Comparative Kinetic Analysis of Reversible Intermolecular Electron-Transfer Reactions between a Series of Pentaammineruthenium Complexes and Cytochrome *c*

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Received November 23, 1994[⊗]

In this kinetic and thermodynamic study, the reversible outer-sphere electron-transfer reactions between a series of $Ru(NH_3)_5L^{3+/2+}$ complexes (L = etpy, py, lut) (etpy = 4-ethylpyridine; py = pyridine; lut = 3,5-lutidine) and cytochrome *c* were investigated as a function of ionic strength, buffer, pH, temperature, and pressure. Due to the low driving forces of these systems, it was possible to study all the reactions in both redox directions. The observed rate constants for various L are correlated on the basis of the ability of ligands on the ruthenium complex to penetrate the heme groove on cytochrome *c*. The measurements as a function of pressure enabled the construction of volume profiles for all investigated systems. The activation volumes for all of these processes are very similar: between −14.9 and −17.8 $cm^3\ mol^{-1}$ for the reduction and between +14.7 and +17.8 $cm^3\ mol^{-1}$ for the oxidation of the protein by $Ru(NH_3)_5L^{2+/3+}$, respectively. The overall reaction volume varies between 27 and 35 $cm^3\ mol^{-1}$, from which it follows that the transition state lies exactly halfway between reactant and product states on a volume basis in all cases. There is good agreement throughout between kinetic and thermodynamic data.

Introduction

Electron transfer plays an important role in biological processes such as respiration and photosynthesis. Redox reactions between pairs of donors and acceptors can occur over long distances (≥10 Å) in biological systems. A good example is the redox protein cytochrome *c*. It is a relatively small protein with a MW of ca. 12 400 which undergoes a reversible Fe(II)/Fe(III) redox reaction. Electron-transfer reactions of cytochrome *c* have been widely studied and remain subjects of continued interest. For example, intra- and intermolecular electron transfer studies have been performed using pulse radiolysis, flash photolysis, or stopped-flow techniques on cytochrome *c* and redox center-modified cytochrome *c*.[1−6]

Previous kinetic studies have shown that cytochrome *c* is oxidized by a large number of redox complexes such as $Co(phen)_3^{3+}$ and $Ru(NH_3)_5py^{3+}$ (py = pyridine) via outer-sphere mechanisms.[7,8] The reaction site is expected to be in the vicinity of the partially exposed heme edge. It has been proposed[8] that the π-conjugated pyridine ligand in the latter system is able to penetrate into the interior of the protein, whereas $Ru(NH_3)_6^{3+}$ is not able to penetrate into the protein surface. Furthermore, it was proposed that the access of the complex to this heme edge depends on the properties of the reactants, i.e. size, charge, and surface properties.[8] In this work, the substituents on the pyridine ring were varied in order to determine if interactions between the amino acid side chain on the protein and the pyridine ring can affect the reaction. If the substituted pyridine ring is not able to penetrate completely into the pocket close to the heme edge, the reaction rate will probably be unusually low due to the increase in distance between the redox centers.

This study includes a detailed kinetic and thermodynamic analysis of the electron-transfer reactions between cytochrome *c* and several pentaammineruthenium complexes. Due to the low driving force of these systems, we were able to follow the reactions in both directions. The combination of activation volumes for the forward and reverse reactions, together with the overall reaction volume determined for these reactions, enabled us to construct volume profiles for the overall processes. In a previous study[9] we showed that the transition state for the $Ru(NH_3)_5$(isonicotinamide)$^{2+/3+}$/cytochrome *c* system lies halfway between the reactant and product states on a volume basis. This is in agreement with theoretical predictions based on the Marcus theory. The main volume changes were assumed to arise from electrostriction effects on the metal complex, since cytochrome *c* shows only a very small volume change during the redox process.[10] Modifications of the ligand on the ruthenium ammine complexes may affect the penetration in the precursor complex as outlined above and so influence the position of the transition state in terms of "early" or "late" along the reaction coordinate for the electron-transfer process, which should clearly show up in the volume profile.

Experimental Section

Materials. Horse heart cytochrome *c* (type VI, Sigma) was purified and reduced as reported previously.[9] The concentrations of the cytochrome solutions were determined by UV/vis spectroscopy. All

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

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0020-1669/96/1335-1564$12.00/0

ruthenium complexes were prepared by the method of Ford et al.[9,11] and purified by eluting the compound from an SP-Sephadex C-25 column with a gradient of 0.1−0.5 M trifluoroacetic acid. The absence of impurities such as the pentaammineaquaruthenium(III) ion was confirmed by cyclic voltammetry using a BAS 100 electrochemical analyzer. All chemicals used for the experiments were of analytical grade.

Measurements. UV/vis spectra at ambient pressure were recorded on a Hewlett Packard HP8452 spectrophotometer. The UV/vis spectra at high pressure were recorded on Zeiss DMR 10 and Cary 1 spectrophotometers equipped with a high-pressure cell for pressures up to 200 MPa.[12] The kinetic traces for the oxidation or reduction of cytochrome *c* were recorded at 550 nm. All kinetic measurements at ambient pressure were performed on a Durrum D110 stopped-flow instrument. For the high-pressure measurements, a homemade high-pressure stopped-flow system was used.[13,14] All instruments were thermostated at ±0.1 °C. The kinetic traces, consisting of 1000 points per trace, were collected and stored on an IBM-compatible computer using Biologic (Claix, France) software. The rate constants were calculated using the OLIS KINFIT program (Bogart, Georgia). All kinetic traces showed excellent first-order behavior over 3−4 half-lives. The quoted rate constants are the mean of at least six kinetic runs. The corresponding errors are the standard deviation of the mean value.

All solutions were saturated with argon to avoid oxidation of the reactants by dissolved oxygen. The ruthenium complex solutions were protected from light to avoid decomposition via photoaquation.[15] The solutions were transferred into the stopped-flow unit using Hamilton gastight syringes. All experiments were performed in 50 mM Tris buffer, pH 7, and 50 mM$LiClO_4$, unless otherwise indicated.

Results and Discussion

Reactions of cytochrome *c* with metal complexes such as $Co(phen)_3^{2+/3+}$, $Co(terpy)_2^{2+/3+}$, or $Ru(NH_3)_5py^{2+/3+}$ (phen = 1,10-phenanthroline; terpy = 2,2′:6′,2″-terpyridine) are known to be of the outer-sphere type.[5,6,16] This mechanism includes the formation of a precursor complex due to electrostatic interactions (K_p) with a subsequent, often rate-determining electron-transfer step k_{ET}, followed by the final dissociation to form the products. The precursor complex formation step is normally too weak to reach a saturation effect in the rate constant at high complex concentrations. Even oxidation of cytochrome *c* by $Fe(CN)_6^{3-}$, in which formation of the precursor complex should be more favorable because of electrostatic interaction between oppositely charged ions, showed no limiting value of the rate constant at high $Fe(CN)_6^{3-}$ concentrations[5,6,17] in stopped-flow experiments. In such cases, the precursor formation constant can only be estimated using a theoretical approach. With the aid of the Fuoss equation[18,19] this results in a precursor formation constant (K_p) of ca. 3 M^{-1} for the reaction of cytochrome *c* with positively charged metal complexes like $Ru(NH_3)_5py^{2+/3+}$. For the reaction of cytochrome *c* with $Fe(CN)_6^{4-/3-}$, a K_p value of ca. 200 M^{-1} is calculated. This is in good agreement with the experimentally obtained value of 285 M^{-1} from NMR measurements reported for the reduction of cytochrome *c* by $Fe(CN)_6^{4-}$ [6] and indicates that the Fuoss equation is also valid for systems involving proteins, thus illustrating the importance of electrostatic interactions for these processes. For the reactions studied in this investigation, the K_p value is small due to the same type of charge for both reactants and the observed second-order rate constant for the outer-sphere mechanism is equal to the product K_pk_{ET}.

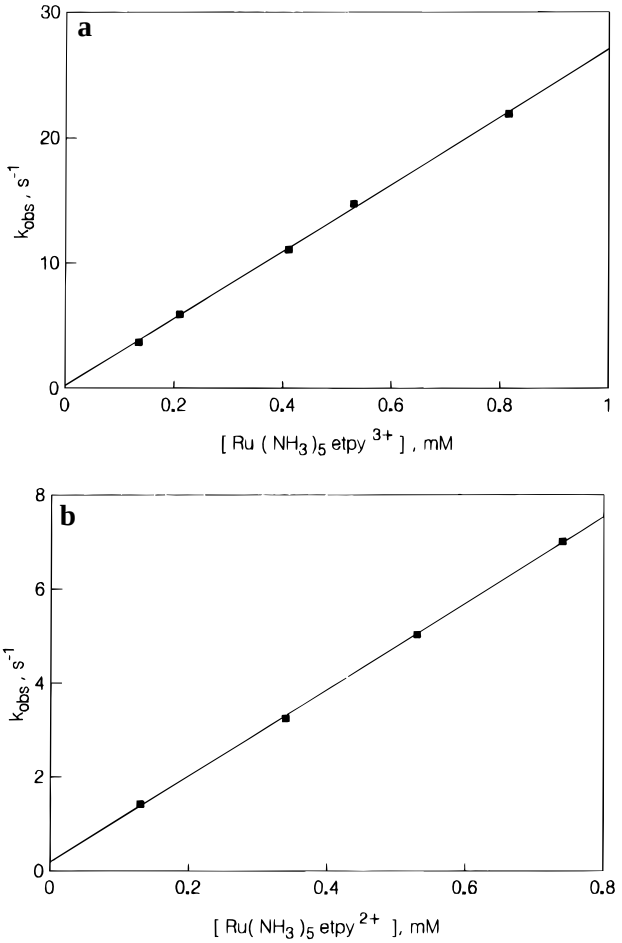

Figure 1. Concentration dependence of k_{obs} for the reduction of cyt c^{III} (a) and oxidation of cyt c^{II} (b) by $Ru(NH_3)_5etpy^{2+/3+}$. Experimental conditions: [cyt $c^{III/II}$] = 1.0 × 10^{-5} M, pH = 7.2, ionic strength = 0.1 M, [Tris] = 0.05 M, [$LiNO_3$] = 0.05 M, λ = 550 nm, T = 25.0 °C.

General Observations. In this study, we report the results for the redox reaction between cytochrome *c* and several pentaammine(L)ruthenium complexes, L = py, etpy, lut (py = pyridine; etpy = 4-ethylpyridine; lut = 3,5-lutidine), as shown in reaction 1. Due to the low driving force of these systems

$$Ru^{III}a_5L^{3+} + \text{cyt } c^{II} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} Ru^{II}a_5L^{2+} + \text{cyt } c^{III} \qquad (1)$$

(≈0.1 eV), we were able to follow these reactions in both directions. The kinetics of these reactions were followed spectrophotometrically at 550 nm. We observed a decrease in absorbance as a function of time for the oxidation of the protein and an increase in absorbance for the reverse reaction, in agreement with the spectra of ferri- and ferrocytochrome *c*. The rate constants determined at this wavelength agree very well with the values determined by monitoring the Soret bands at 420 nm. All kinetic measurements were performed under pseudo-first-order conditions with an excess of $Ru^{II/III}$. The plots of k_{obs} versus [$Ru^{II/III}$] are linear for all complexes in both redox directions (for example see Figure 1 for the etpy complex). The second-order rate constant (K_pk_{ET}) was obtained from the slope of the plot of k_{obs} versus [$Ru^{II/III}$].

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Table 1. Summary of Rate and Activation Parameters for the Electron-Transfer Reaction between Cytochrome *c* and Several Pentaammineruthenium Complexes (a = NH_3): $Ru^{III}a_5L^{3+}$ + $cytc^{II}$ ⇌ $Ru^{II}a_5L^{2+}$ + cyt c^{III}

reaction	k, M^{-1} s^{-1} [a]	$\Delta H^{\ddagger}$, kJ mol^{-1}	$\Delta S^{\ddagger}$, J K^{-1} mol^{-1}	$\Delta V^{\ddagger}$, cm^3 mol^{-1}	$\Delta \bar{V}$, cm^3 mol^{-1}	$-\Delta G$, eV	K_E[b]	K_{TD}[b]	K_{KIN}[b]
$Ru^{III}a_5$lut + cyt c^{II}	$(2.7 \pm 0.1) \times 10^4$	35.4 ± 0.3	-41 ± 1	$+16.9 \pm 1.4$	33.6 ± 1.7[c]	0.025	2.6	2.6 ± 0.1	2.9 ± 0.4
$Ru^{II}a_5$lut + cyt c^{III}	$(9.4 \pm 0.5) \times 10^3$	21 ± 1	-99 ± 5	-17.8 ± 1.6	34.7 ± 2.1[d]				
$Ru^{III}a_5$etpy + cyt c^{II}	$(2.68 \pm 0.05) \times 10^4$	29 ± 2	-61 ± 7	$+14.7 \pm 0.9$	26.9 ± 1.8[c]	0.024	2.5	2.2 ± 0.4	2.9 ± 0.1
$Ru^{II}a_5$etpy + cyt c^{III}	$(9.2 \pm 0.1) \times 10^3$	25 ± 2	-86 ± 6	-14.9 ± 1.1	29.6 ± 1.4[d]				
$Ru^{III}a_5$py + cyt c^{II}	$(4.9 \pm 0.1) \times 10^4$	28 ± 1	-64 ± 5	$+17.4 \pm 1.5$	33.4 ± 1.9[c]	0.038	4.4	6.4 ± 2.1	4.6 ± 0.4
	5.96×10^3 [e]	33.4[e]	−58.5[e]						
$Ru^{II}a_5$py + cyt c^{III}	$(1.05 \pm 0.05) \times 10^4$	33 ± 4	-59 ± 13	-17.7 ± 0.8	35.1 ± 1.7[d]				
$Ru^{III}a_5$isn + cyt c^{II} [f]	1.15×10^5	22 ± 1	-75 ± 3	$+16.0 \pm 0.9$	31 ± 1[c]	0.115	88	71 ± 7	75.7
					26.4 ± 0.9[g]				
$Ru^{II}a_5$isn + cyt c^{III} [f]	$(1.5 \pm 0.1) \times 10^3$	28 ± 4	-87 ± 12	-17.2 ± 1.5	33.2 ± 1.7[d]				
$Ru^{II}a_6$ + cyt c^{III} [h]	3.8×10^4	12	−117			0.210			
	6.7×10^4 [i]	3.8 ± 0.8[i]	-142 ± 4[i]	-15.6 ± 0.6[i]					

[a] Reaction conditions: $T = 25$ °C, $\mu = 0.1$ M, [cyt *c*] = 1×10^{-5} M, [Tris] = 0.05 M, [$LiClO_4$] = 0.05 M, pH = 7.1, $\lambda = 550$ nm. [b] Equilibrium constant for the oxidation of cytochrome. [c] Reaction volume determined spectrophotometrically for the oxidation of cytochrome *c*. [d] Reaction volume determined kinetically for the oxidation of cytochrome *c*. [e] pH = 5.3 (acetate), $\mu = 0.1$ M, ref 8. [f] Reference 9. [g] Reference 10. [h] Reference 45. [i] Reference 4.

In theory it should be possible to correlate the intercept and the slope with the overall equilibrium constant. In these systems this is not possible to do so, due to non-pseudo-first-order conditions for the reverse reaction, which result in deviations at low Ru^{III} concentrations, i.e. inaccurate intercepts. As suggested before,[9] it would be more accurate to perform measurements in the presence of Ru^{II} to also ensure pseudo-first-order conditions for the reverse reaction. Unfortunately, under such conditions the absorbance changes become too small to obtain accurate data. The forward and reverse reactions were followed as a function of temperature (20−40 °C) and pressure (0.1−100 MPa), and the results are reported as Supporting Information. The estimated rate and activation parameters are summarized in Table 1.

In the case of each ruthenium complex, the observed rate constants for the forward and reverse reactions can be correlated with the equilibrium constant determined from the reduction potential and from spectral measurements as a function of complex concentration. Table 1 shows that there is an excellent agreement between the kinetically determined equilibrium constant ($K = k_f/k_b$) and the value obtained from thermodynamic measurements.

The rates for the pentaammine(L)ruthenium complexes (L = lut, etpy) are very similar for both redox directions, as expected from the very similar driving forces. The rates for the oxidation of cytochrome *c* by the isonicotinamide complex[9] are faster by a factor of 4 and those for the py complex are faster by a factor of 2 than the rates for the lutidine and the etpy complexes by virtue of the higher driving forces. For the reverse step (reduction of the protein) the isonicotinamide complex reacts 6 times slower than the etpy complex. Surprisingly, the rate constants for the oxidation of cytochrome *c* by $Ru(NH_3)_5py^{3+}$ in our case ($k = 4.9 \times 10^4$ M^{-1} s^{-1}) are a factor of 9 larger than the value reported by Cummins and Gray[8] ($k = 5.96 \times 10^3$ M^{-1} s^{-1}) at the same ionic strength. In contrast to our study, Cummins and Gray[8] performed the measurements in acetate solutions at pH 5.3. Reactions of cytochrome *c* are known to depend strongly on the composition of the solution. Cummins and Gray[8] found that the rate for this reaction is 50% larger in phosphate buffer (pH 6.5, $\mu = 0.1$ M) than in the acetate buffer (pH = 5.3, $\mu = 0.1$ M). This difference was attributed to a specific interaction between phosphate ion and cytochrome *c* which results in a reduced effective charge on cytochrome *c*. We used $LiClO_4$ to adjust the ionic strength in these studies because it is known that K^+ and Na^+ bind to ferrocytochrome *c*.[21] The real reason for this observed difference is not clear. The activation entropy and enthalpy we determined are close to the values reported by Cummins and Gray[8] in acetate solution at pH 5.3.

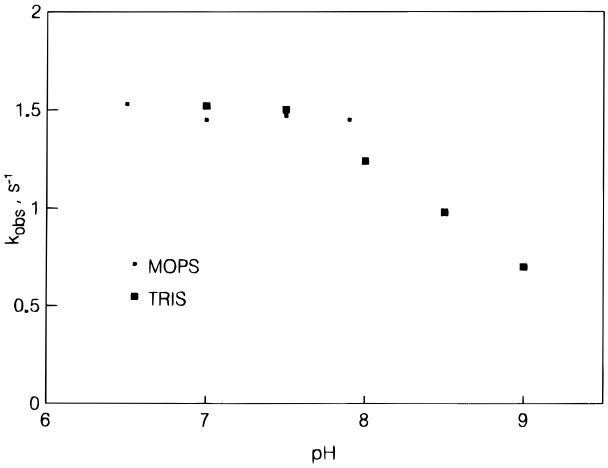

Figure 2. pH dependence of k_{obs} for the reduction of cyt c^{III} by $Ru(NH_3)_5etpy^{2+}$. Experimental conditions: [cyt $c^{III/II}$] = 1.0×10^{-5} M, ionic strength = 0.1 M, [Tris], [mops] = 0.05 M, [$LiNO_3$] = 0.05 M, $\lambda = 550$ nm, $T = 25.0$ °C.

To check the pH dependence of these processes, we studied the effect of pH on the reaction of cytochrome *c* with $Ru(NH_3)_5$-$etpy^{2+}$ in different buffers. We used mops, Tris, bistris, hepes, and mopso buffers. At pH 7.1, the observed rates for bistris and Tris were very similar and slightly higher than the rates for hepes, mops, and mopso. The rates were found to be independent of pH in the range 6.5−7.5 for the reaction in both directions. The rate decreases at higher pH (>8.0) (Figure 2), presumably due to the formation of the alkaline forms of cytochrome *c*.[22] This rate decrease is in agreement with the decrease in the reduction potential at higher pH.[22]

In order to estimate the distance between the redox centers in the precursor complex, which is usually assumed to be the sum of the radii of the redox partners,[23] we performed measurements at different ionic strengths. The ionic strength dependence of rate constants is given by the Brönsted−Bjerrum equation (eq 2).[24] In this equation, z_a and z_b represent the

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$$\log k = \log k_0 + \frac{2z_a z_b A\mu^{1/2}}{1 + \sigma_{12} B\mu^{1/2}} \quad (2)$$

charges of the reactants, A and B are constants, μ is the ionic strength, and σ_{12} is the contact distance of the reactants. In general, this equation can only be used for ionic strengths up to 0.01 M because it is based on low ionic strength assumptions (Debye−Hückel); therefore, the results are expected to deviate at high ionic strength. However, many publications have demonstrated that this equation also gives good results for higher ionic strength.[8,25−27] In our experiments, ionic strengths of up to 0.2 M were used. A good agreement between theory and experiment was reported by Cummins and Gray[8] for the oxidation of cytochrome c^{II} by $Ru(NH_3)_5py^{3+}$ (up to $\mu = 0.5$ M). For the oxidation of the etpy complex, we observed an increase in the rate constant with increasing ionic strength. From the fit of our data to eq 2, a contact distance σ_{12} of 1.82 ± 0.06 nm was obtained (Figure 3). This experimental value is slightly smaller than the sum of the radii (2.01 nm),[5,8] indicating that there may be some penetration (up to 2 Å) of the pyridine ring or NH_3 group into the protein surface. On the basis of our data, this can only be a suggestion due to the expected deviations at high ionic strength.

Ligand Effects. Previously,[5,6] it has been shown that $Co(phen)_3^{3+}$, $Ru(NH_3)_5bm^{2+}$ (bm = benzimidazole), and $Ru(NH_3)_5py^{3+}$ all use the same mechanism for the electron-transfer reactions with cytochrome *c*. The process involves the exposed heme edge, according to modification studies by Sykes and coworkers.[28] The heme edge lies in a cleft which is 1−2 Å deep, consistent with the penetration depth estimated above. For the complexes studied here, the observed rates depend on the driving force as predicted by the Marcus theory. This agreement supports the assumption of similar mechanisms in all of the present cases. The direct interaction between the pyridine and the heme edge provides the closest approach and the highest degree of coupling for the electron-transfer process.

It is possible to see the effect of penetration on electron-transfer rates by comparing the observed rates for the $Ru(NH_3)_6^{2+/3+}$ and $Ru(NH_3)_5L^{2+/3+}$ complexes with cytochrome *c* with the relative rates predicted by the Marcus theory on the basis of driving force. In one formalism,[29] the rate of intermolecular electron transfer k may be expressed as the product of the equilibrium quotient for formation of the precursor complex, K_p, and the rate of electron transfer between the members of the complex, k_{ET}, viz. $k = K_p k_{ET}$.

$$K_p = 4\pi\sigma_{12}^2(\delta\sigma)\exp(-w_{12}/RT) \quad (3)$$

$$k_{ET} = \kappa_{el}\nu_n \exp(-\Delta G^*_r/RT) \quad (4)$$

In eq 3, σ_{12} is the separation distance in the precursor complex, $\delta\sigma$ is the distance range over which electron transfer may occur (typically taken to be 0.08 nm),[29] and w_{12} is the work required to bring the reactants together into the complex (eq 10; vide infra). The effect on K_p by substitution of a substituted pyridine ligand for ammonia in complexes of the type $Ru(NH_3)_5L^{2+/3+}$ are expected to be small, since σ_{12} increases by only 0.05 nm.[29]

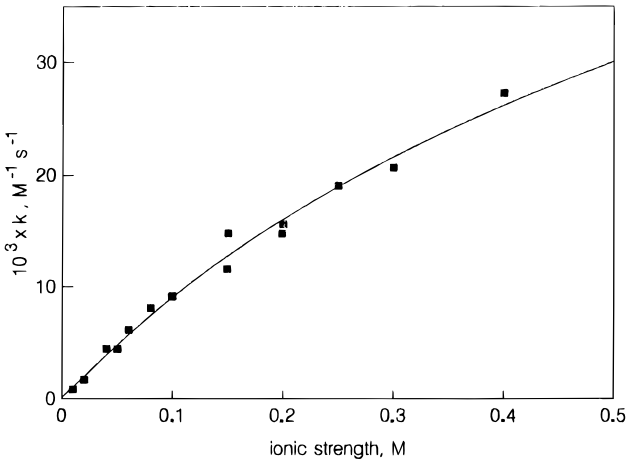

Figure 3. Ionic strength dependence of k_{obs} for the reduction of cyt c^{III} by $Ru(NH_3)_5etpy^{2+}$. Solid line represents the fit to eq 2. Experimental conditions: [cyt $c^{III/II}$] = 1.0×10^{-5} M, pH = 7.2, [Tris] = 0.05 M, ionic strength adjusted with $LiNO_3$, λ = 550 nm, T = 25.0 °C.

On the other hand, k_{ET} is expected to change as substitution of different ligands L changes the driving force ($\Delta G°_{12}$) and thereby affects the free energy barrier to reorganization, ΔG^*_r, according to eq 5 (where the reorganization energy $\lambda_{12} = (\lambda_{cyt} + \lambda_{Ru})/2$). The preexponential term in eq 4 consists of ν_n, the effective nuclear frequency along the reaction coordinate, which should not be sensitive to substitutions in the ligand L, and κ_{el}, the electronic transmission coefficient. If the pyridine ring does enhance the electronic coupling between the redox centers through penetration of the heme-edge groove, the effect will be manifested as an increase in κ_{el} relative to the $Ru(NH_3)_6^{2+}$ case.

$$\Delta G^*_r = (\lambda_{12}/4)\left(1 + \frac{\Delta G°_{12} + w_{21} - w_{12}}{\lambda_{12}}\right)^2 \quad (5)$$

To search for such an effect, we will compare the observed second-order electron-transfer rates in both directions, measured in the same media, between cytochrome *c* and the complexes $Ru(NH_3)_5L^{2+/3+}$ (L = NH_3, etpy, lut, py, isn) while correcting for the differences in driving force according to eq 5. Table 2 summarizes the results. The electrochemically-determined reaction free energies ($\Delta G°_{12}$), estimated reorganization energies (λ_{cyt}, λ_{Ru}) from the literature,[29] and work terms w_{21} and w_{12} (calculated from eq 10; vide infra) were used to calculate the free energy barriers to reorganization (ΔG^*_r) in each case. Calculated rate constants were normalized so that the calculated rate of cytochrome oxidation by $Ru(NH_3)_5(pyridine)^{3+}$ was equal to the observed one:

$$k_{calc,L} = k_{obs,py}\exp\left(-\frac{w_{12} - w_{12,py}}{RT}\right)\exp\left(-\frac{\Delta G^*_{r,L} - \Delta G^*_{r,py}}{RT}\right) \quad (6)$$

where w_{12} = 4.3 kJ mol^{-1} for the reactions involving Ru^{II} and cyt c^{III}, and w_{12} = 5.6 kJ mol^{-1} for the reactions involving Ru^{III} and cyt c^{II}. The effect of this normalization is to set the activationless rate limit ($= K_p\kappa_{el}\nu_n$) equal to 8.2×10^8 M^{-1} s^{-1} for the cytochrome oxidation reactions.

The ratios of the observed rate constants to the calculated ones (Table 2, right column) allow us to make comparisons between different complexes by removing the driving force dependence. All of the substituted pyridines have similar rate ratios, with those of isonicotinamide, 4-ethylpyridine, and 3,5-lutidine slightly more than 60% of the defined value of 1.0 for pyridine. On the other hand, the observed rate for $Ru(NH_3)_6^{2+}$

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Table 2. Estimation of Scaled Rate Constants Corrected for Driving Force

Ru complex[a] and direction of ET	$\Delta G°$,[b] eV	λ_{cyt}, eV	λ_{Ru}, eV	λ_{12}, eV	w_{12},[c] kJ mol^{-1}	ΔG^*_r, eV	k_{obs}, M^{-1} s^{-1}	k_{calc}, M^{-1} s^{-1}	k_{obs}/k_{calc}
$A_5Ru^{III}lut \leftarrow Fe^{II}$	−0.025	1.00	1.20	1.10	5.6	0.256	27 100	38 100	0.71
$A_5Ru^{II}lut \rightarrow Fe^{III}$	0.025	1.00	1.20	1.10	4.3	0.295	9 450	14 400	0.66
$A_5Ru^{III}etpy \leftarrow Fe^{II}$	−0.024	1.00	1.20	1.10	5.6	0.257	26 800	37 400	0.72
$A_5Ru^{II}etpy \rightarrow Fe^{III}$	0.024	1.00	1.20	1.10	4.3	0.294	9 180	14 700	0.63
$A_5Ru^{III}py \leftarrow Fe^{II}$	−0.038	1.00	1.20	1.10	5.6	0.25	48 600	48 600	1.00
$A_5Ru^{II}py \rightarrow Fe^{III}$	0.038	1.00	1.20	1.10	4.3	0.301	10 500	11 100	0.95
$A_5Ru^{III}isn \leftarrow Fe^{II}$	−0.115	1.00	1.20	1.10	5.6	0.216	115 000	193 000	0.60
$A_5Ru^{II}isn \rightarrow Fe^{III}$	0.115	1.00	1.20	1.10	4.3	0.343	1 520	2 050	0.70
$A_6Ru^{II} \rightarrow Fe^{III}$	−0.210	1.00	1.40	1.20	4.3	0.210	38 000	391 000	0.10

[a] A = NH_3. [b] Based on the following potentials vs NHE: cyt *c*, + 0.260 V; $A_5Ru(lut)^{2+/3+}$, 0.285 V (this work); $A_5Ru(etpy)^{2+/3+}$, 0.284 V (this work); $A_5Ru(py)^{2+/3+}$, 0.298 V;[50] $A_5Ru(isn)^{2+/3+}$, 0.375 V.[51] [c] If w_{12} for a given reaction equals 4.3 kJ mol^{-1}, w_{21} = 5.6 kJ mol^{-1}, and vice versa.

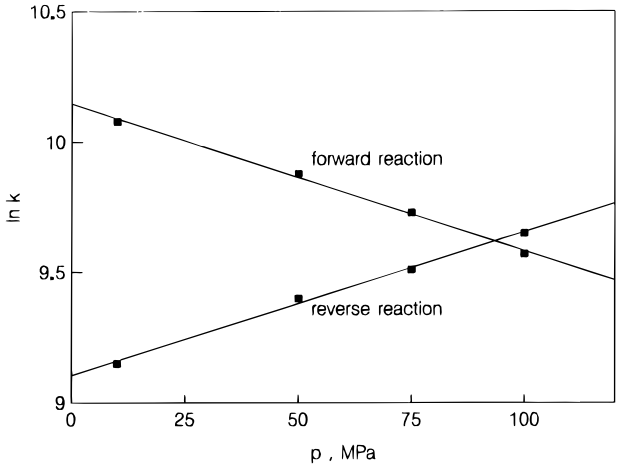

Figure 4. Plot of ln *k* versus pressure for the forward and reverse reactions. For experimental conditions see Figure 1.

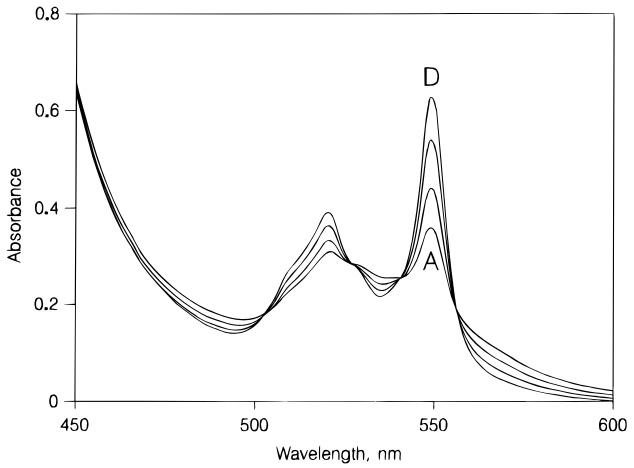

Figure 5. UV/vis spectra of an equillibrium mixture of cyt $c^{II/III}$ and $Ru(NH_3)_5etpy^{2+/3+}$ as a function of pressure: (A) 5 MPa; (B) 50 MPa; (C) 100 MPa; (D) = 150 MPa. Experimental conditions: [cyt c] = 8.5×10^{-6} M, [Ru] = 2.5×10^{-3} M, pH = 7.2, ionic strength = 0.1 M, [Tris] = 0.05 M, [$LiNO_3$] = 0.05 M, *T* = 25.0 °C.

reduction of ferricytochrome *c* (bottom row) is only 10% of the estimated rate based on the pyridine reaction. All other factors being relatively constant, it would appear that the electronic transmission coefficient κ_{el} is up to 10 times larger for the complexes with ligands that can penetrate the heme groove over the hexaammine complex, which cannot. Two other factors may increase the rate of electron transfer in the $Ru(NH_3)_5L^{2+/3+}$, L = py, etpy, lut, and isn, cases over that for $Ru(NH_3)_6^{2+}$. First, specific interactions, such as hydrophobicity, between the pyridine ligand and residues on the surface of the heme-edge groove may increase the equilibrium constant for precursor complex formation, K_p. Second, if the internuclear distance is increased, the outer-sphere contribution to the reorganization energy λ will be reduced, decreasing ΔG^*_r and increasing the electron transfer rate. Both of these factors are consistent with groove penetration as proposed. Using the common values of $\beta = 1.2-1.4$ Å^{-1} for the distance attenuation factor, the distance of closest approach is 1.9−1.7 Å shorter for $Ru(NH_3)_5L^{2+/3+}$ than for $Ru(NH_3)_6^{2+}$, consistent with our other observations.

Pressure Effects. In all cases examined, the oxidation of the protein was decelerated with increasing pressure, resulting in activation volumes of +14.7 ± 0.9 for the etpy complex, +17.4 ± 1.5 for the py complex, and +17.8 ± 1.6 cm^3 mol^{-1} for the lutidine complex. The value for the etpy complex is somewhat lower than in the other cases, including isonicotinamide (Table 1). This trend holds for the reverse reaction, which in all cases is significantly accelerated by pressure. The plots of ln *k* versus pressure are linear within the experimental error limits (for a typical example, see Figure 4 for the etpy system). Again the values are very similar; only the values for the reduction of cytochrome *c* by $Ru(NH_3)_6^{2+}$ and $Ru(NH_3)_5etpy^{2+}$ seem to be smaller. The results show a large volume increase for the forward (spontaneous) reaction. For the reverse reaction, going from Ru^{II} to Ru^{III}, we observe a significantly negative reaction volume. On a volume basis, the transition state for these processes is located halfway between reactant and product states, independent of steric factors or driving force within this low driving force range.

As an independent check of the net reaction data calculated from kinetic results, the spectrum of an equilibrium mixture of each ruthenium complex and cytochrome *c* was recorded at several pressures to obtain the equilibrium constants as a function of pressure (Figure 5). As the pressure increases, the equillibrium in reaction 1 is shifted to the left side in all four investigated systems. An increase in absorbance at 550 nm indicates that the concentration of cytochrome c^{II} increases with increasing pressure. The reaction volume can be calculated from the slope of the plot of ln *K* versus pressure in the usual way[9] (for a typical example, see Figure 6). The values obtained for the three systems are in very good agreement with the corresponding values calculated from the differences in the activation volumes for the forward and reverse reactions. In general, we observe excellent agreement between the reaction volumes obtained from the kinetically determined activation volumes and those obtained from the equilibrium measurements. For the cytochrome *c*/isonicotinamide system a value of 26 cm^3 mol^{-1} was also obtained from electrochemical measurements as a function of pressure.[10]

A volume profile can be constructed from a combination of the kinetic and thermodynamic volume data (a typical example is given in Figure 7). All the volume profiles for the investigated reactions (see the data in Table 1) are very similar

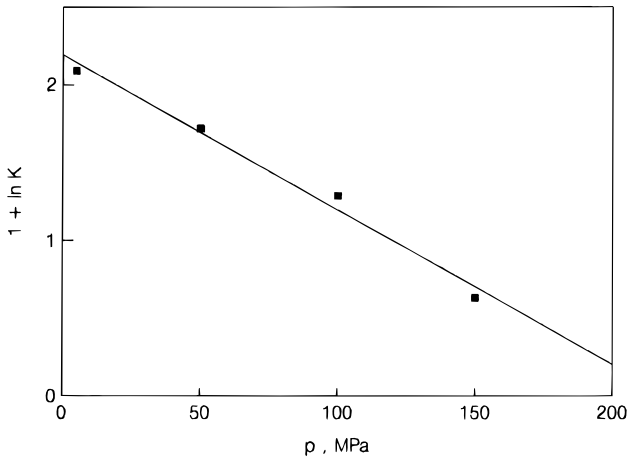

Figure 6. Plot of ln *K* versus pressure. For experimental conditions see Figure 5.

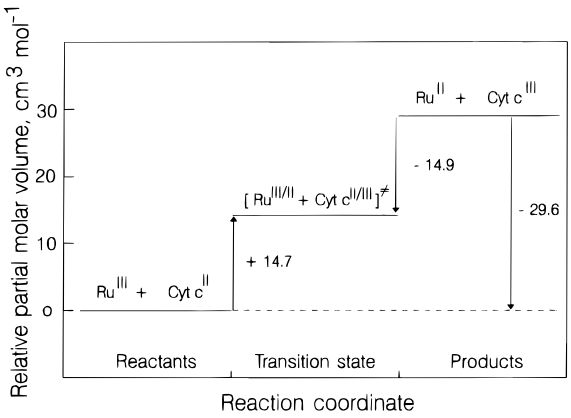

Figure 7. Volume profile for the overall reaction $Ru(NH_3)_5etpy^{3+}$ + cyt $c^{II} \rightleftharpoons Ru(NH_3)_5etpy^{2+}$ + cyt c^{III}.

and clearly indicate that the transition state is located halfway between the reactant and product states on a volume basis in each case. The similarity of the volume profiles is consistent with, but not proof of, a single mechanism for all of the reactions studied here. Previous work has shown that the dominant part of the volume effects in reactions between ammineruthenium complexes and cytochrome *c* is solvent reorganization about the ruthenium center. The transition state occurs at the midpoint of the outer-sphere reorganization coordinate, resulting in the observed volume profiles. It is reasonable to expect that interpenetration of the (substituted) pyridine ligand would result in a negative contribution to the volume of the transition state; however, the heme groove is fully solvated and the water molecules expelled by the ligand penetration would have a positive contribution, which roughly cancels the first effect. Hence, the activation volume for the reduction of ferricytochrome *c* by $Ru(NH_3)_6^{2+}$ is comparable to those for the $Ru(NH_3)_5L^{2+}$ complexes. Interestingly, the net reaction volume change in the ethylpyridine case is about 5 cm^3 mol^{-1} less than those in the other three cases. This result suggests that the ethyl group may disrupt the solvation sphere around the $(NH_3)_5Ru(etpy)^{2+/3+}$ cations, thereby decreasing the change in electrostriction around the ruthenium complex that is associated with the redox process. Further measurements on related ruthenium complexes may substantiate this observation.

Previous experiments[9,10] have shown that most of the volume change during the electron-transfer process in this type of reaction occurs around the ruthenium center rather than on cytochrome *c*. An electrostriction decrease upon reduction of the ruthenium center is primarily responsible for the observed volume effect.[30] The partial molar volume change upon oxidation of cytochrome *c* in this medium is +5 ± 1 cm^3 mol^{-1}.[10]

Temperature Effects. In order to compare the observed activation entropies with those of other reactions, it is necessary to correct for the net reaction entropy ($\Delta S^{\ddagger}_{corr} = \Delta S^{\ddagger}_{obs} - \Delta S°/2$) as shown by Sutin.[31] The corrected values for the oxidation of the protein range from −62 ± 7 for the pyridine complex to −81 ± 6 J K^{-1} mol^{-1} for the isonicotinamide complex. Activation enthalpies are corrected in the same manner. The corrected activation entropies and activation enthalpies fall within the range of many other reactions of metal complexes with metalloproteins.[31]

The systems studied here can be grouped into two categories according to their net reaction thermodynamics. The oxidations of cytochrome *c* by the pyridine ($\Delta H° = -5 \pm 4$ kJ mol^{-1}, $\Delta S° = -5 \pm 14$ J K^{-1} mol^{-1}) and isonicotinamide ($\Delta H° = -6 \pm 4$ kJ mol^{-1}, $\Delta S° = +12 \pm 12$ J K^{-1} mol^{-1}) complexes are exothermic, while the reactions with the 4-ethylpyridine ($\Delta H° = +4 \pm 3$ kJ mol^{-1}, $\Delta S° = +25 \pm 9$ J K^{-1} mol^{-1}) and 3,5-lutidine ($\Delta H° = +14 \pm 1$ kJ mol^{-1}, $\Delta S° = +58 \pm 5$ J K^{-1} mol^{-1}) complexes are entropy-driven. It is interesting to note that complexes which appear to be very similar can have significantly different thermodynamic profiles.

Theoretical Calculations. The Marcus cross relation (eqs 7−9) has been shown[29,31,32] to provide reasonably close agreement between theory and experiment for reactions of this type.

$$k_{12} = (k_{11}k_{22}K_{12}f_{12})^{0.5}W_{12} \tag{7}$$

$$\ln f_{12} = \frac{(\ln K_{12})^2}{4\ln(k_{11}k_{22}/Z^2)} \tag{8}$$

$$W_{12} = \exp\left(-\frac{w_{12} + w_{21} - w_{11} - w_{22}}{2RT}\right) \tag{9}$$

Of the three complexes studied here, only the pyridine complex has a reported self-exchange rate; therefore the estimation of the electron-transfer rate will only be carried out for that case. The cross relation can also be applied to estimate self-exchange rates from observed electron-transfer rates for the pyridine and lutidine complexes. In this formalism, *Z* is the collision frequency (normally taken to be 10^{11} M^{-1} s^{-1}) and the factor *f* is close to 1 for low-driving-force reactions such as the reaction studied in this work. The electrostatic correction term W_{12} is given by eq 9, w_{12} and w_{21} are the electrostatic work terms required to bring the reactants and products, respectively, together in the precursor complex, and w_{11} and w_{22} are the corresponding work terms for the self-exchange reactions (eq 10). z_i and z_j are the charges of the ions, e_o is the electronic

$$w_{ij} = \frac{z_i z_j e_0^{\,2} N_A}{4\pi\epsilon_0\epsilon\sigma_{12}(1 + \kappa\sigma_{12})} \tag{10}$$

charge, ϵ_0 is the permittivity of the vacuum, ϵ is the bulk dielectric constant, σ_{12} is the contact distance of the ions ($\sigma_{12} \approx r_1 + r_2$), and κ is the reciprocal Debye−Hückel length. For aqueous solutions at 25 °C, $\epsilon = 78.5$ and $\kappa = 3.29\,\mu^{1/2}$ nm^{-1}.[33,34]

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To calculate the rate for the reaction between cytochrome $c^{II/III}$ and $Ru(NH_3)_5py^{3+/2+}$ under the experimental conditions in this study, the following values were used. For the Ru(III)/Ru(II) couple: $E°_{11} = 0.318$ V,[35] $k_{11} = 4.3 \times 10^5$ M^{-1} s^{-1},[36] radii 3.5 Å.[8] For cytochrome *c*: $E°_{22} = 0.260$ V,[35] $k_{22} = 350$ M^{-1} s^{-1},[37] radius 1.66 nm,[5,8] charges +7.5/6.5.[21] Using these values ($\mu = 0.1$ M; $T = 25.0$ °C), we obtain $f_{12} \approx 1$, $W_{12} = 1.0$, $w_{12} = 4.3$ kJ mol^{-1}, $w_{21} = 5.6$ kJ mol^{-1}, $w_{11} = 8.8$ kJ mol^{-1}, and $w_{22} = 5.8$ kJ mol^{-1}.

This results in 2.9×10^4 and 5.2×10^3 M^{-1} s^{-1} for k_f and k_b, respectively. These values are in reasonably good agreement with the experimental values of $(4.9 \pm 0.1) \times 10^4$ and $(10.5 \pm 0.5) \times 10^3$ M^{-1} s^{-1} for the forward and reverse reactions, respectively. Using the values calculated for the py complex, we are able to estimate the self-exchange rates for the etpy, lutidine, and isn complexes. This results in $k_{11} = 9.3 \times 10^5$ and 8.8×10^5 M^{-1} s^{-1} for the etpy and lutidine complexes, respectively. For the isn complex we can calculate a value of 5.0×10^5 M^{-1} s^{-1}. In the latter case, we can compare the self-exchange rate with the literature value of 4.3×10^5 M^{-1} s^{-1}.[36] The good agreement indicates that, despite the uncertainties involved in using proteins for cross relation calculations, this approach seems valid for these complexes.

Using the theoretical treatment of Stranks,[38] Swaddle et al.,[39−42] and Wherland et al.,[43,44] it is possible to calculate the activation volume from eq 11. According to this theory the

$$\Delta V^{\ddagger} = \Delta V^{*}_{IR} + \Delta V^{*}_{COUL} + \Delta V^{*}_{DH} + \Delta V^{*}_{SR} + \beta RT + \lambda^{*}\Delta\bar{V} \quad (11)$$

activation volume consists of six components: ΔV^{*}_{IR} is the inner-sphere rearrangement, which is neglected here because the contribution is usually close to zero[38,42] in these systems; ΔV^{*}_{COUL} is the Coulombic term; ΔV^{*}_{DH} includes Debye−Hückel or other electrolyte effects; ΔV^{*}_{SR} is the contribution from the solvent reorganization; and βRT is the contribution from the preexponential part of the work terms (=1.3 cm^3 mol^{-1}). $\lambda^{*}\Delta\bar{V}$ represents the contribution due to the overall volume change. The λ^{*} parameter represents the location of the transition state relative to products and reactants along the reaction coordinate ($0 \le \lambda^{*} \le 1$) and can be calculated according to the Marcus theory (eq 12). For the low-driving-

$$\lambda^{*} = \frac{1}{2}\left(1 + \frac{\Delta G°_{12} + w_{21} - w_{12}}{\lambda}\right) \quad (12)$$

force system of cytochrome $c^{II/III}$ and $Ru(NH_3)_5py^{3+/2+}$, a λ^{*} value of 0.48 can be calculated using $\lambda = 102$ kJ mol^{-1}.[29]

For the forward reaction the following volume contributions were calculated (all values in cm^3 mol^{-1}): $\Delta V^{*}_{COUL} = -5.5$; $\Delta V^{*}_{DH} = +5.5$; $\Delta V^{*}_{SR} = -6.4$; $\lambda^{*}\Delta\bar{V} = 0.48 \times 34 = 16.3$. For the reverse reaction the contributions were as follows (cm^3 mol^{-1}): $\Delta V^{*}_{COUL} = -4.2$; $\Delta V^{*}_{DH} = +4.2$; $\Delta V^{*}_{SR} = -6.4$; $\lambda^{*}\Delta\bar{V} = -0.52 \times 34 = -17.7$. These values result in a $\Delta V^{\ddagger}_{f}$ value of 11.2 and a $\Delta V^{\ddagger}_{b}$ value of -22.8 cm^3 mol^{-1}, respectively. Similar to our earlier work,[9] the theory gives too negative values for both steps. The Coulomb and the Debye−Hückel terms compensate for each other, and only the ΔV^{*}_{SR} and $\lambda^{*}\Delta\bar{V}$ terms determine the calculated value. Since the main volume changes occur only on the metal complex and not on the cytochrome *c*, the ΔV^{*}_{SR} term requires correction. If we perform this correction, the ΔV^{*}_{SR} term is only 17% (ratio between the radius of the metal complex and the radius of the precursor complex) of the calculated ($\Delta V^{*}_{SRc} = 1.1$ cm^3 mol^{-1}). Using this value, we obtain a $\Delta V^{\ddagger}_{f}$ value of 16.5 and a $\Delta V^{\ddagger}_{b}$ value of -17.5 cm^3 mol^{-1}. These results are in excellent agreement with the experimental values and indicate that these calculations are valid not only for model complexes.

Conclusions

The experiments described in this report involve a series of closely related ruthenium complexes and analyze their redox behavior with cytochrome *c*. The observed rate constants for the electron-transfer reactions in both directions can be correlated on the basis of the ability of the ligands on the ruthenium center to penetrate the heme groove on cytochrome *c*. Similar volume profiles were obtained for all these systems. In each case, the activation volume $\Delta V^{\ddagger}_{12}$ is very nearly half of the overall net reaction volume $\Delta\bar{V}$. The fact that the activation volumes are half of the total volume change implies, according to Swaddle's[46] recent treatment of the volume profiles of cross reactions, and keeping in mind the limitations of the Marcus theory when applied to reactions of this type, that the activation volumes of self-exchange for the ruthenium complexes and for cytochrome *c* have to be approximately equal and opposite in sign if, as in the present cases, $\Delta V^{\ddagger}_{12} \approx \Delta\bar{V}/2$ (eq 13).

$$\Delta V^{\ddagger}_{12} = \frac{\Delta V^{\ddagger}_{11} + \Delta V^{\ddagger}_{22} + \Delta\bar{V}}{2} \quad (13)$$

Unfortunately, self-exchange activation volumes are not yet available for either reactant. If such information were available, comparison of the observed and calculated $\Delta V^{\ddagger}_{12}$ values might point toward (or against) the existence of specific interactions in the encounter complex, such as the penetration of the cytochrome's heme-edge groove by pyridyl groups as suggested by Cummins and Gray.[8]

Acknowledgment. R.v.E. gratefully acknowledges financial support from the Deutsche Forschungsgemeinschaft, the Volkswagen Stiftung, and the Fonds der Chemischen Industrie. The work at Brookhaven National Laboratory was carried out under Contract DE-ACO2-76CH-00016 with the U.S. Department of Energy and supported by its Division of Chemical Sciences, Office of Basic Energy Sciences.

Supporting Information Available: Tables containing kinetic data (6 pages). Ordering information is given on any current masthead page.

IC9413528

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