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Intramolecular electron transfer between Ru(I) and Ru(III) and the heme iron of cytochrome *c* labeled with ruthenium(II) polypyridine complexes

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Abstract

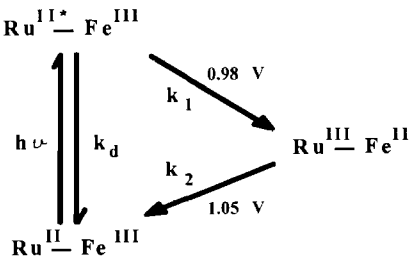
The rates of intramolecular electron transfer between Ru(III) and Fe(II) of three derivatives of cytochrome *c* labeled at lysines 86, 8 and 7 with Ru(4,4′-dicarboxybipyridine)(bipyridine)$_2^{2+}$ have been measured. The respective rate constants are 1.1×10^5, 1.3×10^5 and 6×10^5 s^{-1}. Corresponding rates of electron transfer between Ru(I) and Fe(III) of these derivatives have also been measured. The respective rate constants are 3.3×10^5, 5.7×10^5 and 1×10^6 s^{-1}. In addition the rate constant for a derivative labeled at lysine 87 was determined to be 2.7×10^5 s^{-1}. The two sets of rate constants appeared to be correlated with each other with respect to the location of the ruthenium label. The Ru(I) rate constants are 2–3 times larger than the corresponding Ru(III) rate constants. Preliminary analysis suggests that the observed rate constants are not consistent with the large free energy of reaction expected for Ru(I).

Keywords: Kinetics and mechanism; Intramolecular electron transfer; Cytochrome *c*; Ruthenium complexes; Polypyridine complexes

1. Introduction

Over the past decade considerable attention has been focused on electron-transfer reactions that involve biologically relevant proteins [1–5]. Answers to questions such as how is electron transfer coupled through a protein medium, how does driving force affect the reaction, what factors influence the activation energetics and what features about protein/protein interactions are rate determining have been the goal of this attention [6,7]. Several different strategies have been devised to investigate these questions. Prominent among these strategies is the use of metalloproteins labeled at well defined locations with small metal complexes. Gray and co-workers [8] and Isied et al. [9] pioneered this strategy using ruthenium moieties such as $Ru(NH_3)_5^{2+}$ and $Ru(NH_3)_4L^{2+}$.

More recently we introduced a new series of labels based on Ru(bipyridine)$_3^{2+}$ (Ru(bpy)$_3^{2+}$) which makes use of the well known redox properties of the excited state of ruthenium(II) polypyridine complexes [10–12]. When metalloproteins such as cytochrome *c* or cytochrome b_5 labeled with ruthenium are subjected to a short laser pulse of suitable wavelength the excited state is produced which can be oxidatively quenched by the Fe(III) of the heme center. The quenching is followed by a rapid thermal back reaction which returns the system to its original oxidation states. Thus the rate of electron transfer to and from the heme center can be monitored as shown in Scheme 1. The redox potentials shown in Schemes 1–3 are for the reaction between cytochrome *c* and Ru(4,4′-dicarboxybipyridine)(bipyridine)$_2$ covalently bound to a single surface lysine [11]. In a recent paper [12] we demonstrated the flexibility of this approach by labeling cytochrome b_5 with a series of ruthenium complexes with redox potentials which spanned the expected reorganization energy for electron transfer between the ruthenium complex and the iron heme center. In this way we were



Scheme 1.

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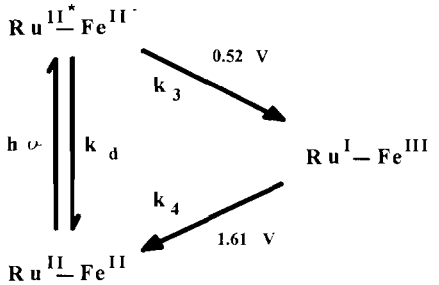
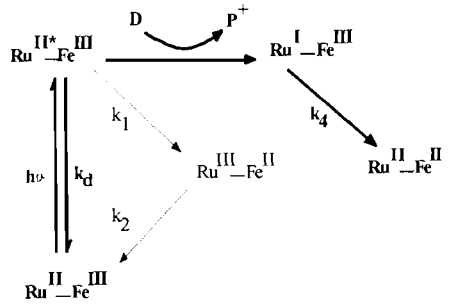
able to show that the rate constants obtained for electron transfer obey the free energy dependence originally predicted by Marcus [13].

This basic series of photoredox reactions also provides a means of measuring the rates of electron transfer between proteins. For these investigations, several reagents have been found which effectively stop the thermal back reaction and provide for the permanent and extremely rapid production of reduced heme. If a suitable metalloprotein is present in solution and this metalloprotein electrostatically binds to the labeled protein then electron transfer between the two proteins can be examined. The approach has been successfully applied to the study of electron transfer between cytochrome *c* and plastocyanin [14], cytochrome *c* peroxidase [15], cytochrome *c* oxidase [16] and cytochrome b_5 [17].

In this paper we will describe some intramolecular electron-transfer reactions between cytochrome *c* and various oxidation states of ruthenium bipyridine complexes covalently bound to surface lysines. Particular emphasis will be placed on the reactions which result from the generation of Ru(I). Two routes are available for the generation of Ru(I). One is shown in Scheme 2 and is analogous to the oxidative path shown in Scheme 1. This route has not yet proven to be experimentally viable. The other is summarized by the reaction sequence in Scheme 3 and appears to work very well. For comparison purposes, we will also report some rate constants for the electron-transfer reactions shown in Scheme 1. These were previously not reported [11] and involve derivatives in which the ruthenium



Scheme 2.



Scheme 3.

complex is in the order of 20 Å away from the porphyrin ring and only very weakly coupled to the iron.

This chemical system is the first in which Ru(I) has been generated in a labeled protein and should provide some unique opportunities for further studies. For example, systematic investigations of the reactions of Ru(I) polypyridine complexes are scarce, unlike those involving Ru(III), and some of the fundamental kinetic parameters such as the reorganization energy are poorly known. It will be shown that the Ru(I) is well behaved on the time scale of the experiments and should be amenable to in-depth studies comparable to those previously reported with Ru(III). In addition, reductive quenching of the ruthenium excited state may provide a means of generating Fe(III) metal centers in metalloproteins. By developing a reaction sequence for the rapid generation of Fe(III) analogous to that described above, electron-transfer reactions between proteins which involve reduction of cytochrome *c* can be investigated. At the present time only proteins which can oxidize ferrocytochrome *c* or b_5 have been suitable for study.

2. Experimental

2.1. Materials

Pure, well characterized samples of cytochrome *c* singly labeled with Ru(4,4′-dicarboxybipyridine)-(bipyridine)$_2^{2+}$ at lysines 86, 87, 8, 7 were available from previous work. Ruthenium labeled lysozyme was also available from previous studies. The preparation of these derivatives has been described by Pan and coworkers [11]. In all cases, similarly labeled samples were chosen from different batches prepared by different workers to insure that the results were representative of the indicated labeled proteins. 3-Dimethylaminobenzoic acid (DMAB), horse heart cytochrome *c* (type VI) and chicken egg white lysozyme were obtained from Sigma Chemical Co. *Para*-anisidine (PAD) was obtained from Aldrich Chemical and used without further purification.

2.2. Flash photolysis

The rate constants for electron transfer were determined from transient absorption measurements obtained by laser flash photolysis. The equipment has been previously described [11]. Briefly, either the third harmonic of Nd:YAG or a flash lamp pumped dye laser (at 450 nm) was used as an excitation source with a pulsed Xe arc lamp as the probe source. In some experiments a tungsten lamp source with a photodiode detector was used. In all others PMT circuitry was employed. Transients were recorded at a variety of

wavelengths. The appearance of Fe(II) cytochrome *c* was determined at 550 and 420 nm and that of Ru(I) at 504 and 556.5 nm (isosbestic points for changes in redox state of cytochrome *c*). The Ru(II) excited state was monitored by the magnitude of bleaching at 434 nm (isosbestic point for changes in redox state of cytochrome *c*).

Flash photolysis solutions contained 0.1 M phosphate buffer at pH 7, 5–20 μM derivatized proteins and 5–20 mM quencher (when used). Concentrated stock solutions of the quenchers were prepared daily. The 300–400 μl samples were held in glass semimicrocuvettes. In all experiments involving Ru(I) the cuvettes were sealed with septum caps and purged with nitrogen. No transients were detected in the presence of dissolved oxygen. In experiments involving Ru(III) dissolved oxygen had no effect on the measured rates.

2.3. Kinetic data analysis

Transients were recorded on a LeCroy 7200 digital oscilloscope interfaced to a personal computer. Data sets contained 2000 data points and were averages of 1 to 20 transients. The kinetic equations which describe Scheme 1 have been reported in detail previously [11]. Under the conditions where $k_1 + k_d \gg k_2$, the absorbance due to the Fe(II) intermediate will decay exponentially with a rate described by k_2. The transient absorbance due to the Ru(I) intermediate in Scheme 3 likewise will decay exponentially with the first-order rate constant k_4. Rate constants were obtained using single and bi-exponential fitting procedures in the program KINFIT distributed by OLIS (On-Line Instrument Service, Jefferson, GA). The amounts of Ru(I) and Fe(II) generated were calculated using extinction coefficients of 12 and 18.7 mM^{-1}, respectively [18,19].

3. Results

The transient absorbance recorded at 550 nm following laser excitation of cytochrome *c* labeled at Lys-86 is illustrated in Fig. 1. The exponential decay in absorbance shown in Fig. 1 corresponds to the back electron transfer reaction characterized by k_2 in Scheme 1. The rate constants for this reaction and that with other cytochrome *c* derivatives are summarized in Table 1. The initial bleaching is caused by a combination of scattered light, emission and the limited response time of the instrument. For this experiment, the detector response was equivalent to a first-order rate constant of 1×10^6 s^{-1} and was chosen to optimize the signal to noise ratio at a response time appropriate for the reactions under investigation. Several control experiments were done to insure that the observed transients were not artifacts of the system. For example, cytochrome *c* derivatives such as Ru-72–cyt *c* show only a small amount of initial bleaching and no transient absorbance under these conditions [11] since the majority of the emission is quenched by internal electron transfer and the lifetime of the transient absorbing intermediate (ferrocytochrome *c*) is only a few hundred nanoseconds. No entry is given for Ru-87–cyt *c* in Table 1 because the electron-transfer reaction appears to be complicated by an additional reaction which does not return the system to the initial redox states within the time frame of the experiment. Apparently, the reaction involves oxidation of an amino acid side chain by Ru(III) which inhibits the thermal back reaction.

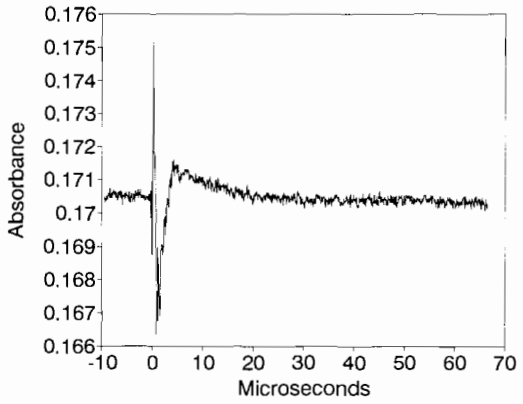


Fig. 1. Transient absorbance recorded at 550 nm following excitation of a 16 μM solution of Ru-86–cyt *c* in 100 mM phosphate buffer, pH = 7, at 22 °C.

Table 1
First-order rate constants[a] for electron transfer from Ru(I) to Fe(III) and from Fe(II) to Ru(III) as a function of label location

Lysine	k_2 Fe(II) to Ru(III) (s^{-1})	k_4 Ru(I) to Fe(III) (s^{-1})	Fe–Ru separation distance[b] (Å)
7	6×10^5	1.2×10^6	9–16
8	1.3×10^5	5.7×10^5	14–19
86	1.1×10^5	3.3×10^5	9–20
87	n.a.	2.7×10^5	10–22

[a]Rate constants were obtained at 22 °C and in solutions containing 100 mM phosphate buffer at pH 7 with estimates of error of ±10%.
[b]Edge to edge distance as described in Ref. [11].

The thermal reactions involving Ru(I) described by k_4 were investigated using the reactions shown in Scheme 3. Our initial investigations were primarily a series of screening studies aimed at selecting reagents which were suitable quenchers. Of the numerous reagents tested two were used in the present study; dimethylaminobenzoic acid (DMAB) and *p*-anisidine (PAD). Stern–Volmer plots of relative emission intensity versus quencher concentration for these two reagents are illustrated in Fig. 2. The plots indicate that both PAD and DMAB quench the ruthenium complex excited

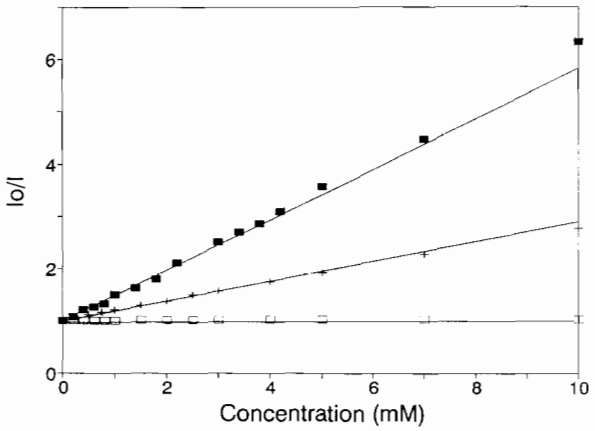


Fig. 2. Stern–Volmer plot of the quenching of emission from ruthenium labeled cytochrome *c*. The solid boxes indicate quenching by *p*-anisidine, the open boxes by aniline and the crosses by dimethylaminobenzoic acid. Steady state emission was monitored at 660 nm with excitation at 450 nm. The solutions contained 3 μm protein in 100 mM phosphate buffer, pH = 7 and were deaerated with N_2. The lines are least-squares fits to the data.

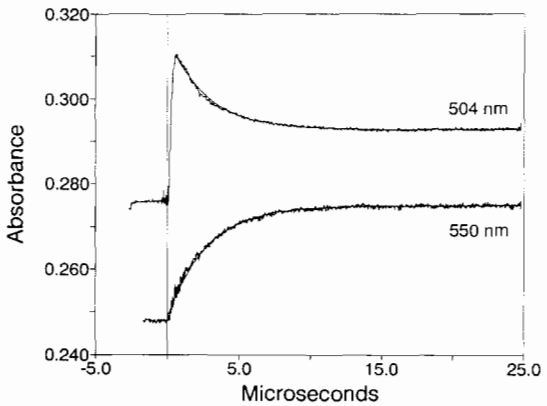


Fig. 3. Transient absorbance following excitation of a 20 μM solution of Ru-86–cyt *c* in 100 mM phosphate buffer, pH = 7, at 22 °C containing 15 mM DMAB and purged with N_2. The upper trace was recorded at 550 nm. The lower trace is the difference of data recorded at 550 nm minus data recorded at 556.5 nm.

state with rates which are potentially competitive with the rate of intramolecular electron transfer (k_1). For example, from the slope of line shown in Fig. 2 (0.19 mM^{-1}) and the emission lifetime in the absence of quencher (300 ns), we find that DMAB quenches Ru-86–cyt *c* with a rate constant of approximately 6×10^8 $s^{-1}\,M^{-1}$. This corresponds to a quenching rate constant of $6 \times 10^6\ s^{-1}$ with [DMAB] = 10 mM, i.e. approximately an order of magnitude greater than k_1. As a control, Fig. 2 also shows data obtained with aniline. Aniline is a poor quencher as expected on the basis of its redox potential. Although this discussion focuses on the Ru-86–cyt *c* derivative only minor differences in quenching are seen within this set of derivatives.

Fig. 3 shows the transient absorbance recorded at 504 nm and the transient obtained by subtracting data obtained at 556.5 nm from that at 550 nm for Ru-86–cyt *c* in the presence of 15 mM DMAB. 504 nm is an isosbestic point for cytochrome *c* and near a maximum in the Ru(I) spectrum. Subtraction of 556.5 nm data from the 550 nm data provides a means of removing the broad band Ru(I) contribution from the transient absorbance observed at 550 nm (the maximum in the cytochrome *c* α band). 556.5 nm is an isosbestic point for cytochrome *c*. The transient absorbance changes at 504 nm show a rapid growth corresponding to the generation of the Ru(I)–Fe(III) intermediate (limited by instrument response). The increase in absorbance is followed by a slow exponential decay corresponding to the depletion of Ru(I), i.e. k_4. The 550–556.5 nm transient absorbance change corresponds to the appearance of Fe(II) cytochrome *c*. The rates of corresponding processes at the two wavelengths are best fit by the same rate constant and are independent of the nature and concentration of the quencher over the range of 5–20 mM. The rate constants were also independent of the protein concentration over the range 5–20 μM. In addition, the magnitude of the transients measured at 504 and 550 nm indicate that the loss of Ru(I) corresponds to an equivalent molar production of Fe(II).

The reader will note that the transient at 504 nm fails to return completely to the preflash baseline over the time period shown. After several milliseconds, however, the system does return to the preflash baseline. Repeated excitation up to a few hundred times produces no detectable permanent change. The additional absorbance is due to quencher radical cations or products derived from that species. We have verified this claim by generating the cation radical of DMAB by the reaction of Ru(III) produced by quenching with peroxydisulfate. In this reaction all of the intermediate products produced do not absorb in the spectral range of 500–560 nm except those derived from DMAB. In these experiments unbound $Ru(bpy)_3^{2+}$ was used but all other conditions were the same. Further evidence for this claim can be found in a comparison with data obtained with the quencher PAD. When used with Ru-86–cyt *c* an absorbing intermediate is still observed but the magnitude is reduced and the decay is substantially faster.

Since it has been reported that Ru(I) complexes of bipyridine are unstable in aqueous solution we also have examined the behavior of the Ru(I)–lysozyme. Lysozyme contains no metal center and presumably no other functional groups which will react with Ru(I). The labeled protein contains a single equivalent of ruthenium complex covalently linked to an unspecified lysine. Fig. 4 shows the transient absorbance obtained at 505 nm. The transient shows the rapid production and slow decay of Ru(I) as expected. In this case the Ru(I) intermediate presumably decays by a second-

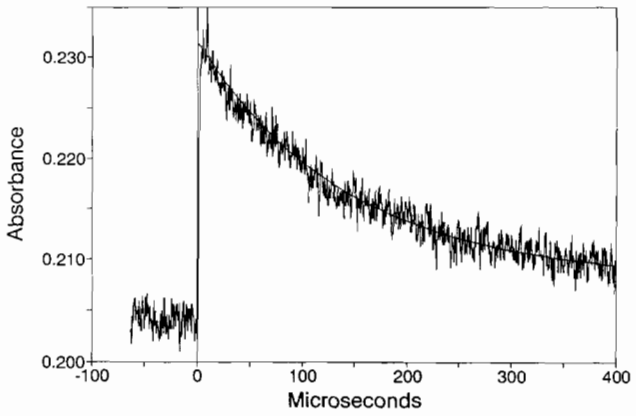


Fig. 4. Transient absorbance following excitation of a solution containing 20 μM Ru–lysozyme with 20 mM DMAB in 100 mM phosphate buffer and deaerated with N_2. The trace was recorded at 505 nm.

order back reaction with the quencher oxidation products. Under the conditions of these experiments, which also correspond to those used with cytochrome *c*, the half-life of the Ru(I) intermediate is 1.2×10^{-4} s. This is much longer than the half-lives of Ru(I) intermediates observed in the presence of the iron heme center. The observed half-life is consistent with a near diffusion controlled second-order rate constant commonly observed with aniline based quenchers [20].

4. Discussion

Previously we reported the preparation and characterization of a series of cytochrome *c* derivatives labeled at single surface lysine residues [10]. The series was based on the formation of an amide bond between the carboxylate group of Ru(4,4′-dicarboxybipyridine)(bipyridine)$_2^{2+}$ and the terminal amine of surface lysines. Electron transfer from the ruthenium complex to the heme iron center can be initiated by photoexcitation of the ruthenium complex as indicated by Scheme 1. In this scheme the Fe(III) of cytochrome *c* functions as an oxidative quencher for the excited state of the ruthenium complex. The rate constant k_d describes all processes other than electron transfer that return the excited state to the ground state. In the four derivatives described in the present work k_d varies over the range of $2–5\times10^6$ s^{-1} and is comparable in magnitude to that measured in ruthenium labeled lysozyme. We have concluded from these measurements that energy transfer is not a significant mode of decay for the ruthenium labeled proteins in the +3 oxidation state [11]. The redox potentials of the species involved provide for an overall free energy of reaction of 0.98 V in the forward direction and 1.05 V versus NHE for the reverse reaction. The rate constants, k_d, k_1 and k_2, for derivatives labeled at lysines 72, 13, 27, 25 and 7 were successfully determined and reported previously [11].

By analogy, a reductive quenching pathway is described in Scheme 2. In this scheme, only the initial redox state of the heme is different. The overall free energy of reaction in the forward direction is 0.52 V and 1.61 V versus NHE [20] for the reverse direction. Numerous previous attempts in our laboratory have been unsuccessful in demonstrating the proposed reaction scheme with these derivatives. In the past we have attributed this difficulty to the relatively small driving force and corresponding small rate constant for the forward reaction. In order to produce a detectable amount of the Ru(I) intermediate, the forward reaction must be competitive with the natural decay rate (k_d) and the rate of the thermal back reaction (k_4). If either k_d or k_4 is significantly larger (i.e. more than five-fold) than k_3, then the signal amplitude will be very small. We assumed that energy transfer to Fe(II) heme was not important in Scheme 2. This assumption is based only on the fact that the oxidative scheme did not suffer from this problem and ignores the fact that Fe(II) heme may have much more energetically favorable receptor states [21].

The cyclic Schemes 1 and 2 can be elaborated by the addition of external quenchers and scavengers. For example, addition of an efficient reductive quencher for Ru(II)* is shown in Scheme 3. This scheme provides a means of measuring k_4, the rate constant for the reaction of Ru(I) with Fe(III). In this reaction sequence the initial redox state of the protein is Fe(III) which is much easier to maintain experimentally than the reduced state. There are some restrictions which must be considered in the application of this reaction scheme. Obviously the quencher must be compatible with Fe(III) cytochrome *c*. In other words, it must have a reduction potential more positive than +0.26 V versus NHE. It must also be sufficiently reactive to compete with k_1 and k_d; the direct intramolecular quenching and the natural decay paths, respectively. In the present study this last criterion was greatly relaxed by focusing on those derivatives in which the label is far removed from the heme. A search of over 30 different candidates provided two quenchers which met all of the requirements and proved successful experimentally. These were dimethylaminobenzoic acid and *p*-anisidine. Using these quenchers we were able to determine the rate constants for electron transfer from Ru(I) to Fe(III) in four different derivatives. As expected the rate constants summarized in Table 1 were independent of the choice of quencher.

Table 1 also contains rate constants for electron transfer from Fe(II) to Ru(III) for the same series of cytochrome *c* derivatives. These rate constants were not available from previous studies since the flash photolysis equipment used earlier was unable to resolve

the very small signals. These rate constants are of interest as a comparison to the Ru(I) rate constants and in the context of the previously measured Ru(III)/Fe(II) rate constants since these greatly extend the range of distance and reactivities in the series.

A pairwise comparison of the k_2 and k_4 values listed in Table 1 shows that, for each derivative, the rate constants for the reaction of Ru(I) with Fe(III) are 2–3 times larger than the rate constants for the reaction of Ru(III) with Fe(II). These results are not consistent with the very high free energy of the Ru(I)/Fe(III) reaction and the expected similarity in electronic coupling between the Ru(I)/Fe(III) and Ru(III)/Fe(II) reactions. A more quantitative view of the problem can be obtained by an examination of the free energy dependence of the reactions and calculations of some representative electronic coupling parameters. The free energy dependence can be described by Eq. (1), according to the semiclassical treatment of electron transfer developed by Marcus [13].

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

In this equation, λ is the reorganization energy, ΔG is the overall free energy of reaction and H_{AB} is a measure of the electronic coupling between the redox centers. The equation predicts that the rate constant will increase with the increasing free energy of reaction up to a maximum where $\lambda = -\Delta G^{\circ\prime}$. Further increases in the free energy will retard the rate of reaction. The predicted free energy dependence has been experimentally verified by Closs and Miller [22] and recently by Scott et al. [12] for intramolecular electron transfer in cytochrome b_5 labeled with a series of ruthenium polypyridine complexes.

The magnitude of the rate constants are determined by the free energy, the reorganizational energy and the electronic coupling element, H_{AB}. The free energies for the reactions are shown in Schemes 1 and 2. The reorganization energies for the two reactions reported in Table 1 are probably in the range of 0.7 to 0.9 eV, based on previous studies of cytochrome *c*, Ru(III) and Ru(I) [7,23,24]. Using a value of 0.8 eV for the reorganizational energy, the rate constants given in Table 1, and Eq. (1) we obtain 1.3, 1.5, 2.4 and 2.9 cm^{-1} for H_{AB} for the reactions of Ru(I) with Fe(III) in Ru-87–cyt *c*, Ru-86–cyt *c*, Ru-8–cyt *c* and Ru-7–cyt *c*, respectively. These values are significantly higher than any previously reported value for intermolecular electron transfer in labeled proteins and are not consistent with the expected coupling between Ru(I) and Fe(III) nor with that between Ru(III) and Fe(II). In fact, an electronic coupling element of this magnitude would indicate that the redox centers are coupled by an equivalent of 6–8 covalent bonds [7,25]. For comparison, $H_{AB} = 0.25$ cm^{-1} for the electron transfer between the heme iron of cytochrome b_5 and ruthenium complexes bound to Cys-65 in which the redox centers are coupled by 12 covalent bonds [12]. In the four derivatives listed in Table 1, the minimum separation distances are 9–14 Å and there are no short covalent coupling paths between the redox centers which could provide for strong electronic coupling. If that were the case then this coupling path would also be available for the reaction of Ru(III) with Fe(II). A similar calculation for the reaction of Ru(III) with Fe(II) in Ru-86–cyt *c* yields a value of 0.03 cm^{-1} for H_{AB} which is more consistent with expectations. For example, Meade et al. [25] reported that $H_{AB} = 0.03$ cm^{-1} for electron transfer between Fe(III) of cytochrome *c* and Ru(II)$(NH_3)_5$ bound to His-33. In this derivative (Rua_5(His 33)–cyt *c*), the separation distance between the redox centers is also about 12 Å and the electronic coupling is weak due to the lack of a direct covalent bonded pathway between the centers. Wuttke et al. [26] have estimated that the electronic coupling in Rua_5(His 33)–cyt *c* is equivalent to a 20 Å separation.

The difficulties presented by the reactions of Ru(I) may stem from the fact that the reactions do not show the inverted behavior predicted by Eq. (1). The extremely large redox potentials calculated for the reactions of Ru(I) should place the reactions well into the inverted region and should result in reactions which are slower that those of Ru(III). The fact that the reactions of Ru(I) are faster than predicted may indicate that the electron-transfer reactions of Ru(I) have sufficient energy to involve an excited state of cytochrome *c* [21]. If this were the case then the driving force for the reaction would be significantly lower than 1.6 V. Clearly additional data will be required to define the free energy dependence before further insights can be gained. These experiments are currently in progress.

In conclusion, we have described a means of photochemically inducing electron transfer from a Ru(I) complex covalently linked to the surface to the iron heme center of cytochrome *c*. The electron-transfer reactions are well behaved in terms of a lack of complicating side reactions but have larger rate constants than expected based on reasonable assessments of the reorganizational energies and magnitudes of electronic coupling.

Acknowledgements

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