Effect of Binding Cytochrome *c* and Ionic Strength on the Reorganizational Energy and Intramolecular Electron Transfer in Cytochrome b_5 Labeled with Ruthenium(II) Polypyridine Complexes

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Abstract: The rates of intramolecular electron transfer in T65C cytochrome b_5 labeled with three different ruthenium polypyridine complexes at Cys-65 have been measured over a range of ionic strengths in the presence and absence of cytochrome *c*. The redox potentials of the ruthenium complexes provide for a range of free energies of reaction which span the reorganizational energy of the system. The system obeys the familiar dependence of ln k_{obs} on free energy of reaction predicted by Marcus (Marcus, R. A. *J. Chem. Phys.* **1956**, *24*, 966). The reorganization energies for these reactions obtained at 0 and 300 mM NaCl in the absence of cytochrome *c* are 1.02 and 0.93 eV, respectively. The corresponding electronic coupling elements calculated as H_{AB} from $k_{max} = 2.0 \times 10^7$ and 1.4×10^7 s^{-1}are 0.29 and 0.21 cm^{-1}, respectively. Surprisingly, the rate constants obtained with cytochrome *c* bound to cytochrome b_5 ([NaCl] = 0) are the same as those obtained at [NaCl] > 150 mM in the absence of cytochrome *c*. These rate constants are, however, very different from those obtained at low [NaCl] in the absence of cytochrome *c*. We suggest that these results can be explained in terms of an alteration of the solvent reorganizational barrier either by binding of cytochrome *c* and exclusion of water from the binding domain or by the influence of sodium and chloride ions on the solvent in close proximity to the redox centers.

Introduction

Does the intrinsic reorganizational barrier to electron transfer of a protein change when the protein binds to its redox partner? Such a scenario is easily imagined given that solvent reorganization is a dominant term in the overall reorganizational barrier[1] and that binding of one protein to another is expected to exclude water molecules from the protein:protein interface. Gray and Malmström,[2] for example, suggested that the reorganizational energy may be reduced significantly because of changes in the environment surrounding the redox centers if water is excluded. In keeping with this view, Rogers et al.[3] have demonstrated that solvent is excluded from the cytochrome b_5/cytochrome *c* interface upon binding. McLendon[4] has also suggested that a large reorganizational energy may be associated with the intracomplex electron transfer between docked proteins due to the repolarization of the proteins.

In the past we have labeled the T65C variant of cytochrome b_5 with several ruthenium polypyridyl complexes in order to investigate the internal electron transfer between the ruthenium label and the heme iron of the cytochrome b_5.[5] The electron-transfer reactions could be described by the semiclassical Marcus theory,[6] which can be summarized, for reactions between two redox centers held at a fixed distance, by the equation

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

where H_{AB} describes the electronic coupling between the redox centers and λ describes the reorganizational energy. From a plot of ln k_{et} versus $-\Delta G^\circ$, we were able to determine the reorganizational energy for the labeled cytochrome b_5.

In this paper, we wish to describe an experiment designed to test the hypothesis that the reorganizational barrier of a protein is altered by the binding of its redox partner. The experiment is based on the use of the well-defined cytochrome b_5/cytochrome *c* system and the exploitation of ruthenium-labeled T65C cytochrome b_5. The ruthenium-labeled T65C has a well-defined 12 covalent bond pathway between the axial histidine of the heme iron and the bipyridine ligand of the ruthenium complex, and the location of the label does not interfere with the protein:protein binding site. This system provided a means of measuring the reorganizational energy barrier by examining the rate constants for electron transfer over a wide range of free energies by altering the reduction potential of the ruthenium complex. Furthermore, protein:protein binding can be controlled by the ionic strength of the reaction solution. This has enabled us to determine the reorganization barrier of the protein with and without its natural redox partner present. As a control, we have also investigated the effect of ionic strength on the electron-transfer rates of labeled cytochromes b_5 in the absence of cytochrome *c*.

As indicated, the cytochrome b_5/cytochrome *c* system has been extensively studied.[7] The crystal structures for the oxidized and

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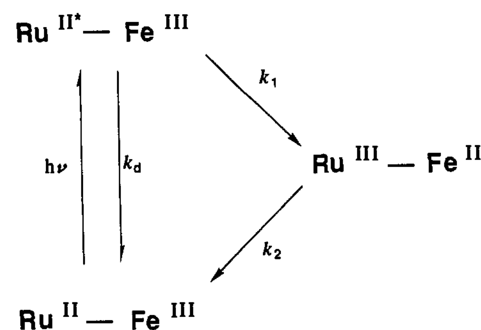
reduced forms for both cytochrome b_5[8] and cytochrome c[9] are known. There are no major structural differences between the two oxidation states for either protein which could contribute significantly to the reorganizational energy. NMR studies have been done for cytochrome b_5,[10] and cytochrome c,[11] and for the cytochrome b_5/cytochrome c complex.[12] The self-exchange electron-transfer reactions of cytochrome b_5 and cytochrome c have been investigated by Dixon et al.[13] Salemme and co-workers[14] have also thoroughly investigated the molecular dynamics of docking cytochrome c to cytochrome b_5 by molecular modeling. Cytochrome b_5 is a particularly good candidate for the experiments described since the heme is highly exposed[13a] and exclusion of water from the heme domain should result in a large change in the solvent reorganizational energy.

Willie et al. have used flash photolysis of ruthenium polypyridyl-labeled T65C variant[15] of cytochrome b_5 to investigate the intracomplex electron transfer between cytochrome b_5 and native cytochromes c[16] and variants of cytochrome c.[17] In these studies, the labeled T65C cytochrome b_5 variant binds cytochrome c in a native-like manner. The native-like binding domain of labeled T65C cytochrome b_5 has also been confirmed by comparison to ruthenium polypyridyl-labeled T73C cytochrome b_5 variant,[18] which has identical intracomplex electron-transfer rate constants.

Experimental Section

Materials. The T65C variant was prepared as described by Stayton et al.[15] 4-(Hydroxymethyl)-4′-methylbipyridine(bisbipyridine)ruthenium(II)$^{2+}$ was prepared as described by Geren et al.[19] [4-(Hydroxymethyl)-4′-methylbipyridine]bis(4,4′-dimethylbipyridine)ruthenium(II)$^{2+}$ and [4-(hydroxymethyl)-4′-methylbipyridine]bipyridine(2,2′-bipyrimidine)ruthenium(II)$^{2+}$ were prepared as described by Scott et al.[5] All ruthenium compounds were purified by chromatography on 2.5 × 16 cm alumina columns with an eluent of 1:1 acetonitrile/methylene chloride. The purified compounds were then brominated via thionyl bromide as described by Scott et al.[5] to produce 4-(bromomethyl)-4′-methylbipyridine(bisbipyridine)ruthenium(II)$^{2+}$, [4-(bromomethyl)-4′-methylbipyridine]bis(4,4′-dimethylbipyridine)ruthenium(II)$^{2+}$, and [4-(bromomethyl)-4′-methylbipyridine]bipyridine(2,2′-bipyrimidine)ruthenium(II)$^{2+}$, respectively. Ru-65-cyt b_5 was prepared by the reaction of T65C cytochrome b_5 with 4-(bromomethyl)-4′-methylbipyridine(bisbipyridine)ruthenium(II)$^{2+}$ in 50 mM sodium borate buffer at pH 9 by a method similar to that described by Geren et al.[19] Me_5Ru-65-cyt b_5 and bpymRu-65-cyt b_5 were made by analogous procedures described by Scott et al.[5] The abbreviations Ru-65-cyt b_5, Me_5Ru-65-cyt b_5, and bpymRu-65-cyt b_5 describe a variant of cytochrome b_5 (T65C) labeled at the sulfur of Cys-65 with 4-(bromomethyl)-4′-methylbipyridine-(bisbipyridine)ruthenium(II)$^{2+}$, [4-(bromomethyl)-4′-methylbipyridine]-bis(4,4′-dimethylbipyridine)ruthenium(II)$^{2+}$, or [4-(bromomethyl)-4′-methylbipyridine]bipyridine(2,2′-bipyrimidine)ruthenium(II)$^{2+}$, respectively.

Scheme 1



Horse cytochrome c (type VI) and yeast iso-1-cytochrome c (type VIIIB) were obtained from Sigma Chemical Co. The yeast cytochrome c was treated with a minimum amount of dithiothreitol to reduce the heme iron and any disulfide cross-linked dimers. The reduced yeast cytochrome c solution was passed through a Bio-Gel P-2 column to remove excess dithiothreitol and stored in the reduced form under N_2. The oxidized yeast cytochrome c was produced by adding a trace amount of cytochrome oxidase to the reduced yeast cytochrome c.

Characterization of Labeled Cytochrome b_5. The labeled proteins were purified and characterized as described by Scott et al.[5] After purification, the derivatized proteins were concentrated and washed twice with a 20:1 v/v ratio of 1 mM sodium phosphate (pH 7) to labeled protein solution using Amicon concentrators.

Electrochemistry. Redox potentials of the free ruthenium complexes and the heme groups in the labeled proteins were determined as previously described.[5] Cyclic voltammetry was not performed on the labeled proteins due to interference by solvent oxidation at the potentials of interest and the low concentrations achievable. However, the emission maximum determined at 77 K, which exhibits a strong correlation with $E_{1/2}$,[20] was the same for Ru-65-cyt b_5, Me_5Ru-65-cyt b_5, and bpymRu-65-cyt b_5 and the corresponding unbound ruthenium complexes. The previously reported emission maxima were determined in 1:1 H_2O/ethylene glycol. The current emission values for the free complexes and derivatized proteins were obtained in 1:1 100 mM sodium phosphate (pH 7)/ethylene glycol. Of the ruthenium complexes, only Ru(bpy)(bpym)(mebpyOH)$^{2+}$ is affected by pH since the 2,2′-bipyrimidine ligand has two uncoordinated ring nitrogens which can be protonated. The previously reported emission maximum for Ru(bpy)(bpym)(mebpyOH)$^{2+}$ is not correct.[5] The reported emission value was an artifact which was misassigned due to the low level of emission exhibited by this complex. The current emission value for Ru(bpy)(bpym)(mebpyOH)$^{2+}$ is the same as that reported by Meyer and co-workers.[21]

Flash Photolysis Experiments. Transient absorbance measurements were performed by laser flash photolysis as described previously.[5,22] Other than minor base-line corrections, the rate constants indicated in Scheme 1 were determined as previously described[5,22] except in the case of bpymRu-65-cyt b_5. In the previous paper,[5] we determined the rate constants for bpymRu-cyt b_5 from quantum efficiencies. Due to improved signal to noise, we are now able to fit the bpymRu-65-cyt b_5 data in the same manner as the other derivatives. The reactions of the heme group in each derivative were monitored at 424 and 556 nm and that of the ruthenium

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Table 1. 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]	–0.56	1.96

[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 as previously described.[5] [d] Emission energies determined from emission maxima recorded at 77 K in ethylene glycol/100 mM sodium phosphate (pH 7) frozen glass. [e] Potential measured in acetonitrile and corrected to 100 mM phosphate.

complexes at the heme isobestic points 439 and 547 nm. Emission decay kinetics were determined as previously described.[22] All measurements in aqueous solutions were in 1 mM sodium phosphate (pH 7) with 5–20 μM protein and 0–300 mM NaCl. In the experiments with cytochrome b_5 and cytochromes c, the molar ratio of cyt b_5 to cyt c ranged from 1:1.3 to 1:2, respectively, to ensure a 1:1 ratio of cyt b_5 to cyt c. No difference in rates was observed for 1:1.3 ratios and 1:2 ratios. Measurements were made in both air-saturated and nitrogen-purged solutions. No difference was observed. All experiments were carried out at 22 °C.

Statistical Analysis. Rate constants obtained at 0 and 300 mM NaCl were fit to eq 1 using the Marquardt–Levenberg method in the program PSI-Plot from Poly Software International. Identical results were obtained by manual fits using a spreadsheet program or the curve-fitting routines in SigmaPlot from Jandel Scientific. The fitted parameters were H_{AB} and λ. The reported best fit parameters are followed in the text by the standard deviations in parentheses. The standard deviations, confidence intervals, and correlation coefficients were calculated according to methods contained in PSI-Plot and are described in the user's manual. The standard deviations of the rate constants were estimated from the means of numerous determinations and are reported in Table 2.

Results

Three derivatives of T65C cytochrome b_5 having a ruthenium complex covalently bonded through a thioether link at cysteine 65 were prepared as described by Scott et al.[5] The purification and characterization of all three ruthenium-labeled cytochromes b_5 have been described previously.[5,16] The redox potentials and emission energies of the ruthenium complexes are summarized in Table 1. Since the emission maxima at 77 K for the ruthenium complexes attached to cytochrome b_5 are the same as the emission maxima of the unbound ruthenium complexes, the electronic properties of the ruthenium complexes do not appear to be perturbed by the covalent link between the 4-methyl group of the bipyridine and Cys-65.

Scheme 1 illustrates the overall electron-transfer reaction sequence along with the relevant rate constants. The excited state of the ruthenium complex was produced with a short laser pulse. The excited state 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. In Scheme 1, 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 can be followed by monitoring at the appropriate wavelengths.[5,22] Figure 1 shows transients at 424 nm, which corresponds to the appearance and disappearance of Fe(II). The production of Fe(II) was also monitored at 556 nm. The disappearance and reap-

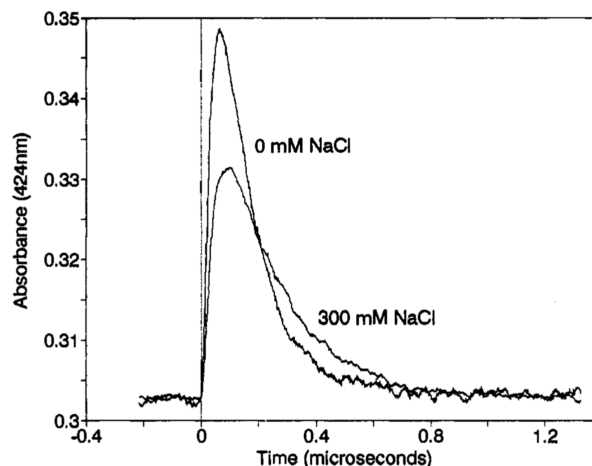

Figure 1. Transient absorbance of a 10 μM solution of Me_5Ru-65-cyt b_5 monitored at 424 nm in 1 mM sodium phosphate buffer (pH 7) with 0 and 300 mM NaCl.

pearance of Ru(II) and Ru(II)* were monitored at 439 and 547 nm. The rate constants for Ru-65-cyt b_5, Me_5Ru-65-cyt b_5, and bpymRu-65-cyt b_5 were obtained by simultaneously fitting the transient absorbance and emission data as previously described.[22]

Since it has been shown that cytochrome c and cytochrome b_5 form a 1:1 complex at low ionic strength, which is dissociated with increasing ionic strength,[7b,10c,12] we manipulated the extent of protein:protein binding by the omission or addition of NaCl to the reaction medium. As a control, the electron-transfer rate constants for the labeled cytochromes b_5 without cytochrome c present were determined over a range of NaCl concentrations. Interestingly, the rate constants for the ruthenium-labeled cytochromes b_5 alone were not independent of ionic strength, as initially expected. Examples of the transients collected at 0 and 300 mM NaCl are shown in Figure 1. The differences in the transients, while small, are clearly evident and very reproducible. The ionic strength dependence of the calculated rate constants k_1 and k_2 are illustrated in Figures 2a and 3a, respectively. The same general trend in k_2 was observed with Me_5Ru-65-cyt b_5, Ru-65-cyt b_5, and bpymRu-65-cyt b_5, and therefore, the later two have been omitted from Figure 3a for clarity. In each case, the rate constant k_2 declined steadily from a maximum at low ionic strength to a minimum constant value above 150 mM NaCl. The rate constant k_1 for Me_5Ru-65-cyt b_5 also showed a similar trend with added salt, but the k_1 for Ru-65-cyt b_5 was independent of [NaCl] and the k_1 for bpymRu-65-cyt b_5 increased with added salt within the uncertainty of the measurements.

The effect of binding cytochrome c on the rate constants k_1 and k_2 for Me_5Ru-65-cyt b_5 and Ru-65-cyt b_5 are summarized in Figures 2b and 3b. Due to signal to noise limitations, data for the bpymRu-65-cyt b_5/cytochrome c complex were not obtained. In Figure 2b, the rate constant k_1 for Me_5Ru-65-cyt b_5 increases to a maximum around 20 mM and then decreases to a constant value at salt concentrations > 150 mM. The rate constant k_1 for Ru-65-cyt b_5 with cytochrome c is essentially constant. Figure 3b illustrates the change in the rate constant k_2 with salt concentration for Me_5Ru-65-cyt b_5/cytochrome c complexes, which is similar to the trend in k_1 for these systems. Again, the trend in k_2 for Ru-65-cyt b_5/cytochrome c is similar to Me_5-Ru-65-cyt b_5/cytochrome c and is not shown. There is no noticeable difference in the rate constants for the reduced and oxidized yeast cytochrome c/Me_5Ru-65-cyt b_5 complexes, confirming that electron transfer to cytochrome c does not occur under these conditions. No difference in rates was observed with various b_5:c ratios.

Discussion

In an earlier publication[5] we described the determination of the rate constants for electron transfer between the heme iron of

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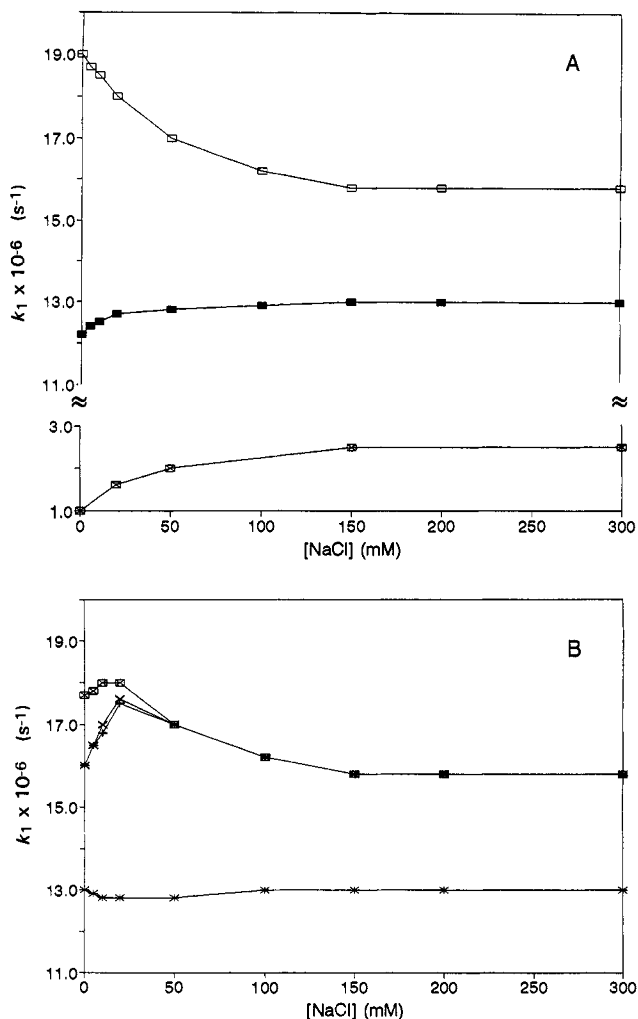

Figure 2. Trend in rate constant k_1 as described in Scheme 1. (A) Data without cytochrome c present: Me_5Ru-cyt b_5 (□), Ru-cyt b_5 (■), and bpymRu-cyt b_5 ( ) (B) Data with cytochrome(s) c present: Me_5Ru-cyt b_5 with horse cyt c ( ), Me_5Ru-cyt b_5 with reduced yeast cyt c (+), Me_5Ru-cyt b_5 with oxidized yeast cyt c (×), and Ru-cyt b_5 with oxidized yeast cyt c (*).

cytochrome b_5 and a ruthenium complex covalently linked to residue Cys-65. In that study we were able to show that the system obeyed the free energy dependence suggested by Marcus.[6] The reorganization energy and the electronic coupling were also determined.

The use of ruthenium-modified cytochrome b_5 has provided us with means of addressing some additional questions. Specifically, we wanted to examine the question of protein binding and to what extent binding of a protein can alter the reorganizational barrier to electron transfer. We have elected to attempt to answer this question by an examination of the ruthenium-modified cytochrome b_5/cytochrome c complex as illustrated in Figure 4. In this system, we have three potential redox sites: the ruthenium complex, the cytochrome b_5 heme, and the cytochrome c heme. The redox reaction of interest is that between the ruthenium complex and the cytochrome b_5 heme. Although thermodynamically allowed, electron transfer from ferrocytochrome b_5 to ferricytochrome c is not likely to occur since it is not competitive with the thermal back-reaction to the ruthenium complex due to the longer distances involved. Previous studies[16,17] have shown that direct electron transfer between the ruthenium complex attached to cytochrome b_5 and the heme of cytochrome c does not take place.

The description of the system in terms of intrinsic reorganizational barriers, determined from self-exchange measurements

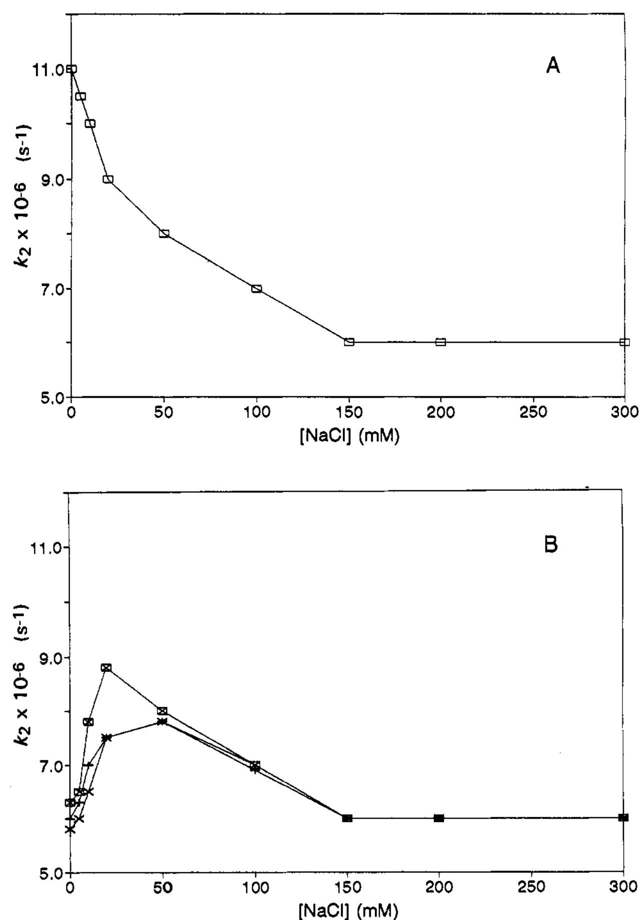

Figure 3. Trend in rate constant k_2 as described in Scheme 1. (A) Data for Me_5Ru-cyt b_5 (□) without cytochrome(s) c present. (B) Data with cytochrome(s) c present: Me_5Ru-cyt b_5 with horse cyt c ( ), Me_5Ru-cyt b_5 with reduced yeast cyt c (+), and Me_5Ru-cyt b_5 with oxidized yeast cyt c (×).

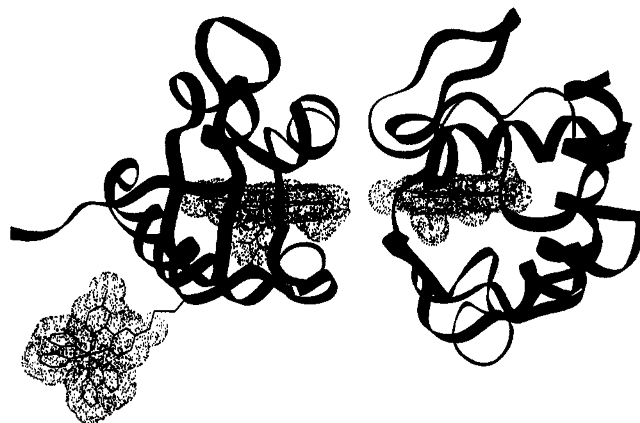

Figure 4. Molecular model of the Ru-65-cytochrome b_5/cytochrome c complex. The orientation of the complex is the same as the orientation for the complex between native cytochrome b_5 and cytochrome c proposed by Salemme.[14] 4-Methyl-4′-methylbipyridine ligand of the ruthenium complex was bonded to the sulfur of Cys-65 using the X-ray structure of cytochrome b_5[8c] in the Brookhaven Protein Data Bank.[23]

as originally described by Marcus,[6] is instructive. The intrinsic reorganizational barrier of a protein is determined from rate measurements which presumably describe electron transfer between like proteins at some optimal distance for efficient electron transfer. In proteins such as cytochrome c and cytochrome b_5, which are both highly charged, it is unlikely that the protein: protein interaction in a self-exchange electron-transfer complex will be very strong. The solvation of the free proteins and those

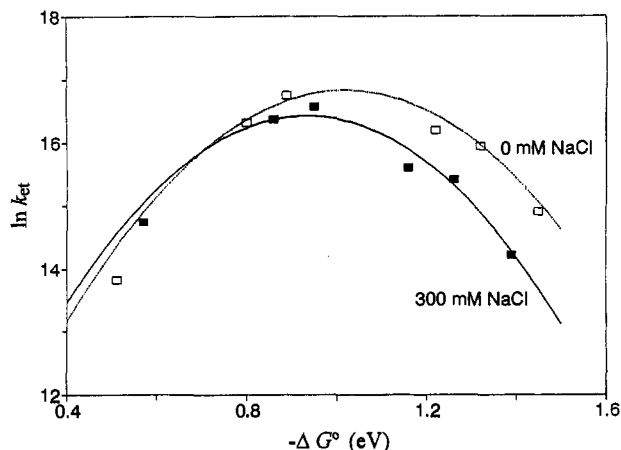

Figure 5. Plot of free energy of reaction versus natural logarithm of the rate constant for electron transfer k_{et} described by eq 1. The solid line shows the theoretical dependence with a reorganizational energy of 0.93 eV and preexponential term of 1.4×10^7 s^{-1} for the data obtained in 300 mM NaCl (■). The dotted line shows the theoretical dependence with a reorganization energy of 1.02 eV and preexponential term of 2.0×10^7 s^{-1} for the data obtained in 0 mM NaCl (□).

in the complex are likely to be similar. In such a case, the cross relation, $\lambda_{12} = (\lambda_{11} + \lambda_{22})/2$, suggested by Marcus[6] is probably a good first-order approximation. Indeed, measurements of the reorganizational energy for electron transfer between the ruthenium complex and the heme of cytochrome b_5 agree with published values for the intrinsic barriers and the cross relation.[13b]

Cytochrome *c* and cytochrome b_5 form a strong 1:1 complex, which has been the focus of several experimental and modeling studies.[7,12,14,16,17] If measurements of the electron transfer between the ruthenium complex and the heme of cytochrome b_5 are done with cytochrome *c* bound to cytochrome b_5, the intrinsic barrier for cytochrome b_5 may not apply since the solvation around the heme site may be significantly altered by the binding of cytochrome *c*. Gray and Malmström,[2] for example, predicted that in the case of cytochrome *c* covalently bonded to ammine complexes of ruthenium the reorganization barrier to electron transfer would decrease by as much as 1 eV if water were removed from the outer spheres of the donor or acceptor.

Experimentally, the electron transfer between the covalently linked ruthenium complexes and cytochrome b_5 can be monitored in the presence of varying amounts of bound cytochrome *c* by taking advantage of the dependence of the protein:protein association constant on ionic strength or simply the concentration of added NaCl. Initially, we expected the reaction in the absence of cytochrome *c* to be relatively insensitive to the [NaCl]. In fact, the effects of added salt on the measured rate constants are comparable in magnitude to that observed with bound cytochrome *c*.

Cytochrome b_5 without Cytochrome *c*. Rate constants for electron transfer between the heme of cytochrome b_5 and three different covalently linked ruthenium complexes were determined over a wide range of [NaCl]. The redox potentials of the three ruthenium complexes span a range of free energies of reaction which bracket the reorganization energy of the reaction. Plots of ln k_{et} versus $-\Delta G°$ obtained at 0 and 300 mM added NaCl are illustrated in Figure 5 and both show the familiar inverted parabolic dependence predicted by Marcus.[6] The individual rate constants are summarized in Table 2. Previously, we have shown that the excited-state reaction and the thermal back-reaction appear to have very similar electronic coupling and reorganization energy, and thus it is appropriate to combine the rate constants of both reactions (k_1's and k_2's) to describe the free energy dependence.[5] The best fits to the data in Figure 5 indicate that the reorganization energies for these reactions obtained at 0 and 300 mM added NaCl are 1.02(0.016) and 0.93(0.014) eV, respectively. The corresponding electronic coupling element calculated as H_{AB} are 0.29(0.01) and 0.21(0.01) cm^{-1} at these ionic strengths ($k_{max} = 2.0 \times 10^7$ and 1.4×10^7 s^{-1}). The reduction potentials of cytochrome b_5 at 2 and 300 mM ionic strength are approximately −50 and +10 mV vs NHE, respectively,[24] and have been incorporated into the data in Figure 5. Chiorboli et al.[25] did not observe any changes in the electronic properties of $Ru(bpy)_3^{2+}$ with various salt concentrations; therefore, no corrections for the effect of ionic strength have been made for the ruthenium redox potentials.

Given the magnitude of the differences in the measured rate constants, the assumption that the data are best described by two different fit lines may be questioned. This conclusion, however, is supported by several points. The first is that statistical analysis of the fits to two curves shows that the fit parameters are significantly different. Specifically, the standard deviation in λ is 0.016 and 0.014 eV for the two fitted curves and indicates that the fitted parameters are different at better than a 95% confidence level. The standard deviations in H_{AB} for two fitted curves also indicate that the two sets of data represent independent populations with a confidence level of greater than 95%. Alternatively, the correlation coefficient for a fit to a single curve is only 0.81, whereas it is 0.97 for fits to two curves. Again, this measure of goodness of fit shows that a fit to two separate curves is the better choice. The second and equally persuasive evidence is the trends in the differences in the rate constants obtained at high and low [NaCl]. The differences in the rate constants for corresponding reactions are small at the intersection point but large and of *opposite* sign at the extremes in free energy. For example, the rate constants (k_2) for the thermal reaction of Ru-65-cyt b_5 decrease as the concentration of salt is increased. The excited-state rate constants (k_1) for this derivative change very little over this range. And finally, the excited-state rate constants for bpymRu-65-cyt b_5 *increase with increasing* [NaCl]. In addition, even though Figure 5 shows data taken at only two salt concentrations, the salt dependence of each rate constant shows a smooth change between 0 and 300 mM NaCl, as illustrated in Figures 2, 3, 6 and 7. The individual rate constants have a standard deviation of 5% or less (except for bpymRu-65-cyt b_5, which is larger) and were determined both by sequential addition and from random additions of NaCl.

There are very few reports of the effect of ionic strength on rates of electron-transfer reactions in which the reactants are covalently linked and presumably do not involve diffusion. Elliott and co-workers[26] have investigated the effect of ionic strength on electron transfer between ruthenium complexes and covalently linked diquats. In that study, the authors attributed the effect to changes in the conformation distribution of the covalently linked species which would provide for a different distribution of electronic couplings. Meyer and co-workers[27] have investigated ionic strength effects on the intramolecular electron-transfer reactions of $[Re^I(bpy)(CO)_3(py\text{-}PTZ)](PF_6)$ in the context of energy gap law relationships. In this case, the rate constants increased with increasing ionic strength in a nonlinear manner. This was rationalized in terms of stabilization of the anion–cation pair with increasing ionic content of the solution and the resulting decrease in the energy gap. Lewis and Obeng[28] concluded, from a study of the effect of ionic strength on the intervalence transfer band of a binuclear ruthenium compound in dimethyl sulfoxide and *N*-methylformamide, that the dielectric continuum model

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Table 2. Rate Constants for the Photoinduced Electron-Transfer Reactions of the T65C Cytochrome b_5 Derivatives in 1 mM Sodium Phosphate Buffer (pH 7) and Various Concentrations of Sodium Chloride

[NaCl], mM	Me_5Ru-65-cyt b_5[a] k_1, s^{-1}	Me_5Ru-65-cyt b_5[a] k_2, s^{-1}	Ru-65-cyt b_5[a] k_1, s^{-1}	Ru-65-cyt b_5[a] k_2, s^{-1}	bpymRu-65-cyt b_5[b] k_1, s^{-1}	bpymRu-65-cyt b_5[b] k_2, s^{-1}
0	1.90×10^7	1.10×10^7	1.22×10^7	8.5×10^6	1.0×10^6	3.0×10^6
300	1.58×10^7	6.0×10^6	1.30×10^7	5.0×10^6	2.5×10^6	1.5×10^6
0 + cyt *c*	1.60×10^7	5.8×10^6	1.30×10^7	4.8×10^6		

[a] Standard deviation of rate constants is 5% or less. [b] Standard deviation of rate constants is 20%.

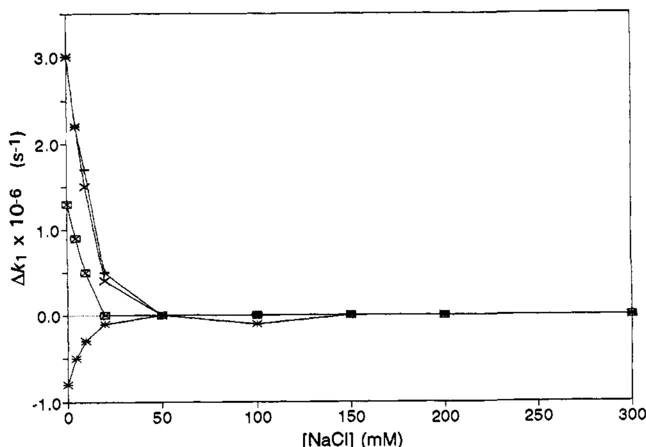

Figure 6. Difference in rate constant k_1: Me_5Ru-cyt b_5 alone minus Me_5Ru-cyt b_5 with horse cyt *c* ( ), Me_5Ru-cyt b_5 alone minus reduced yeast cyt *c* (+), Me_5Ru-cyt b_5 alone minus oxidized yeast cyt *c* (×), and Ru-cyt b_5 alone minus Ru-cyt b_5 with oxidized yeast cyt *c* (✱).

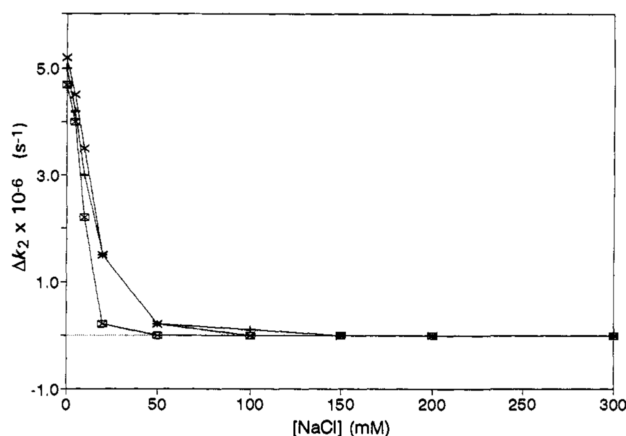

Figure 7. Difference in rate constant k_2: Me_5Ru-cyt b_5 alone minus Me_5Ru-cyt b_5 with horse cyt *c* ( ), Me_5Ru-cyt b_5 alone minus reduced yeast cyt *c* (+), and Me_5Ru-cyt b_5 alone minus oxidized yeast cyt *c* (×).

was inappropriate since it did not account for ion-pairing. Piotrowiak and Miller[29] have also investigated the effects of counterions in electron-transfer reactions. All of these studies were carried out in nonaqueous solvents in which ion-pairing is more pronounced at low ion concentrations than in water.

In the present system we have been able to measure the rates of a series of similar reactions which span a range of free energies comparable in size to the reorganization barrier. Consequently, we have also been able to show that changes in both the electronic and nuclear terms contribute to the differences in rate constants obtained at different ionic strengths. The basis for the effects, however, are not clear. Ruthenium-labeled T65C cytochrome b_5 has a direct 12 covalent bond pathway between the axial histidine of the heme iron and the attached bipyridine ligand, which should remain constant regardless of ionic strength. NMR studies of cytochrome b_5 in 0.2 M deuterated phosphate buffer[10b] and in ^2H_2O[10a] are essentially the same, indicating that there are no major structural changes in cytochrome b_5 with salt. The absence of changes in the electronic properties of $Ru(bpy)_3^{2+}$ with various salt concentrations suggests that there are no significant structural changes in the ruthenium complexes.[24] Since there are only very minor structural changes in either the ruthenium complexes[24] or cytochrome b_5,[10a,b] any contributions to the inner-sphere components of the reorganizational energy are expected to be small and any changes brought about by the addition of NaCl should be insignificant. The small difference in electronic coupling indicated by the data shown in Figure 5 is consistent with this view. If a simple exponential distance dependence model (β = 1.0 $Å^{-1}$) is used, the difference in electronic coupling obtained at 0 and 300 mM NaCl corresponds to a calculated distance change of 0.4 Å. This calculation is at best an indicator of the maximum distance change since it is very probable that the difference in H_{AB} is, in part, a result of small changes in donor and acceptor energy levels since the redox potential of the heme does change with ionic strength.

It is known that solvent reorganization makes a major contribution to the free energy of activation in the system under investigation. It is therefore reasonable, given the electrostatic nature of this aspect of the reaction energetics, to assume that ionic strength should play a role in determining the solvent barrier. In the simplest model,[6] in which the solvent is treated as a dielectric continuum with no contribution from ionic strength, λ_{outer} is described by eq 2.

$$\lambda_o = (\Delta e)^2\left[\frac{1}{2a_1} + \frac{1}{2a_2} - \frac{1}{r}\right]\left[\frac{1}{D_{op}} - \frac{1}{D_s}\right] \tag{2}$$

If we assume that the radii of the donor and acceptor (a_1 and a_2) and the distance between donor and acceptor (r) remain constant at 0 and 300 mM NaCl, then changes in λ_o should be due to changes in D_{op} and D_s, which are the optical and static dielectric constants. Neither the bulk solution refractive index nor the static dielectric constants in 0 and 300 mM NaCl[30] change sufficiently to account for the observed shift in λ. Clearly, the model is too simple to provide any microscopic insight into the source of the ionic strength dependence. Many authors[31] have suggested a more accurate description of proteins in solution would account for the interaction between the surface charges, ions, and solvent.

Qualitatively, one might envision the charged surface of cytochrome b_5 as an electrode because of the high density of charged residues surrounding the exposed heme edge. According to the Gouy and Chapman diffuse layer theory,[32] at a low ionic strength of 1 mM the effect of the charged surface extends out approximately 100 Å and this effect rapidly diminishes with increasing ionic strength. The effect of the charged surface may manifest itself in a variety of forms. One manifestation may be dielectric saturation of some fraction of the solvent near the

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charged surface which would directly impact on the solvent reorganizational energy. The extent would be influenced by the ionic strength. We intend to focus further attention on this aspect of the reaction and look for specific ion effects indicative of ion-pairing, as suggested by Meyer and co-workers,[27] Lewis and Obeng,[28] and Piotrowiak and Miller.[29]

Cytochrome b_5 with Cytochrome *c*. The effect of binding cytochrome *c* on the rates of electron transfer between the ruthenium complexes and the heme of cytochrome b_5 is clearly evident in Figures 2 and 3. This is further illustrated by the plots in Figures 6 and 7, in which the differences between the rate constants obtained without cytochrome *c* and those with cytochrome *c* present are plotted. As noted earlier, the extent of binding is expected to decrease with added NaCl until effectively no protein:protein complexes are formed and the difference in the observed rate constants falls to 0. Horse heart ferricytochrome *c*, yeast ferricytochrome *c*, and yeast ferrocytochrome *c* were examined. Consistent with previous investigations,[16,17] the data indicate that horse heart cytochrome *c* binds less strongly than yeast cytochrome *c*. Little difference is observed between oxidized and reduced forms of yeast cytochrome *c*, which indicates that the binding for both forms is similar and that electron transfer from the cytochrome b_5 heme to the cytochrome *c* heme is not competitive with the thermal back-reaction to the ruthenium complex.

Surprisingly, the rate constants obtained under conditions of low [NaCl] at which cytochrome *c* is expected to bind to cytochrome b_5 are the same as those obtained without cytochrome *c* present at high salt concentrations. Since the measured rate constants with cytochrome *c* bound to cytochrome b_5 are essentially identical to those measured at high salt concentrations in the absence of cytochrome *c*, the 300 mM NaCl data shown in Figure 5 are indicative of the reorganizational energy and electronic coupling found when cytochrome *c* is bound. The redox potential of cytochrome b_5 with bound cytochrome *c* has been determined to be approximately +10 mV vs NHE,[33] which is the same as the redox potential for cytochrome b_5 at high ionic strength. As previously indicated, the difference in electronic coupling suggested by the data shown in Figure 5 is small. This is consistent with the expectation that electronic coupling is predominantly governed by the 12 covalent bond link between the ruthenium complex and the heme, which should be unaffected by the binding of cytochrome *c*.

A small but measurable change in the reorganizational energy is observed upon binding cytochrome *c*. Is this small change consistent with the idea of reducing the solvent reorganization energy by the exclusion of water by bound cytochrome *c*? We have attempted to address this point at least to the level of a correct order of magnitude by the following simple approximation.

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If eq 2 is simplified to $\lambda_o = S[1/D_{op} - 1/D_s]$, where the variable S is a constant which contains the geometric information of eq 2, and if we assume that the measured reorganizational energy contains only a 0.1-eV contribution from inner contributions, then a 0.4–0.5-eV reduction in reorganizational energy is expected if the dielectric of the entire surrounding medium is reduced to a value of 4.[31b] In other words, the entire solvation shell of water is replaced by something with a dielectric constant similar to the interior of a protein. The observed change in reorganizational energy of 0.09 eV, upon binding cytochrome *c*, is about 25% of this value. At this crude level of approximation, a 25% decrease is consistent with a large reduction in the dielectric constant of the fraction of the medium surrounding the heme of cytochrome b_5 which is covered when cytochrome *c* binds. The effect of changing the dielectric constant on the reorganizational energy is analogous to that reported in the classic papers by Closs and Miller.[34] In these calculations we have purposely ignored the details of partitioning the reorganizational energetics into that controlled by low dielectric protein medium surrounding the heme cavity and that influenced directly by the solvent since the partitioning is meaningless at this level of approximation. The problem of a heterogeneous dielectric environment has been very recently addressed by Furuki et al.[35]

In conclusion, we suggest that the changes in the rates of electron transfer observed when cytochrome b_5 is bound to cytochrome *c* or when cytochrome b_5 is placed in solutions of different ionic strengths can be rationalized in terms of solvent reorganization. In one case the dielectric of the medium is lowered compared to that of water by the binding of cytochrome *c* and exclusion of water from the binding domain. In the other case the barrier is lowered by the influence of sodium and chloride ions on the solvent in close proximity to the redox centers. It would appear that these effects are dominant at the exposed heme of cytochrome b_5 since the effects observed with added salt are the same as those observed with binding of cytochrome *c*, which is some distance away from the ruthenium label. Further studies aimed at confirming this suggestion are currently under way.

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Supplementary Material Available: Tables III and IV, consisting of rate constants for ruthenium-labeled cytochromes b_5 without and with cytochromes *c* present, respectively (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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