Electron Transfer in Ruthenium-Modified Proteins

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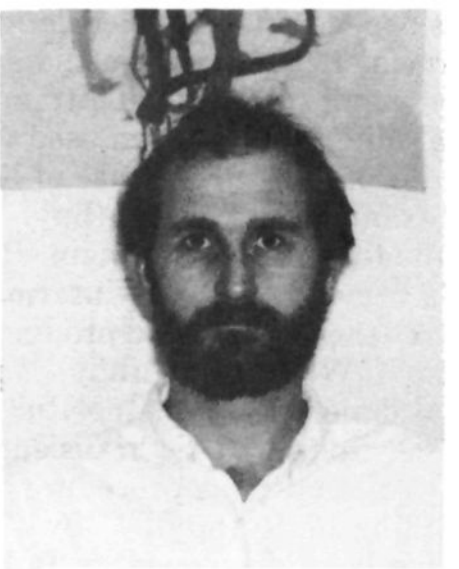
Contents

I. Introduction

The electron-transfer (ET) reactions of metalloproteins are potentially far more complex than those of small molecules. The structural intricacies of proteins are largely responsible for the added complexity. Reactive centers are surrounded by polypeptide matrices that shield these sites from solvent and separate them from their redox partners. The nonuniform charge distributions on the protein surfaces create anisotropic interactions between redox partners, confounding the interpretation of bimolecular reactions.[1,2] Covalent or electrostatic coupling of redox partners can be exploited to simplify the problem but does not eliminate all of the complicating factors in metalloprotein ET reactions.[3-5] The energetics and dynamics of nuclear reorientations accompanying protein ET reactions continue to be a source of inquiry.[6,7] Multiple conformational states in the polymer surrounding the redox sites create the potential for "gating" and directional electron transfer.[8,9] The peptide matrix also separates the redox sites, leading to questions of how protein ET can be efficient over such long (>10 Å) distances.[10-14] One line of research aimed at addressing many of the fundamental issues in protein ET involves the use of proteins modified by the coordination of Ru complexes to surface amino acid residues.

Many excellent reviews of ET in metalloproteins have been published in the past 10 years;[15] the scope of this paper will therefore be limited to a review of electron-transfer reactions in Ru-modified metalloproteins, the first report of which appeared in 1982.[16] Variations on the original work include modifications of the Ru-coordination sphere to effect changes in reaction driving force and nuclear reorganization energy, changes in the point of Ru attachment to assess the roles of distance and intervening medium in coupling electron donors and acceptors, and extension to a variety of proteins with different active sites.[15g] This work has provided many insights and spawned several new lines of inquiry into protein ET reactions. Abbreviations used in this paper are as follows: cyt *c*, cytochrome *c*; Mb, myo-



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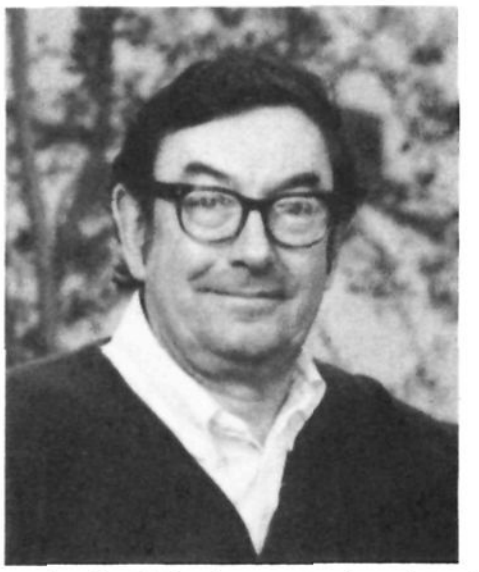

Harry B. Gray received a B.S. degree in chemistry from Western Kentucky University in 1957. Under the direction of Fred Basolo and Ralph Pearson, he received a Ph.D. degree in chemistry from Northwestern University in 1960. He then moved to the University of Copenhagen to work with Carl Ballhausen as an NSF Postdoctoral Fellow. He joined the chemistry faculty at Columbia University in 1961. In 1966, he moved to the California Institute of Technology, where he is the Arnold O. Beckman Professor of Chemistry and the Director of the Beckman Institute.

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globin; Tb_5, trypsin-solubilized cytochrome b_5; DPb_5, deuteroporphyrin-substituted Tb_5; LMb_5, mutant lipase solubilized cytochrome b_5; a, NH_3; bpy, 2,2′-bipyridine; His, histidine; Lys, lysine; k_B, Boltzmann's constant, **d** = edge-to-edge donor–acceptor separation.

II. Measurements

Ru-modified proteins are members of a large class of donor–acceptor complexes in which the two redox sites are covalently linked (D–A).[17-22] When neither redox site in such a complex has a long-lived excited state, ET measurements depend upon bimolecular generation of a kinetic intermediate. This intermediate then relaxes to the thermodynamic product via intramolecular ET. Photochemical initiation has been used with Ru–ammine-modified proteins. In the case of Ru_a_5(His33)–Fe–cyt *c*, for example, excited $Ru(bpy)_3^{2+}$ injects an electron into the fully oxidized protein (Ru^{III}, Fe^{III}) to yield Ru^{II}–Fe^{III}–cyt *c* (kinetic intermediate) and Ru^{III}–Fe^{II}–cyt *c* (thermodynamic product) with a 4:1 branching ratio.[16] When the $Ru(bpy)_3^{3+}$ is scavenged by a sacrificial donor (EDTA), the $Ru^{II} \rightarrow Fe^{III}$ ET reaction can be monitored by transient absorption spectroscopy. An analogous procedure based on reductive quenching of $Ru(bpy)_3^{2+*}$ by fully reduced protein also has been developed.[23] Pulse-radiolytic generation of reducing or oxidizing radicals, which inject electrons into or remove electrons from Ru-modified proteins, has also found application in these studies.[24-26]

The bimolecular method of generating a kinetic intermediate has a relatively low upper limit for measurable intramolecular ET rates ($\leq 10^4$ s^{-1}). A significant improvement results when a long-lived excited state can be prepared on one of the redox sites. The excited redox site, being both a better electron donor *and* acceptor than the ground-state species, can transfer electrons to or from the redox partner.[3-5,17-21] There is, in principle, no upper limit on rates that can be measured by direct photoinduced ET, but the lower limit is always fixed by the intrinsic decay rate of the excited center. A bonus from these measurements is that two ET rates often can be determined: those of the photoinduced ET *and* thermal electron-hole recombination reactions. Measurements of this type in Ru–ammine-modified proteins have been performed in metal-substituted (e.g., Zn, Mg, Cd, Pt, Pd) heme proteins.[27-32] The triplet excited-state lifetimes of these metalloporphyrins in proteins range from ~ 10 μs (for Pt) to over 10 ms (for Zn, Mg, Cd) and provide a reasonably wide observation window for intramolecular ET.

Long-lived excited states are also found in Ru^{II}–bpy complexes, though the lifetimes of these excited molecules are substantially shorter (≤ 1 μs) than those of closed-shell metalloporphyrins.[33] Ru–bpy complexes have, nevertheless, been bound to surface amino acid residues and used in studies of intramolecular ET.[34] The lower limits for measurable direct photoinduced ET rates are higher ($\geq 10^5$ s^{-1}) than those with excited metalloporphyrins. This lower limit can be extended by using a flash–quench technique.[35] In this approach, instead of intramolecular ET quenching the excited metal complex, bimolecular ET with a reagent in solution accelerates excited-state deactivation. The oxidized or reduced protein-bound metal complex is then free to transfer electrons from or to the protein active site. If the intramolecular ET does not compete effectively with the electron-hole recombination reaction with quencher, then irreversible quenchers can be employed. Thus, with Ru–bpy complexes covalently bound to proteins, intramolecular ET rates can be measured on time scales ranging from picoseconds to seconds.

III. Interpretation

The experimental results will be discussed in the context of semiclassical ET theory, which describes the rate constant for nonadiabatic reaction between a donor and acceptor held at fixed distance and orientation:[15b]

$$k_{ET} = (4\pi^3/h^2\lambda k_B T)^{1/2}(H_{AB})^2 \exp[-(\Delta G^\circ + \lambda)^2/4\lambda k_B T] \quad (1)$$

The tunneling matrix element H_{AB} is a measure of the electronic coupling between the reactants and the products at the transition state. The magnitude of H_{AB} depends upon donor–acceptor separation and orientation and the nature of the intervening medium. The exponential term in eq 1 reflects the interplay between reaction driving force ($-\Delta G^\circ$) and nuclear reorganization energy (λ). Various approaches have been used to test the validity of eq 1 and to extract the ET parameters H_{AB} and λ. Driving-force studies have proven to be one reliable approach, and several such studies will be discussed in this paper.

In the nonadiabatic limit, the probability is quite low that reactants will crossover to products at the transition-state configuration.[15b] This probability depends upon the electronic hopping frequency (determined by H_{AB}) and upon the frequency of motion along the reaction coordinate.[36] When solvent reorientation dominates λ, the nuclear reorientation time scale is believed to be given by the solvent longitudinal dielectric relaxation time, τ_L. The nonadiabatic limit for ET results when $H_{AB}^2 \ll \{\lambda\hbar/4\pi\tau_L\}^{1/2}$.[36] Water reorients very rapidly ($\tau_L \approx 0.5$ ps[37]), and the solvent-controlled adiabatic limit results when $H_{AB} \gg 80$ cm^{-1}. Conversely, when $H_{AB} \ll 80$ cm^{-1}, eq 1 should adequately describe the ET kinetics. Reorientation of the peptide matrix introduces complications in protein ET. Time scales for this nuclear motion are much slower than the τ_L for water.[38] In situations where slow peptide motions dominate λ, much smaller values of H_{AB} are necessary to achieve the "solvent-controlled" adiabatic limit.

In simple models, the electronic-coupling strength is predicted to decay exponentially with increasing donor–acceptor separation:[15b,39]

$$H_{AB} = H_{AB}^\circ \exp[-\tfrac{1}{2}\beta(\mathbf{d} - \mathbf{d}^\circ)] \quad (2)$$

In eq 2, H_{AB}° is the electronic coupling at close contact ($\mathbf{d}^\circ$) and β is the rate of decay of coupling with distance (**d**). Studies of the distance dependence of ET rates in donor–acceptor complexes, and of randomly oriented donors and acceptors in rigid matrices, have suggested $0.8 \leq \beta \leq 1.2$ $Å^{-1}$.[20,22,40-43] Donor–acceptor electronic coupling in small complexes can be interpreted equally well in terms of simple exponential decay with distance (eq 2) or with the number of chemical bonds in the bridge between redox sites.[22] This situation arises from the fact that the direct distance between redox sites tends to be proportional to the number of intervening chemical bonds. The medium separating two redox

TABLE I. Rate Constants and Activation Parameters for Intramolecular ET Reactions of Ru(His)-Modified Cytochrome *c*

electron transfer	$-\Delta G°$, eV	k_{ET}, s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, eu
	His33 Derivatives (**d** = 11.1 Å)[a]			
$Ru_5(His)^{2+} \rightarrow Fe^{III}$ [b]	0.18 (2)	$3.0\ (5) \times 10^{1}$	2.0 (5)	−43 (5)
$Ru_4(isn)(His)^{2+} \rightarrow ZnP^{+}$ [c]	0.66 (5)	$2.0\ (2) \times 10^{5}$	<0.5	−35 (5)
$ZnP^{*} \rightarrow Ru_5(His)^{3+}$ [d]	0.70 (5)	$7.7\ (8) \times 10^{5}$	1.7 (4)	−27 (5)
$Ru_4(py)(His)^{2+} \rightarrow ZnP^{+}$ [c]	0.74 (5)	$3.5\ (4) \times 10^{5}$	<0.5	−34 (5)
$ZnP^{*} \rightarrow Ru_4(py)(His)^{3+}$ [c]	0.97 (5)	$3.3\ (3) \times 10^{6}$	2.2 (4)	−22 (5)
$Ru_5(His)^{2+} \rightarrow ZnP^{+}$ [d]	1.01 (5)	$1.6\ (4) \times 10^{6}$		
$ZnP^{*} \rightarrow Ru_4(isn)(His)^{3+}$ [c]	1.05 (5)	$2.9\ (3) \times 10^{6}$	<0.5	−30 (5)
$Fe^{II} \rightarrow Ru(bpy)_2(im)(His)^{3+}$ [e]	0.74 (2)	$2.6\ (3) \times 10^{6}$		
	His39 Derivatives (**d** = 12.3 Å)[e,f]			
$Ru_4(isn)(His)^{2+} \rightarrow ZnP^{+}$	0.66 (5)	$6.5\ (7) \times 10^{5}$	−1.7 (4)	−39 (5)
$ZnP^{*} \rightarrow Ru_5(His)^{3+}$	0.70 (5)	$1.5\ (2) \times 10^{6}$	1.3 (3)	−27 (5)
$Ru_4(py)(His)^{2+} \rightarrow ZnP^{+}$	0.74 (5)	$1.5\ (2) \times 10^{6}$	−1.8 (4)	−37 (5)
$ZnP^{*} \rightarrow Ru_4(py)(His)^{3+}$	0.97 (5)	$8.9\ (9) \times 10^{6}$	0.2 (2)	−27 (5)
$Ru_5(His)^{2+} \rightarrow ZnP^{+}$	1.01 (5)	$5.7\ (6) \times 10^{6}$	−0.2 (2)	−29 (5)
$ZnP^{*} \rightarrow Ru_4(isn)(His)^{3+}$	1.05 (5)	$1.0\ (1) \times 10^{7}$	0.2 (2)	−27 (5)
	His62 Derivatives (**d** = 14.8 Å)[e]			
$Ru_5(His)^{2+} \rightarrow Fe^{III}$ [g]	0.20 (2)	1.7 (1)		
$ZnP^{*} \rightarrow Ru_5(His)^{3+}$ [h]	0.70 (5)	$6.5\ (7) \times 10^{3}$	1.4 (3)	−37 (5)
$Ru_4(py)(His)^{2+} \rightarrow ZnP^{+}$ [h]	0.74 (5)	$8.1\ (8) \times 10^{3}$		
$ZnP^{*} \rightarrow Ru_4(py)(His)^{3+}$ [h]	0.97 (5)	$3.6\ (4) \times 10^{4}$		
$Ru_5(His)^{2+} \rightarrow ZnP^{+}$ [h]	1.01 (5)	$2.0\ (2) \times 10^{4}$	0.7 (7)	−37 (5)

[a] Reference 46. [b] Reference 16. [c] Reference 31. [d] Reference 27. [e] Reference 35. [f] Reference 32. [g] Reference 52. [h] Reference 53.

sites in a protein, however, is a heterogeneous array of bonded and nonbonded interactions. The covalently bonded path between donor and acceptor can be a tortuous route involving many more bonds than would be found in a typical synthetic D–A complex with comparable separation. Beratan and Onuchic have developed a formalism that describes the medium between redox sites in a protein in terms of "unit blocks" connected together to form a physical pathway for ET.[12] A unit block may be a covalent bond, a hydrogen bond, or a through-space jump, each with a corresponding decay factor. At intermediate D–A distances, a single pathway tends to dominate the coupling and H_{AB} can be written as the product of the decay factors for each block in the pathway. By scaling H-bonds and through-space jumps to the number of covalent bonds that would give a comparable decay in coupling strength, pathways can be described in terms of a number of *effective bonds* (n_{eff}) between the redox sites.

IV. Cytochrome c

A. His33 Derivatives

The first work on the ET reactions of Ru-modified proteins involved horse heart cytochrome *c* modified by coordination of pentaammineruthenium to His33 (Figure 1).[16,44] The rate of intramolecular ET from $Ru_5(His33)^{2+}$ to the ferriheme (T = 298 K), measured using photochemical techniques, is 30(5) s^{-1} (Table I).[16] The reaction exhibits a rather small activation enthalpy (2 kcal mol^{-1}) and a large negative activation entropy (−43 eu). Measurements of the temperature dependences of the $Ru_5(His)^{3+/2+}$ and $Fe^{3+/2+}$ potentials in $Ru_5(His33)$–Fe–cyt *c* have provided estimates of $\Delta G°$ [−4.3(2) kcal mol^{-1}, 298 K], $\Delta H°$ [−11.9(10) kcal mol^{-1}], and $\Delta S°$ [−26(3) eu] for the $Ru^{II} \rightarrow Fe^{III}$ intramolecular ET reaction. Given these thermodynamic quantities and the temperature dependence (2–40 °C) of the ET rate in $Ru_5(His33)$–Fe–cyt *c*, it is possible to extract values of λ and H_{AB} from eq 1. Nonlinear least-squares

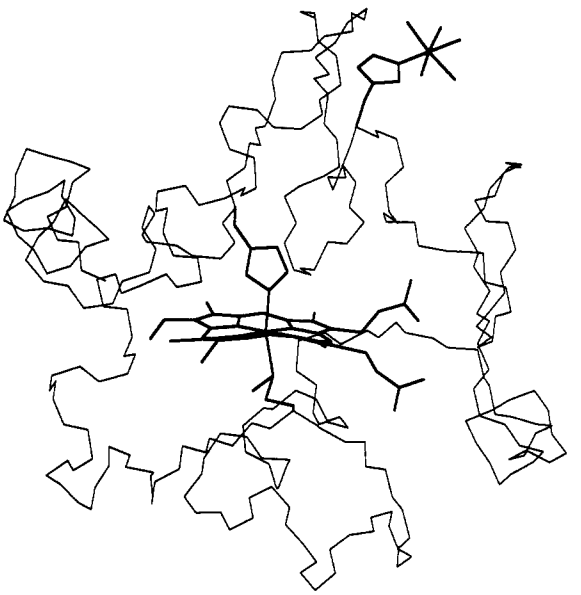

Figure 1. Peptide-backone structure of $Ru_5(His33)$–Fe–cyt *c*. This derivative was prepared by reaction of $Ru_5(OH_2)^{2+}$ with Fe^{II}–cyt *c* for 24 h at room temperature. The pure singly modified derivative was isolated by ion-exchange chromatography and extensively characterized by spectroscopic and chemical methods.[44]

fits to the data suggest λ = 1.2 eV and H_{AB} = 0.03 cm^{-1}.[35b] This value of the reorganization energy is quite close to that predicted by the Marcus cross relation[15b] [$\lambda_{12} = {}^1/_2(\lambda_{11} + \lambda_{22})$] using the reorganization energies for the Fe(III/II)–cyt *c* (λ_{11} = 1.04 eV) and $Ru_5(py)^{3+/2+}$ (λ_{22} = 1.20 eV) self-exchange reactions.[15b,45]

A value of β = 2.0 $Å^{-1}$ can be extracted from eq 2 for the $Ru_5(His33)$–Fe–cyt *c* system by taking **d** = 11.1 Å[46] and **d°** = 3.0 Å[15b] and assuming that H_{AB}° = 200 cm^{-1}.[35b] The large value of β suggested by the $Ru_5(His33)$–Fe–cyt *c* temperature-dependence data indicates a faster decay of electronic coupling with donor–acceptor separation than found in small-molecule systems. Alternatively, the large apparent β could suggest that, owing to the inhomogeneity of the intervening medium,

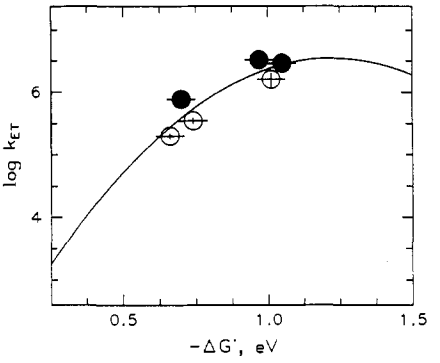

Figure 2. Free-energy dependence of intramolecular ET rates in Rua_4L(His33)–Zn–cyt *c*. Solid curve is the best fit to eq 1 using the parameters $\lambda = 1.2$ eV and $H_{AB} = 0.12$ cm^{-1}. (●) Photoinduced reactions; (○) recombination reactions.

the effective distance for ET is greater than the 11.1-Å direct separation. These early data, then, provided some evidence that donor–acceptor electronic coupling in protein systems might not be described by the simple expression in eq 2.

A clear understanding of the electronic-coupling strengths in metalloprotein ET reactions depends upon reliable values of λ and H_{AB}. It is clear from eq 1 that, in addition to studies of temperature dependences, ET parameters can also be extracted from studies of the driving-force dependence of ET rates. In the low-driving-force regime ($-\Delta G^{\circ}/\lambda \ll 1$), the variation of rate with free energy does not strongly depend upon λ [i.e., $\partial(\ln k_{ET})/\partial(\Delta G^{\circ}) \approx 1/2k_BT$], and it is difficult to obtain a good value for this parameter. Better values of λ and H_{AB} can be obtained from high-driving-force measurements (i.e., $\lambda \approx -\Delta G^{\circ}$). In this region, the driving-force curve flattens out and ET rates approach their maximum values.

It is difficult to prepare a Ru–ammine complex of Fe–cyt *c* in which the driving force for intramolecular ET is much greater than 0.2 eV. Substitution of the native Fe center in cytochrome *c* with Zn,[47] however, has provided an avenue to high-driving-force intramolecular ET. The lowest triplet excited state of the Zn–porphyrin in Zn–cyt *c* has a 15-ms lifetime and is a potent reductant ($E^{\circ} = -0.62$ V vs NHE).[27] The rates of direct photoinduced ET and thermal recombination have been measured for three Rua_5L(His33)–Zn–cyt *c* proteins (L = NH_3, pyridine, isonicotinamide), spanning a 0.39-eV range in ΔG° (–0.66 to –1.05 eV, Table I).[27,31] Fits of these data to eq 1 yield $\lambda = 1.10$ eV and $H_{AB} = 0.12$ cm^{-1} for the photoinduced reactions and $\lambda = 1.19$ eV and $H_{AB} = 0.09$ cm^{-1} for the recombinations. The ET parameters are not extremely sensitive to the nature of the reaction (photoinduced or recombination), and these reactions can be adequately described by a single pair of parameters: $\lambda = 1.15(10)$ eV and $H_{AB} = 0.1(2)$ cm^{-1} (Figure 2). The value of H_{AB} in Ru(His33)–Zn–cyt *c* is about 3 times that estimated for Ru(His33)–Fe–cyt *c*. The difference may be the result of variations in coupling to Ru between the porphyrin-localized states in Zn–cyt *c* and the metal-localized states in Fe–cyt *c*.

The similarity in reorganization energies for the Ru–Fe–cyt *c* and Ru–Zn–cyt *c* intramolecular ET reactions is to be expected. The total reorganization energy is a sum of inner-sphere (λ_i) and outer-sphere (λ_o) elements. Inner-sphere contributions arise from nuclear rearrangements in the Ru–ammine and metallo-porphyrin complexes accompanying electron transfer. These rearrangements are rather small and have been estimated to contribute no more than 0.2 eV to λ for both Ru–Fe–cyt *c* and Ru–Zn–cyt *c*.[31] There are two sources of outer-sphere rearrangements: the solvent and the peptide matrix. Calculations based on a single-sphere dielectric continuum model[48] indicate a 0.6-eV contribution to λ_o from the solvent.[31] From the structures of ferri- and ferrocytochromes *c*, the peptide contribution to λ_o has been calculated to be about 0.2 eV.[7] The sum of these individual components (1.0 eV) is in good agreement with the experimentally derived reorganization energy for the Ru–M–cyt *c* (M = Fe, Zn) systems.

There have been persistent suggestions that the ET step is not rate-limiting in the redox reactions of cytochrome *c*.[49] The variation of ET rate with driving force in Ru–Zn–cyt *c*, however, does not support this hypothesis. (It can be argued, however, that Zn–cyt *c* is not an appropriate model for the redox reactions of native cytochrome *c*.) Additional evidence bearing on this point has come from experiments in which intramolecular ET at high driving force in native Fe cytochrome *c* has been examined in proteins modified by the coordination of a bis(bipyridine)–Ru group to His33.[49a-c] The $Ru^{3+/2+}$ potentials in these complexes are greater than 1.0 V vs NHE; the driving forces for intramolecular ET with the cytochrome *c* heme are therefore in the same range as those measured with Ru–Zn–cyt *c*. A rate of 55 s^{-1} ($-\Delta G^{\circ} = 0.74$ eV) has been reported in pulse-radiolysis studies of ferroheme oxidation by $Ru(bpy)_2(im)(His33)^{3+}$.[49a-c] The rate of ferriheme *reduction* by $Ru(bpy)_2(im)(His33)^{+}$ was reported to be 2.0×10^5 s^{-1} ($-\Delta G^{\circ} = 1.5$ eV).[49a-c] This disparity between oxidation and reduction rates is far greater than can be accounted for by the driving-force dependence described in eq 1. Similar rates of ferroheme oxidation were reported for $Ru(bpy)_2(OH_2)(His33)^{3+}$, $Ru(bpy)_2(pyridine)(His33)^{3+}$, and Ru(bpy)(2,2′,2″-terpyridine)$(His33)^{3+}$–Fe–cyt *c* complexes.[49a-c] It was suggested that this behavior indicates "gated" or directional electron transfer in cytochrome *c*.[49] Recently, however, it has been shown using flash–quench techniques that the rate constant for oxidation of the ferroheme by $Ru(bpy)_2(im)(His33)^{3+}$ is actually 2.6×10^6 s^{-1} (Table I).[35a] The data clearly demonstrate that there is no ferroheme oxidation proceeding at a slower (i.e., 55 s^{-1}) rate (Figure 3). This observation is in much better agreement with the Ru–Zn–cyt *c* data and with the pulse-radiolysis measurement of the rate of ferriheme reduction by $Ru(bpy)_2(im)(His33)^{+}$. In light of this result, the slow rates of ferroheme oxidation by the other Ru–bpy–$His33^{3+}$ complexes must be viewed with suspicion. Currently then, there are no unequivocal data indicating gated or directional electron transfer in Ru-modified cytochrome *c*: observed ET rates are all consistent with a rate-limiting electron-transfer step.

An interesting aspect of electron transfer in Ru(bpy)$_2$(im)(His33)–Fe–cyt *c* is the possibility of a substantially lower reorganization energy than was found in Ru–ammine derivatives (1.2 eV). The major contribution to λ in Ru–Zn–cyt *c* arises from reorientation of solvent molecules around the Ru complex in response to the electron transfer. Replacing the hydrophilic

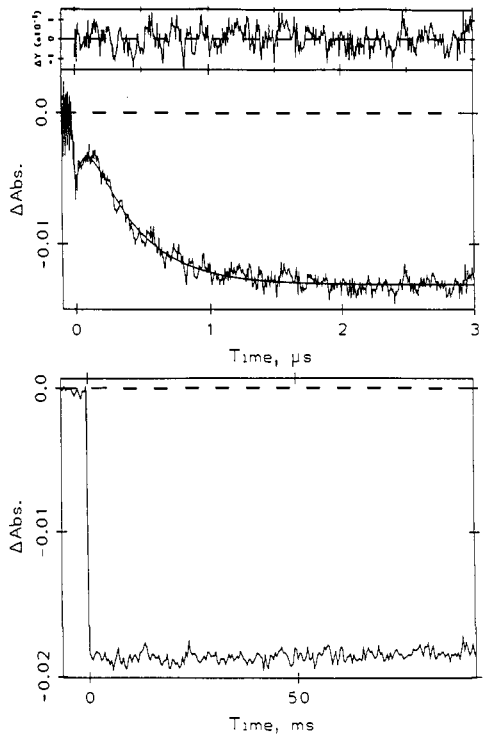

Figure 3. Transient kinetics following laser flash excitation of a mixture of $Ru(bpy)_2(im)(His33)-Fe^{II}$–cyt *c* and Rua_6^{3+}. (Top) 550-nm kinetics recorded with a 3-μs time window. Smooth lines are fits to a biexponential decay function. The faster component corresponds to decay of the excited Ru complex (k_{obsd} = 1.3 × 10^7 s^{-1}); the slower component arises from the intramolecular ET reaction [k_{obsd} = 2.6(3) × 10^6 s^{-1}]. (Bottom) 550-nm kinetics recorded with a 100-ms time window demonstrating that there is not a slow intramolecular oxidation of Fe^{II} by Ru^{III}.

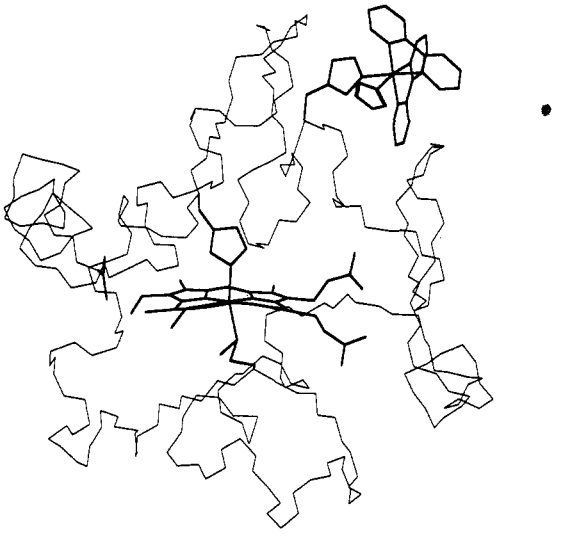

Figure 4. Energy-minimized structure of $Ru(bpy)_2(im)$-(His33)–Fe–cyt *c*. The structure was computed using BIOGRAF, version 2.1 (Biodesign, Inc.), and the coordinates from the X-ray crystal structure of horse heart ferricytochrome *c*.[46] Coordinates for the Ru complex were adapted from the structure of $Ru(bpy)_2Cl_2$.[50]

ammine ligands with more hydrophobic bipyridine ligands (Figure 4) will lower this reorganization energy: the λ of the self-exchange reaction of $Rua_5(py)^{3+/2+}$ (λ_{22} = 1.2 eV) is substantially greater than that of $Ru(bpy)_3^{3+/2+}$ (λ_{22} = 0.6 eV). The Marcus cross-relation (using λ_{11} = 1.0 eV for cytochrome *c*) suggests a value of λ_{12} = 0.8 eV for intramolecular ET in $Ru(bpy)_2$-(im)(His33)–Fe–cyt *c*. An important advantage of the reduced reorganization energy in the bis(bipyridine)–Ru-modified proteins is that the inverted region for ET (i.e., $-\Delta G° > \lambda$) is more accessible.

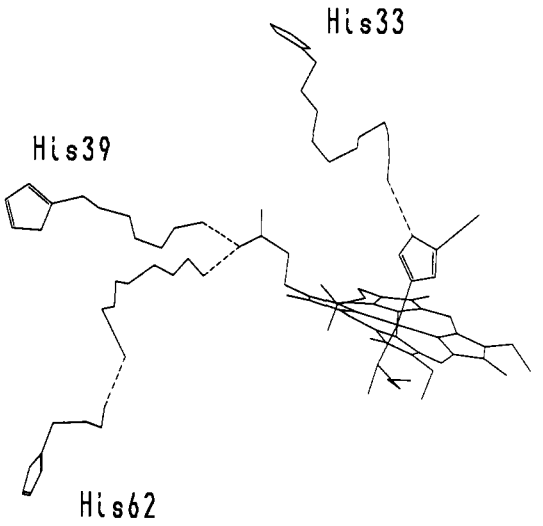

Figure 5. Predicted electronic coupling pathways in Ru(His33)-, Ru(His39)-, and Ru(His62)-modified cytochrome *c*. Covalent bonds are depicted as solid lines, and H-bonds are shown as dashed lines.

B. Other His Derivatives

His39. Ru–ammine complexes have been bound to His39 of Zn-substituted cytochrome *c* from *Candida krusei*.[32,51] Intramolecular ET rates (Table I) are approximately 3 times faster than those of corresponding reactions in His33 derivatives of horse heart cytochrome *c*. The variation of rates with driving force in these derivatives suggests a 1.2(1)-eV reorganization energy, indistinguishable from that found in the His33-modified proteins. The faster ET rates have been attributed to stronger donor–acceptor electronic coupling in the His39-modified protein.[32]

The direct D–A distances in Ru(His33)–Zn–cyt *c* and Ru(His39)–Zn–cyt *c* are at variance (11.1 and 12.3 Å, respectively) with the 2-fold larger H_{AB} for the His39 system. The pathway model is somewhat more consistent with the data: both the His33 and His39 pathways consist of 11 covalent bonds and 1 H-bond (Figure 5). The n_{eff} values for His33 and His39 are 13.9 and 14.0 bonds, respectively.[35b]

His62. Site-directed mutagenesis creates many new opportunities for studying electron transfer in Ru-modified proteins. A yeast (*Saccharomyces cerevisiae*) cytochrome *c* variant has been characterized with a surface histidine at position 62.[52] The Rua_5(His62) derivative of this mutant protein was prepared, and the rate of electron transfer from Ru^{II} to Fe^{III} was found to be 1.7 s^{-1} (Table I).[52] Rua_5(His62) and $Rua_4(py)$-(His62) derivatives of Zn-substituted *S. cerevisiae* cytochrome *c* have also been examined. The rates of the photoinduced and thermal recombination reactions are more than 2 orders of magnitude slower than the rates of analogous reactions in His33 derivatives of horse heart cytochrome *c*.[53] The driving-force data are more limited than for the other His derivatives of cytochrome *c* but again suggest that $\lambda \approx 1.2$ eV. The slower rates for the His62 derivatives are attributed to weaker electronic coupling. The direct D–A separation is 14.8

TABLE II. Maximum Rates, D–A Distances, Coupling Strengths, and Effective Bonds in Pathways for $Ru_4L(His)$-Modified Cytochrome *c*

	k_{max}, s^{-1}	d,[d] Å	H_{AB}, cm^{-1}	n_{eff},[d] bonds
His39[a]	1.4×10^7	12.3	0.24	14.0
His33[b]	2.9×10^6	11.1	0.11	13.9
His62[c]	2.0×10^4	14.8	0.01	20.6

[a] Reference 32. [b] Reference 31. [c] Reference 53. [d] Reference 35b.

TABLE III. Rate Constants and D–A Separations in Ru(Lys)-Modified Cytochrome *c*[a]

modified residue	d, Å	k_{ET}, s^{-1}: $*Ru(bpy)_2(dcbpy)^{2+} \rightarrow Fe^{III}$	k_{ET}, s^{-1}: $Fe^{II} \rightarrow Ru(bpy)_2(dcbpy)^{3+}$
Lys27	6–12	$2.0\ (3) \times 10^7$	$3.0\ (5) \times 10^7$
Lys13	6–10	$1.6\ (3) \times 10^7$	$2.6\ (5) \times 10^7$
Lys72	8–16	$1.4\ (3) \times 10^7$	$2.4\ (5) \times 10^7$
Lys25	9–16	$1.0\ (3) \times 10^6$	$1.5\ (3) \times 10^6$
Lys7	9–16	$3\ (1) \times 10^5$	$6\ (2) \times 10^5$

[a] References 34 and 54.

Å, while the effective number of bonds in the pathway is 20.6 (Table II).[35b] By both measures, it is reasonable to expect the His62 ET reactions to be substantially slower than those found in His33 or His39 derivatives.

C. Lysine Derivatives

Individual lysine residues have been modified with $Ru(bpy)_2(dcbpy)^{2+}$ complexes (dcbpy = dicarboxybipyridine) via an amide linkage.[34] The large number of surface lysines on cytochrome *c* (17) at widely varying distances from the heme creates the potential for an extensive study of the distance dependence of the ET rate. Ten derivatives have been prepared; photoinduced ET and thermal recombination rates have been measured in five of them. Photoinduced rates change from 3×10^5 to 2×10^7 s^{-1}; recombination rates vary from 6×10^5 to 3×10^7 s^{-1} (Table III).[34,54] One shortcoming of this system is the conformational flexibility of the lysine side chain. Molecular mechanics modeling studies indicate that the *uncertainties* in D–A separations range from 4 to 8 Å. This uncertainty makes it difficult to correlate these data with measurements for Ru(His)-modified proteins.

The Ru–Lys derivatives have found important applications in studies of ET in protein–protein complexes.[54,55] Photoinduced ET from the Ru complex rapidly injects an electron into the heme center of cytochrome *c*. Sacrificial donors scavenge the Ru^{III} complex, inhibiting the recombination reaction and leaving the ferroheme available to react with complexed redox partners. Experiments of this type have been performed with plastocyanin and cytochrome *c* oxidase as electron acceptors.[54,55] The interprotein ET rates vary with the site of Lys modification, suggesting that the Ru label may be modifying the protein binding. The protein–protein ET results are set out in Table IV.

V. Myoglobin

A. His48 Derivatives

Myoglobin is an oxygen-storage protein with 153 amino acids and a heme prosthetic group.[56] Five His residues on the surface of sperm-whale myoglobin have been labeled with Ru–ammine complexes.[57,58] This multiplicity of His residues introduces significant complications into the preparation and purification of Ru-modified proteins. The major products are the His48-modified Mb, and most of the ET studies have been performed with these derivatives. Unlike cytochrome *c*, the heme is not covalently bound to the protein in Mb. This feature greatly facilitates metal substitution and has enabled the preparation of Ru–ammine-modified proteins with six different metalloporphyrin active sites. The intramolecular ET rates for these derivatives are set out in Table V.[28-30,57-59]

In the case of cytochrome *c*, there was good reason to expect that the reorganization energy for the ET reactions of the Zn-substituted protein would be nearly the same as that of the native Fe protein. This, however, is not likely to be true in myoglobin: Fe^{II}–Mb is a five-coordinate complex which, upon one-electron oxidation, binds a water molecule to form a six-coordinate species.[56] This change in coordination number should be reflected in a greater reorganization energy in native Fe–Mb or even ET rates limited by ligand binding or dissociation. The electron-transfer reactions

TABLE IV. Rate Constants for ET from Ru(Lys)-Modified Ferrocytochrome *c* to Complexed Acceptors[a]

modified residue	k_{ET}, s^{-1}: [Ru–Fe–cyt *c*]: [plastocyanin]	k_{ET}, s^{-1}: [Ru–Fe–cyt *c*]: [cyt *c* oxidase]
Lys7	$1.3\ (1) \times 10^3$	
Lys8	$1.5\ (1) \times 10^3$	
Lys13	$1.9\ (2) \times 10^3$	6.0×10^2
Lys25	$8.2\ (6) \times 10^2$	
Lys27	$5.3\ (7) \times 10^2$	3.0×10^3
Lys72	$8.0\ (5) \times 10^2$	
Lys86	$1.0\ (1) \times 10^3$	

[a] References 54 and 55.

TABLE V. ET Rates in Ru(His)-Modified Myoglobin

electron transfer	$-\Delta G°$, eV	k_{ET}, s^{-1}
His48 Derivatives (d = 12.7 Å)[a]		
$H_2P^* \rightarrow Ru_5(His)^{3+}$ [a]	0.39 (2)	$7.6\ (8) \times 10^2$
$Ru_4(isn)(His)^{2+} \rightarrow ZnP^+$ [b]	0.61 (2)	$1.4\ (5) \times 10^4$
$PdP^* \rightarrow Ru_5(His)^{3+}$ [c]	0.64 (2)	$9.1\ (9) \times 10^3$
$Ru_4(py)(His)^{2+} \rightarrow ZnP^+$ [b]	0.69 (2)	$2.0\ (5) \times 10^4$
$CdP^* \rightarrow Ru_5(His)^{3+}$ [a,d]	0.79 (2)	$4.5\ (5) \times 10^4$
$MgP^* \rightarrow Ru_5(His)^{3+}$ [a,d]	0.81 (2)	$9.5\ (9) \times 10^4$
$ZnP^* \rightarrow Ru_5(His)^{3+}$ [b]	0.82 (2)	$7.0\ (7) \times 10^4$
$PdP^* \rightarrow Ru_4(py)(His)^{3+}$ [c]	0.91 (2)	$9.0\ (9) \times 10^4$
$Ru_5(His)^{2+} \rightarrow MgP^+$ [d]	0.91 (2)	$4.9\ (5) \times 10^4$
$Ru_5(His)^{2+} \rightarrow CdP^+$ [d]	0.92 (2)	$2.1\ (2) \times 10^5$
$Ru_5(His)^{2+} \rightarrow ZnP^+$ [d]	0.96 (2)	$1.5\ (5) \times 10^5$
$ZnP^* \rightarrow Ru_4(py)(His)^{3+}$ [b]	1.09 (2)	$2.0\ (2) \times 10^5$
$ZnP^* \rightarrow Ru_4(isn)(His)^{3+}$ [b]	1.17 (2)	$2.9\ (3) \times 10^5$
$Ru_5(His)^{2+} \rightarrow Fe^{III}(CN\text{-}Br)$ [e]	0.09 (2)	2.0 (5)
$Fe^{II}(CN\text{-}Br) \rightarrow Ru_4(isn)(His)^{3+}$ [e]	0.26 (2)	5.5 (5)
$Fe^{II} \rightarrow Ru_5(His)^{3+}$ [f]	0.02 (2)	$4.0\ (5) \times 10^{-2}$
$Fe^{II} \rightarrow Ru_4(py)(His)^{3+}$ [g]	0.28 (2)	2.5 (5)
$Fe^{II} \rightarrow Ru_4(isn)(His)^{3+}$ [e]	0.35 (2)	3.0 (4)
His81 Derivative (d = 19.3 Å)[h]		
$ZnP^* \rightarrow Ru_5(His)^{3+}$	0.82 (2)	$8.6\ (10) \times 10^1$
His116 Derivative (d = 20.1 Å)[h]		
$ZnP^* \rightarrow Ru_5(His)^{3+}$	0.82 (2)	$8.9\ (3) \times 10^1$
His12 Derivative (d = 22.0 Å)[h]		
$ZnP^* \rightarrow Ru_5(His)^{3+}$	0.82 (2)	$1.0\ (1) \times 10^2$

[a] Reference 30. [b] Reference 58. [c] Reference 29. [d] Reference 59. [e] Reference 61. [f] Reference 57. [g] Reference 23. [h] Reference 28.

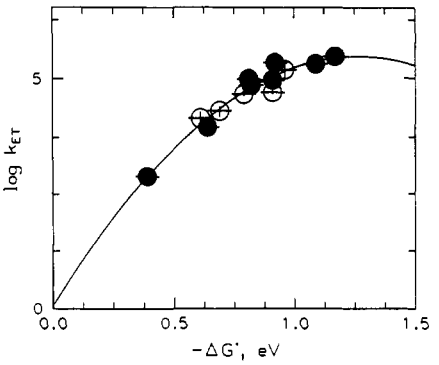

Figure 6. Free-energy dependence of intramolecular ET rates in Ru(His48)-modified Mb. Solid line is a fit to eq 1 using the parameters $\lambda = 1.26$ eV and $H_{AB} = 0.03$ cm^{-1}. (●) Photoinduced reactions; (○) recombination reactions.

of Ru-modified, metal-substituted Mb, however, are not accompanied by changes in metal coordination, and it is reasonable to expect that they can be described by a single set of ET parameters. The rates of 13 different ET reactions in Ru-modified, metal-substituted Mb have been reported (Table V), spanning nearly 0.8 eV in driving force. Fitting the photoinduced and thermal recombination rates to eq 1 yields $\lambda = 1.26$ eV and $H_{AB} = 0.03$ cm^{-1} (Figure 6).[59]

It has been shown that cyanogen bromide modification of His64 in the distal heme pocket of myoglobin inhibits coordination of a water ligand to the ferric heme.[60] It is likely, then, that the reorganization energy for ET in cyanogen bromide treated Ru(His48)–Fe–Mb will be nearly the same as that of the metal-substituted myoglobins. Fitting the two ET rates measured for this system (Table V) to eq 1, holding λ fixed equal to 1.26 eV, yields an electronic-coupling matrix element of 0.01 cm^{-1}.[61] As in the case of Ru-modified cytochrome *c*, the apparent coupling strength in Ru–ammine/iron–heme reactions is somewhat smaller than that found for reactions involving Ru–ammines and metal-substituted porphyrins.

Three ET rates have been measured with Ru–ammines bound to His48 of native Fe myoglobin (Table V).[23,57] The reorganization energy for these reactions can be estimated by assuming that the coupling strength is the same as that found in the cyanogen bromide treated systems (0.01 cm^{-1}) and optimizing λ. The data suggest $\lambda = 1.48$ eV,[59] a 0.2-eV increase over the value found in systems in which there is no change in coordination number.

B. Other His Derivatives

Electron-transfer kinetics have been examined in three additional Ru–pentaammine derivatives of sperm-whale Mb (His81, His116, His12). The D–A separations in these derivatives are substantially longer (19.3, 20.1, 22.0 Å, respectively) than that (12.7 Å) of the His48-modified Mb. Consequently, rates of photoinduced ET from ZnP* to these Ru^{III} complexes are at least 2 orders of magnitude slower than the corresponding His48 rate.[28] The reported ET rates for all three derivatives are quite similar, though the D–A distance increases by nearly 3 Å from His81 to His12. It is possible that bimolecular ET reactions contributed to the observed kinetics in this system. If so, then the 100-s^{-1} rate for the His12 system (and possibly for His81 and His116 as well) represents an upper limit to the true intramolecular ET rate.

TABLE VI. ET Rates in Ru(His)-Modified Proteins

electron transfer	$-\Delta G^\circ$, eV	k_{ET}, s^{-1}	*d*, Å
cytochrome b_5[a]			
Fe^{II}-Tb_5 → Ru_5(His26)$^{3+}$	0.08 (2)	1.4 (1)	12.1
Fe^{II}-LMb_5 → Ru_5(His26)$^{3+}$	0.10 (2)	5.9 (5)	12.0
Fe^{II}-DPb_5 → Ru_5(His26)$^{3+}$	0.13 (2)	0.2 (1)	12.9
azurin[b]			
Ru_5(His83)$^{2+}$ → Cu^{II}	0.28 (2)	1.9 (4)	12.0
plastocyanin[c]			
Ru_5(His59)$^{2+}$ → Cu^{II} (*A. variabilis*)	0.26 (2)	<0.08	12
Ru_5(His59)$^{2+}$ → Cu^{II} (*S. obliquus*)	0.28 (2)	<0.26	12
stellacyanin[d]			
Ru_5(His32,100)$^{2+}$ → Cu^{II}	0.26 (2)	0.05	(16.1)
HiPIP[e]			
Ru_5(His42)$^{2+}$ → $[Fe_4S_4]^{3+}$	0.27 (2)	18 (2)	7.9
cytochrome c_{551}[f]			
Ru_5(His47)$^{2+}$ → Fe^{III}	0.20 (2)	13 (2)	7.9

[a] Reference 63. [b] Reference 64. [c] Reference 25b. [d] Reference 26. [e] Reference 25c. [f] Reference 25a.

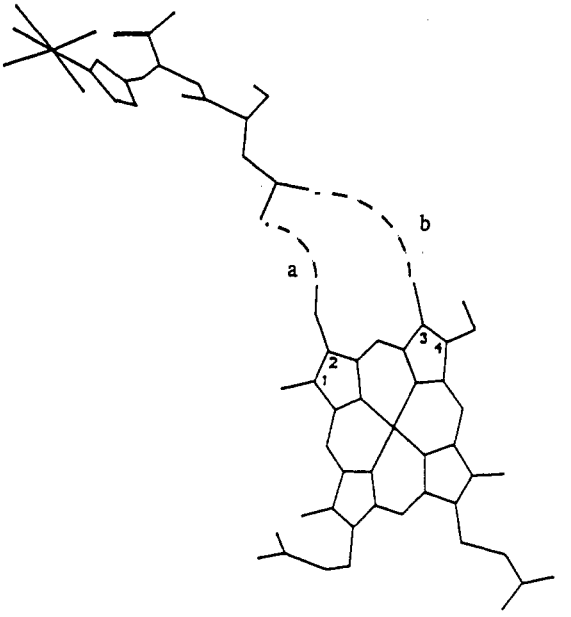

Figure 7. Best pathway through-space jump from Leu25 to the heme in Ru(His26)-modified cytochrome Tb_5 (a) and DPb_5 (b).

VI. Other Systems

A. Cytochrome b_5

Three surface His residues of Tb_5 have been modified by coordination to Ru–pentaammine complexes (His15, His80, His26).[62] Rates of intramolecular ET from Fe^{II} to Ru^{III} have been measured in three His26 derivatives: [Ru_5(His26)–Tb_5]; mutant (Asn57 to Asp, Gln13 to Glu, Glu11 to Gln, His15 to Asn, His80 to Asn) lipase-solubilized cyt b_5 [Ru_5(His26)LMb_5]; and DPb_5 [Ru_5(His26)DPb_5].[63] ET rates vary by more than an order of magnitude for the three proteins (Table VI), and the variations do not correlate with differences in driving force or estimated D–A separations. The pathway model has been invoked to account for the differences in rates. A critical through-space jump (from Leu25 to the heme) in the pathway from His26 to the heme is not constant in the three different pro-

teins (Figure 7). The dramatic reduction in rate in Rua_5(His26)DPb_5 has been attributed to the absence of the heme 2-vinyl group, which is the terminus of the Leu25 to heme through-space jump in the other two proteins.[63] A longer jump to the heme 3-methyl is predicted for Rua_5(His26)DPb_5, leading to a slower ET rate.

B. Copper Proteins

Azurin. Pseudomonas aeruginosa azurin has been derivatized at His83 with a Ru–pentaammine complex. Flash-photolysis measurements indicate that the rate of $Ru^{II} \rightarrow Cu^{II}$ ET is 1.9(4) s^{-1} and is virtually independent of temperature between 265 and 325 K.[64] The temperature dependence of the *P. aeruginosa* azurin potential[65] permits an estimate of the ET parameters from the variation in k_{ET} with temperature. The best fit of these data suggests $\lambda = 0.9$ eV and $H_{AB} = 4 \times 10^{-4}$ cm^{-1}.[35b] It is difficult to rationalize these substantial reductions in λ and H_{AB} (compared to His33 cytochrome *c* values). The direct donor–acceptor separation in this system (12.0 Å) is quite close to that of the His33 and His39 derivatives of cytochrome *c*, and it is not clear why H_{AB} would decrease by nearly 2 orders of magnitude. The pathway model does, however, predict $n_{eff} = 18.5$ bonds in Ru(His83)–azurin.[35b] This 4.5-bond increase over His33 and His39 cytochrome is consistent with a 25-fold decrease in H_{AB} (assuming a decay of 0.5 for each bond[12]). Though the electron self-exchange rate in *P. aeruginosa* azurin is substantially larger than that in cytochrome *c* (10^6 and 10^3 M^{-1} s^{-1}, respectively),[66,67] this is most likely the result of differences in the pre-exponential factor rather than a lower reorganization energy. It is clear that driving-force data are necessary to determine the best values of λ and H_{AB} in this system. Probably the most important lesson to be taken from these data is that considerable caution must be exercised when ET parameters are extracted from temperature dependences of rates.

Plastocyanin. His59 has been modified in two different species of algal plastocyanin: *Anabaena variabilis* and *Scenedesmus obliquus*.[25b] D–A separations in both are estimated to be about 12 Å, and the driving forces for ET from Ru^{II} to Cu^{II} are 0.26 and 0.28 eV, respectively. Hence, the distances and driving forces are similar to those found in His83-modified azurin and His33- and His39-modified cytochrome *c*. It is remarkable, then, that the ET reactions in the two plastocyanin derivatives are so slow (Table VI): <0.08 s^{-1} for *A. variabilis* and <0.26 s^{-1} for *S. obliquus*.[25b] It is not likely that these slow rates are attributable to a significant increase in λ; a substantial reduction in H_{AB} seems a more reasonable explanation. A 3–5-fold drop in H_{AB} compared to azurin (assuming identical values of λ) is necessary to explain the slower ET rates.

Stellacyanin. Rhus vernicifera stellacyanin is a blue copper protein with two surface His residues (His32, His100) that have been modified by coordination to Ru–pentaammine complexes.[26] Intramolecular ET from Ru^{II} to Cu^{II} was examined in the doubly modified derivative. The first-order rate constant for ET was reported to be 0.05 s^{-1} (Table VI). Since stellacyanin has not been structurally characterized, it is difficult to compare this rate to those for the other blue copper systems. On the basis of a *model* of the stellacyanin structure, a 16.1-Å D–A distance has been estimated.[26] The driving force for this ET reaction (0.10 eV) is somewhat smaller than that for the other blue copper derivatives. The combination of the longer distance and lower driving force could explain the relatively slow intramolecular ET in this system.

Intramolecular ET in blue copper proteins has not been studied as extensively as ET in the heme proteins. One reason for this is the difficulty in examining ET rates at high driving forces. The limited set of ET experiments on Ru–ammine-modified blue copper proteins offers some intriguing puzzles and clearly demonstrates the need for more data (especially driving-force dependences) to define better the electron-transfer parameters in these proteins.

C. HiPIP

High-potential iron–sulfur protein (HiPIP) from *Chromatium vinosum* has been modified with Ru–pentaammine at His42.[25c] This is a particularly interesting derivative because of the very short D–A distance (7.9 Å). The obvious prediction is that the $Ru^{II} \rightarrow [Fe_4S_4]^{3+}$ ($-\Delta G° = 0.27$ eV) ET rate in this derivative would be much higher than that found in any of the previous Ru-modified proteins. The surprising result, however, is that the ET rate is just 18 s^{-1} (Table VI).

D. Cytochrome c_{551}

A short D–A separation (7.9 Å) is also found in the Ru–pentaammine–His47 derivative of cytochrome c_{551} from *Pseudomonas stutzeri*. The driving force for intramolecular ET from Ru^{II} to the ferriheme is 0.2 eV, and the ET rate is reported to be 13 s^{-1}.[25a] As with HiPIP, the ET rate at ~8 Å is not substantially faster than that found in other systems with ~12-Å D–A separations. This result is puzzling. Crystal structures of both oxidized and reduced forms have been solved for *P. aeruginosa* cytochrome c_{551}; no large differences were found for the two oxidation states.[68] The structures are, in fact, remarkably similar to those of cytochrome c. The solvent exposure of the heme in cytochrome c_{551} is somewhat greater and could account for some increase in λ, but it is difficult to envision a change in λ large enough to explain the observed ET rate.

VII. Comparisons

On the basis of the few systems in which a reliable number has been extracted, $\lambda = 1.2$ eV appears to be a reasonable value for Ru–ammine-modified proteins. Perhaps due to lack of data and limited precision in the derived parameters, λ has not been found to be particularly sensitive to D–A separation or site of modification. In fact, the simple Marcus cross-relation provides a reasonably good estimate of the reorganization energies in these reactions. Since outer-sphere reorganization seems to dominate, changes in the Ru-coordination sphere (ammine → bipyridine) appear to have the greatest impact on λ.

Unlike the reorganization energy, the electronic-coupling strengths in the Ru-modified proteins show a great deal of variability. Equation 2 expresses a simple distance dependence for H_{AB} that adequately describes ET in model D–A complexes with values of β between

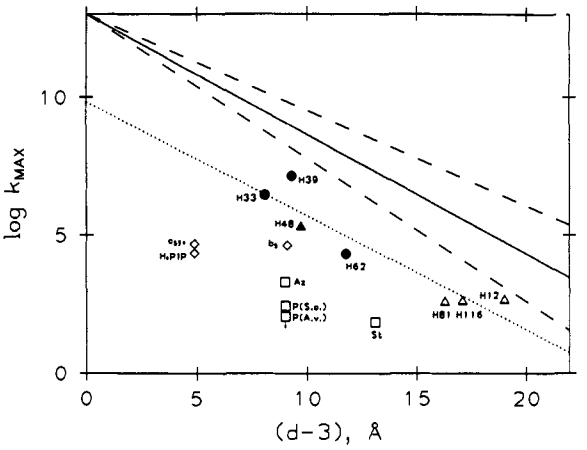

Figure 8. Plot of log k_{MAX} vs D–A distance (d-3) for Ru-modified proteins. Solid and dashed lines represent eq 2, β = 1.0 and 0.8, 1.2 Å^{-1}; dotted line represents the best fit to eq 2 for all of the cytochrome *c* and Mb ET data (H = His). Solid symbols indicate systems in which λ was estimated from a driving-force study. Open symbols indicate that an assumed value for λ (1.2 eV) was used to estimate k_{MAX}. (●) Cytochrome *c*; (▲, △) Mb; (□) blue copper proteins [Az, His83 derivative of azurin; P(S.o.), P(A.v.), *S. obliquus* and *A. variabilis* His59 derivatives of plastocyanin; St, His32,100 derivative of stellacyanin]; (◇) c_{551}, His47 derivative of cytochrome c_{551}; b_5, His26 derivative of cytochrome b_5; HiPIP, His42 derivative of high-potential iron–sulfur protein.

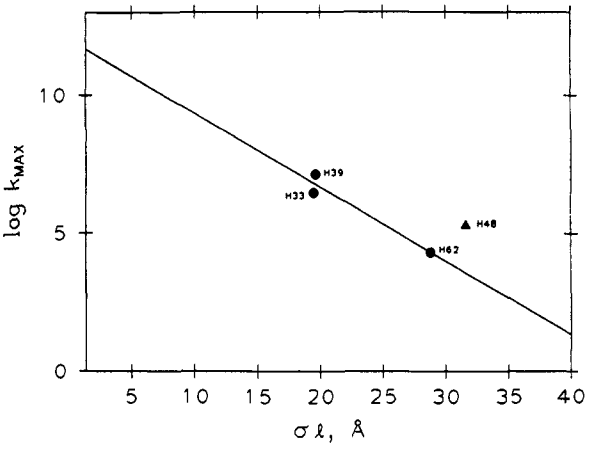

Figure 9. Plot of log k_{MAX} vs the tunneling length, $\sigma\ell$ (=η_{eff} × 1.4 Å/bond), of the physical pathway between donor and acceptor for three Ru-modified derivatives of cytochrome *c* (●) and one Mb derivative (▲). The solid line is a linear least-squares fit to the three cytochrome *c* points.

0.8 and 1.2 Å^{-1}. This distance dependence, assuming a maximum ET rate of 10^{13} s^{-1} at close contact (**d** = 3 Å), is represented by the solid (β = 1.0 Å^{-1}) and dashed (β = 0.8, 1.2 Å^{-1}) lines in Figure 8. Estimates of maximum ET rates (i.e., the rate at $-\Delta G^\circ = \lambda$) for Ru-modified proteins are plotted as a function of D–A separation (λ was assumed to be 1.2 eV for the cases in which this parameter has not been determined experimentally). It is clear that all of the maximum rates lie below the values predicted by eq 2 and that there is no simple correlation. A roughly linear distance dependence is found using just the cytochrome *c* and Mb points (with $\beta \approx 1.0$ Å^{-1}), but this line is displaced below that predicted by eq 2. The vertical displacement corresponds to a 3-order-of-magnitude decrease in k_{MAX}. This line predicts that the maximum ET rate at close contact is $<10^{10}$ s^{-1}, a rate well below that found in many D–A systems.[17,18,69] The obvious conclusion is that, for a given D–A separation, the electronic coupling in the Ru-modified proteins is substantially weaker than that predicted by a simple exponential decay with distance.

The Beratan–Onuchic pathway model predicts the failure of exponential-decay correlations based on edge–edge distances. According to this model, maximum ET rates correlate with the effective number of bonds in the pathway. [Multiplying n_{eff} by a canonical value of 1.4 Å/bond gives a tunneling length ($\sigma\ell$) that replaces **d** in rate–distance correlations.] Maximum ET rates in the three Ru-modified cytochromes *c* are plotted against $\sigma\ell$ in Figure 9. A linear least-squares fit to these three points gives the solid line with a slope of 0.6 Å^{-1}. Though the data are limited, it is important to note that the intercept at one bond (i.e., 1.4 Å) corresponds to a maximum ET rate of 4.6×10^{11} s^{-1}, which is in reasonable agreement with results from complexes with short D–A separations.[17,18,69]

The maximum ET rate for His48-modified Mb (n_{eff} = 22.6 bonds)[35b] is also plotted in Figure 9. This Mb point lies substantially above the line based on the cyt *c* data and clearly indicates a problem with the pathway model. In the simple form of this model, a single route is assumed to dominate the D–A coupling. The pathway-searching algorithm tends to support this assumption in cytochrome *c*, where single-coupling paths stand out. In Mb, however, the pathway-searching algorithm identifies many nearly equivalent pathways: the one used for the point in Figure 9 represents the best route, but there are several close competitors. The problem is again the tunneling distance: with many nearly equivalent paths contributing to D–A coupling, n_{eff} will be substantially below 22.6 bonds for His48 Mb. Efforts are being made to refine the pathway model to accommodate multiple paths.[70] If enough paths contribute to the overall electronic coupling in a given protein, the composition of any one path becomes relatively unimportant and tunneling lengths should closely parallel edge–edge distances.

VIII. Acknowledgment

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IX. References

(1) (a) Butler, J.; Chapman, S. K.; Davies, D. M.; Sykes, A. G.; Speck, S. H.; Osheroff, N.; Margoliash, E. *J. Biol. Chem.* **1983**, *258*, 6400–6404. (b) Osheroff, N.; Brautigan, D. L.; Margoliash, E. *J. Biol. Chem.* **1980**, *255*, 8245–8250. (c) Ferguson-Miller, S.; Brautigan, D. L.; Margoliash, E. *J. Biol. Chem.* **1978**, *253*, 149–159.
(2) Brunschwig, B. S.; DeLaive, P. J.; English, A. M.; Goldberg, M.; Gray, H. B.; Mayo, S. L.; Sutin, N. *Inorg. Chem.* **1985**, *24*, 3743–3749.
(3) (a) Natan, M. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1989**, *111*, 6468–6470. (b) McGourty, J. M.; Peterson-Kennedy, S. E.; Ruo, W. Y.; Hoffman, B. M. *Biochemistry* **1987**, *26*, 8302–8312. (c) Peterson-Kennedy, S. E.; McGourty, J. L.; Kalweit, J. A.; Hoffman, B. M. *J. Am. Chem. Soc.* **1986**, *108*, 1739–1746.
(4) Nocek, J. M.; Liang, N.; Wallin, S. A.; Mauk, A. G.; Hoffman, B. M. *J. Am. Chem. Soc.* **1990**, *112*, 1623–1625. (b) Liang, N.; Mauk, A. G.; Pielak, G. J.; Johnson, J. A.; Smith, M.; Hoffman, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1249–1252.
(5) McLendon, G. *Acc. Chem. Res.* **1988**, *21*, 160–167.
(6) Garg, A.; Onuchic, J. N.; Ambegaokar, V. *J. Chem. Phys.* **1985**, *83*, 4491–4503.

(7) Churg, A. K.; Weiss, R. M.; Warshel, A.; Takano, T. *J. Phys. Chem.* **1983**, *87*, 1683–1694.
(8) (a) Hoffman, B. M.; Ratner, M. R. *J. Am. Chem. Soc.* **1987**, *109*, 6237–6243. (b) Hoffman, B. M.; Ratner, M. R. *J. Am. Chem. Soc.* **1988**, *110*, 8267.
(9) Brunschwig, B. S.; Sutin, N. *J. Am. Chem. Soc.* **1989**, *111*, 7454–7465.
(10) (a) Kuki, A.; Wolynes, P. G. *Science* **1987**, *236*, 1647–1652. (b) Gruschus, J. M.; Kuki, A. *J. Am. Chem. Soc.* **1992**, submitted for publication.
(11) Siddarth, P.; Marcus, R. *J. Phys. Chem.* **1990**, *94*, 2985–2989.
(12) (a) Beratan, D. N.; Onuchic, J. N.; Betts, J. N.; Bowler, B. E.; Gray, H. B. *J. Am. Chem. Soc.* **1990**, *112*, 7915–7921. (b) Onuchic, J. N.; Beratan, D. N. *J. Chem. Phys.* **1990**, *92*, 722–733. (c) Beratan, D. N.; Onuchic, J. N. *Photosynth. Res.* **1989**, *22*, 173–186. (d) Beratan, D. N.; Onuchic, J. N.; Hopfield, J. J. *J. Chem. Phys.* **1987**, *86*, 4488–4498.
(13) Larsson, S. *J. Am. Chem. Soc.* **1981**, *103*, 4034–4040.
(14) Christensen, H. E. M.; Conrad, L. S.; Mikkelsen, K. V.; Nielsen, M. K.; Ulstrup, J. *Inorg. Chem.* **1990**, *29*, 2808–2816.
(15) A short list includes: (a) Dreyer, J. L. *Experientia* **1984**, *40*, 653–675. (b) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322. (c) McLendon, G. *Acc. Chem. Res.* **1988**, *21*, 160–167. (d) Williams, R. J. P. *Biochem. Int.* **1989**, *18*, 475–499. (e) Huber, R. *Eur. J. Biochem.* **1990**, *187*, 283–305. (f) Boxer, S. G. *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 267–299. (g) Bowler, B. E.; Raphael, A. L.; Gray, H. B. *Progr. Inorg. Chem.* **1990**, *38*, 259–322.
(16) (a) Winkler, J. R.; Nocera, D. G.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1982**, *104*, 5798–5800. (b) Nocera, D. G.; Winkler, J. R.; Yocom, K. M.; Bordignon, E.; Gray, H. B. *J. Am. Chem. Soc.* **1984**, *106*, 5145–5150.
(17) Fox, L. S.; Kozik, M.; Winkler, J. R.; Gray, H. B. *Science* **1990**, *247*, 1069–1071.
(18) (a) Gaines, G. L.; O'Neil, M. P.; Svec, W. A.; Niemczyk, M. P.; Wasielewski, M. R. *J. Am. Chem. Soc.* **1991**, *113*, 719–721. (b) Wasiewlewski, M. R.; Minsek, D. W.; Niemczyk, M. P.; Svec, W. A.; Yang, N. C. *J. Am. Chem. Soc.* **1991**, *112*, 2823–2824. (c) Wasielewski, M. R.; Niemczyk, M. P.; Johnson, D. G.; Svec, W. A.; Minsek, D. W. *Tetrahedron* **1989**, *42*, 4785–4806. (d) Wasielewski, M. R.; Johnson, D. G.; Svec, W. A.; Kersey, K. M.; Minsek, D. W. *J. Am. Chem. Soc.* **1988**, *110*, 7219–7221. (e) Wasielewski, M. R. *Photochem. Photobiol.* **1988**, *47*, 923–929. (f) Wasielewski, M. R.; Niemczyk, M. P.; Svec, W. A.; Pewitt, E. B. *J. Am. Chem. Soc.* **1985**, *107*, 1080–1082. (g) Wasielewski, M. R.; Niemczyk, M. P.; Svec, W. A.; Pewitt, E. B. *J. Am. Chem. Soc.* **1985**, *107*, 5562–5563.
(19) (a) Gust, D.; Moore, D. A. *Top. Curr. Chem.* **1991**, *159*, 103–151. (b) Gust, D.; Moore, T. A. *Science* **1990**, *248*, 199–201. (c) Gust, D.; Moore, T. A. *Science* **1989**, *244*, 35–41. (d) Gust, D.; Moore, T. A.; Makings, L. R.; Liddell, P. A.; Nemeth, G. A.; Moore, A. L. *J. Am. Chem. Soc.* **1986**, *108*, 8028–8031.
(20) (a) Kroon, J.; Verhoeven, J. W.; Paddon-Row, M. N.; Oliver, A. M. *Angew. Chem.* **1991**, *30*, 1358–1361. (b) Warman, J. M.; Hom, M.; Paddon-Row, M. N.; Oliver, A. M.; Kroon, J. *Chem. Phys. Lett.* **1990**, *172*, 114–119. (c) Oevering, H.; Verhoeven, J. W.; Paddon-Row, M. N.; Warman, J. M. *Tetrahedron* **1989**, *45*, 4751–4766. (d) Paddon-Row, M. N.; Oliver, A. M.; Warman, J. M.; Smit, K. J.; DeHaas, M. P.; Oevering, H.; Verhoeven, J. W. *J. Phys. Chem.* **1988**, *92*, 6952–6962. (e) Oevering, H.; Paddon-Row, M. N.; Heppener, M.; Oliver, A. M.; Cotsaris, E.; Verhoeven, J. W.; Hush, N. S. *J. Am. Chem. Soc.* **1987**, *109*, 3258–3269.
(21) Leland, B. A.; Joran, A. D.; Felker, P. M.; Hopfield, J. J.; Zewail, A. H.; Dervan, P. B. *J. Phys. Chem.* **1985**, *89*, 5571–5573.
(22) Closs, G. L.; Miller, J. R. *Science* **1988**, *240*, 440–447.
(23) Lieber, C. M.; Karas, J. L.; Gray, H. B. *J. Am. Chem. Soc.* **1987**, *109*, 3779–3780.
(24) (a) Bechtold, R.; Gardineer, M. B.; Kazmi, A.; van Hemelryck, B.; Isied, S. S. *J. Phys. Chem.* **1986**, *90*, 3800–3804. (b) Isied, S. S.; Keuhn, C.; Worosila, G. *J. Am. Chem. Soc.* **1984**, *106*, 1722–1726. (c) Isied, S. S.; Worosila, G.; Atherton, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 7659–7661.
(25) (a) Osvath, P.; Salmon, G. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1988**, *110*, 7114–7118. (b) Jackman, M. P.; McGinnis, J.; Powls, R.; Salmon, G. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1988**, *110*, 5880–5887. (c) Jackman, M. P.; Lim, M.-C.; Sykes, A. G.; Salmon, G. A. *J. Chem. Soc., Dalton Trans.* **1988**, 2843–2850.
(26) Farver, O.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6968–6972.
(27) Elias, H.; Chou, M. H.; Winkler, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 429–434.
(28) Axup, A. W.; Albin, M.; Mayo, S. L.; Crutchley, R. J.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 435–439.
(29) Karas, J. L.; Lieber, C. M.; Gray, H. B. *J. Am. Chem. Soc.* **1988**, *110*, 599–600.
(30) Cowan, J. A.; Upmacis, R. K.; Beratan, D. N.; Onuchic, J. N.; Gray, H. B. *Ann. N. Y. Acad. Sci.* **1989**, *550*, 68–84.
(31) Meade, T. J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 4353–4356.
(32) Therien, M. J.; Selman, M. A.; Gray, H. B.; Chang, I.-J.; Winkler, J. R. *J. Am. Chem. Soc.* **1990**, *112*, 2420–2422.
(33) Hoffman, M. Z.; Bolletta, F.; Moggi, L.; Hug, G. L. *J. Phys. Chem. Ref. Data* **1989**, *18*, 219–543.
(34) (a) Durham, B.; Pan, L. P.; Long, J. E.; Millett, F. *Biochemistry* **1989**, *28*, 8659–8665. (b) Pan, L. P.; Durham, B.; Wolinska, J.; Millett, F. *Biochemistry* **1988**, *27*, 7180–7184.
(35) (a) Chang, I.-J.; Gray, H. B.; Winkler, J. R. *J. Am. Chem. Soc.* **1991**, *113*, 7056–7057. (b) Bjerrum, M. J.; Casimiro, D. R.; Chang, I.-J.; Colón, J. L.; DiBilio, A. J.; Wuttke, D. S.; Winkler, J. R. Unpublished results.
(36) (a) Jortner, J.; Bixon, M. *J. Chem. Phys.* **1988**, *88*, 167–170. (b) Rips, I.; Jortner, J. *J. Chem. Phys.* **1987**, *87*, 2090–2104.
(37) Jarzęba, W.; Walker, G. C.; Johnson, A. E.; Kahlow, M. A.; Barbara, P. F. *J. Phys. Chem.* **1988**, *92*, 7039–7041.
(38) Bashkin, J. S.; McLendon, G.; Mukamel, S.; Marohn, J. *J. Phys. Chem.* **1990**, *94*, 4757–4761.
(39) Hopfield, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3640–3644.
(40) (a) Vassilian, A.; Wishart, J. F.; van Hemelryck, B.; Schwarz, H.; Isied, S. S. *J. Am. Chem. Soc.* **1990**, *112*, 7278–7286. (b) Isied, S. S.; Vassilian, A.; Wishart, J. F.; Creutz, C.; Schwarz, H.; Sutin, N. *J. Am. Chem. Soc.* **1988**, *110*, 635–637.
(41) Miller, J. R.; Beitz, J. V.; Huddleston, R. K. *J. Am. Chem. Soc.* **1984**, *106*, 5057–5068.
(42) Strauch, S.; McLendon, G.; McGuire, M.; Guarr, T. *J. Phys. Chem.* **1983**, *87*, 3579–3581.
(43) (a) Dorfman, R. C.; Lin, Y.; Zimmt, M. B.; Baumann, J.; Domingue, R. P.; Fayer, M. D. *J. Phys. Chem.* **1988**, *92*, 4258–4260. (b) Domingue, R. P.; Fayer, M. D. *J. Chem. Phys.* **1985**, *83*, 2242–2251.
(44) Yocom, K. M.; Shelton, J. B.; Shelton, J. R.; Schroeder, W. E.; Worosila, G.; Isied, S. S.; Bordignon, E.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 7052–7055. (b) Yocom, K. M. Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1981.
(45) Brown, G. M.; Sutin, N. *J. Am. Chem. Soc.* **1979**, *101*, 883–892.
(46) Direct D–A separations are taken as the shortest edge–edge distance based on crystal structure coordinates and energy-minimized models in which Ru complexes have been coordinated to amino acid side chains. The value of **d** for Ru_{65}-(His33)–Fe–cyt *c* was determined using the recent horse heart cytochrome *c* crystal structure: Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585–595.
(47) (a) Vanderkooi, J. M.; Ericińska, M. *Eur. J. Biochem.* **1975**, *60*, 199–207. (b) Fisher, W. R.; Taniuchi, H.; Anfinsen, C. B. *J. Biol. Chem.* **1973**, *248*, 3188–3195.
(48) Brunschwig, B. S.; Ehrenson, S.; Sutin, N. *J. Phys. Chem.* **1986**, *90*, 3657–3668.
(49) (a) Isied, S. S. In *Metals in Biological Systems*; Sigel, H., Sigel, A., Eds.; Dekker: New York, 1991; Vol. 27, pp 1–56. (b) Isied, S. S. In *ACS Advances in Chemistry Series*; Johnson, M. K., King, R. B., Kurtz, D. M., Kutal, C., Norton, M. L., Scott, R. A., Eds.; American Chemical Society: Washington, DC, 1990; No. 226, pp 91–100. (c) Isied, S. S. In *ACS Advances in Chemistry Series*; Bolton, J. R., Mataga, N., McLendon, G., Eds.; American Chemical Society: Washington, DC, 1991; No. 228, pp 229–245. (d) Bechtold, R.; Kuehn, C.; Lepre, C.; Isied, S. S. *Nature* **1986**, *322*, 286–288.
(50) Eggleston, D. S.; Goldsby, K. A.; Hodgson, D. J.; Meyer, T. J. *Inorg. Chem.* **1985**, *24*, 4573–4580.
(51) Selman, M. A. Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1989.
(52) Bowler, B. E.; Meade, T. J.; Mayo, S. L.; Richards, J. H.; Gray, H. B. *J. Am. Chem. Soc.* **1989**, *111*, 8757.
(53) Therien, M. J.; Bowler, B. E.; Selman, M. A.; Gray, H. B.; Chang, I.-J.; Winkler, J. R. In *ACS Advances in Chemistry Series*; Bolton, J. R., Mataga, N., McLendon, G., Eds.; American Chemical Society: Washington, DC, 1991; No. 228, pp 191–200.
(54) Millett, F.; Durham, B. In *Metals in Biological Systems*; Sigel, H., Sigel, A., Eds.; Dekker: New York, 1991; Vol. 27, pp 223–264.
(55) Pan, L. P.; Frame, M.; Durham, B.; Davis, D.; Millett, F. *Biochemistry* **1990**, *29*, 3231–3236.
(56) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in their Reactions with Ligands*; North-Holland Publishing: Amsterdam, 1971.
(57) Crutchley, R. J.; Ellis, W. R.; Gray, H. B. *J. Am. Chem. Soc.* **1985**, *107*, 5002–5004.
(58) (a) Karas, J. L. Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1989. (b) Moss, M.; Haymore, B. L. Unpublished results.
(59) Zewert, T. E.; Wong, L. L.; Upmacis, R. K.; Cowan, J. A.; Karas, J. L.; Winkler, J. R.; Gray, H. B. Unpublished results.
(60) (a) Kamiya, N.; Shiro, Y.; Iwata, T.; Iizuka, T.; Iwasaki, H. *J. Am. Chem. Soc.* **1991**, *113*, 1826–1829. (b) Morishima, I.; Shiro, J.; Wakino, T. *J. Am. Chem. Soc.* **1985**, *107*, 1063–1065.

(c) Shiro, Y.; Morishima, I. *Biochemistry* **1984**, *23*, 4879–4884.
(61) Zewert, T. E. Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1990.
(62) Jacobs, B. A. Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1991.
(63) Jacobs, B. A.; Mauk, M. R.; Funk, W. D.; MacGillivray, R. T. A.; Mauk, A. G.; Gray, H. B. *J. Am. Chem. Soc.* **1991**, *113*, 4390–4394.
(64) Kostič, N. M.; Margalit, R.; Che, C.-M.; Gray, H. B. *J. Am. Chem. Soc.* **1983**, *105*, 7765–7767.
(65) Margalit, R.; Kostič, N. M.; Che, C.-M.; Blair, D. F.; Chang, H.-J.; Pecht, I.; Shelton, J. B.; Shelton, J. R.; Schroeder, W. A.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6554–6558.
(66) (a) Gupta, R. K.; Koenig, S. H.; Redfield, A. G. *J. Magn. Reson.* **1972**, *7*, 66–74. (b) Concar, D. W.; Hill, A. O.; Moore, G. R.; Whitford, D.; Williams, R. J. P. *FEBS Lett.* **1986**, *206*, 15–19.
(67) (a) Uğurbil, K.; Mitra, S. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2039–2043. (b) Groeneveld, C. M.; Canters, G. W. *J. Biol. Chem.* **1988**, *263*, 167–173. (c) Groeneveld, C. M.; Ouwerling, M. C.; Erkelens, C.; Canters, G. W. *J. Mol. Biol.* **1988**, *200*, 189–199.
(68) Matsuura, Y.; Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1982**, *156*, 389–409.
(69) Holten, D.; Hoganson, C.; Windsor, M. W.; Schenck, C. C.; Parson, W. W.; Migus, A.; Fork, R. L.; Shank, C. V. *Biochim. Biophys. Acta* **1980**, *592*, 461–477.
(70) Beratan, D. N.; Onuchic, J. N. Unpublished results.