Intramolecular Electron Transfer in Cytochrome b_5 Labeled with Ruthenium(II) Polypyridine Complexes: Rate Measurements in the Marcus Inverted Region

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Abstract: Three derivatives of recombinant T65C-cytochrome b_5 were produced by labeling the single sulfhydryl group at Cys-65 with (4-(bromomethyl)-4′-methylbipyridine)bis(bipyridine)ruthenium(2+), (4-bromomethyl-4′-methylbipyridine)bis(4,4′-dimethylbipyridine)ruthenium(2+), and (4-(bromomethyl)-4′-methylbipyridine)(bipyrimidine)-(bipyridine)ruthenium(2+). The ruthenium labels are linked to the heme iron through a well-defined 12-covalent-bond path and provide a range of free energies of reaction which bracket the expected reorganizational energy of 0.94 eV. The rate constants for photoinduced electron transfer from Ru(II*) to the ferric heme were 1.4×10^7, 1.7×10^7, and 6×10^5 s^{-1}, respectively, for the three different labeled proteins. The rate constants for the thermal back-reaction from the ferrous heme to Ru(III) were 6.0×10^6, 5.4×10^6, and 2.3×10^6 s^{-1}, respectively. The rate constants show an inverse dependence on driving force, as predicted by Marcus (Marcus, R. A. *J. Chem. Phys.* **1957**, *26*, 867–871), with an electronic coupling term consistent with parameters for covalent bond coupling suggested by Beratan et al. (Beratan, D. N.; Onuchic, J. N.; Betts, J. N.; Bowler, B. E.; Gray, H. B. *J. Am. Chem. Soc.* **1990**, *112*, 7915–7920). The electronic coupling is also consistent with a simple exponential decay model with $\beta = 1.4$ Å and a through space separation of 12 Å. The rate constants were independent of temperature over the range +25 to −90 °C.

Over the past two decades a considerable effort has been made to better understand electron transfer processes in proteins.[1] A variety of strategies have been employed in studies of this problem, including labeling of structurally characterized proteins or genetically altered proteins with ruthenium(II) amine or bipyridine complexes,[2] with cobalt cage complexes,[3] and by substitution of metals in the heme ring.[4,5] The results of most of these studies have been examined in the context of the semiclassical theory suggested by Marcus,[6,7] which can be summarized, for reactions between two redox centers held at a fixed distance, by the equation

$$k_{et} = \frac{4\pi^2}{h} H_{AB}{}^2 \frac{1}{(4\pi\lambda RT)^{1/2}} \exp[-(\Delta G^{\circ\prime} + \lambda)^2/4\lambda RT] \quad (1)$$

where H_{AB} describes the electronic coupling between the redox centers and λ describes the nuclear reorganizational energy.

Early studies by Gray and co-workers[4,8] and Isied and co-workers[9] focused on the dependence of the rate of electron transfer on the distance between the two redox centers. These studies seemed to indicate that a simple exponential dependence adequately accommodated the observed rates. The work with proteins was paralleled by studies of synthetic models with rigid spacers by Closs, Miller, and co-workers and several other investigators.[10–13] One frequently encountered problem in these investigations was the correlation of through-space separation distance with the number of covalent bonds separating the redox centers.

The problem of electronic coupling of redox centers through a protein has been addressed by Beratan et al., Siddarth and Marcus, Kuki, and others.[14] Beratan and co-workers[14a–d] have suggested a pathways model in which the electronic coupling between redox centers in a protein can be described by a combination of through-bond, through-hydrogen-bond and through-space contributions which maximize the overall electronic coupling. Gray and co-workers in a series of papers involving predominantly cytochrome *c* have demonstrated that this approach does provide a better explanation of the kinetic data than the direct through-space distance approach in some cases.[1,15]

Other studies of the electron-transfer processes in metalloproteins have focused on the free energy dependence.[2] Much of the emphasis in these investigations has been on finding systems which clearly show the quadratic dependence on driving force

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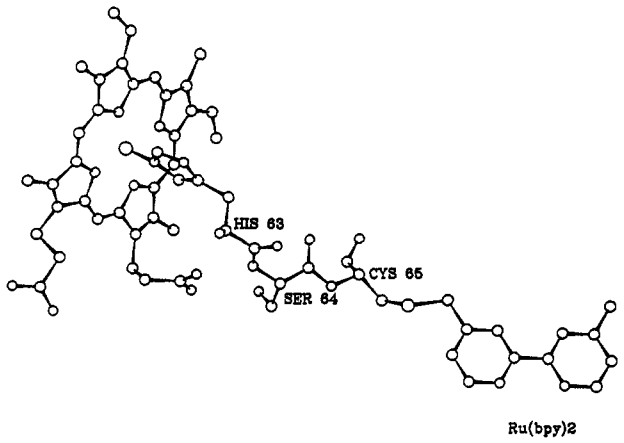

Figure 1. Heme and residues 63, 64, and 65 of cytochrome b_5 with 4-methylene-4′-methylbipyridine fragment bonded to sulfur of Cys-65 using the X-ray structure of cytochrome b_5[27] in the Brookhaven Protein Data Bank.[28] Native threonine 65 was replaced with cysteine and the bipyridine added using the Biosym molecular modeling package. The carbonyls of the amino acids are labeled, and all hydrogens have been deleted for clarity.

predicted by eq 1. This has proven to be a difficult task, and only a few synthetic systems were found until recently.[13,16,17] Continuing work in this area has been driven by several important features of reactions at high driving force. First, systems which clearly show the quadratic dependence on $\Delta G°$ predicted by eq 1 also provide the most definitive determinations of λ, the nuclear reorganization energy. Secondly, when $\Delta G° = -\lambda$, eq 1 is reduced to the preexponential term, which allows investigators to focus on the remaining electronic coupling term. Thirdly, it appears that the inverted behavior may be important in reducing the extent of charge recombination in photosynthetic reaction centers.[18]

The following is a report on a system prepared by covalently bonding various derivatives of tris(bipyridine)ruthenium(II) to a mutant of cytochrome b_5, T65C, which has been genetically engineered to provide an optimal point of attachment for the ruthenium label.[19] The covalent bond is through a thioether link formed with the cysteine sulfhydryl group at position 65, which is two residues away from one of the ligating histidines at position 63. This provides a well-defined 12-covalent-bond path (measured from the edge of the bipyridine ligand to the axial histidine of the heme iron) between the redox centers which is illustrated in Figure 1. Electron transfer can be initiated by photoexcitation of the ruthenium complexes which are strong reductants in the excited state. The reorganizational barriers for the 2+*/3+ and the 2+/3+ couples of the ruthenium complexes are in the range 0.5–0.6 eV. This feature coupled with the high over all free energy for the reaction results in a system which clearly shows the predicted inverse parabolic dependence on $\Delta G°$. In addition, the system requires no external reagents, and thus measurements can be made at low temperatures or in frozen matrices to further define the reaction energetics. Kinetic measurements over the temperature range +25 to −90 °C are described.

Experimental Section

Materials. T65C was prepared as described by Stayton et al.[20] Ru-65-cyt b_5 was prepared by the reaction of T65C cytochrome b_5 with (4-(bromomethyl))-(4′-methylbipyridine)bis(bipyridine)ruthenium(II)(2+) as described by Geren et al.[21] T65C cytochrome b_5 labeled with (4-(bromomethyl)-4′-methylbipyridine)bis(4,4′-dimethylbipyridine)ruthenium(II)(2+) (Me_5Ru-65-cyt b_5) was prepared by analogous procedures using 4,4′-dimethylbipyridine in place of bipyridine in each step of the synthesis. The same procedure was followed in the preparation of T65C labeled with (4-(bromomethyl)-4′-methylbipyridine)(bipyridine)(bipyrimidine)ruthenium(II)(2+) (bpymRu-65-cyt b_5). Ru(bpy)Cl_3 was prepared as described by Krause.[22]

Ru(bpy)(bipyrimidine)(4-(bromomethyl)-4′-methylbipyridine)Br_2. Ru(bpy)Cl_3 (2.6 g, 7.2 mmol) was mixed with pyridine (4 mL, 50 mmol) in 125 mL of water with 40 mL of ethanol and refluxed for 24 h. The mixture was evaporated to dryness and washed with ether to remove traces of pyridine. The solid was mixed with 4-(hydroxymethyl)-4′-methylbipyridine (1.42 g, 7.1 mmol),[21] 50 mL of water, and 10 mL of ethanol and heated on a steam bath for 2 h. Concentrated hydrochloric acid (100 mL) was added and the mixture refluxed for 24 h and allowed to stand for 2 weeks. The black solid was recovered by vacuum filtration, washed with water, 2-propanol, and acetone, and air dried. Cyclic voltammetry at this stage indicated that the product was Ru(bpy)(4-(hydroxymethyl)-4′-methylbipyridine)Cl_2 as expected. Ru(bpy)(4-(hydroxymethyl)-4′-methylbipyridine)Cl_2 (0.88 g, 1.6 mmol) was then refluxed with 2,2′-bipyrimidine (0.3 g, 1.9 mmol) in 40 mL of water for 2 h. After cooling, 1 mL of a concentrated solution of NH_4PF_6 was added to precipitate the product. The product was recovered by vacuum filtration, washed with water and ether, and air dried. The product was further purified by chromatography on a 2.5 × 16 cm alumina column with 1:1 acetonitrile/methylene chloride as the eluent. The Ru(bpy)(4-(hydroxymethyl)-4′-methylbipyridine)(bipyrimidine)$(PF_6)_2$ (0.08 g, 88 mmol) was stirred with 2 mL of thionyl bromide for 18 h and finally precipitated by adding the mixture to ether dropwise. The complex was recovered as the bromide salt, since most of the PF_6^- decomposed during the reaction. The identity of the complex was checked before and after bromination by comparison of the ^1H NMR, redox potentials, and visible absorption spectrum to literature values[23] for [Ru(bpy)$_2$(2,2′-bipyrimidine)]$(PF_6)_2$.

Characterization of Labeled Cytochrome b_5. The labeled proteins were purified using a Waters 625 LC system equipped with a DEAE 8HR column and were eluted with a linear gradient from 20 to 500 mM sodium phosphate, pH 7. The product peak was concentrated and washed twice with 100 mM sodium phosphate buffer, pH 7, using Amicon concentrators. The location of the ruthenium label was determined by digesting the labeled proteins with 10% *Staphylococcus aureus* protease in 50 mM sodium phosphate (pH 7.8). HPLC of the digest was performed as described by Geren et al.[21] using two detectors in series set at 210 and 450 nm. The chromatograms of the digested proteins all showed one major peak and some minor peaks which contained ruthenium. An Applied 473A protein sequencer was used to determine that the major peptide was DVGHS[C]DARE and the minor peptides were DVGHS[C]D, VGHS[C]D, and NFEDVGHS[C]DARE. Native Cys-65 was missing from the sequence of each peptide, indicating that Cys-65 was the site of the labeling. The visible absorption spectra of the labeled proteins were the same as the sum of the absorption spectra of the 1:1 mixtures of the complexes and the native T65C. The redox potential of the heme iron in each of the labeled cytochromes b_5 was +15 ± 10 mV in 100 mM sodium phoshate buffer (pH 7), which compares favorably to +10 mV for the native cytochrome b_5.[24]

Electrochemistry. Redox potentials of the free ruthenium complexes were determined by cyclic voltammetry using a platinum bead working electrode, a saturated sodium chloride calomel reference electrode, and a platinum wire auxiliary electrode in either 100 mM aqueous sodium phosphate buffer (pH 7) or 100 mM TBAH in acetonitrile. The reduction

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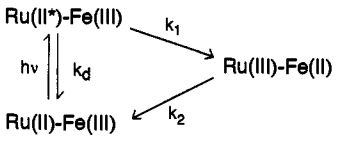
Scheme I



potentials of the heme groups in the labeled proteins were determined spectrophotometrically as previously described.[19] Cyclic voltammetry was not performed on the labeled proteins due to interference by solvent oxidation at the potentials of interest and the low concentrations attainable. However, the emission maximum at 77 K, which is sensitive to the redox potential of the ruthenium complex, was the same for Ru-65-cyt b_5 and the free complex.

Flash Photolysis Experiments. Transient absorbance measurements were performed by laser flash photolysis of 300-μL samples contained in 1-cm-path glass semimicrocuvettes. The samples were excited at 90° with respect to the probe beam with the third harmonic from an Nd:YAG Quanta-Ray DCR 1. The pulsed probe beam, detector, and associated electronics were the same as previously described.[25] The signals were recorded with a LeCroy 7200 series digital oscilloscope and transferred to a PC for analysis, Transients were stored either as single transients or as averages of 20. No differences between the single-shot and averaged transients were noted other than the expected improvement in signal to noise ratio, and no degradation of the transients or absorbance spectra was observed in samples subjected to hundreds of laser flashes. The rate constants indicated in Scheme I were determined as previously described[25] except in the case of bpymRu-65-cyt b_5. In this case $k_d \gg k_1$ and the initial absorbance changes are dominated by the ruthenium excited-state transient absorbance. The small amount of Fe(II) produced decayed exponentially with a rate described by k_2. The forward rate constant k_1 was calculated from the ratio of Ru(II)* to Fe(II) produced immediately after the laser pulse and the relation between the quantum yields, $\Phi_{Fe(II)} = \Phi_{Ru(II)^*} k_1/(k_1 + k_d)$. The reactions of the heme group in each derivative were monitored at 424 and 556 nm, and that of the ruthenium complexes, at the heme isobestic points 439 and 547 nm. Emission decay kinetics were determined as previously described.[25] All measurements in aqueous solutions were in 100 mM sodium phosphate (pH 7) with 5–20 μM protein. Measurements were made in both air-saturated and nitrogen-purged solutions. No difference was observed.

Low-Temperature Kinetic Measurements. The flash photolysis equipment was the same as that described above. The sample was cooled by regulating the passage of cooled dry nitrogen around the cuvette as described by Churg et al.[26] The cuvette holder was held in an aluminum box 10 × 10 × 25 cm. The temperature was measured using an Omega CN380 series temperature controller fitted with a T type thermocouple emersed in the solution. Emission decay measurements were made by attaching a PMT housing with a low-pass filter over one of the openings in the aluminum box provided for the probe beam of the flash photolysis system. The cryosolvent was 2:1 ethylene glycol/100 mM aqueous sodium phosphate, pH 7.

Results

T65C cytochrome b_5 was reacted with three different ruthenium(II) complexes containing 4-(bromomethyl)-4′-methylbipyridine. The reaction with (4-(bromomethyl)-4′-methylbipyridine)bis(bipyridine)ruthenium(II)(2+) and the resultant labeled protein Ru-65-cyt b_5 has been described previously.[19] Reactions with the other two complexes yielded proteins covalently bonded to the ruthenium complexes through thioether links at cysteine 65, as in the previous example. The location of the label in each case was verified using HPLC of *S. aureus* protease digests followed by sequencing of the ruthenium-containing peptide fractions. The integrity of the proteins after labeling was confirmed by comparison of the visible spectra and the heme redox potentials. In addition, studies of the binding and electron-transfer reactions of Ru-65-cyt b_5 with cytochrome *c* indicated that there were no significant conformational differences between the labeled protein and native cytochrome b_5 in solutions of moderate ionic strengths.[19]

Table I. Reduction Potentials vs NHE and Emission Energies for the Free Ruthenium(II) Complexes

complex[a]	$E_{1/2}(2+/3+)$, V[b]	$E_{1/2}(2+^*/3+)$, V[c]	emission E_{0-0}, eV[d]
$Ru(bpy)_2(mebpyOH)$	1.27	−0.85	2.12
$Ru(dmbpy)_2(mebpyOH)$	1.17	−0.94	2.11
Ru(bpy)(bpym)(mebpyOH)	1.40[e]	−0.34	1.74

[a] All complexes have 2+ charge and mebpyOH is 4-(hydroxymethyl)-4′-methylbipyridine. [b] Standard deviation for reduction potentials is 5 mV. [c] Calculated from 2+/3+ potentials and emission energies (see text for details). [d] Emission energies determined from emission maxima recorded at 77 K in ethylene glycol/water frozen glass. [e] Potential measured in acetonitrile and corrected to 100 mM phosphate.

The synthesis, characterization, and photochemistry of the ruthenium complexes as well as a number of other potential candidates have been thoroughly described in the literature.[23] The ruthenium complexes selected in this study were chosen to span a range of redox potentials that bracketed the expected reorganizational energy of 1 eV and for their compatability with the reaction conditions (in particular, stability in the bromination step and adequate solubility in water),

It was not possible to accurately measure the redox potentials of the ruthenium complexes attached to the proteins either by the technique used for the heme iron or by cyclic voltammetry because of the high positive potentials required and interference from the aqueous solvent. The redox potentials and the visible absorption spectra of the ruthenium complexes, however, appear to be insensitive to the nature of the substituents on the 4-methyl of the bipyridine and should not be strongly affected by the covalent link to the protein (e.g. the bromo and hydroxy forms of the complexes have the same $E_{1/2}$). In addition, the emission maximum at 77 K, which is sensitive to the redox potential of the ruthenium complex, was the same for Ru-65-cyt b_5 and the free complex. The redox potentials of the free ruthenium complexes in 100 mM sodium phosphate buffer are reported in Table I. The excited-state potentials were calculated from the luminescence maxima obtained at 77 K in an ethylene glycol/100 mM sodium phoshate buffer glass, as described by Kalyanasundarum,[23b] The emission energies are indicated in Table I.

Excitation of the ruthenium complexes with a short laser pulse produced an excited state which was oxidatively quenched through electron transfer to the heme iron. This was followed by a rapid thermal back-reaction between Ru(III) and Fe(II) which returned the system to its original redox states. The overall reaction is indicated in Scheme I, where k_1 is the rate constant for the excited-state electron transfer and k_2 is the rate constant for the thermal back electron-transfer reaction. All other reactions which return the excited state to the ground state are characterized by the rate constant k_d.

The reaction sequence has been thoroughly investigated in other systems[25] and was shown to apply by monitoring the reaction at appropriate wavelengths. For example, Figure 2 illustrates the transient absorbance at 556 nm and the transient difference spectrum taken shortly after excitation which confirms the transient production of Fe(II). Figure 3 illustrates typical transients observed at 424 nm which correspond to the appearance and disappearance of Fe(II). The rate constants were obtained by simultaneusly fitting the transient absorbance and emission data as previously described[25] except with bpymRu-65-cyt b_5. In

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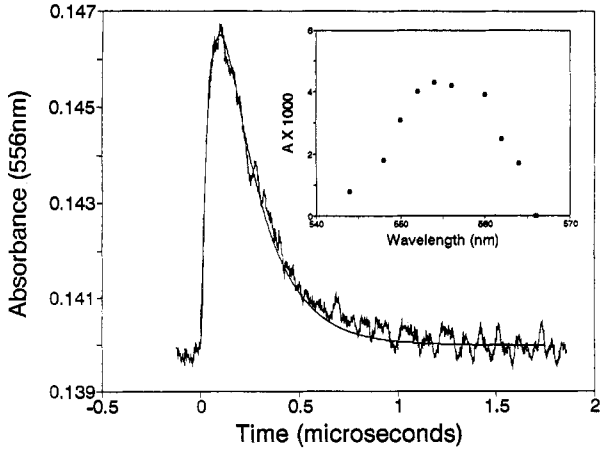

Figure 2. Absorption transient recorded at 556 nm with a 20 μM solution of Ru-65-cyt b_5 in 100 mM phosphate buffer, pH 7, at 22 °C. The smooth curve is the best fit of the data to eq 2 in ref 25. The inset shows the transient difference spectrum under identical conditions determined by measuring the absorbance change between 0 and 30 ns after the laser pulse.

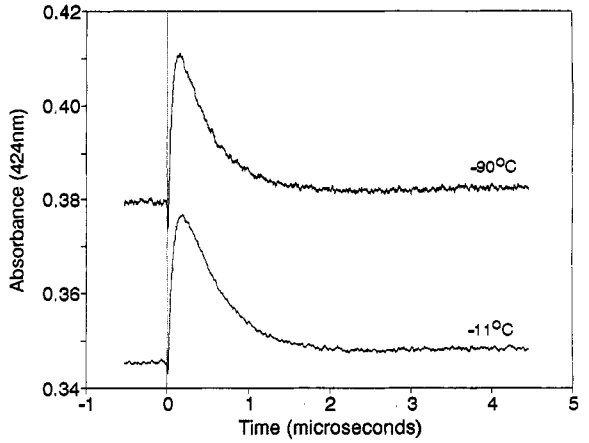

Figure 3. Transient absorbance of a 10 μM solution of Me_5Ru-65-cyt b_5 in 2:1 ethylene glycol/100 mM sodium phosphate buffer (pH 7) monitored at 424 nm. Upper trace was taken at -90 °C and the lower trace at -11 °C.

this case, $k_d \gg k_1$ and the initial part of transient absorbance profile was dominated by excited-state absorbance. The thermal back-reaction, however, is slow compared to k_d and well separated from the transient absorbance due to the excited state of the ruthenium complex. The rate constant k_2 was obtained by treating the transient absorption decay after the laser pulse as a simple exponential. The forward rate constant k_1 was calculated from the ratio of Ru(II)* and Fe(II) produced immediately after the laser pulse. All of the rate constants are summarized in Table II.

In order to investigate the temperature dependence of these reactions, the solvent was changed to 2:1 ethylene glycol/100 mM sodium phosphate buffer (pH 7), Me_5Ru-65-cyt b_5 was well behaved in this solvent and showed transient absorbance profiles at all wavelengths similar to those obtained in 100 mM sodium phosphate buffer. Both Ru-65-cyt b_5 and bpymRu-65-cyt b_5 exhibited transient behavior similar to that observed in 100 mM sodium phosphate buffer, but the nature of the transients changed with continued exposure to light (room lighting was the major contributor). This was only a minor problem with Ru-65-cyt b_5, and experiments could be successfully completed if the samples were protected from light. The difficulty was severe with bpymRu-65-cyt b_5, and accurate rate constants could not be obtained in this solvent. The problem appears to be a result of oxidation of the solvent by the Ru(III) intermediate, since the severity correlates with the oxidizing strength of ruthenium complexes and the transient absorbance changes indicate that Fe(II) is formed but not reoxidized. The visible spectrum of solutions containing Me_5Ru-65-cyt b_5 held in the dark over the temperature range +25 to -73 °C showed no change in the soret band over a period of 4 h. The rate constants are listed in Table II.

Table II. Rate Constants[a] for the Photoinduced Electron-Transfer Reactions of the T65C Cytochrome b_5 Derivatives in 100 mM Sodium Phosphate Buffer (pH 7) and 2:1 Ethylene Glycol/100 mM Sodium Phosphate Buffer

	k_1, s^{-1}	k_2, s^{-1}	k_d, s^{-1}
Complex at +22 °C in Aqueous Buffer			
Ru-65-cyt b_5	1.4×10^7	6.0×10^6	3.0×10^1
Me_5Ru-65-cyt b_5	1.7×10^7	5.4×10^6	2.3×10^6
bpymRu-65-cyt b_5	6×10^5	2.3×10^6	5×10^7
Complex at +25 °C in Glycol/Water			
Ru-65-cyt b_5	4.0×10^6	3.2×10^6	4.7×10^6
Me_5Ru-65-cyt b_5	6.2×10^6	2.7×10^6	5.0×10^6
Complex at -90 °C in Glycol/Water			
Ru-65-cyt b_5	4.0×10^6	3.2×10^6	4.7×10^6
Me_5Ru-56-cyt b_5	6.0×10^6	2.5×10^6	5.0×10^6

[a] Standard deviation of rate constants is 10%.

The rates of the reactions measured in the cryosolvent were temperature independent over the temperature range +25 to -90 °C. Examples of transients collected at two different temperatures are shown in Figure 3. In each case, the best fit kinetic parameters obtained at the higher temperature were also the best or very close to the best fits for the low-temperature data. Given a conservative error estimate of 20% in the determination of rate constants, the enthalpy of activation is less than 100 cal.

The decay rates for emission of the free ruthenium complexes decreased by about 25% over this temperature range, which is characteristic of tris(bipyridine)ruthenium(II) complexes.[23] The decay rates for emission from the labeled proteins, which are governed by $k_1 + k_d$, exhibited similar changes with temperature, and the changes can be attributed entirely to variations in k_d.

Discussion

The ability to produce site-specific mutants of cytochrome b_5[20] coupled with the labeling with ruthenium polypyridine complexes first described by Geren et al.[21] and expanded in this report provides an excellent test environment for the investigation of electron-tranfer reactions in a protein matrix. The free energy of reaction and the distance separating the redox centers (and to an increasing extent the nature of the protein media within the space between the redox centers) are the experimental parameters most often used to probe electron-transfer processes in terms of eq 1. These parameters are well defined in the systems described here. NMR studies indicate that synthetic rat cytochrome b_5 is very similar structurally to beef liver cytochrome b_5 as is the T65C mutant protein used in this investigation.[20] Cysteine 65 lies just outside the conserved patch of negative charge, 11 Å from the nearest heme edge, and provides a point of attachment for the ruthenium label which is only 12 bonds removed from the ligating histidine 63. The chemistry utilized in the labeling step is extremely flexible and allows complexes to be tailored to experimental questions. In the current study, three complexes were chosen which have redox potentials such that the free energies of reaction bracket the expected reorganizational energy.

Figure 4 illustrates an overall fit of the rate constants determined with this system to eq 1. The only adjustable parameter used to generate the solid "best fit" line was the preexponential term. The reorganization barrier λ was determined from literature values of 1.3 eV for cytochrome b_5[15,29] and 0.54 and 0.56 eV for the

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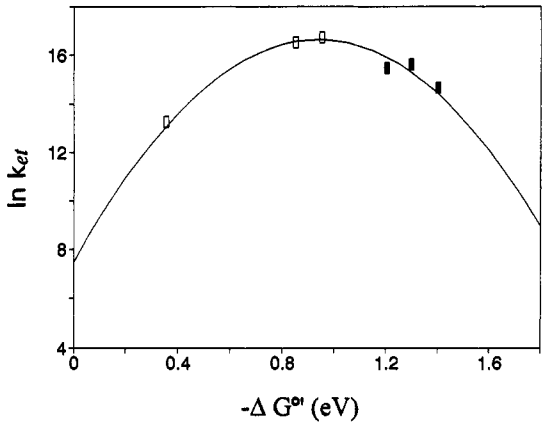

Figure 4. Plot of free energy of reaction versus natural logarithm of the rate constant for electron transfer k_{et} described by eq 1. The solid line shows the theoretical dependence with a reorganizational energy of 0.94 eV and preexponential term of 1.7×10^7 s^{-1}. The open boxes are for the reactions involving the excited state, $k_{et} = k_1$, and the filled boxes are for the thermal back-reaction, $k_{et} = k_2$, all determined at 22 °C.

2+/3+ and 2+*/3+ couples for the ruthenium complexes,[30] respectively, This later estimate is taken from data for tris(bipyridine)ruthenium(II)(2+), which should be very similar to the complexes used in this investigation.

Plots such as that given in Figure 4 are difficult to make, since it must be assumed that both the reorganizational energies and the electronic coupling of the reactants are the same or very similar. In the present example, the reorganizational barriers meet this criteria but it is not clear that the electronic coupling terms for the excited-state reactions and the ground-state reactions also meet this criteria. On the other hand, some recent experimental data on related systems indicate that the electronic terms are sufficiently similar to allow the comparison made in Figure 4. In the present system the electronic contribution from the ruthenium complexes is only a small part of the overall coupling path which is, otherwise, constant. The situation is similar to that reported by Meade et al.[31] with zinc-substituted cytochrome *c* labeled with ruthenium ammine complexes. In this example, the preexponential terms for the excited-state and ground-state reactions, with redox centers separated by distances comparable to the present case, were 3.3×10^6 and 2.0×10^6, respectively. Yonemoto et al.[17] recently reported a factor of 10 difference between excited-state and ground-state coupling with reactions of tris(bipyridine)ruthenium(II) separated by a single methylene group from modified viologens. In this case, where only a single methylene group separates the redox centers, there are relatively few bonds contributing to the overall electronic coupling and differences in orbital overlap to the ruthenium complex (excited state vs ground state) may have a larger impact. Finally, given the literature values for the reorganizational barriers, the best fit of the data given in Figure 4 is obtained with identical preexponential terms for the excited-state and ground-state reactions. The very small activation energies are also consistent with reactions near the activationless regime.

The preexponential term in eq 1, which describes the electronic coupling between the ruthenium complexes and the heme iron, determined from the maximum of the plot in Figure 4, is 1.7×10^7 s^{-1}, or by substitution of the appropriate constants, $H_{AB} = 0.25$ cm^{-1}. In the past, investigations of the electronic coupling between redox centers were focused on the through-space distance. More recently Beratan and co-workers[14] have suggested a hierarchical coupling model which allows for through-bond, through-hydrogen-bond, and through-space coupling. Beratan and co-workers[14c] have developed procedures for chosing the best path or maximum electronic coupling between the redox sites, In the proteins described in this paper the ruthenium complexes in the labeled proteins are two amino acids away from the ligating histidine 63 and have an obvious coupling path containing only 12 covalent bonds with no hydrogen bonds or through space jumps. Using the procedure described by Beratan et al.[14a] for the calculation of the relative electronic coupling term for covalent bonds, with $e_c = 0.6$ and a scalar for the one-bond limit of 3×10^{12} s^{-1} as used by Wuttke et al.[15b] for cytochrome *c*, a preexponential term of 1.4×10^7 s^{-1} ($H_{AB} = 0.23$ cm^{-1}) was calculated, which is remarkably similar to the observed coupling. Alternatively, a simple exponential decay model[32] with $\beta = 1.4$ Å^{-1} and a frequency factor of 3×10^{12} s^{-1} yields a preexponential term of 1.1×10^7 s^{-1} ($H_{AB} = 0.20$ cm^{-1}). The good agreement between the two models is expected given the mathematical similarity when only covalent bonds are involved in the coupling. Wuttke and co-workers[15b] have noted that in cytochrome *c* labeled with different ruthenium complexes the electronic coupling was weaker than that predicted by the exponential decay model when through-space jumps or hydrogen bonding appeared in the calculated pathways.

Most of the previous measurements of electron transfer in proteins labeled with ruthenium complexes have involved best coupling paths which included some noncovalently linked contributions.[14a,15b] The present system is well defined in most respects and appears to utilize only covalent bonds in coupling the redox centers. As such, the experimental data presented here is complimentary to other nonprotein systems with all covalent bonds[33] and may be useful in providing a calibration for the covalent bond component in calculations such as those described by Beratan and co-workers.[14]

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