CHAPTER FOUR

Controlling Electron Hopping

4.1 ABSTRACT

Systematic perturbations were made to the working hopping system Re(dmp)$(CO)_3$(H124)/W122/Az(Cu^+). All together, eight metal-modified azurins were made for the studies. Ruthenium and rhenium labels are attached to H124 or H126; tryptophan and 3-nitrotyrosine are installed at the 122 site. More often than not, electron transfer is observed between the amino acid and the metal label. However, subsequent electron transfer from the copper to the oxidized amino acid does not occur, except in the case of one mutant, Re126/W122/Az(Cu^+). The electron transfer kinetics observed on this system indicate that a second hopping system has been discovered.

4.2 INTRODUCTION

The System

With the working hopping system Re(dmp)$(CO)_3$(H124)/W122/Az(Cu^+) in hand, attention turned towards: 1) installing a nitrotyrosine at the 122 site and observing hopping through the tyrosine analog; 2) perturbing the pseudo-stacking interaction that had been observed between the W122 and the dmp ligand of the rhenium (**Chapter 3**, Structural Characterization); and 3) challenging the robustness of the working hopping system by changing the driving forces of the first electron transfer reaction and the overall process. To that end, the proteins studied were still on the Met121 arm; labeling

was done on either the 124 site or the 126 site, which is estimated to be another 5 Å away from the copper center. A high-potential ruthenium label was used to perturb the reduction potential of the metal label and 3-nitrotyrosine was also installed in the 122 site to perturb the potential at the amino acid site.

Chapter Outline

The chapter is divided into two components. In the first, the metal labels and intermediates are changed, keeping the two sites constant: the metal label is installed at H124 and the intermediate amino acid is installed at the 122 site. In the second part of the chapter, the label is moved two amino acid residues farther down the Met121 arm, increasing distance between the two metal and label another ~5 Å.

4.3 RESULTS AND DISCUSSION

The Importance of Reduction Potentials in Hopping

The proteins discussed in this section include Ru124/W122/Az(Cu^{2+}), Ru124/W122/Az(Zn^{2+}), Ru124/F122/Az(Zn^{2+}), Ru124/$YNO_2$122/Az(Cu^{2+}), and Re124/$YNO_2$122/Az(Cu^{2+}). These proteins were prepared using the methods described in **Chapter 2**. Zn^{2+}-substituted azurins are used for control measurements; Zn^{2+} does not react, and so any electron transfer observed in these systems should be between the aromatic amino acid residue and the metal label.

Ru124/W122/Az(Cu^{2+})

In the first system discussed, a high-potential ruthenium dye $[Ru(trpy)(tfmbpy)]^{2+}$ replaces the Re at the 124 site.

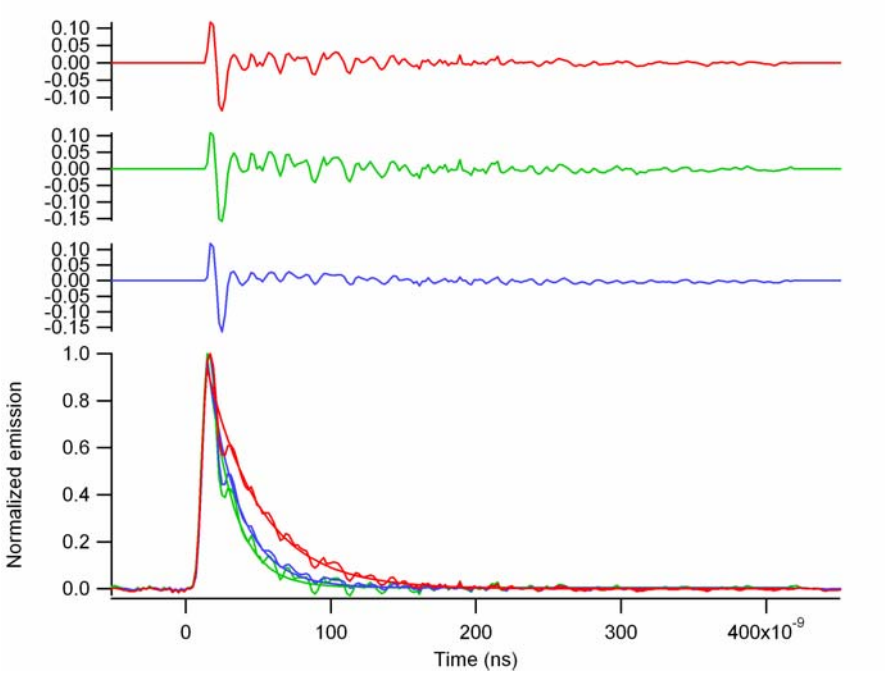


Figure 4.1. Time-resolved emission of Ru124/W122/Az(Cu^{2+}) and (Cu^{+}). 10 μM Ru124/W122/Az(Cu^{2+}), 25 mM KP_i, pH 7.14. λ_{ex} = 490 nm, λ_{em} = 700 nm. Red trace is Ru model compound, $[Ru(trpy)(tfmbpy)(im)](PF_6)_2$ in 25 mM KP_i, pH 7.16, τ = 37 ns. Green trace is Ru124/W122/Az(Cu^{2+}), τ = 18 ns. Blue trace is Ru124/W122/Az(Cu^{+}), τ = 18 ns.

The time-resolved emission data on Ru124/W122/Az(Cu^{+}) and Ru124/W122/Az(Cu^{2+}) both show the same diminished lifetime of the excited state $*Ru^{2+}$ compared to that of the model compound (**Figure 4.1**). This indicates that the excited state is being quenched in the system. Because the lifetime was the same in both Cu^{+} and Cu^{2+} states, the quenching is likely not due to copper. In comparison, the $*Ru^{2+}$ in Ru124/F122/Az(Zn^{2+}) was measured to have a lifetime of 40 ns, indicating that the diminishment of lifetime is likely due to the W122. Time-resolved absorption data recorded at the 510 nm wavelength, which tracks the ground state Ru^{2+}, first displays a

bleach, and then quick recovery (**Figure 4.2**). It is perhaps unfair to discuss the lifetimes extracted from the fitting of this data; the signal is quite small, and lifetimes on the order of 10 ns do test the instrument's response. However, it is clear from the time-resolved absorption spectroscopy that Ru^{2+} is recovered faster in these systems than it is in the model compound. More importantly, no prolonged bleaching is evident in these systems, indicating that the subsequent oxidation of Cu^{+} in the Cu^{+} case does not occur, as Ru^{2+} would not recover so quickly if it did. Rather, what likely happens is that tryptophan oxidizes the $*Ru^{2+}$, and very quick recoupling occurs, faster than the initial quench. This back reaction is still faster than deprotonation of the tryptophan radical cation (**Scheme 4.2**).

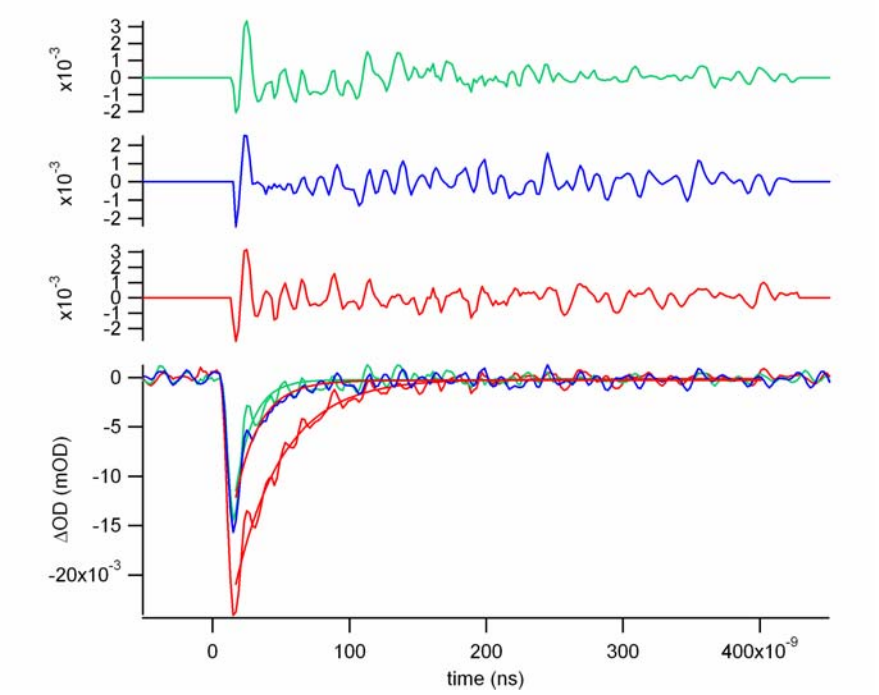
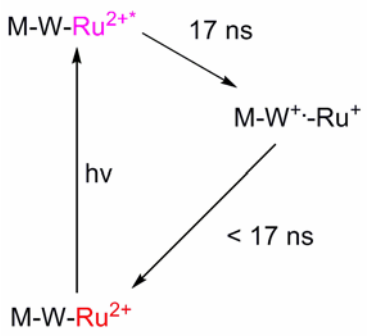


Figure 4.2. Transient absorption of Ru124/W122/Az(Cu^{2+}) and (Cu^{+}). λ_{ex} = 490 nm, λ_{obs} = 510 nm. Red trace is Ru model compound, [Ru(trpy)(tfmbpy)(im)]$(PF_6)_2$ in 25 mM KP_i, pH 7.16, τ = 37 ns. Green trace is 10 μM Ru124/W122/Az(Cu^{2+}), 25 mM KP_i, pH 7.16, τ = 12 ns. Blue trace is 10 μM Ru124/W122/Az(Cu^{+}), 25 mM KP_i, pH 7.16, τ = 17 ns.



Scheme 4.1. Events after sample excitation in Ru124/W122/Az($Cu^{2+/+}$) systems. Colors indicate species observed spectroscopically.

Re124/$YNO_2$122/Az(Cu^{2+})

In this system, the perturbation was at the amino acid site; the rhenium label was installed at 124, and 3-nitrotyrosine was installed at the 122 site.

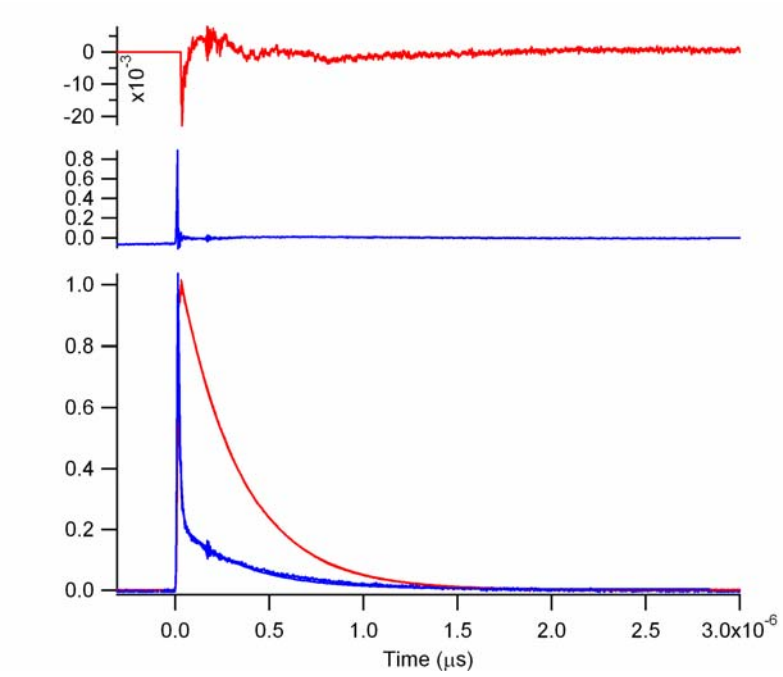
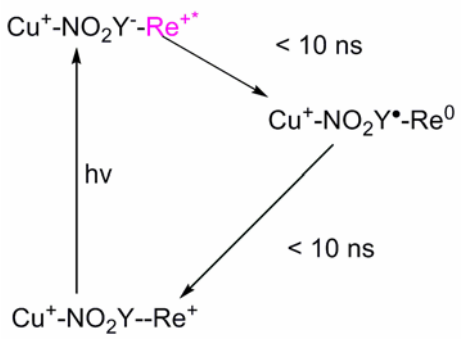


Figure 4.3. Time-resolved emission of Re124/$YNO_2$122/Az(Cu^{+}). ~40 μM Re124/$YNO_2$122/Az(Cu^{2+}), 25 mM KP_i, pH 7.8. λ_{ex} = 355 nm, λ_{em} = 560 nm. Red trace is Re model compound, [Re(dmp)$(CO)_3$(im)]OTf in 25 mM KP_i, pH 7.8, τ = 320 ns. Blue trace is Re124/$YNO_2$122/Az(Cu^{+}), which was fit to a function with two decays, one set at 320 ns. τ = 10 ns

Time-resolved emission data revealed extremely promising results (**Figure 4.3**). While the excited state $*Re^{+}$ of the model compound lives quite long, the $*Re^{+}$ in $Re124/YNO_2122/Az(Cu^{+})$ does not live long at all; indeed, if the data were fit to a function with biphasic decay, the faster phase is fitted to a value of around 10 ns, which challenges the instrument response of the Nanosecond-I laser setup. It is unsurprising that the transient absorption studies done at 434 nm to track the YNO_2^{-} state garnered inconclusive, small bleaches that were too narrow to be considered more than laser scatter; the kinetics are likely too fast for accurate data to be obtained on the 10 ns laser setup. Disappointingly, no Cu^{2+} generation was found when probed for at 632.8 nm, so no subsequent electron transfer between the copper and oxidized nitrotyrosyl radical is observed. Time-resolved emission data done on the 10 ps laser was attempted but frustrated when it was observed that the sample had undergone some aggregation (owing to excessive exposure to H_2/Pt reduction conditions).

The extremely efficient quenching of the $*Re^{+}$ state indicates that electron transfer between the nitrotyrosine residue and the $*Re^{+}$ is much more favorable than that seen in the Re124/W122 case; this is possible if the driving force of this electron transfer is no longer near that of the $*Re^{+}$, but rather, larger and downhill, making the transfer closer to activationless in barrier. $*Re^{+}$ emission data and lack of Cu^{2+} formation indicate that quick charge recombination of the reduced Re^{0} and $YNO_2^{\bullet}$ occurs, owing to a combination of the close distance between the metal label and amino acid residue, and a better driving force for the charge recombination than $Cu^{+}/YNO_2^{\bullet}$ electron transfer.



Scheme 4.2. Events after sample excitation in Re124/YNO$_2$122/Az(Cu$^+$) systems. Colors indicate species observed spectroscopically.

Ru124/YNO$_2$122/Az(Cu^{2+})

In this system, the perturbation was at the both the label and amino acid sites; a ruthenium label was installed at 124, and 3-nitrotyrosine was installed at the 122 site.

Because E°(*Ru^{2+}/Ru$^+$) did not seem to be as high as E°(*Re$^+$/Re^0), it was hoped that more spectroscopic information could be garnered on these systems; the driving force of the initial electron transfer would not be as high, slowing the electron transfer enough for detection with the 10 ns laser system. This hypothesis was true to a certain extent; quenching of the excited state was clear. However, to extract truly accurate kinetics data on this system, measurements done on a faster laser system will be necessary.

Studies done on the Ru124/YNO$_2$122/Az(Cu^{2+}) system were conducted at pHs 4.71 and 7.71 in the hopes of proving NO$_2$Y$^-$ as an electron donor in the quenching of the *Ru^{2+}; at pH 4.71, the nitrotyrosine residue would be protonated, and so would likely not participate in electron transfer.

So it was surprising when it was observed that both protonated and deprotonated samples exhibited quenching of the *Ru^{2+} excited state, regardless of the copper oxidation state (**Figures 4.4 and 4.5**). The fits to the faster decays in both emission and

transient absorption are for mere comparison; again, these lifetimes challenge the response time of the instrument; for data that appropriately details the events of $*Ru^{2+}$, measurements ought to be done with a faster laser system. Quenching in either pH is within error, so it is also unfair to suppose that one sample displays quenching to a greater extent to the other. What *is* clear from **Figures 4.4 and 4.5**, however, is that the $*Ru^{2+}$ is quenched at both pH 4.71 and 7.71. No Cu^{2+} formation was observed at either pH in the Cu^{+} measurements, indicating that the process is once again restricted to electron transfer between the metal label and the residue. To probe for NO_2Y^{-} bleaching, the transient absorption was measured at 434 nm in the pH 7.71 samples. Unfortunately, the broad MLCT of the Ru^{2+} overlaps at this wavelength, so it is unclear whether or not the bleach observed at this wavelength is from the ruthenium or from the nitrotyrosine; ironically, this is a case where the very reason the ruthenium label was pursued frustrated efforts to understand the data.

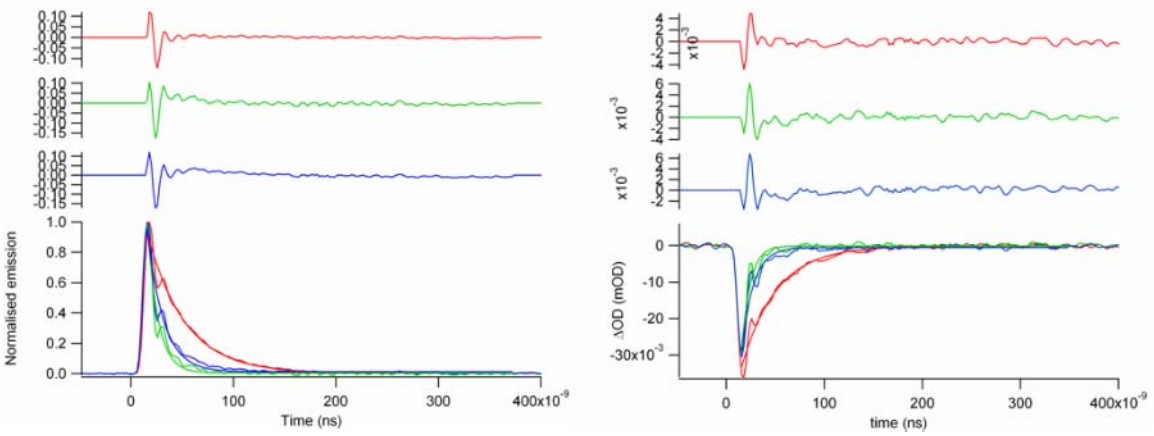


Figure 4.4. Time-resolved UV-VIS Spectroscopy of Ru124/$YNO_2$122/Az(Cu^{2+}) at pH 4.7 and 7.7. 10 μM Ru124/$YNO_2$122/Az(Cu^{2+}), 25 mM KP_i, pH 7.71 (blue), 25 mM NaOAc, pH 4.71 (green). Red trace is Ru model compound, [Ru(trpy)(tfmbpy)(im)]$(PF_6)_2$ in 25 mM KP_i, pH 7.71. **Left:** λ_{ex} = 490 nm, λ_{em} = 700 nm. Each are fit to single exponential decay. Label τ = 36 ns, pH 4.71 τ = 10 ns, pH 7.71 τ = 15 ns. **Right:** λ_{ex} = 490 nm, λ_{obs} = 510 nm. Label τ = 35 ns, pH 4.71 τ = 8 ns, pH 7.71 τ = 11 ns

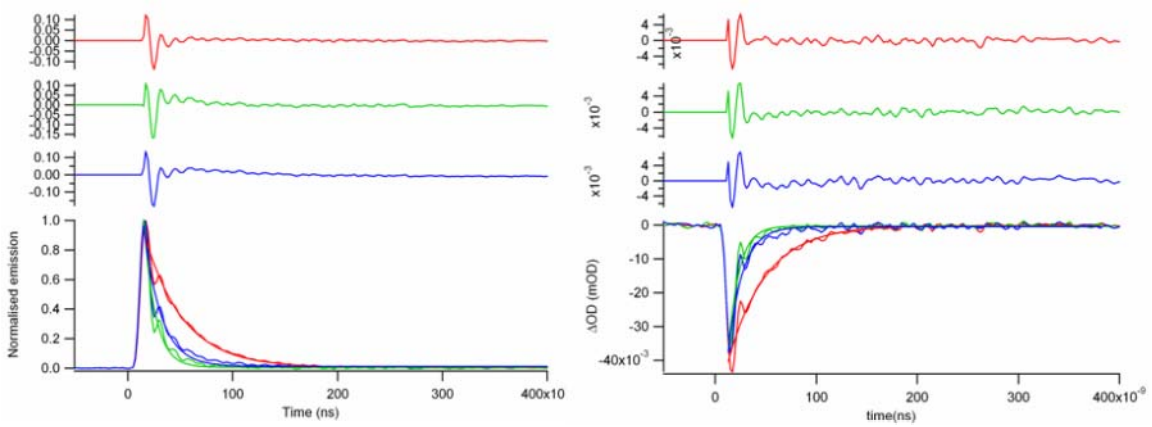


Figure 4.5. Time-resolved UV-VIS Spectroscopy of Ru124/$YNO_2$122/Az(Cu^+) at pH 4.7 and 7.7. 10 μM Ru124/$YNO_2$122/Az(Cu^{2+}), 25 mM KP_i, pH 7.71 (blue), 25 mM NaOAc, pH 4.71 (green). Red trace is Ru model compound, [Ru(trpy)(tfmbpy)(im)]$(PF_6)_2$ in 25 mM KP_i, pH 7.71. **Left:** λ_{ex} = 490 nm, λ_{em} = 700 nm. Each are fit to single exponential decay. Label τ = 36 ns, pH 4.71 τ = 11 ns, pH 7.71 τ = 16 ns. **Right:** λ_{ex} = 490 nm, λ_{obs} = 510 nm. Label τ = 37 ns, pH 4.71 τ = 11 ns, pH 7.71 τ = 14 ns

Still, the data is confusing; is the $*Ru^{2+}$ quenched by protonated or deprotonated nitrotyrosine? An argument can be made that at pH 7.71, not all the nitrotyrosine is deprotonated, and that what quenching is observed is from the protonated state. That is a possibility. Another is that the reduction potentials of both states are conducive to electron transfer. The conservative, completely correct assertion that can be made is that quenching occurs; the tyrosine analog 3-nitrotyrosine can participate in electron transfer. It is unclear what protonation state the residue is in, and there is even confusion of whether the nitrotyrosine is giving up or gaining the electron.

The Importance of Distance in Hopping

The proteins discussed in this section include Ru126/W122/Az(Cu^{2+}), Re126/W122/Az(Cu^{2+}), and Re126/F122/Az(Cu^{2+}). These proteins were prepared using the methods described in **Chapter 2**.

Ru126/W122/Az(Cu^{2+})

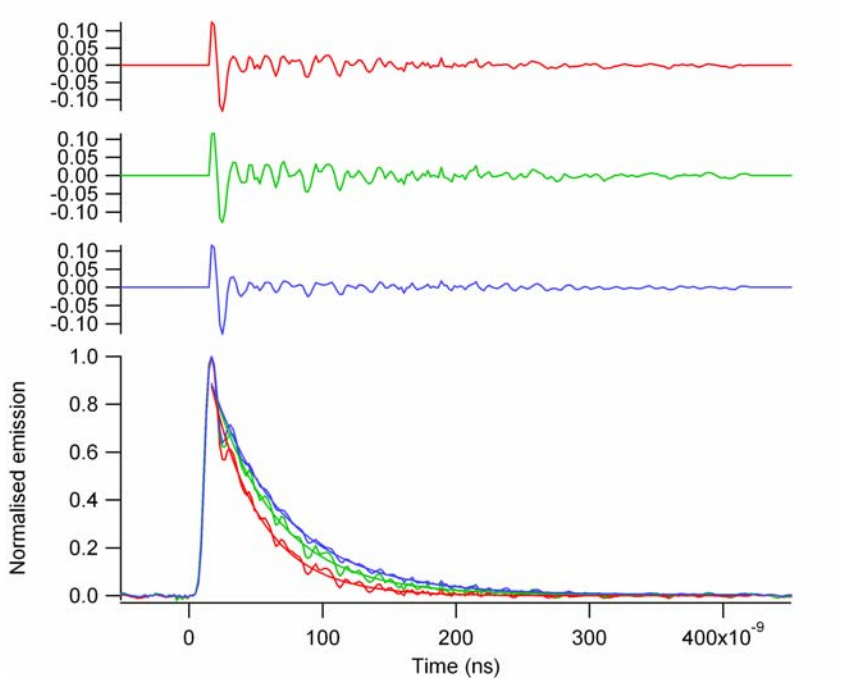


Figure 4.6. Time-resolved emission of Ru126/W122/Az(Cu^{2+}) and (Cu^{+}). 10 μM Ru126/W122/Az(Cu^{2+}), 25 mM KP_i, pH 7.14. λ_{ex} = 490 nm, λ_{em} = 700 nm. Red trace is Ru model compound, [Ru(trpy)(tfmbpy)(im)]$(PF_6)_2$ in 25 mM KP_i, pH 7.16, τ = 37 ns. Green trace is Ru126/W122/Az(Cu^{2+}), τ = 48 ns. Blue trace is Ru126/W122/Az(Cu^{+}), τ = 52 ns.

The attraction of labeling at the 126 site is that it is hoped that by placing the label farther away, the previously observed charge recombination between $W^{\bullet+}$ and Ru^{+} would be slowed enough so that $Cu^{+}/W^{\bullet+}$ electron transfer could be observed. Any pseudo π-stacking interaction between the metal label's ligand and the tryptophan would be disrupted.

The emission data of Ru126/W122/Az($Cu^{2+/+}$) indicate that attaching the label at the 126 site placed the ruthenium too far away from the tryptophan for electron transfer to occur; the electronic coupling between the two centers is too small. The lifetime of the excited state *Ru^{2+} was long, and showed no quenching (**Figure 4.6**). Quenching the *Ru^{2+} state to generate the higher-potential Ru^{3+} was once more considered as an option. The other option was to install the rhenium label at the 126 site instead, taking advantage of its higher reduction potential.

Re126/W122/Az(Cu^{2+})

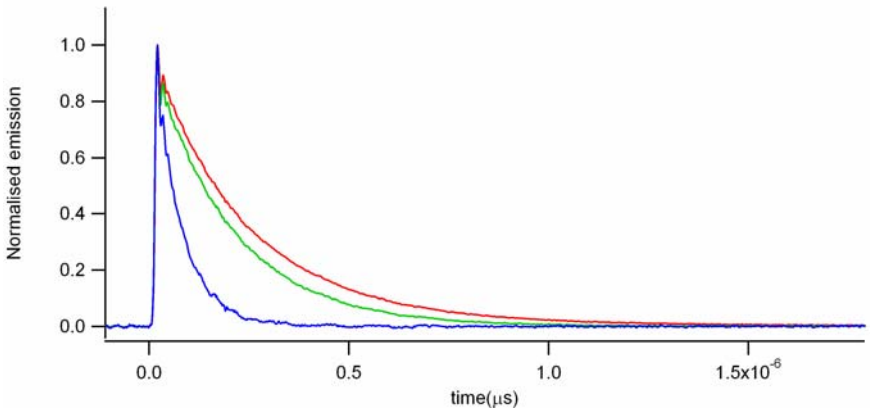


Figure 4.7. Time-resolved emission of Re126/W122/Az(Cu^{2+}) and (Cu^{+}). 10 μM Re126/W122/Az(Cu^{2+}), 25 mM KP_i, pH 7.28. λ_{ex} = 355 nm, λ_{em} = 560 nm. Red trace is Re126/W122/Az(Cu^{2+}). Green trace is Re126/W122/Az(Cu^{+}). Blue trace is Re126/W122/Az(Cu^{+}) + 10 mM $Ru(NH_3)_6Cl_3$.

The emission studies on the *Re^{+} excited state indicate that, when substituted at the 126 site, the rhenium label is simply too far away the participate in hopping the way it did in the working Re124/W122/Az(Cu^{+}) system (**Figure 4.7**). There was no quenching of the *Re^{+} in either Cu^{2+} or Cu^{+} protein. The quencher $Ru(NH_3)_6^{3+}$ had been utilized before for flash-quench experiments with the Re-modified azurin,[1] and so 10 mM

quencher was added to the sample to access the higher potential Re^{2+} state. Accessing this state drove the quick generation of Cu^{2+} within 100 ns (**Figure 4.8**).

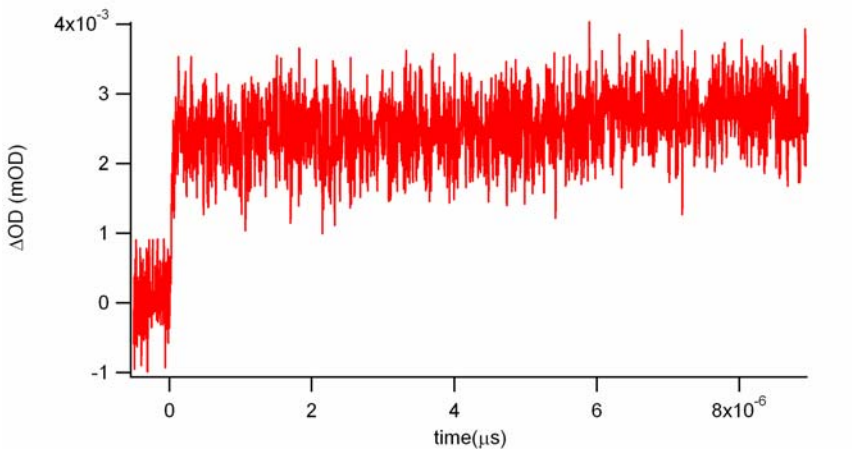
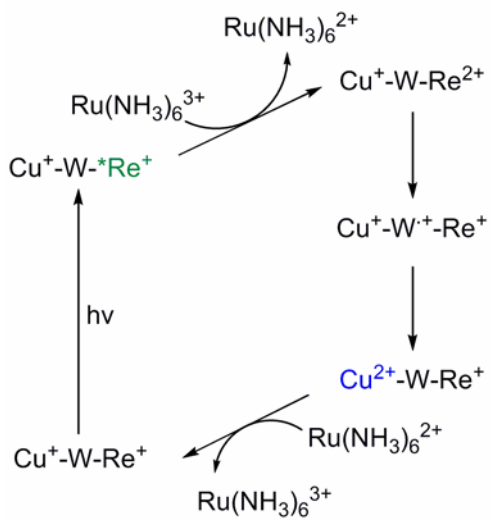


Figure 4.8. Transient absorption of Re126/W122/Az(Cu^{+}) with quencher. 10 μM Ru124/W122/Az(Cu^{+}), 10 mM $Ru(NH_3)_6Cl_3$, 25 mM KP_i, pH 7.28. λ_{ex} = 355 nm, λ_{obs} = 628 nm

The 100 ns generation of Cu^{2+} is much faster than the previously observed single-step electron transfer, which was measured to be on the order of hundreds of microseconds.[1,2] The presence of the tryptophan proved to be essential for Cu^{2+} formation, studies executed on the Re126/F122/Az(Cu^{+}) system did not reproduce the quick Cu^{2+} generation. The generation of Cu^{2+} could be accomplished through the multistep tunneling mechanism shown in **Scheme 3**. Upon excitation, the excited $*Re^{+}$ is oxidized by the quencher $Ru(NH_3)_6^{3+}$, generating the high-potential Re^{2+} state. The rhenium oxidizes the tryptophan, which in turn oxidizes the copper. The system is eventually reduced to its ground state through charge recombination with $Ru(NH_3)_6^{2+}$.

Structural characterization is needed to obtain the electron transfer distances. It is likely this system does not exhibit the same pseudo π-stacking to bring the metal label and tryptophan closer together.



Scheme 4.3. Events after sample excitation in Re126/W122/Az(Cu^+) with $Ru(NH_6)_3^{3+}$. Colors indicate species observed spectroscopically.

4.4 CONCLUSIONS

The experiments done to perturb the working Re124/W122/Az(Cu^+) system clearly demonstrate that a balance of driving force and distance between the three redox sites is necessary to achieve observable hopping kinetics. 3-Nitrotyrosine was shown to participate in electron transfer reactions, and it is clear that further investigations need to be made to understand its behavior. Shifting the labeling site two residues away shut down the original multistep tunneling mechanism; the rhenium and tryptophan were too far apart for electron transfer to occur. However, by oxidizing the excited state rhenium to its high-potential 2+ state, a new hopping system was discovered.

4.5 EXPERIMENTALS

The metal-modified proteins were prepared as described in **Chapter 2**. Laser spectroscopy studies were carried out as described in **Chapter 2**.

4.6 REFERENCES

(1) Miller, J. E., California Institute of Technology, 2003.

(2) Miller, J. E.; Di Bilio, A. J.; Wehbi, W. A.; Green, M. T.; Museth, A. K.; Richards, J. R.; Winkler, J. R.; Gray, H. B. *BBA Bioenergetics* **2004**, *1655*, 59–63.