAphen})]^3^+$ data in both timescale and wavelength profile, and suggest oxidation of the heme active site. The last TA feature is significantly affected by buffer pH, over the pH range of 6-8; the amplitude of the 440 nm feature is greatest at high pH (Figure 2.16).

Figure 2.15. Single-wavelength transient absorption following flash-quench ($\lambda_{\text{ex}}$= 480 nm) of Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ at pH 8. 420 nm (blue), 390 nm (dark blue), 440 nm (red). Inset: TA at 420 nm for Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ (blue) and [Ru$^{II}(\text{bpy})_2(\text{Aphen})]^2^+$ (orange).
Figure 2.16. pH dependence of RuK97C-P450BM3 transient absorption features. pH 6 (red), pH 7 (green), pH 8 (blue). Top: 390 nm. Bottom: 440 nm.
Kinetics Analysis of TA Data

In order to determine the number of kinetic phases, and therefore the number of potential intermediates formed by flash-quench, we performed a truncated generalized singular value decomposition analysis (tgSVD) of the TA data (Regularization Tools, Per Christian Hansen, see sample script in Appendix D) (Figure 2.17). The plot of the tgSVD shows the magnitude (y-axis) of the contribution of each rate constant \( k \) (x-axis) to the overall fitting of the transient absorption data. Grouping of the rate constants into five clusters indicates that as many as five distinct kinetic phases contribute to the recovery of TA signals to baseline.

**Figure 2.17.** tgSVD of TA data for Ru\(^{II}\)K97C-Fe\(^{III}\)P450 with [Ru(NH\(_3\))\(_6\)]\(^{3+}\) at six wavelengths. black: 390 nm; blue: 400 nm; cyan: 410 nm; green: 420 nm; yellow: 430 nm; red: 440 nm, in 50 mM borate buffer, pH 8.
The position of each of the clusters also gives a first-order estimate of the rate constant for each kinetics phase. These estimates greatly facilitate the success of multiexponential fitting procedures. Starting with these rate constants, we have performed a global least-squares fitting of the TA data recorded at six wavelengths (390, 400, 410, 420, 430, 440 nm), to a sum of five exponentials with amplitude coefficients \( \rho_{1-5} \) and observed rate constants \( \gamma_{1-5} \) (Equation 1.1) (see a sample fitting script in Appendix D).

**Equation 1.1:**

\[
TA(\lambda_n) = \rho_{n1} \exp(-\gamma_{1} t) + \rho_{n2} \exp(-\gamma_{2} t) + \rho_{n3} \exp(-\gamma_{3} t) + \rho_{n4} \exp(-\gamma_{4} t) + \rho_{n5} \exp(-\gamma_{5} t)
\]

Based on our interpretation of the nature and decay rate of the first transient absorption signal (*vide infra*), we can fix the first observed rate constant as that for the luminescence quenching (obtained from monoexponential fitting of the luminescence decay at 630 nm). The remaining four rate constants are extracted from the global fitting (Figure 2.18), and are listed in Table 2.2. We find analogous trends in the data from pH 6 to 8.
Figure 2.18. Global fitting of Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ TA data at pH 8. Light blue: data. Dark blue dashed: global fit.

Table 2.2. Observed rate constants ($\gamma_{1-5}, \text{s}^{-1}$) extracted from global fitting of single-wavelength TA at six wavelengths (390-440 nm).

<table>
<thead>
<tr>
<th>pH</th>
<th>$\gamma_1$</th>
<th>$\gamma_2$</th>
<th>$\gamma_3$</th>
<th>$\gamma_4$</th>
<th>$\gamma_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2.5(3)$\times$10$^7$</td>
<td>1.5(5)$\times$10$^6$</td>
<td>2.0(3)$\times$10$^5$</td>
<td>2(1)$\times$10$^4$</td>
<td>6(1)$\times$10$^3$</td>
</tr>
<tr>
<td>7</td>
<td>2.4(3)$\times$10$^7$</td>
<td>1.5(6)$\times$10$^6$</td>
<td>1.5(3)$\times$10$^5$</td>
<td>1.2(3)$\times$10$^4$</td>
<td>7(2)$\times$10$^3$</td>
</tr>
<tr>
<td>8</td>
<td>3.0(4)$\times$10$^7$</td>
<td>2(5)$\times$10$^6$</td>
<td>1.0(3)$\times$10$^5$</td>
<td>4(1)$\times$10$^4$</td>
<td>3(1)$\times$10$^3$</td>
</tr>
</tbody>
</table>

2.4. Discussion

The five distinct kinetics phases suggest that six distinct species are formed following excitation of the Ru photosensitizer. The first transient species is easily identified as excited *Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$, by comparison to TA signals of the un-
quenched Ru-P450 conjugate and of the free, quenched photosensitizer. The second species is formed in the quenching reaction with $[\text{Ru(NH}_3)_6]^{3+}$, and can be identified as $\text{Ru}^{\text{III}}_{\text{K97C}}\text{-Fe}^{\text{III}}_{\text{P450}}$ using similar logic. The final species formed is $\text{Ru}^{\text{II}}_{\text{K97C}}\text{-Fe}^{\text{III}}_{\text{P450}}$, since all TA signals return to baseline; this, along with sample stability over thousands of flash-quench cycles, suggests full reversibility of the photo-triggered reaction. Three transient species remain to be identified; these are most likely associated with oxidation of the P450 heme center, as they are distinct from any TA signals observed for the free photosensitizer. We refer to these species as $\text{P450(OX1-3)}$ in the discussion below.

2.4.1. Kinetics Model

We have developed a sequential kinetics model for the $\text{Ru}^{\text{II}}_{\text{K97C}}\text{-Fe}^{\text{III}}_{\text{P450}}$ TA data (Figure 2.19).

![Scheme for photochemical oxidation of cytochrome P450](image)

**Figure 2.19.** Scheme for photochemical oxidation of cytochrome P450. Elementary rate constants $k_0$-$k_7$ are shown for the proposed reactions. Unidentified oxidized P450 species ($\text{P450(OX1-3)}$) are highlighted in red.

We have solved the rate law for this model, allowing us to express the observed rate constants ($\gamma_1$-$5$) and amplitude coefficients ($\rho_1$-$5$) in terms of nine elementary rate constants ($k_0$, $k_1$, $k_2$, $k_3$, $k_4$, $k_5$, $k_6$, $k_7$) and the initial $*\text{Ru}^{\text{II}}_{\text{K97C}}\text{-Fe}^{\text{III}}_{\text{P450}}$ concentration. This method is used to extract unscaled difference spectra for each transient species (Figure 2.20, see Appendix C for details).
This system is underdetermined; there are nine elementary rate constants in the kinetics model, but only five observed rate constants can be determined from exponential fitting. Therefore, the parameters in the kinetics model cannot be determined by the TA data alone. Values for $k_1$, $k_3$, $k_5$, and $K_{eq} = k_6/k_6$ must be supplied in order to determine molar difference spectra for the six intermediate species. The known spectra of *Ru$^{II}$- and Ru$^{III}$-diimine species constrain the possible values of $k_1$ and $k_3$. The balance between $k_5$ and $k_7$ has no effect on the relative difference spectra extracted from the data, so $k_5$ was set equal to $k_7$. The equilibrium constant $K_{eq}$ was optimized to provide the best agreement between the transient difference spectra recorded at the three different pH values (Figure 2.20).

**Figure 2.20.** Extracted difference spectra of intermediate species. **Top:** Ru-based intermediates. **Bottom:** Oxidized P450 intermediates (P450$^{OX1-3}$).
Table 2.3. Extracted kinetics parameters. **Top**: Rate constants $k$ (s$^{-1}$) for the kinetics model of flash-quench. **Bottom**: $K_{eq}=k_5/k_6$; $\phi_{ET}$ and $\phi_{ET}$ are the yields for formation of *Ru$^{$II$}$ and Ru$^{$III$}$, respectively.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_0$</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_3$</th>
<th>$k_4$</th>
<th>$k_5$</th>
<th>$k_6$</th>
<th>$k_7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>$5 \times 10^6$ (0.3 \times 10^6)</td>
<td>$2 \times 10^7$ (0.3 \times 10^7)</td>
<td>$1 \times 10^5$ (0.4 \times 10^5)</td>
<td>$4 \times 10^5$ (0.8 \times 10^5)</td>
<td>$2 \times 10^4$ (0.2 \times 10^4)</td>
<td>$7 \times 10^4$ (1.6 \times 10^4)</td>
<td>$1 \times 10^4$ (0.5 \times 10^4)</td>
<td>$1.5 \times 10^4$ (0.7 \times 10^4)</td>
</tr>
<tr>
<td>7</td>
<td>$5.5 \times 10^6$ (0.3 \times 10^7)</td>
<td>$1.5 \times 10^7$ (0.3 \times 10^7)</td>
<td>$1 \times 10^6$ (0.6 \times 10^6)</td>
<td>$5.5 \times 10^5$ (0.3 \times 10^5)</td>
<td>$1.5 \times 10^5$ (0.1 \times 10^5)</td>
<td>$8 \times 10^4$ (0.3 \times 10^4)</td>
<td>$7 \times 10^3$ (3 \times 10^3)</td>
<td>$4.5 \times 10^3$ (1 \times 10^3)</td>
</tr>
<tr>
<td>8</td>
<td>$6 \times 10^6$ (0.3 \times 10^6)</td>
<td>$1.5 \times 10^7$ (0.6 \times 10^7)</td>
<td>$1 \times 10^6$ (0.6 \times 10^6)</td>
<td>$8.5 \times 10^5$ (2 \times 10^5)</td>
<td>$1 \times 10^5$ (0.2 \times 10^5)</td>
<td>$4 \times 10^4$ (0.3 \times 10^4)</td>
<td>$3.5 \times 10^3$ (0.7 \times 10^3)</td>
<td>$3.5 \times 10^2$ (0.8 \times 10^2)</td>
</tr>
</tbody>
</table>

The difference spectra of *Ru$^{$II$}$$_{K97C}$-Fe$^{$III$}$$_{P450}$ and Ru$^{$III$}$$_{K97C}$-Fe$^{$III$}$$_{P450}$ extracted from the kinetics analysis exhibit bleaching at 430 nm of the Ru$^{2+}$ MLCT absorption band. Of primary interest are the difference spectra corresponding to intermediates labeled P450$_{OX1-3}$.

As discussed in Chapter 1, flash-quench oxidation of horseradish peroxidase (HRP) and microperoxidase-8 (MP-8) first proceed by transient oxidation of the porphyrin ring (Fe$^{III}$-OH$_2$(P*)); subsequent internal rearrangement and deprotonation led to the ferryl, Fe$^{IV}$=O(P), product (CII). The initial porphyrin radical in HRP and MP-8 are also characterized by a bleach of the heme Soret.$^{15,16}$ The blue shift in absorption for these porphyrin radical intermediates is also consistent with synthetic models of Fe(III)-porphyrin cation radicals.$^{26}$

The spectra of P450$_{OX1}$ and P450$_{OX2}$ are quite similar, and are also characterized by a bleach of the Soret absorption band (centered at 420 nm). The spectrum of P450$_{OX2}$ displays somewhat more absorbance at 390 nm than that of P450$_{OX1}$, but
otherwise closely resembles that of P450OX1. Reasoning by analogy to our results on the oxidation of HRP and MP8, and the similarity of their difference spectra, we suggest that P450OX1 and P450OX2 correspond to six-coordinate porphyrin radical cations: RuII-K97C-FeIII(OH2)P*+(A)P450 and RuII-K97C-FeIII(OH2)P*(B)P450.

The apparent equilibrium constant between P450OX2 and P450OX3 varies with pH ($K_{eq}$: 0.8, pH 6; 2.9, pH 7; 10, pH 8), suggesting that a proton is lost in the formation of P450OX3. Moreover, the difference spectrum for this species indicates a red-shifted Soret absorption band analogous to that reported for the FeIV(OH)P center in CPO CII,27 as well as photochemically-generated CII in HRP and MP-8.

Hence, we suggest that P450OX3 is RuII-K97C-FeIV(OH)P$_P$450. Internal charge transfer in FeIII(OH2)P*+(B)P450 is accompanied by rapid loss of a proton (possibly to water), producing FeIV(OH)P$_P$450. The formation of flash-quench generated CII in HRP was slower ($k_{obs}$ of 4.1 s$^{-1}$) due to rate-limiting water ligation. CII formation in P450 proceeds on the millisecond timescale because a water molecule already occupies the sixth coordination site of the ferric heme.

The specific rate of FeIII(OH2)P*+(A)P450 formation in our conjugate is comparable to that found for reconstituted myoglobin containing a heme tethered directly to Ru(diimine)$_2$$^{2+}$.28 This observation suggests that a favorable pathway couples the P450 porphyrin to RuIII, possibly involving the Trp96-heme propion ate hydrogen bond.29 The conversion of RuII-K97C-FeIII(OH2)P*+(A)P450 to RuII-K97C-FeIII(OH2)P*(B)P450 may be a consequence of changes in polypeptide or solvent conformation in the P450 heme pocket.50
Figure 2.21. Photo-triggered cycle for flash-quench oxidation of Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$.

All of these intermediates are short-lived; transient absorbance features return to baseline within 500 ms, indicating recovery of resting state Ru$^{II}_{K97C}$-Fe$^{III}(OH_2)P_{P450}$. We have modeled this process as recovery from both the ferryl species ($k_7$, Ru$^{2+}_{K97C}$-Fe$^{IV}(OH)P_{P450}$) and its porphyrin radical cation precursor ($k_5$, Ru$^{II}_{K97C}$-Fe$^{III}(OH_2)P^*_{(B)P450}$) (Fig. 4), but it is not possible to determine the two rate constants since equilibration between Ru$^{II}_{K97C}$-Fe$^{III}(OH_2)P^*_{(B)P450}$ and Ru$^{II}_{K97C}$-Fe$^{IV}(OH)P_{P450}$ is faster than the ground-state recovery process. The precise nature of the resting state recovery process remains unclear. It should be described by second-order reaction kinetics, but the experimental data are better modeled by a simple exponential process. Nevertheless, [Ru(NH$_3$)$_6$]$^{2+}$ seems to be involved in the recovery reaction because in the presence of [Ru(NH$_3$)$_6$]$^{3+}$ as quencher, transient absorption data are consistent over the course of multiple hours, and the Soret absorption band appears relatively unaffected after many rounds of flash-quench excitation. However, similar measurements with an irreversible quencher
([Co(NH$_3$)$_5$Cl]$^{3+}$) induce bleaching of the Soret band and rapid sample degradation.

### 2.5. Concluding Remarks

We have developed a flash-quench method to oxidize the buried resting ferric aquo state of the P450-BM3 heme domain to CII without the use of reactive oxygen species (O$_2$, H$_2$O$_2$). The catalytic cycle runs in reverse by photochemically splitting water at the heme site. It is likely that the observed ferryl species is protonated over the pH range of 6-8, as consistent with the current view of chloroperoxidase and P450 CII. The finding that internal oxidation of the iron center is rate limiting has allowed us to observe porphyrin radical cation intermediates. As porphyrin oxidation occurs on the microsecond timescale, we can reasonably expect a second round of flash-quench on photochemically generated CII to produce CI.

Efforts toward this second oxidation step will be greatly facilitated by a better understanding of the coupling pathway between the photosensitizer and heme, and development of a system in which CII is stable on the order of seconds-to-minutes. Examination of the role of W96 and implementation of the photochemical system in the thermophilic P450 CYP119 are described in Chapter 3.

### 2.6. Acknowledgments

This effort would not have been possible without the ground work laid by Lionel Cheruzel, including design of the mutant, development of labeling protocols, crystalization the Ru-P450 conjugate, and patient training of graduate students (myself). Jay Winkler’s guidance and insights were critical for navigating the
intricate details of data analysis, fitting, and kinetics modeling. The synthesis of photosensitizer model compounds (iodo-free [Ru(bpy)$_2$(Aphen)]$^{2+}$), and acquisition of steady state fluorimetry data, was done by Katja Luxem.

2.7. Materials and Methods

Chemicals

Buffer salts were obtained from J.T. Baker. Dicyclohexylcarbodiimide (DCC), iodoacetic acid, and 5-amino-1,10-phenanthroline were obtained from Sigma Aldrich. Ru(2,2'-bipyridine)$_2$Cl$_2$ and [Ru(NH$_3$)$_6$]Cl$_3$ were obtained from Strem Chemicals; these were used without further purification. Mutagenesis primers were obtained from Operon. Solutions were prepared using 18 MΩcm water unless otherwise noted.

Procedures

A detailed description of experimental protocols can be found in Appendix B. A brief description of the procedures is given below, highlighting any deviations from the general protocol. All images of protein crystal structures in this Chapter were made using PyMol graphics software for Mac.

2.7.1. Ru photosensitizer

Synthesis

This photosensitizer was synthesized according to published procedures. Briefly, iodoacetic anhydride was formed by addition of N,N’-Dicyclohexylcarbodiimide (DCC) to a solution of iodoacetic acid in ethyl acetate. Following removal of urea by filtration and evaporation to dryness, the iodoacetic anhydride in acetonitrile
was added to a solution of 5-amino-1,10-phenanthroline in acetonitrile and stirred overnight at room temperature. Solid product (5-iodoacetamido-1,10-phenanthroline, (IAphen)) was refluxed with Ru(bpy)$_2$Cl$_2$ in methanol for 3 hours; a color change from purple to red is observed. After cooling and filtration, product can be precipitated by addition of concentrated aqueous NH$_4$PF$_6$. Alternatively, the compound can be concentrated and redissolved in water without further purification. The starting material impurities do not interact with P450, and do not appear to impact the labeling process.

Characterization

The photosensitizer was characterized by nuclear magnetic resonance (NMR), steady-state luminescence, and transient luminescence and absorption.

2.7.2. Mutagenesis and expression of P450-BM3 mutants

Plasmid

The recombinant P450-BM3 heme domain, consisting of the first 463 residues with an N-terminal 6-histidine tag, was obtained courtesy of Andrew Udit (Occidental College, Los Angeles California), within the pCWori$^+$ vector, which also contains genes for ampicillin resistance and IPTG induction.

Mutagenesis

The triple mutant C62A/C156S/K97C was made using Qiagen QuikChange site-directed mutagenesis using the following primers:

CTAATTAAAGAAGCAGCCGATGAATCACG (C62A),
CGATTGGTCTTAGCGGCTTTAAC (C156S),

was added to a solution of 5-amino-1,10-phenanthroline in acetonitrile and stirred overnight at room temperature. Solid product (5-iodoacetamido-1,10-phenanthroline, (IAphen)) was refluxed with Ru(bpy)$_2$Cl$_2$ in methanol for 3 hours; a color change from purple to red is observed. After cooling and filtration, product can be precipitated by addition of concentrated aqueous NH$_4$PF$_6$. Alternatively, the compound can be concentrated and redissolved in water without further purification. The starting material impurities do not interact with P450, and do not appear to impact the labeling process.

Characterization

The photosensitizer was characterized by nuclear magnetic resonance (NMR), steady-state luminescence, and transient luminescence and absorption.

2.7.2. Mutagenesis and expression of P450-BM3 mutants

Plasmid

The recombinant P450-BM3 heme domain, consisting of the first 463 residues with an N-terminal 6-histidine tag, was obtained courtesy of Andrew Udit (Occidental College, Los Angeles California), within the pCWori$^+$ vector, which also contains genes for ampicillin resistance and IPTG induction.

Mutagenesis

The triple mutant C62A/C156S/K97C was made using Qiagen QuikChange site-directed mutagenesis using the following primers:

CTAATTAAAGAAGCAGCCGATGAATCACG (C62A),
CGATTGGTCTTAGCGGCTTTAAC (C156S),
GCTGGACGCATCAAAAAAATTGGTGCAAAGCGC (K97C). The P450 gene was sequenced (Laragen) to verify successful mutagenesis.

Expression

P450 enzymes were overexpressed in *E. coli*. Briefly, overnight Luria Bertani medium cultures were used to inoculate 1x Terrific Broth induction cultures with added mineral supplements, shaken at 37 °C. At an OD(600 nm) ~1, the temperature was lowered to 30 °C, expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG), and α-aminolevulenic acid was added. After expression for 24 or 40 hours (for P450 BM3 and CYP119, respectively), cells were harvested by centrifugation and stored at −80 °C.

Extraction and purification

P450 enzymes were extracted by sonication in the presence of protease inhibitors (benzamidine hydrochloride and Pefabloc SC hydrochloride). After centrifugation, the supernatant was purified by nickel immobilized metal affinity chromatography on a batch column. Samples were further purified by FPLC anion exchange chromatography and gel filtration.

Purity was determined by UV-vis absorption (A<sub>418/A<sub>280</sub>), SDS-PAGE, and mass spectrometry. Dithiothreitol (DTT) was added to protein not intended for immediate use, and samples were flash-frozen in liquid nitrogen and stored at −80°C.
2.7.3. Ru-P450 conjugation

The complex $[\text{Ru(bpy)}_2(\text{IAphen})]^2^+ \ (\text{IAphen} = 5$-iodoacetamido-1,10-phenanthroline) was covalently coupled to triple mutant C62A/C156S/K97C (abbreviated as tK97C) at Cys97 to give the conjugate Ru$^{II}_{\text{K97C}}$-Fe$^{III}_{\text{P450}}$.

Briefly, approximately three-fold excess of $[\text{Ru}^{II}(\text{bpy})_2(\text{IAphen})]^2^+$ was added to a $\sim 10 \ \mu\text{M}$ P450 solution in 20 mM Tris buffer, pH 8. The reaction solution shaken gently for $\sim 4$ hours at 4 °C in the dark, followed by desalting to remove excess photosensitizer and purification of Ru-labeled and unlabeled enzymes by anion exchange chromatography on an MonoQ or HiPrepQ FPLC column.

This conjugate was characterized by mass spectrometry, UV-Vis and luminescence spectroscopies (steady state, time-resolved), and X-ray crystallography. The conjugate Ru$^{II}_{\text{K97C}}$-Fe$^{III}_{\text{P450}}$ demonstrates activity in the hydroxylation of lauric acid via the peroxide shunt.$^{31}$

2.7.4. Crystallization and structure determination

Crystals of Ru$^{II}_{\text{K97C}}$-Fe$^{III}_{\text{P450}}$ were obtained by the sitting-drop vapor diffusion method: 27 mg/ml Ru$^{II}_{\text{K97C}}$-Fe$^{III}_{\text{P450}}$ in 10 mM potassium phosphate, pH 8.4 was mixed with a crystallization well solution of 2 M (NH$_4$)$_2$SO$_4$ (w/v) in a 1:1 ratio (v/v). Crystals formed over a period of 2 days at 4 °C, and were flash frozen directly from the crystallization solution. X-ray diffraction data were collected at 100 K using beamline 7-1 at the Stanford Synchrotron Radiation Laboratory. Diffraction data were processed with Mosflm and Scala. Initial model for the Ru$^{II}_{\text{K97C}}$-Fe$^{III}_{\text{P450}}$ structure was derived from the palmitic acid-bound P450-BM3 structure (pdb ID 2UWH) by molecular replacement using Molrep. Coot and Refmac5 were used for model fitting and refinement. The final models were
validated using the programs Procheck, Sfcheck and Molprobity. Most of the above processes were done with the graphical interface to the CCP4 program suite. All structural graphics were generated using the Pymol Graphics System.

Statistics for data collection and refinement are shown in the Table S1. Atomic coordinates and structure factors were deposited in the Protein Data Bank under the entry 3NPL.

2.7.5. Preparation of laser samples

Laser samples were composed of either Ru$^{II}_{K97C}$-Fe$^{III}_{P450}$ or the model complex [Ru(bpy)$_2$(IAphen)]$^{2+}$ ($\sim$10 μM), with and without oxidative quencher (17 mM [Ru(NH$_3$)$_6$]Cl$_3$) in buffered solution (pH 6: 20 mM sodium acetate; pH 7: 20 mM sodium acetate; pH 8: 50 mM sodium borate or 50 mM Tris; additionally, each buffer contained sodium chloride). Samples were placed in a high-vacuum four-sided quartz cuvette, equipped with a small stir bar. Deoxygenation was achieved via gentle pump-backfill cycles with argon.

For acquisition of time-resolved fluorescence and transient absorption data, samples were excited with 8 ns laser pulses at 480 nm. Luminescence decays were monitored at 630 nm. Single wavelength transient absorption (TA) kinetics were monitored every 10 nm from 390-440 nm, averaging ~500 shots per wavelength. Data from five separate timescales (2 μs, 40 μs, 400 μs, 10 ms, and 500 ms) were collected, log-compressed, and spliced together to produce full kinetics traces. See Appendix D for log-compression and splicing scripts in Matlab.
2.8. References


(14) Sutin, N.; Creutz, C. Properties and Reactivities of the Luminescent Excited States of Polypyridine Complexes of Ruthenium(II) and Osmium(II).
Inorganic and Organometallic Photochemistry; Advances in Chemistry; American Chemical Society: Washington, DC, 1978; Vol. 1.


