CHAPTER ONE

Background & Research Plan

1.1 STATEMENT OF INTENT

Long-range electron transfer is a central component of processes that are essential for biological function. While many studies have been made to understand electron transfer in proteins, biologically efficient electron transfer at distances exceeding 25 Å remains unobserved in these experiments, and hence unresolved. It is proposed that long-range electron transfer is in actuality multistep electron tunneling. What is reported in this thesis is the design and synthesis of many protein systems for the purpose of studying multistep electron tunneling in azurin, two of which conclusively demonstrate the postulated phenomenon. This chapter gives a brief summary of current electron transfer theory, an overview of the metal-modified metalloprotein program, and outlines the various aspects of the research plan taken in this project.

1.2 ELECTRON TRANSFER IN PROTEINS

Electron Transfer Theory

Though not understood, the importance of electron transfer reactions in proteins has always been noted: in 1941, Szent-Györgyi observed that "electrons wander directly from enzyme to enzyme" in redox enzymes that were immobilized in membranes.[1] This electron "wandering" was much debated; popular models included electron "packets" traveling through a semiconductive of the protein medium,[1] as well as conformational changes bringing electron donors and acceptors in close contact for the reaction to occur.[2] In 1974, Hopfield proposed that the electrons tunneled through the protein medium the same way that particles could tunnel through energy barriers.[3] It is now accepted that electrons tunnel through a protein medium that is highly tuned to facilitate the efficient transfer of electrons. But what makes an electron transfer efficient?

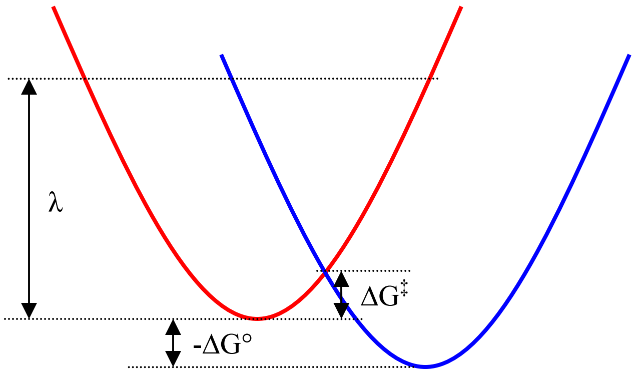

Figure 1.1. Potential energy curves. Representation of reactant (red) and product (blue) potential energy curves, with activation barrier ($\Delta G^{\ddagger}$), driving force ($-\Delta G^{\circ}$), and reorganization energy (λ) noted

A central tenet of electron transfer theory is the Franck-Condon principle, which states that because electrons move much faster than nuclei, the nuclei remain fixed during the actual reaction; therefore, the transition state of the reaction must be in a nuclear-configuration space where the reactant and product states are degenerate (in **Figure 1.1**, where the two energy curves intersect).[4,5] And so, the kinetics of the reaction are dependent on the activation barrier ($\Delta G^{\ddagger}$). According to Marcus theory, the activation barrier for adiabatic electron transfer reactions depends on the driving force ($-\Delta G^{\circ}$) and reorganization energy (λ).[6] The driving force is the difference between the reduction potentials of the electron donor (D) and acceptor (A). The reorganization energy comprises inner-sphere (ligand) and outer-sphere (solvent) nuclear rearrangements that accompany the electron transfer. In **Figure 1.1**, it is the energy of the reactants at the equilibrium nuclear configuration of the products. It can be observed from the exponential term in **Equation 1.1** that at low driving forces ($-\Delta G° < \lambda$), the rates increase with $-\Delta G°$. The rate will reach a maximum where the two values are equal, and then will decrease as $-\Delta G°$ continues to increase, which is also known as the inverted effect. A direct lesson from Marcus theory is that the nuclear rearrangements that accompany an electron transfer must be compensated by the reaction driving force.

In proteins, electron transfers are usually over fairly long distances. Electronic interaction between the two sites is weak, and the transition state must be formed many times before the electron transfer actually occurs, rendering the process non-adiabatic. This consideration is noted in the pre-exponential factor of **Equation 1.1**, in the electronic coupling matrix term H_{AB}.

$$k_{ET} = \sqrt{\frac{4\pi^3}{h^2 \lambda RT}} H_{AB}{}^2 \exp\left(-\frac{\left(\Delta G° + \lambda\right)^2}{4\lambda RT} \right) \qquad \text{(Eq 1.1)}$$

The electronic coupling matrix element is a description of how much overlap there is between the localized donor and acceptor wave functions; the more overlap, the better the electronic interaction. In proteins, H_{AB} is quite small. The distance dependence can be mathematically expressed, utilizing a decay factor, β, that was estimated by Hopfield to be approximately 1.4 Å^{-1} (**Equation 1.2**).[3] The larger β is, the more dependent on distance the coupling is.

$$H_{AB}(r) = H_{AB}(r_0) \exp\left(-\frac{\beta(r - r_0)}{2} \right) \qquad \text{(Eq 1.2)}$$

It is clear from **Equation 1.2** that the electron coupling between the electron donor and acceptor exhibits an exponential dependence on the distance between the two redox centers. This exponential dependence translates into an exponential dependence on distance for the rate of the electron transfer as well.

Is there a β that is universal to all proteins, or is β specific to each protein, each possible electron tunneling pathway? Though there was considerable argument for the former,[7] it has been observed the latter suggestion is a more accurate approximation: the bridging medium that connects donor and acceptor mediates the electronic coupling via superexchange.[8] Mathematically speaking, the medium is broken into *n* identical repeat units, and the electronic coupling matrix element is thereby described as a function of the coupling between the redox sites and their bridge (h_{Db}, h_{bA}), the coupling between the bridging units themselves (h_{bb}), and the energy required to actually place an electron or hole on the bridge ($\Delta\varepsilon$) (**Equation 1.3**).

$$H_{AB} = \frac{h_{Db}}{\Delta\varepsilon}\left(\frac{h_{bb}}{\Delta\varepsilon}\right)^{n-1} h_{bA} \qquad \text{(Eq. 1.3)}$$

Equation 1.3 as applied to biological systems is more useful for philosophical exercises than accurate calculation, because the bridging protein medium is in actuality a complex array of bonded and non-bonded contacts. In this large array, which route does the electron take? A general approach, taken by the persistent proponents of the universal β, has conceded the heterogeneity of the medium by including a modification to their theory, packing density parameter ρ (on a scale of 0 (vacuum) to 1.0 (completely packed medium)). This ρ was found to be on average 0.76 with a (rather large) standard deviation of about 0.10.[9] While this approach can offer a general idea of electron transfer kinetics, it does not offer the complete picture of the tunneling medium. The tunneling pathway model, which takes the more atomistic view, breaks the extensive arrays down into components linked by covalent bonds, hydrogen bonds, and through-space jumps.[10–16] Each component is assigned is own decay constant (ε_C, ε_H, ε_S, respectively). A structure-dependent searching algorithm is used to identify the tunneling pathway that best couples the two redox sites. The total electron coupling is expressed as a repeated product of the couplings for the individual components (**Equation 1.4**).

$$H_{AB} \propto \Pi \varepsilon_C \Pi \varepsilon_H \Pi \varepsilon_S \quad \text{(Eq 1.4)}$$

In summary, to render electron transfer efficient in biological systems, the protein fold creates a balance of driving force and reorganization energy. It provides adequate electronic coupling between the donor and acceptor, a well-engineered system of covalent bonds, hydrogen bonds, and through-space jumps through which the electron can tunnel.

Electron Transfer Experiments: Metal-Modified Metalloproteins

The Gray group has a long-term goal of empirically demonstrating the considerable amount of theory that has been proposed to describe electron transfer in biological systems.[17–20] Such experiments must involve the systematic manipulation of the parameters, driving force, reorganization energy, and electronic coupling.

The Gray group's plan of attack is to surface-label metalloproteins with redox-active metal complexes and to study the intramolecular electron transfer between the two metal centers. The electron transfer would be induced by laser excitation, and because the each metal had its own optical signature in each of its various oxidation states, the state of the metals could be monitored over time. By changing the label or the metal resident to the protein, the driving force (-ΔG°) can be changed. By changing the sites of labeling (by installing histidine at various positions using site-directed mutagenesis), the distance and therefore the electronic coupling (H_{AB}) can be varied.

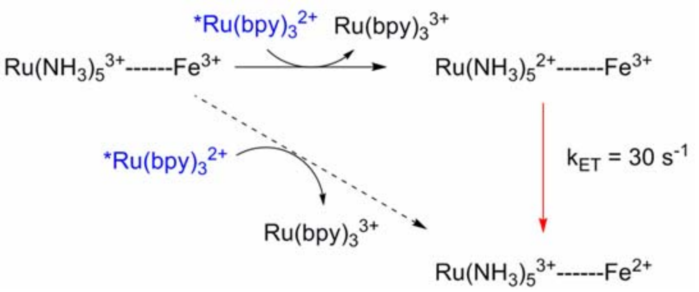

Figure 1.2. Electron transfer between $Ru(NH_3)_5^{2+}$ and Fe^{3+}. Scheme of first reported intramolecular electron transfer obtained through the use of metal-modified metalloproteins. In the study, $Ru(bpy)_3^{2+}$ was excited, generating its high-potential excited stated (highlighted in blue). It was found that both the $Ru(NH_3)_5^{3+}$ and Fe^{3+} could oxidixe $*Ru(bpy)_3^{2+}$, but that the quenching by ruthenium was faster, generating the kinetic product $Ru(NH_3)^{2+}$ in fivefold excess over the thermodynamic product Fe^{2+}, demonstrated in the figure by the hashed arrow for the slower, less-dominant phase. EDTA was utilized to scavenge $Ru(bpy)_3^{3+}$ to prevent back reaction, so that the kinetics of the intramolecular electron transfer could be observed.

The first of these systems was reported on in 1982: cytochrome *c* was labeled with $Ru(NH_3)_5^{3+}$ at the His33 site.[21] Reports on the phototriggered electron transfer quickly followed.[22,23] The electron transfer was initiated by the excitation of the photosensitizer $Ru(bpy)_3^{2+}$ with a 532 nm laser pulse; in its long-lived excited state, $*Ru(bpy)_3^{2+}$ is an excellent reducing agent. It donates an electron to the modified metalloprotein (PFe^{3+}-Ru^{3+}) system (**Figure 1.2**). While it does donate electrons to both the iron and the ruthenium label, it was found that $*Ru(bpy)_3^{2+}$ quenching generates the reduced ruthenium complex in fivefold excess to the reduced iron product (denoted with a solid arrow in the figure). By utilizing a $Ru(bpy)_3^{3+}$ scavenger (ethylene diammine tetraacetic acid), the kinetics of the intramolecular electron transfer from the Ru^{2+} to Fe^{3+}

could be revealed and monitored. The rate of this electron transfer was determined to be 30 s^{-1}.

The utility of diimine ligands proved to be an important and useful modification to the program. The reorganization energy that accompanied Ru-diimine$^{3+/2+}$ electron transfer was observed to be smaller than that measured for $Ru(NH_3)_5^{3+/2+}$, which allowed for investigations into the inverted region (where $-\Delta G° > \lambda$).[24] Furthermore, the ruthenium-diimine photosensitizers could be directly attached to the protein (still labeling at histidine sites) and so the complications of intermolecular electron transfer could be avoided. Finally, the diimine ligands allowed for facile manipulation of the reduction potential of the label, which allowed for a systematic approach to studying the effects of driving force on the kinetics of electron transfer.[25] It was from these variations that the empirical demonstration of the inverted effect was observed (**Figure 1.3**).

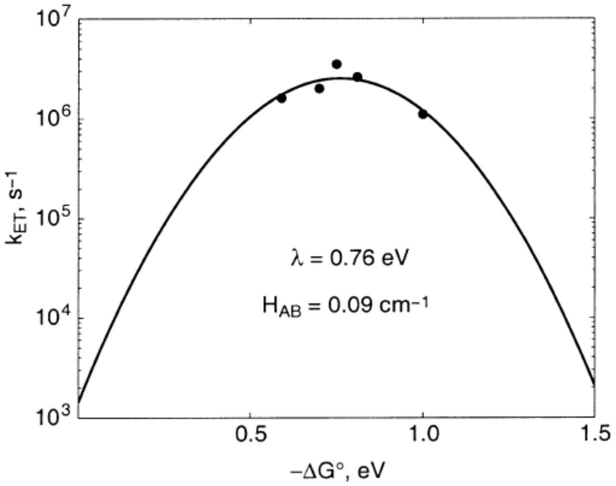

Figure 1.3. Driving force dependence of electron transfer rates in Ru-His33 cytochrome *c*. Solid line is the best fit to **Equation 1.1**; values calculated for λ and H_{AB} shown.

The metal-modified metalloprotein program has gone on to demonstrate the importance of tunneling pathways in determining electron transfer kinetics[18,26] (**Figure**

1.4). **Figure 1.4** summarizes the study of activationless ($-\Delta G^\circ = \lambda = \sim 0.8$ eV) electron transfer in the metal-modified metalloprotein program. In these cases, because the driving force and reorganization energy are approximately equal, the exponential term in **Equation 1.1** has the value of 1, and so any change on the rate of electron transfer/tunneling should be solely dependent on the electronic interactions between the centers (i.e., H_{AB}, and, in turn, distance).

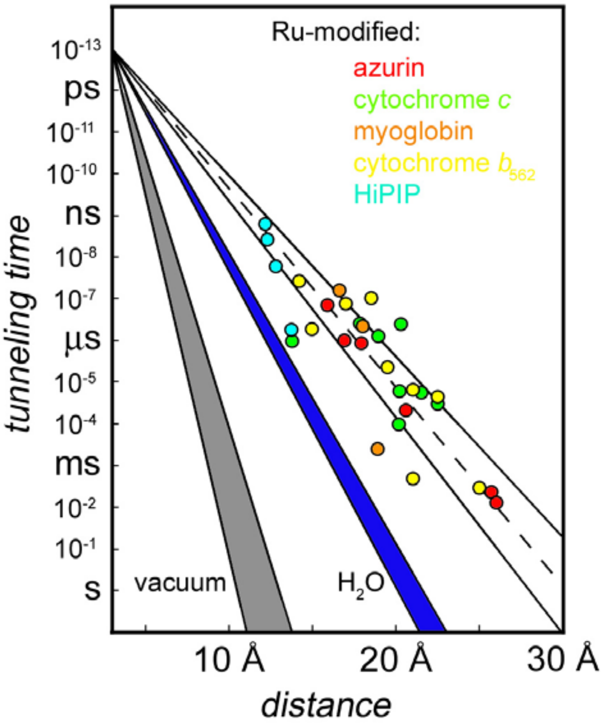

Figure 1.4. Tunneling timetable for activationless electron transfer in five different proteins (indicated above). The tunneling time is plotted in logarithmic scale against the distance traversed by the electron.[20]

In **Figure 1.4**, the tunneling times of the electron transfers in the proteins studied are plotted logarithmically against the distance between the redox centers; one should note that β is the slope of the various lines. The data display a few marked characteristics: 1) tunneling through proteins is more efficient than tunneling through vacuum or water (β is smaller for proteins than for the other two). This is because the protein fold lowers the reorganization energy of the electron transfer event by excluding water and utilizing an expanded network of hydrogen bonds to minimize the reorganization of ligands about the metal during electron transfer;[27] 2) the protein data points are all scattered around an average β of 1.1 Å^{-1}, which is close to the β = 1.0 Å^{-1} value found for the superexchange-mediated electron tunneling across saturated alkane bridges.[28,29] This similarity indicates that the electronic coupling in proteins is similar to the electron coupling in alkane chains, which is not completely surprising; 3) though some electron transfers happen over the same distance (i.e., ~21 Å), the kinetics of the electron transfer can vary up to three orders of magnitude; the range and the scatter that is observed across all proteins demonstrate the effect tunneling pathways have on electron transfer/tunneling kinetics.

An examination of this tunneling timetable reveals a limitation of experiments executed thus far; efficient electron transfer in proteins has been demonstrated for distances up to 15 Å in these studies. But according to the timetable, electron tunneling at longer distances take on the order of milliseconds to seconds to complete. It remains mysterious how nature can convey electrons over distances of over 30 Å on a much faster timescale in the processes that sustain life in cells.

1.3 LONG-RANGE ELECTRON TRANSFER IN PROTEINS

Photosynthesis and respiration are complementary energy transduction processes that utilize long-range electron transfer.[19,30–32] In respiration, hydrogen atoms are abstracted from organic molecules, stored, then passed into the respiratory chain, a system of membrane-bound proteins located in cell organelles, mitochondria, or the cell membrane. The hydrogen atoms are split into protons and electrons; protons are sequestered to one side of the membrane, while electrons are passed through the chain to eventually reduce oxygen into water. The proton gradient that is generated is utilized to generate adenosine triphosphate (ATP), which serves as the currency for energy in living cells. In the light reactions of photosynthesis, photons from the sun trigger the separation of charge in a system of membrane-bound proteins. Water is oxidized to oxygen, and electrons are passed through the system to eventually generate the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) which is utilized later in the dark reactions of photosynthesis to fix carbon dioxide.

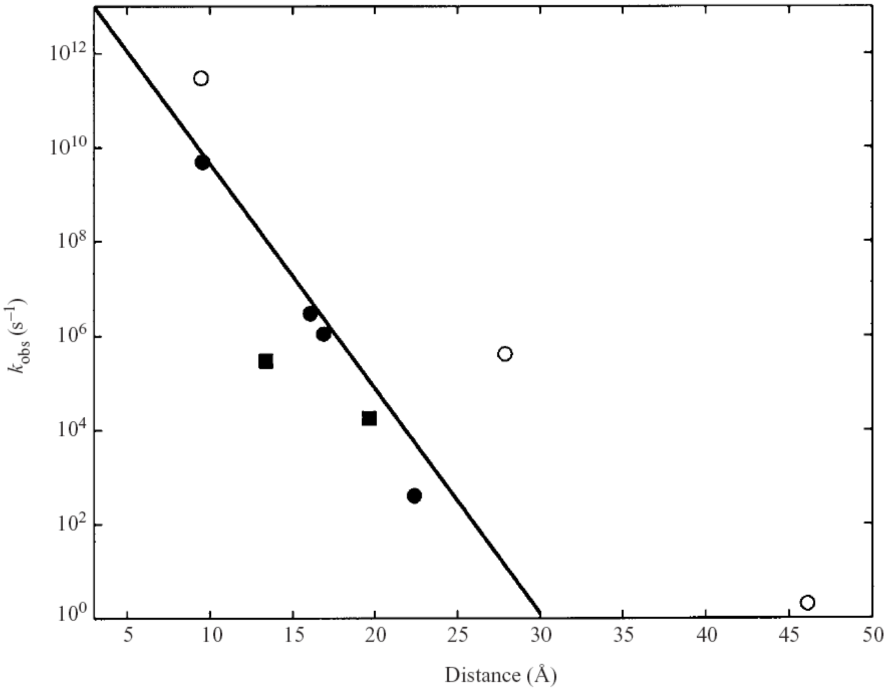

Figure 1.5. Distance dependence of observed electron transfer rates in cytochrome *c* oxidase (squares) and bacterial photosynthetic reaction centers (circles). Open circles represent transfers where multistep tunneling may be in operation.[19]

The observed electron transfer kinetics in bacterial photosynthetic reaction centers and cytochrome *c* oxidase (where oxygen is reduced to water in the respiratory chain) are plotted against the average $\beta = 1.1$ Å^{-1} value below in **Figure 1.5**.[19,33–36] One can clearly see that many of the electron transfer reactions lie very closely to the line, revealing just how well tuned this biological machinery is to serve its function! Intriguingly, three of the data points (open circles) lie well above the $\beta = 1.1$ Å^{-1} line, orders of magnitude faster than would be expected for activationless electron transfer. It is speculated that these faster kinetics can be accessed through a multistep tunneling mechanism.[34,37,38]

By this mechanism, the bridging protein medium not only electronically couples the electron donor and acceptor; it (in particular, an amino acid in the bridge) is also oxidized and reduced. Participation of this amino acid renders the long-range electron transfer a multistep tunneling process, also known as "hopping". A long-distance transfer is now broken into multiple electron tunneling steps, or "hops", which are separated by redox-active intermediates. Because electron transfer rates are exponentially dependent on distance, the kinetics of multiple short electron transfers will be orders of magnitude faster than the kinetics of one long single-step electron transfer between the donor and acceptor. It is now the latest goal of the metal-modified metalloprotein program to engineer systems to exhibit this behavior, lending experimental support towards the hypothesis.

1.4 PROTEIN-BASED RADICALS

Which amino acids can be utilized as intermediates in multistep tunneling? Amino acid radicals are actually quite common, and their roles in biology (beyond the role in photosynthesis and respiration proposed above in the previous section) include DNA biosynthesis and repair, metabolism of assorted biomolecules, hormone synthesis, and disproportionation of hydrogen peroxide.[39] Observed amino acid radicals in these proteins include glycines, cysteines, tyrosines, tryptophans, and post-translationally modified tyrosines and tryptophans. Reduction potentials for some of the amino acids in aqueous media have been measured (and remeasured, occasionally, as there has been debate, especially over tyrosine and tryptophan) (**Table 1.1**).

Radical	$E^{\circ\prime}$	Reference
Gly$^{\bullet}$	1.22 V (pH 10.5)	a
Cys$^{\bullet}$	1.33 V	b
Tyr$^{\bullet}$	0.93 V	c,d
Trp$^{\bullet}$	1.01 V, 1.05 V	c,d

Table 1.1. Measured reduction potentials of natural amino acids in solvated environments (*v.* NHE, unless otherwise specified, at pH 7). [a]Zhao, R.; Lind, J.; Merenyi, G.; Eriksen, T.E. *J. Am. Chem. Soc.* **1994**, *116*, 12010–12015. [b]Sudhar, P.S.; Armstrong, D.A. *J. Phys. Chem.* **1987**, *91*, 6532–6537. [c]Determined by cyclic voltammetry in: Harriman, A. *J. Phys. Chem.* **1987**, *91*, 6102–6104. [d]Determined by pulse radiolysis in: DeFelippis, M.R.; Murthy, C.P.; Faraggi, M.; Klapper, M.H. *Biochemistry* **1989**, *28*, 4847–4853.

An examination of **Table 1.1** reveals that the amino acids that are easiest to oxidize are tyrosine and tryptophan. These amino acids have been found at strategic locations in proteins that exhibit efficient long-range electron transfer: photosystem II,[40,41] class I ribonucleotide reductase,[42] and DNA photolyase,[43,44]. In these cases, there have already been extensive spectroscopic characterizations of tyrosine-based and tryptophan-based radicals in these sites. It is clear that these two amino acids present likely candidates through which multistep tunneling occurs, so they have been the focus of the multistep tunneling program for some time now.

1.5 MULTISTEP TUNNELING IN THE GRAY GROUP

The plan to demonstrate multistep tunneling in proteins in the Gray group is fairly straightforward; take one of the previously synthesized systems, install a tyrosine or tryptophan between the metal label and the metal resident to the protein, and demonstrate that the kinetics of electron transfer in this system are significantly enhanced (**Figure 1.6**).

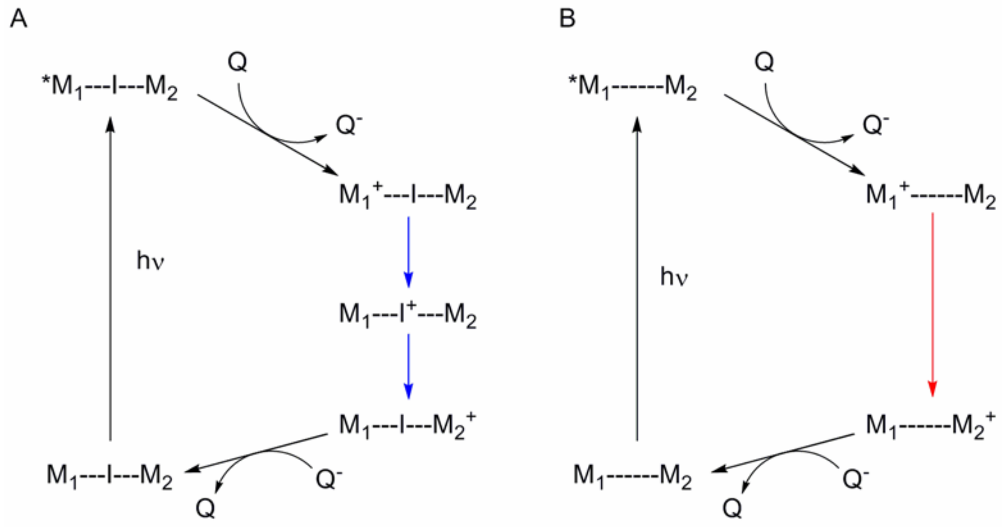

Figure 1.6. Possible plan for studying multistep tunneling in proteins. M_1 is the photosensitizer, I is the intermediate amino acid, and M_2 is the metal that is resident to the protein. **A.** Multistep tunneling: the photosensitizer is excited and, in its excited state, oxidized by an external quencher. Two electron transfers follow (blue arrows): intermediate to M_1, M_2 to I^+. Eventually, the M_2^+ is reduced by reduced quencher. **B.** Single-step tunneling: the photosensitizer is excited and, in its excited state, oxidized by an external quencher. One electron transfer (red arrow) occurs between the two redox centers. Eventually, M_2^+ is reduced by reduced quencher. It is hoped that M_2^+ will form quicker in system **A**.

Because the systems on *Pseudomonas aeruginosa* azurin exhibit very well-behaved kinetics (red data points in **Figure 1.4**),[45–47] it was selected as the protein on which the multistep tunneling experiments would be executed. Initial attempts were conducted by former graduate students Drs. William A. Wehbi[48] and Jeremiah E.

Miller,[49] as well as post-doctoral scholar Dr. Malin Abrahamsson. They labeled their proteins with the high-potential photosensitizer Re(dmp)$(CO)_3^+$, which is an excellent oxidant in either its excited state or oxidized 2+ state. Wehbi focused his studies on tyrosine (though he also did some work with cysteine), while Miller and Abrahamsson focused on tryptophan.

It was soon found, however, that, these studies were not as straightforward as previously supposed; upon oxidation, both aromatic amino acids become extremely susceptible to deprotonation, generating neutral radicals. The deprotonated amino acid radicals have lower reduction potentials, and so the driving force is not high enough to drive the subsequent electron transfer.

Circumventing the problem of deprotonation could be done in one of two ways: 1) find a system where the deprotonation of the radical cation would occur on a slower timescale than the subsequent electron transfer; or 2) find a system that was already deprotonated. Both systems have been examined in this thesis, and hopping has been probed through tyrosine, tryptophan, and 3-Nitrotyrosine (**Figure 1.7**).

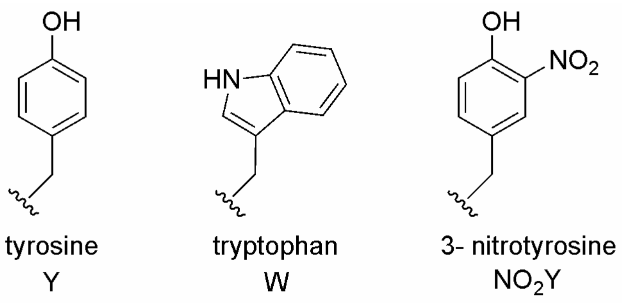

Figure 1.7. Hopping residues studied

1.6 RESEARCH OUTLINE

At the time I began my research, the research plan had been modified in two ways: first, high-potential ruthenium sensitizers would be pursued; second, the tyrosine analog 3-nitrotyrosine was to be employed as the newest hopping candidate. My research later incorporated studies utilizing both rhenium and tryptophan.

High-Potential Ruthenium Sensitizers

While the previously utilized rhenium sensitizers had the appropriate potentials for hopping studies, their optical inactivity limited the information that could be gained on their redox states. Re^0 could be traced at 500 nm, but both Re^+ and Re^{2+} were optically silent. Ruthenium dyes were an attractive alternative, because their absorbance was quite substantial in the 500 nm region,[25] and minimal in the 620 nm region, where the Cu^{2+} center of azurin absorbs.[50,51] The only limitation was that the ruthenium labels previously utilized in the metal-modified metalloprotein program were not of a high enough potential to drive electron transfer to intermediate amino acid residues. Therefore, the first goal was to install electron withdrawing groups onto the ligand to raise the potential of the metal. Chapter two summarizes the synthesis and characterization of three high-potential ruthenium photosensitizers, one of which is utilized in chapters four and five.

3-Nitrotyrosine as the Intermediate

Because deprotonation of the radical cations of both tryptophan and tyrosine appeared to complicate hopping studies, it was proposed to perturb the pK_a of the protons by substituting onto the aromatic ring of the amino acid. If the pK_a were lowered enough, the studies could be conducted with the amino acid in only one protonation state.

Synthetic protocols for the nitration of tyrosines using tetranitromethane have been used since their development in the late 1960s.[52–58] Moreover, the Gray group has also had experience and success with the protocol: Dr. Jennifer C. Lee utilized 3-nitrotyrosine in her studies of α-synuclein structure.[58] 3-nitrotyrosine's proton has a pK_a of around 7,[54] so it is very feasible to work with the amino acid in its deprotonated state for hopping studies. The deprotonated 3-nitrotyrosininate has a reduction potential of about 1.07 V *v.* NHE,[59] which is close to that of tyrosine and tryptophan, so it should participate as an intermediate in hopping systems. Deprotonated 3-nitrotyrosinate absorbs at 428 nm, which offers a spectroscopic handle for the oxidation state of the intermediate amino acid residue. These advantages and details all made 3-nitrotyrosine an extremely attractive target for use in the engineered hopping systems. I was successful in installing the nitro group onto tyrosines in multiple sites of the protein, and was able to demonstrate that the residue could participate in redox chemistry on the protein; discussion of the protocol, and the results from the nitrotyrosine mutants are in Chapters Two and Four.

Hopping Systems

At the time I joined the multistep tunneling program, a successful hopping system had just been discovered by Dr. Malin Abrahamsson. H124/W122/All-Phe azurin was modified with $Re(dmp)(CO)_3$. When the rhenium label was excited, it induced a nearly 20 Å multistep electron transfer that occurred within 50 nanoseconds! Because the system was the first of its kind, as much information on it had to be obtained as possible; samples were sent to Brian Crane at Cornell University, so that structural data could be obtained. The kinetics data was confirmed by ultrafast time-resolved infrared spectroscopy, done by Tony Vlček at Queen Mary, University London. I got involved on the project when temperature studies and ultrafast UV-Vis spectroscopy studies also had to be carried out on the system. Chapter Three summarizes the conclusions obtained from my data.

Inspired by this data, I expanded my studies into other systems based on this one to figure out what made hopping in this system work so well. I manipulated potentials of both metal label and intermediate, and varied distance as well. It was through these investigations that I discovered another promising hopping system. These pursuits are discussed in Chapter Four.

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