d^8-d^9 configuration, it is not possible with the limited information in hand to rule out entirely the latter ground state.

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Kinetics of Intramolecular Electron Transfer from Ru^{II} to Fe^{III} in Ruthenium-Modified Cytochrome *c*

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Abstract: The kinetics of intramolecular electron transfer in $Ru(NH_3)_5$(histidine-33)$^{2+}$-ferricytochrome *c* (PFe^{III}-Ru^{II}) and intermolecular electron transfer from $Ru(NH_3)_5L^{2+}$ (L = histidine, imidazole, NH_3) to ferricytochrome *c* (PFe^{III}) have been studied by transient absorption and stopped-flow spectroscopic techniques. Electron transfer from electronically excited $Ru(bpy)_3^{2+}$ (bpy = 2,2'-bipyridine) to PFe^{III}-Ru^{III} produces PFe^{III}-Ru^{II} in fivefold excess to PFe^{II}-Ru^{III}, and in the presence of EDTA (which rapidly reduces $Ru(bpy)_3^{3+}$) the PFe^{III}-Ru^{II} decays mainly by intramolecular electron transfer to PFe^{II}-Ru^{III}. At pH 7 (μ = 0.1 M) the rate constant (30 (3) s^{-1}, 23 °C) does not vary substantially over the temperature range 0-80 °C, thereby allowing an upper limit of 1.5 kcal mol^{-1} to be placed on $\Delta H^{\ddagger}$. Above 80 °C, intramolecular $Ru^{II} \rightarrow Fe^{III}$ electron transfer is not observed, owing to the displacement of methionine-80 from the iron coordination sphere. Combining the activation enthalpy of the intramolecular electron-transfer reaction with the redox thermodynamic parameters for the $Ru(NH_3)_5L^{2+}/PFe^{III}$ system allows an upper limit of 8 kcal mol^{-1} to be placed on the $PFe^{III/II}$ reorganizational enthalpy. The very small activation enthalpies for both the intramolecular and intermolecular electron-transfer reactions demonstrate that the enthalpic contribution to the free energy of formation of the $Ru(NH_3)_5L^{2+}/PFe^{III}$ precursor complex is almost zero. The activation entropy for the intramolecular process (-48 (2) eu) is more negative than that for the $Ru(NH_3)_5His^{2+}/PFe^{III}$ reaction (-36 (1) eu), which suggests that the electron-transfer distance is shorter than 11.8 Å in the intermolecular precursor complex.

Introduction

Several research groups have begun to report the results of experiments that bear directly on the role of redox-site separation distance on metalloprotein electron-transfer rates.[2-6] An approach that we have found attractive is to measure the electron-transfer kinetics in semisynthetic systems in which redox-active inorganic reagents are bound covalently to specific residues on the polypeptide chain of a structurally well-characterized electron-transfer metalloprotein. In these semisynthetic systems the electron-transfer distances are fixed and known.

It has been shown that horse heart ferricytochrome *c* (PFe^{III}) reacts with excess $Ru(NH_3)_5OH_2^{2+}$ to produce pentaammine(histidine-33)ruthenium(III)-ferricytochrome *c* (PFe^{III}-Ru^{III}) (Figure 1).[2,3,7] Preliminary experiments with the modified protein demonstrated that intramolecular electron transfer from Ru^{II} to PFe^{III} proceeds at a significant rate.[2,3a] We now have completed a thorough study of the intramolecular electron-transfer kinetics in this semisynthetic system. The interpretation of the results has been aided by an investigation of the thermodynamics as well as the kinetics of the intermolecular electron-transfer reaction between $Ru(NH_3)_5His^{2+}$ and PFe^{III}.

Experimental Section

Materials. All protein and buffer solutions were prepared by using distilled water that was purified by passage through a Barnstead Nanopure water purification system. Phosphate buffers were prepared from analytical grade reagents, and HEPES buffers were prepared from the sodium salt and acid forms of HEPES (Calbiochem). Horse heart cytochrome *c* (type VI; Sigma Chemical Co.) was purified on a CM-cellulose (Watman CM52) column prior to use in order to remove deamidated forms of the protein.[8]

The chloride salt of hexaammineruthenium(II), prepared by the method of Lever and Powell,[9] was recrystallized according to the following procedure. Two grams of crude product was dissolved in 20 mL of boiling aqueous ammonia (15%). To ensure complete reduction of the ruthenium, zinc powder was added to the ammonia solution, which was then filtered while hot. The filtrate was placed under an argon counterflow, and NH_4Cl was added to the solution. After the solution was cooled to 0 °C, the yellow-orange precipitate was collected, washed with cold aqueous ammonia and cold acetone, and dried under vacuum. $[Ru(NH_3)_5Cl]Cl_2$ was prepared from $[Ru(NH_3)_6]Cl_2$ (Matthey-Bishop)[10] and recrystallized from 0.1 M HCl.[11] $[Ru(NH_3)_5His]Cl_3 \cdot H_2O$ was obtained from the reaction of L-histidine (Sigma) with $[Ru(NH_3)_5Cl]Cl_2$[12] over zinc amalgam and was purified by ion-exchange chromatog-

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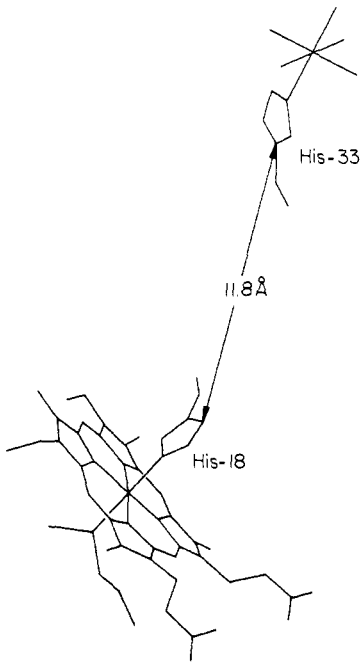

Figure 1. Structural model for selected parts of $Ru(NH_3)_5$(histidine-33)–ferricytochrome *c* based on coordinates for the tuna protein (Swanson, R.; Trus, B. L.; Mandel, N.; Mandel, G.; Kallai, O. B.; Dickerson, R. E. *J. Biol. Chem.* **1977**, *252*, 759–775). In this view we have assumed that the imidazole of His-33 is coincident with the five-membered ring of Trp-33 in the tuna structure.

raphy followed by recrystallization from an acetone/water solution. The chloride salt of $Ru(NH_3)_5Im^{3+}$ (Im = imidazole) was obtained from the reaction of $[Ru(NH_3)_5Cl]Cl_2$ and Im over zinc amalgam followed by air oxidation.[13] 4,4′-Bipyridine dihydrate and EDTA (Baker) were recrystallized from water. The sodium salt of diethylenetriaminepentaacetic acid (Aldrich) was prepared by neutralization of a solution of the acid with NaOH. Crystalline tris(2,2′-bipyridine)ruthenium(II) chloride was obtained from Sigma and used without further purification.

The semisynthetic protein PFe^{III}–Ru^{III} was prepared according to the published procedure[7] with slight modification. The reaction of $Ru(NH_3)_5OH_2^{2+}$ with cytochrome *c* was allowed to proceed for 24 h in HEPES buffer (pH 7.0, μ = 0.1 M) after which the reaction was terminated by separation on a Sephadex-G25 column. The cytochrome fraction collected from the column was then equilibrated against water. This modification increased the yield of PFe^{III}–Ru^{III} by a factor of 3.

All electrochemical and kinetic experiments were performed on freshly prepared semisynthetic protein.

Instrumentation and Methods. Stopped-flow kinetics of the reduction of PFe^{III} by $Ru(NH_3)_5His^{2+}$ were measured with a Durrum Model D-110 spectrophotometer that had been modified for anaerobic manipulations.[14,15] Sodium phosphate buffer solutions (μ = 0.1 M, pH 7.0) of PFe^{III} (4–6 μM) were deoxygenated by direct argon bubbling for 15 min prior to kinetic measurements. A $Ru(NH_3)_5His^{2+}$ stock solution was prepared by adding zinc amalgam to deoxygenated sodium phosphate buffer solutions (μ = 0.1 M, pH 7.0) of the Ru^{III} salt. Solutions for stopped-flow measurements were obtained by transferring the appropriate aliquot of Ru^{II} stock solutions via a gas-tight syringe equipped with a Millipore filter to deoxygenated buffer to give the desired $Ru(NH_3)_5His^{2+}$ concentration (ϵ_{260} = 3620 M^{-1} cm^{-1}, ϵ_{280} = 3160 M^{-1} cm^{-1}).[12] The temperature bath surrounding the syringes containing the reactant solutions was maintained to ±0.1 °C (Forma-Scientific bath, Gorman-Rupp circulating pump). Reactant solutions were allowed to equilibrate with the temperature bath for a minimum of 15 min prior to kinetic measurements. The rate of PFe^{III} reduction was monitored at 550 nm ($\Delta\epsilon_{550}$ = 18 500 M^{-1} cm^{-1}).[16] Absorbance changes of 0.05–0.10 were monitored, and $Ru(NH_3)_5His^{2+}$ concentrations were in pseudo-first-order excess over PFe^{III} concentrations. Rate constants for the observed kinetic measurements were calculated by utilizing an iterative nonlinear least-squares method based on the Newton–Gauss approach (KINPRO).[14] The observed rate constant reported at each reductant concentration represent the average of at least four reproducible kinetic runs. A weighted least-squares program (KI2FIT) was used to calculate the second-order rate constant, and the temperature dependence of the rate was fit to the Eyring expression by a weighted least-squares program (EYRFIT) to yield values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.[14]

Rate constants for the quenching of the $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine) excited state ($Ru(bpy)_3^{2+*}$) were determined by measuring its lifetime as a function of quencher concentration. The emission lifetime measurements were performed with a pulsed laser system built at Caltech. The excitation source was a Quanta Ray DCR-1 Nd:YAG laser frequency doubled with a Quanta Ray HG-1 harmonic generator followed by a Quanta Ray PHS-1 prism harmonic separator to produce a 532-nm pulse of 8-ns (fwhm) duration at 10 Hz. Light emitted from the sample was collected at 90° to the excitation beam with a collimating lens (*f*/1.3) and then focused by a second lens (*f*/6.5) through a Corning 3-67 color filter onto the entrance slit of a MacPherson 0.35-m monochromator. $Ru(bpy)_3^{2+}$ luminescence was monitored at 650 nm and detected by a Hamamatsu Model R928 photomultiplier tube, and the signal passed through a LeCroy Model VV101ATM amplifier to the 50-Ω impedance input of a Biomation Model 6500 waveform recorder. Laser triggering, data acquisition, and data analysis were controlled with a Digital Model PDP11/03-L computer. In luminescence quenching experiments with $Ru(NH_3)_5His^{3+}$, a solution of $[Ru(bpy)_3]Cl_3$ in phosphate buffer (μ = 0.1 M, pH 7.0) in a vacuum flask with a side-arm 1-cm path-length fluorescence cuvette was degassed on a vacuum line with five freeze–pump–thaw cycles. The quencher concentrations were adjusted by sequential additions of $[Ru(NH_3)_5His]Cl_3 \cdot H_2O$ under vacuum. Lifetimes were determined from least-squares fits to data averaged from 500 laser pulses. Samples of PFe^{III} and PFe^{III}–Ru^{III} were prepared for quenching experiments by lyophilization of a known quantity of protein in a 10-mL round-bottom flask fitted with a small tube (30 mm long, 3 mm diameter) on the side. All further manipulations were performed under a nitrogen atmosphere in a Vacuum Atmosphere Co. HE-43-2 Dri Lab plus HE-493 Dri Train inert-atmosphere box. The protein was dissolved with a known volume (initial volume, 200 μL) of vacuum degassed stock solution of $Ru(bpy)_3^{2+}$ in phosphate buffer (μ = 0.1 M, pH 7.0), and dilutions were performed by successive additions of aliquots from this stock solution. The $Ru(bpy)_3^{2+}$/protein solution was laser irradiated in the side-arm tube portion of the flask, and lifetimes were determined from least-squares fits to data averaged from 500 laser pulses.

Transient absorption experiments were performed with the laser excitation and detection systems described above but with a different optical train. In these experiments the laser excitation beam and a probe beam intersected in a 160° angle at a sample contained in a 1-mm path length cuvette oriented with its face normal to the probe beam direction. The probe beam originated from a 150-W Xe arc lamp in an Oriel Model 6137 universal lamp housing with a *f*/1.0 UV grade fused silica lens, passed through a Corning 3-71 color filter, and focused onto the sample with a *f*/7.8 lens. The beam was mechanically shuttered just before the sample with a Uniblitz Model 2118 shutter and Uniblitz Model DS 122B shutter drive unit. After emerging from the sample, the probe beam passed through a collimating lens (*f*/4.0) was deflected by two mirrors and focused by a *f*/4.0 lens through a Corning 3-67 color filter onto the entrance slit of the monochromator. The output from the photomultiplier tube was monitored with a Tektronix Model P6201 probe and fed into the 50 Ω impedance input of the Biomation waveform recorder. Laser triggering, shutter timing, data acquisition, and data analysis were controlled by the PDP 11/03-L computer. Known quantities of protein were prepared for transient absorption experiments by lyophilization, then dissolved in degassed $Ru(bpy)_3^{2+}$/phosphate buffer in the inert-atmosphere box, and transferred to air-tight 1-mm cuvettes. Data were averaged over 1000 laser pulses. Laser power was optimized before each set of data was collected. A correction for a small base line transient signal was performed by recording with the laser beam blocked, one set of base line data for each set of real data. This base line trace was subtracted from the observed trace to produce a corrected transient decay signal.

Flash photolysis experiments were performed on an apparatus constructed at Caltech and described previously.[17] This instrument was modified slightly by the inclusion of a DC voltage offset circuit at the output of the photomultiplier tube in order to permit greater vertical sensitivity on the Biomation waveform recorder. Corning 3-71 or 3-75 color filters were used for excitation filtering. Samples for these experiments were contained in an air-tight cylindrical cell (15 cm long, 0.8

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Table I. Thermodynamic Parameters for the Reduction of PFe^{III} and $Ru(NH_3)_5His^{3+}$ in Aqueous Sodium Phosphate (pH 7, μ = 0.1 M)

	$PFe^{III/II}$ [a]	$PFe^{III/II}$–Ru^{III}	$Ru(NH_3)_5$-$His^{3+/2+}$ [b]
$E°$, mV vs. NHE (25 °C)	260 (2)	270 (2)	80 (5)
$\Delta S°$, eu	−28.5 (12)	−29.2 (8)	−3.4 (20)
$\Delta S°_{rc}$, eu	−12.9 (12)	−13.6 (8)	12.2 (20)
$\Delta G°$, kcal mol^{-1}	−6.0 (5)	−6.22 (5)	−1.96 (12)
$\Delta H°$, kcal mol^{-1}	−14.5 (4)	−14.9 (3)	−3.0 (8)

[a] Reference 19. [b] $NaClO_4$, 0.1 M. [c] $\Delta S°_{rc} = S°_{red} - S°_{ox}$.

cm diameter) equipped with a water cooling jacket. A known quantity of protein in phosphate buffer (μ = 0.1 M, pH 7.0) was lyophilized and then dissolved in the appropriate volume of degassed water (to maintain the phosphate buffer conditions) under a nitrogen atmosphere in the glovebox. Aliquots from this protein stock solution were mixed with known quantities of a degassed $Ru(bpy)_3^{2+}$/phosphate buffer solution to yield solutions for flash photolysis that were between 1 and 5 μM in protein and had an absorbance approximately equal to 1 at 450 nm (1-cm path length). Na_2EDTA was dissolved directly in the flash cell with the $Ru(bpy)_3^{2+}$/protein/phosphate buffer solution (30 mg/11 mL). The temperature of the flash photolysis solution was maintained by circulating a 50:50 water/ethylene glycol solution through the cooling jacket. Temperatures were measured after each experiment by immersion of a copper/constantan thermocouple directly into the solution used for flash photolysis measurements. Data were hand digitized, and rate constants were determined from nonlinear least-squares fits.

Differential pulse voltammetry was performed with a Princeton Applied Research (PAR) Model 174A polarographic analyzer (57-ms pulse width) at a scan rate of 2 mV s^{-1} and a 0.5-s drop time. The electrochemical cell was of standard H cell design with a glass-sintered fine frit separating the working and reference electrode compartments. These compartments were enclosed within a glass reservoir through which a constant temperature water bath (25.0 ± 0.1 °C) flowed. A three-way stopcock valve allowed either the top or the bottom of the working electrode compartment to be purged with argon to ensure anaerobic electrochemical measurements. The electrode system consisted of a gold button (2-mm diameter) working electrode (Bioanalytical, Inc.), a Pt wire auxiliary electrode, and a saturated sodium chloride calomel (SSCE) reference electrode. Electrochemical measurements on the semisynthetic protein were performed in sodium phosphate buffer (μ = 0.1 M, pH 7.0) solutions containing 0.1 M $NaClO_4$ supporting electrolyte and 0.01 M 4,4′-bipyridyl, which acts as an electron-transfer mediator between cytochrome *c* and the solid gold electrode.[18] Protein concentrations were typically between 1 and 3 mM. Working solutions were degassed for 15 min by gentle argon bubbling and then blanketed by an argon flow during electrochemical measurements. The gold button electrode was polished with fine alumina in a water suspension prior to immersion into the electrochemical solution.

Nonisothermal cyclic voltammetric experiments were performed with a PAR 173 potentiostat/galvanostat and PAR 175 universal programmer using a doubly jacketed H-cell. The electrode system was the same as that used in the differential pulse experiments. The temperature of the reference electrode compartment was maintained at 25.0 (1) °C, and that of the working electrode compartment was varied from 5.0 to 40.0 (1) °C.

Optically transparent thin-layer electrolysis (OTTLE) cells in a nonisothermal electrochemical cell configuration were used to determine formal electrode potentials for the heme center in PFe^{III}–Ru^{III}. The OTTLE cells and spectrochemical procedures for metalloproteins have been described elsewhere.[19] A gold electroformed mesh (60% transmittance) was used as the working electrode material. The SSCE reference electrode was maintained at 25.0 ± 0.1 °C. Sodium phosphate buffer solutions (μ = 0.1 M, pH 7.0) for spectroelectrochemical measurments were 0.16 mM in PFe^{III}–Ru^{III}. A mediator titrant, $[Ru(NH_3)_5(py)](ClO_4)_3$ (0.8 mM), was employed for the PFe^{III}–Ru^{III} experiment. Standard reaction entropies and enthalpies were determined from plots of the formal electrode potential vs. temperature. Formal electrode potentials and *n* values were determined from Nernst plots at eight different temperatures (0–40 °C). Seven data points were included in each Nernst plot.

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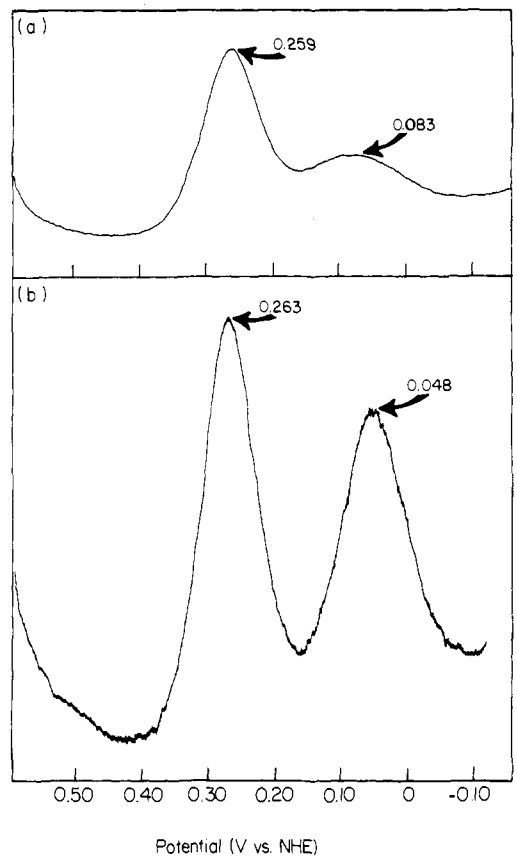

Figure 2. Nonisothermal differential pulse voltammograms for a sodium phosphate buffer solution (pH 7.0, μ = 0.1 M) containing PFe^{III}–Ru^{III} (5 mg/mL), $NaClO_4$ (0.1 M), and 4,4′-bipyridyl (0.01 M) at (a) 25.0 (2) °C and (b) 5.0 (2) °C (potential vs. NHE (25 °C)). Voltammogram b displayed at twice the sensitivity of a.

Electronic absorption spectra were measured on Cary 17 and Cary 219 spectrometers.

Results and Discussion

The thermodynamic parameters derived from the temperature dependence of the reduction potential of the heme center in PFe^{III}–Ru^{III} along with the corresponding parameters for PFe^{III} are set out in Table I. Because the high optical density of the heme protein precluded spectroelectrochemical determination of the reduction potential of the ruthenium center, differential pulse (DP) voltammetry was employed to obtain $E°(Ru^{III/II})$ in P-Fe^{III}–Ru^{III}. Although the DP voltammogram at 25 °C is distinctly asymmetric, with the $Ru(NH_3)_5(His\text{-}33)^{3+/2+}$ charge-transfer wave being considerably broader and smaller in height than the heme center wave (Figure 2a), the two peaks are well separated and indicate reduction potentials at the ruthenium and heme centers of 80 (5) and 260 (5) mV (vs. NHE), respectively. Notably, as the temperature of the system is lowered, the DP voltammogram becomes more symmetric; at 5 °C, the shapes of the two waves in the DP are nearly identical (Figure 2b). Additionally, the shapes and positions of the DP waves are independent of potential scan direction at all temperatures investigated.

The temperature dependence of the $Ru(NH_3)_5His^{3+/2+}$ redox couple was determined by nonisothermal cyclic voltammetry (Table I). The free energy change ($\Delta G°$) for the PFe^{III}/Ru-$(NH_3)_5His^{2+}$ electron-transfer reaction is −4.0 (2) kcal mol^{-1} at 25 °C, with $\Delta H°$ = −11.5 (10) kcal mol^{-1} and $\Delta S°$ = −25.1 (30) eu. Taking the $Ru(NH_3)_5His^{3+/2+}$ results as the reference standard for $Ru(NH_3)_5(His\text{-}33)^{3+/2+}$, the corresponding parameters for the intramolecular electron-transfer reaction (PFe^{III}–Ru^{II} → PFe^{II}–Ru^{III}) are as follows: $\Delta G°$ = −4.3 (2) kcal mol^{-1} at 25 °C; $\Delta H°$ = −11.9 (10) kcal mol^{-1}; and $\Delta S°$ = −25.8 (30) eu.

Stopped-flow absorbance-time kinetic data for the reduction of PFe^{III} by excess $Ru(NH_3)_5L^{2+}$ (L = Im, His, NH_3) in phosphate buffer solutions show first-order kinetics for greater than

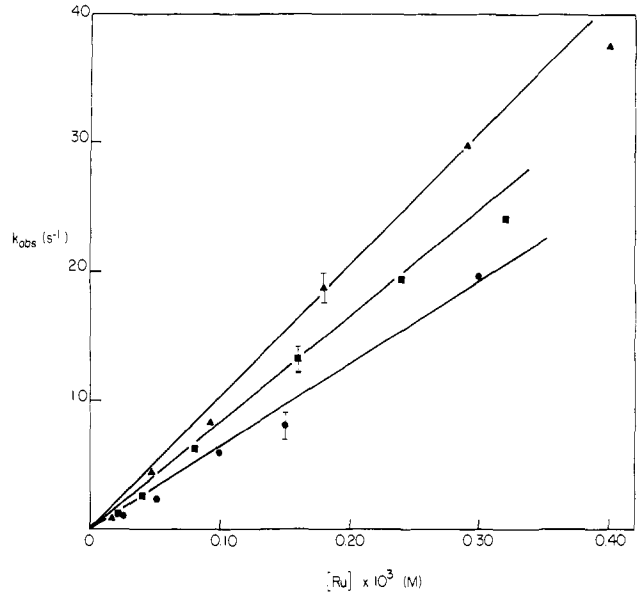

Figure 3. The dependence of the observed rate constants for the reduction of ferricytochrome *c* on the concentration of $Ru(NH_3)_5L^{2+}$ at 25 °C in sodium phosphate buffer (pH 7.0, μ = 0.1 M): (●), L = NH_3; (▲), L = Im; (■), L = His.

Table II. Rate Constants and Activation Parameters for the Reduction of PFe^{III} by $Ru(NH_3)_5L^{2+}$ in Aqueous Sodium Phosphate (pH 7, μ = 0.1 M)

	L		
	NH_3	Im	His
k, M^{-1} s^{-1} (25 °C)	6.70 (14) × 10^4	1.05 (3) × 10^5	8.51 (10) × 10^4
$\Delta H^{\ddagger}$, kcal mol^{-1}	0.91 (15)	1.3 (7)	0.19 (22)
$\Delta S^{\ddagger}$, eu	−34 (1)	−32 (2)	−36 (1)

90% of the reaction. In all cases, the observed first-order rate constant varies linearly with the reductant concentration and extrapolates to zero (Figure 3).[20] Eyring plots for a 10–40 °C temperature range reveal only a slight dependence of the observed rate constant on temperature (Figure 4). The rate constants and activation parameters for these reactions are given in Table II.

Photochemical kinetic methods were employed in the study of PFe^{III}–Ru^{III} electron-transfer chemistry. PFe^{III}, $Ru(NH_3)_5His^{3+}$, and PFe^{III}–Ru^{III} all quench the long-lived electronic excited state of $Ru(bpy)_3^{2+}$ ($Ru(bpy)_3^{2+*}$) with rate constants (determined by the Stern–Volmer lifetime quenching method) of 2.5 × 10^8, 1.2 × 10^9, and 7.8 × 10^8 M^{-1} s^{-1}, respectively.[21] The transient difference spectrum resulting from laser flash photolysis of a $PFe^{III}/Ru(bpy)_3^{2+}$ solution (pH 7.0, μ = 0.1 M; phosphate) exhibits a prominent absorption maximum at 550 nm attributable to PFe^{II}, indicating that electron-transfer contributes significantly to the quenching mechanism.[2] The ruthenium-modified protein quenches $Ru(bpy)_3^{2+}$ luminescence three times faster than native protein, yet a $\Delta OD(PFe^{II})/\Delta OD(PFe^{II}$–$Ru^{III})$ ratio of 2.6 is obtained from two $Ru(bpy)_3^{2+}$ solutions containing equimolar concentrations of PFe^{III} and PFe^{III}–Ru^{III}. Apparently, though PFe^{III}–Ru^{III} is a more efficient quencher of $Ru(bpy)_3^{2+*}$ than is PFe^{III}, fewer of the quenching events are direct electron transfers from the excited metal complex to the heme center. The simplest explanation of this result is that, with the modified protein, most of the quenching arises from electron transfer to $Ru(NH_3)_5$-

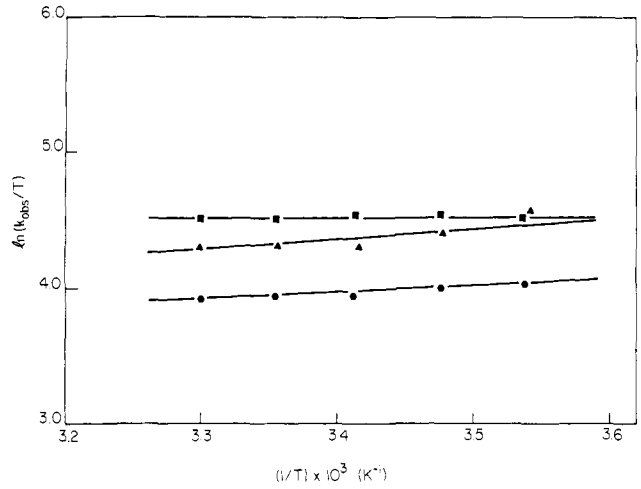

Figure 4. Eyring plots of the rate constant data for the reduction of ferricytochrome *c* in sodium phosphate buffer (pH 7.0, μ = 0.1 M): (●), $[Ru(NH_3)_6^{2+}]$ = 1.0 × 10^{-4} M; (▲), $[Ru(NH_3)_5Im^{2+}]$ = 4.7 × 10^{-5} M; (■), $[Ru(NH_3)_5His^{2+}]$ = 4.4 × 10^{-5} M.

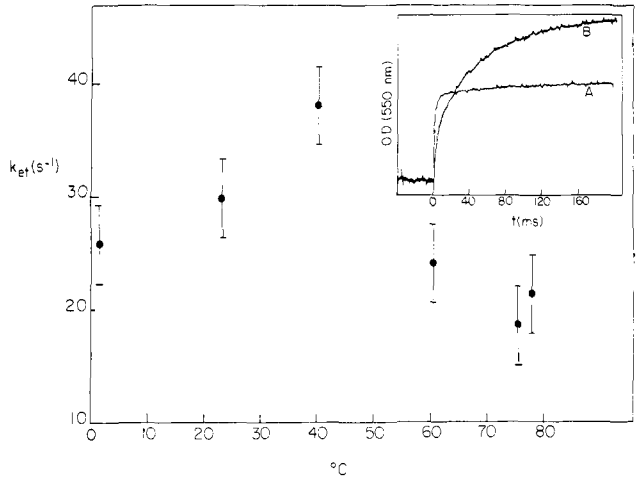

Figure 5. Temperature dependence of the rate constant for the intramolecular electron-transfer reaction PFe^{III}–Ru^{II} → PFe^{II}–Ru^{III} at pH 7 (μ = 0.1 M). Inset: traces of the change in 550-nm optical density resulting from flash photolysis of (A) $PFe^{III}/Ru(bpy)_3^{2+}$/EDTA and (B) PFe^{III}–$Ru^{III}/Ru(bpy)_3^{2+}$/EDTA in aqueous sodium phosphate (pH 7, μ = 0.1 M) at 23 °C. The vertical axis refers to light intensity at the detector and t = 0 on the horizontal axis coincides with the flash pulse. The intensities of the two traces have been normalized to reflect the differences in quenching rate constants.

$(His\text{-}33)^{3+}$ rather than to PFe^{III}. Indeed, the ratio of initial transient signals permits dissection of $k_q(PFe^{III}$–$Ru^{III})$ into quenching rate constants for the individual ruthenium and iron centers (6.6 × 10^8 and 1.2 × 10^8 M^{-1} s^{-1}, respectively).[22] In other words, quenching of $Ru(bpy)_3^{2+*}$ by PFe^{III}–Ru^{III} yields the kinetic product PFe^{III}–Ru^{II} in roughly a fivefold excess over the thermodynamic product PFe^{II}–Ru^{III}. That k_q for both the iron and ruthenium sites is smaller in PFe^{III}–Ru^{III} than in either individual species is probably due to the greater work required to assemble

(20) The results of our investigation of the kinetics of reduction of horse heart ferricytochrome *c* by $Ru(NH_3)_6^{2+}$ at pH 7.0 and 25 °C accord closely with those obtained by Ewall and Bennett (Ewall, R. X.; Bennett, L. E. *J. Am. Chem. Soc.* **1974**, *96*, 940–942).

(21) Quenching of $Ru(bpy)_3^{2+*}$ by PFe^{III} has been studied previously (Sutin, N. In "Bioinorganic Chemistry-II"; Raymond, K. N., Ed.; American Chemical Society: Washington, D.C., 1977; *Adv. Chem. Ser.* pp 156–172. McLendon, G.; Lum, V. R.; English, A. M.; Gray, H. B., unpublished results).

(22) The ratio $OD(PFe^{III})/OD(PFe^{III}$–$Ru^{III})$ will just be equal to the ratio of the quantum yields for formation of reduced iron in the two species (neglecting the back reaction)

$$\frac{\Delta OD(PFe^{III})}{\Delta OD(PFe^{III}\text{–}Ru^{III})} = \frac{k_q}{k_q^{Fe}} \frac{k_1 + k'_q[PFe^{III}\text{–}Ru^{III}]}{k_1 + k_q[PFe^{III}]} = \frac{k_q\tau}{k_q^{Fe}\tau'}$$

where k_q is the quenching rate constant of PFe^{III}, k'_q is the quenching rate constant of PFe^{III}–Ru^{III} and is equal to the sum of the quenching rate constants for the iron and ruthenium sites, k_q^{Fe} and k_q^{Ru}, respectively, and k_1 is the decay constant of $Ru(bpy)_3^{2+}$ in the absence of quenchers. This equation can be solved for k_q^{Fe} and evaluated with parameters from the transient absorption experiment.

the higher charged reactants.[23] Although the magnitudes of the 550-nm transient absorption signals of PFe^{III} and PFe^{III}–Ru^{III} are quite different, their decay kinetics are indistinguishable. We conclude, therefore, that intramolecular electron transfer from $Ru(NH_3)_5(His\text{-}33)^{2+}$ to the PFe^{III} heme center is slower than the back electron transfer to $Ru(bpy)_3^{3+}$.

The laser experiments suggest that, if the back-reaction between $Ru(bpy)_3^{3+}$ and PFe^{III}–Ru^{II} could be inhibited, intramolecular electron transfer from ruthenium to iron should be readily detected[24] and that the time scale for this reaction is compatible with conventional microsecond flash photolysis. It is known that EDTA, at neutral or greater pH, rapidly reduces $Ru(bpy)_3^{3+}$, yielding, initially, an amine-free radical that, upon one-electron oxidation, decomposes to produce ethylenediaminetriacetic acid, formaldehyde, and carbon dioxide.[25-28] The disodium salt of EDTA was therefore chosen as a $Ru(bpy)_3^{3+}$ scavenger. Flash photolysis of a phosphate buffer solution containing $Ru(bpy)_3^{2+}$ and PFe^{III} produces a small transient absorption at 550 nm that rapidly decays nearly to the preflash base line. The trace of ΔOD (550 nm) vs. time resulting from flash photolysis of $Ru(bpy)_3^{2+}$, PFe^{III}, and Na_2EDTA ($[Ru] \approx [Fe] \approx 5 \times 10^{-6}$ M; $[EDTA] \approx 5 \times 10^{-3}$ M) in phosphate buffer is shown in trace A in the inset in Figure 5. The instantaneous increase in optical density immediately after the flash arises from direct reduction of PFe^{III} by $Ru(bpy)_3^{2+*}$. A slower, secondary process then leads to further production of PFe^{II}. The halftime for this reaction varies approximately inversely with protein concentration and also appears to decrease with increasing temperature. This behavior is indicative of an intermolecular electron-transfer reaction in which PFe^{III} is the oxidant and the reductant is the amine cation radical produced from the oxidation of EDTA by $Ru(bpy)_3^{3+}$. A rate constant of 8×10^7 M^{-1} s^{-1} may be estimated for this reaction from the dependence of the halftime for the secondary production of PFe^{II} on protein concentration.

The trace of ΔOD (550 nm) vs. time resulting from flash photolysis of $Ru(bpy)_3^{2+}$ and PFe^{III}–Ru^{III} is identical with that obtained for native protein. Trace B in the Figure 5 inset, however, illustrates the change in optical density at 550 nm resulting from flash photolysis of a $Ru(bpy)_3^{2+}$/PFe^{III}–Ru^{III}/EDTA solution. As with PFe^{III}, there is production of a small amount of PFe^{II}–Ru^{III} immediately after the flash. The subsequent production of reduced iron in the heme center, though, preceeds more slowly and in greater yield with the semisynthetic protein. This production of Fe^{II} in the heme site obeys first-order kinetics over a period of 3 half-lives with a rate constant of 30 (3) s^{-1} (23 °C, $[PFe^{III}$–$Ru^{III}] = 5 \times 10^{-6}$ M). This rate is much slower than that found for the reaction between PFe^{III} and the EDTA radical at comparable protein concentrations. Furthermore, variation of the protein concentration from 2 to 8 μM does not, within our error limits, affect the rate constant, nor does the use of an alternative $Ru(bpy)_3^{3+}$ scavenger, diethylenetriaminepentaacetic acid.[29] The absence of a dependence of the observed rate constant on protein concentration precludes a bimolecular mechanism for PFe^{II}–Ru^{III} production. Rather, the protein concentration independence of the rate constant, coupled with the fact that quenching of $Ru(bpy)_3^{2+*}$ by the modified protein produces a fivefold excess of the kinetic product (PFe^{III}–Ru^{II}), leads to the conclusion that it is the simple intramolecular electron-transfer reaction (PFe^{III}–Ru^{II} → PFe^{II}–Ru^{III}) that proceeds at a rate of 30 (3) s^{-1} at 23 °C.[30]

We are able to place some limits on the activation parameters from measurements of the temperature dependence of the rate constant for the intramolecular electron transfer (Figure 5). The quantity of PFe^{III}–Ru^{II} produced in each flash experiment decreases as the temperature is raised from 0 to 75 °C, as expected from the reduction of the $Ru(bpy)_3^{2+*}$ lifetime over this temperature interval.[31] Analysis of the data over the temperature region (0–40 °C) where the heme *c* coordination unit is fully intact[19,32,33] gives $\Delta S^{\ddagger} = -48$ (2) eu and an upper limit of 1.5 kcal mol^{-1} for $\Delta H^{\ddagger}$ (1.1 (4) kcal mol^{-1}). These values are not substantially different from ones obtained by Isied and co-workers in pulse radiolysis experiments on the modified protein.[3b]

At about 80 °C the flash transient signal of the $Ru(bpy)_3^{2+}$/PFe^{III}–Ru^{III}/EDTA solution changes dramatically, because reduced iron is no longer produced at a rate of 25 s^{-1} (a small amount of reduced iron is produced immediately after the flash, but there is no subsequent production). Upon cooling the solution below 80 °C, however, first-order production of PFe^{II}–Ru^{III} at 25 s^{-1} is again observed in flash photolysis experiments. The high-temperature cutoff of the intramolecular electron transfer is not unexpected, because it is known that methionine-80 (Met-80) is no longer coordinated to the iron center above 82 °C.[32,33] It is certain that the reduction potential of the heme center in PFe^{III}–Ru^{III} will be perturbed substantially upon loss of Met-80 coordination, and it is possible that PFe^{III}–Ru^{II} is the thermodynamic product (as well as the kinetic product) of the $Ru(bpy)_3^{2+*}$ quenching reaction.[34-37]

Our determination of the activation enthalpy for the intramolecular electron-transfer reaction permits us to analyze the various contributions to the activation parameters in the intermolecular reactions between $Ru(NH_3)_5L^{2+}$ and PFe^{III}. Employing Brown and Sutin's precursor complex model for outer-sphere electron-transfer reactions, the observed second-order rate constant for the reaction of $Ru(NH_3)_5L^{2+}$ with PFe^{III}, k_{obsd}, is given by eq 1–3.[38] In the above expressions, k_{et} is the electron-transfer

$$k_{obsd} = K_0 k_{et} \tag{1}$$

$$k_{et} = (k_\beta T/h)\kappa \exp[-\Delta G^*/RT] \tag{2}$$

$$K_0 = c_0 \exp[-w_r/RT] \tag{3}$$

rate constant within a $[Ru(NH_3)_5L^{2+}/PFe^{III}]$ precursor complex, which forms with equilibrium constant K_0, ΔG^* is the free energy change required to reach the activated complex from the precursor complex, κ is the probability of electron transfer within the activated complex, w_r is the free energy change associated with the work required to bring the reactants together, and c_0 is a constant characteristic of the particular preequilibrium association model. Substituting eq 2 and 3 into eq 1 yields eq 4. Application of the

$$k_{obsd} = (k_\beta T/h) \exp\{-(\Delta G^* + w_r - RT \ln c_0 - RT \ln \kappa)/RT\} \tag{4}$$

Gibbs–Helmholtz relations ($\Delta H = \partial(\Delta G/T)/\partial(1/T)$: $\Delta S = -\partial(\Delta G/\partial T)$ to the argument of the exponent in eq 4 gives the following expressions for the observed enthalpy and entropy of

(23) At pH 7.0 the net charge on cytochrome *c* is roughly +8 (Goldkorn, T.; Schejter, A. *J. Biol. Chem.* **1979**, *254*, 12562–12566).

(24) A simpler experiment would be to observe intramolecular electron transfer within PFe^{II}–Ru^{III} upon excitation of the heme center. The short excited-state lifetime of the heme center (6 ps) (Juppert, K. D.; Straub, K. D.; Rentzepis, P. M. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 4139–4143) precluded this experiment.

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(27) Miller, D.; McLendon, G. *Inorg. Chem.* **1981**, *20*, 950–953.

(28) McLendon, G.; Smith, M. *Inorg. Chem.* **1982**, *21*, 847–850.

(29) The kinetics for the secondary production of PFe^{II}, however, were different from those obtained with EDTA.

(30) The intramolecular kinetics at 25 °C have been verified in pulse radiolysis experiments (k_{et} = 33 (3) s^{-1}) (Miller, J. R.; McLendon, G.; Winkler, J. R.; Nocera, D. G.; Gray, H. B., to be submitted for publication).

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(34) The Fe–S (Met-80) bond also is ruptured in alkaline solutions of ferricytochrome *c* (lysine-79 may occupy the vacated coordiantion site).[35,36] The reduction potential of the protein at pH 10 is 120 mV.[37]

(35) Davis, L. A.; Schejter, A.; Hess, G. P. *J. Biol. Chem.* **1974**, *249*, 2624–2632.

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(38) Brown, G. M.; Sutin, N. *J. Am. Chem. Soc.* **1979**, *101*, 883–892.

activation (assuming $\partial K/\partial T = 0$):

$$\Delta H^{\ddagger}_{obsd} = \Delta H^{*} + w_r - T(\partial w_r/\partial T) = RT^2(\ln c_0/\partial T) \quad (5)$$

$$\Delta S^{\ddagger}_{obsd} = \Delta S^{*} - \partial w_r/\partial T = R \ln c_0 + R \ln \kappa + RT(\partial \ln c_0/\partial T) \quad (6)$$

In the case of the intramolecular electron-transfer reaction, the reactants are preassembled at a fixed distance ($w_r = 0$; $c_0 = 1$) and

$$\Delta H^{\ddagger}_{obsd}[PFe^{III}\text{–}Ru^{II} \rightarrow PFe^{II}\text{–}Ru^{III}] = \Delta H^{*} \quad (7)$$

The intramolecular electron-transfer experiment establishes that $\Delta H^{*}(PFe^{III}\text{–}Ru^{II} \rightarrow PFe^{II}\text{–}Ru^{III}]$ is less than 1.5 kcal mol^{-1}. In the Marcus framework,[39] this allows us to estimate the protein reorganizational barrier, because ΔH^{*} is expressed as in eq 8,[40]

$$\Delta H^{*} = \frac{\Delta H^{*}_{11}}{2} + \frac{\Delta H^{*}_{22}}{2} + \frac{\Delta H^{\circ}_{12}}{2} \quad (8)$$

where ΔH^{*}_{11} and ΔH^{*}_{22} are the enthalpies of reorganization for the electron exchange reactions of reagents 1 and 2, respectively, and ΔH°_{12} (= −11.9 kcal mol^{-1}) is the standard enthalpy change for the electron-transfer reaction (1 = Fe and 2 = Ru). The reorganizational energy of electron exchange in $Ru(NH_3)_5(py)^{3+/2+}$, which should closely approximate ΔH^{*}_{22}, has been estimated to be 6.9 kcal mol^{-1}.[38] Our determination of ΔH^{*} (<1.5 kcal mol^{-1}) places an upper limit of 8.0 kcal mol^{-1} on the enthalpy of reorganization (inner plus outer sphere)[41] of the $PFe^{III/II}$ electron exchange reaction. It is of special interest that there is now some experimental evidence in support of the very low theoretical value of the $PFe^{III/II}$ electron exchange barrier obtained by Warshel and co-workers[42] in their detailed analysis of the oxidized and reduced cytochrome *c* structures.

The enthalpies of activation for the intermolecular reactions between $Ru(NH_3)_5L^{2+}$ and PFe^{III} are composed of reorganizational contributions, ΔH^{*}, as well as terms arising from the enthalpy of formation of the precursor complex. The values of $\Delta H^{\ddagger}_{obsd}$ for the three intermolecular reactions are all less than 1.3 kcal mol^{-1} (Table II), and we know from the intramolecular electron-transfer reaction that $\Delta H^{*} < 1.5$ kcal mol^{-1}. The contribution to $\Delta H^{\ddagger}_{obsd}$ from the term $RT^2(\ln c_0/\partial T)$ is generally believed to be quite small (<0.3 kcal mol^{-1})[38] and can safely be ignored. Substituting the limiting values $\Delta H^{\ddagger}_{obsd} < 1.3$ kcal mol^{-1} and $\Delta H^{*} < 1.5$ kcal mol^{-1} allows us to estimate that $\Delta H_w = 0 \pm 1.5$ kcal mol^{-1}, where ΔH_w, the enthalpic contribution to the work required to assemble the precursor complex, is defined by eq 9. Our estimate of ΔH_w is in reasonable agreement with the

$$\Delta H_w = w_r - T(\partial w_r/\partial T) \quad (9)$$

value calculated from the Debye–Hückel theory.[43] The activation entropy for the intramolecular reaction is −48 (2) eu, which is considerably more negative than the corresponding intermolecular quantities (Table II). The term in eq 6 most likely to be responsible for the more negative $\Delta S^{\ddagger}$ for the intramolecular process is $R \ln \kappa$.[44] This result suggests, therefore, that κ, the probability of electron transfer within the activated complex, is much smaller for the intramolecular reaction. If we assume that κ depends primarily on the separation of the redox centers,[45] we are able to conclude that *intermolecular* $Ru(NH_3)_5L^{2+}/PFe^{III}$ electron transfer occurs at shorter range than the 11.8-Å fixed distance in the ruthenium-modified protein.

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(39) The near-zero $\Delta H^{\ddagger}$ for electron transfer in $PFe^{III}\text{–}Ru^{II}$ also has been explained quantum mechanically (Freed, K. F. *Chem. Phys. Lett.* **1983**, *97*, 489–493).

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(41) See for example: Sutin, N. *Prog. Inorg. Chem.* **1983**, *30*, 441–498.

(42) Churg, A. K.; Weiss, R. M.; Warshel, A.; Takano, T. *J. Phys. Chem.* **1983**, *87*, 1683–1694.

(43) Using the Debye–Hückel expression for the work term at 25 °C and $\mu = 0.1$ M[38], and assuming a contact distance of 20 Å and charges of 8+ and 2+ for the cytochrome and ruthenium reagents, respectively, we obtain ΔH_w (calcd) = −0.3 kcal mol^{-1} and ΔS_w (calcd) = 5 eu.

(44) Since $\Delta H_w = 0 \pm 1.5$ kcal mol^{-1} and $w_r > 0$ (charged reactants), eq 9 implies that $\Delta S_w(= -\partial w_r/\partial T)$ is very small and probably negative. Two common forms for c_0[38] yield negative values for $\ln c_0$ and $\partial \ln c_0/\partial T$. Therefore, only $\ln \kappa$ can be more negative in the intramolecular reaction.

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