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Electron Transfer from Cytochrome b_5 to Cytochrome *c*

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The reaction of cytochrome b_5 with cytochrome *c* has become a very prominent system for investigating fundamental questions regarding interprotein electron transfer. One of the first computer modeling studies of electron transfer and protein/protein interaction was reported using this system. Subsequently, numerous studies focused on the experimental determination of the features which control protein/protein interactions. Kinetic measurements of the intracomplex electron transfer reaction have only appeared in the last 10 years. The current review will provide a summary of the kinetic measurements and a critical assessment of the interpretation of these experiments.

KEY WORDS: Cytochrome b_5; cytochrome *c;* electron transfer; kinetics; ruthenium.

INTRODUCTION

The reaction of cytochrome b_5 with cytochrome *c* has become an important system for investigating fundamental questions regarding interprotein electron transfer. The prominence of the system in this area is a result of the fact that both proteins are relatively robust and both have been structurally characterized in the reduced and oxidized forms. Salemme (1976) and Wendoloski *et al.* (1987) reported the first computer modeling studies of electron transfer and protein/protein interaction using this system. Northrup *et al.* (1993) have very recently reported an extensive modeling study using Brownian dynamics calculations. Several investigations of the kinetics of electron transfer have been reported as well as extensive experimental studies of the cytochrome b_5/*c* interprotein interactions (see Mauk *et al.* in this volume).

Liver microsomal cytochrome b_5 is a small membrane-bound protein that is involved in NADH-dependent fatty acid desaturation and many cytochrome P-450 catalyzed reactions. It consists of two domains, a hydrophilic domain of 98 amino acids which contains the heme group, and a hydrophobic domain of 36 amino acids which anchors the protein to the membrane of the endoplasmic reticulum (Spatz and Strittmatter, 1971; Ozols and Gerard, 1977). Cytochrome b_5 functions as a mobile carrier on the surface of the membrane, transferring electrons from NADH-cytochrome b_5 reductase to the fatty acid desaturase enzyme (Strittmatter *et al.*, 1974). Its role in the monooxidation of both endogenous and exogenous substrates by cytochromes P-450 is more complex (Holmans *et al.*, 1994). It has been proposed that cytochrome b_5 donates an electron to the substrate-bound oxy form of cytochrome P-450, that it influences the coupling efficiency of electron flow for substrate hydroyxlation, and that it might also cause conformational changes in the structure of cytochrome P-450 that affect catalytic activity (Pompon and Coon, 1984). The mitochondrial outer membrane contains a membrane-bound form of NADH-cytochrome b_5 reductase that is nearly identical to the microsomal enzyme (Borgese and Longhi, 1990). However, outer membrane cytochrome b_5 has only a 58% sequence homology to microsomal cytochrome b_5 and is coded by a separate gene (Lederer *et al.*, 1983). These enzymes are responsible for the rotenone-insensitive NADH

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cytochrome *c* reductase activity of mitochondria. Matlib and O'Brien (1976) have found that cytochrome b_5 is located on the inner surface of the outer membrane, and can transfer an electron to cytochrome *c* present in the intermembrane space at physiological ionic strength.

Mild proteolysis releases the hydrophilic, catalytic domains of both cytochrome b_5 and NADH-cytochrome b_5 reductase from the endoplasmic reticulum membrane (Spatz and Strittmatter, 1973). Erythrocytes contain water-soluble forms of cytochrome b_5 and NADH-cytochrome b_5 reductase that are identical to the hydrophilic domains of the microsomal proteins (Giordano and Steggles, 1993; Shirabe *et al.*, 1989). Cytochrome b_5 transfers an electron from NADH-cytochrome b_5 reductase to methemoglobin to regenerate the functional hemoglobin (Hultquist *et al.*, 1981).

Cytochrome *c* is a globular protein comparable in size to cytochrome b_5. The net charge of cytochrome *c* is + 7 at pH 7 (Moore and Pettigrew, 1990) which contrasts to a charge of −9 for cytochrome b_5 (Eltis *et al.*, 1991). The standard II/III reduction potential of horse heart cytochrome *c* is 0.260 V vs NHE (Moore and Pettigrew, 1990) and that of rat liver microsomal cytochrome b_5 is 0.010 V vs NHE (Reid *et al.*, 1982). Cytochrome *c* is found in the mitochondria where it functions primarily as an electron shuttle between the bc_1 complex and cytochrome *c* oxidase. It is also involved in electron transfer reactions with a number of other metalloproteins, including cytochrome b_5, which are accessible in the intermembrane space of the mitochondria. Structure, function, and properties of cytochrome *c* have been extensively reviewed (Moore and Pettigrew, 1990; Mauk and Scott, 1994; Lehninger, 1964).

In this report we will briefly review some of the attempts to model the electrostatic interaction of cytochrome *c* and cytochrome b_5 (see Mauk *et al.* in this issue for a more detailed review). This will be followed by a review of recent kinetic studies aimed at measuring the rate of electron transfer from cytochrome b_5 to cytochrome *c*. The review will be concluded with a comparison of the kinetic results with the modeling studies.

MODELING STUDIES

The electron transfer reaction between cytochrome *c* and cytochrome b_5 has been described as a model system for the study of protein/protein redox reactions. Both of the reactants are structurally well characterized (Argos and Mathews, 1975; Berghuis and Brayer, 1992) and very amenable to computer and wet laboratory experiments. Salemme (1976) and Wendoloski *et al.* (1987) have done extensive modeling of the protein/protein interaction in the electron-transfer complex. Northrup *et al.* (1993) have also performed Brownian dynamics calculations in conjunction with kinetic measurements obtained with several mutants of yeast iso-1-cytochrome *c* and trypsin-solubilized bovine liver cytochrome b_5. In this later study, the most frequently encountered docking geometries between cytochrome b_5 and yeast iso-1-cytochrome *c* involved interactions Glu48-Arg13, Glu56-Lys87, Asp60-Lys86, and heme-Tml72 (Tml denotes trimethyllysine) with an average electrostatic energy of −13.0 kcal mol^{-1}. A less stable mode of interaction which was the same as that previously suggested by Salemme (1976) was also identified. The electrostatic stabilization energy of this mode was 6.4 kcal mol^{-1} and involved interactions Glu44-Lys27, Glu48-Arg13, Asp60-Tml72, and heme-Lys79. The closest approaches of the two hemes were 12 and 8.4 Å in these two forms, respectively. Figure 1 shows the important electrostatic interactions and the heme orientation in the lowest energy protein complex configuration proposed by Northrup *et al.* (1993) (see contribution from Mauk *et al.* for additional figures).

Many of the salient points of these modeling studies have been investigated by various techniques. For example, chemical modification of lysines 13, 25, 27, or 79 on cytochrome *c* results in a decrease in the rate of reaction with cytochrome b_5 and supports their involvement in binding (Ng *et al.*, 1977; Stonehuerner *et al.*, 1979; Smith *et al.*, 1980). Direct spectroscopic

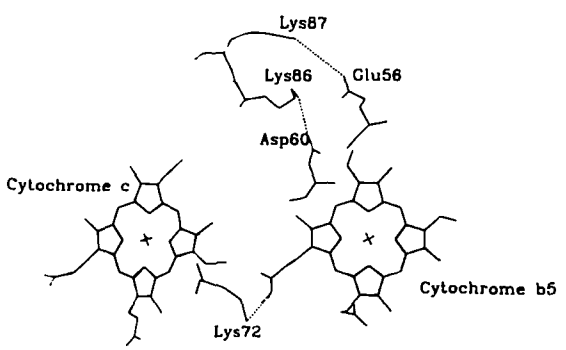


Fig. 1. Partial molecular model of the cytochrome b_5/cytochrome *c* protein complex as calculated by Northrup *et al.* (1993) showing some of the salt bridge forming interactions and the proximity of the two porphyrins.

evidence for the formation of a 1:1 complex at low ionic strength was obtained by Mauk *et al.* (1982). Methylation of the exposed heme propionate group of cytochrome b_5 was found to alter the orientation of the complex (Reid *et al.*, 1984; Mauk and Mauk, 1986; Eltis *et al.*, 1988). Rodgers and Sligar (1991) and Rodgers *et al.* (1988) used site-directed mutagenesis to change specific carboxylate groups to corresponding amides. Decreases in binding strength and specific volume changes associated with these modifications map the interaction domain to that proposed by Salemme. NMR and molecular dynamics studies have indicated that the complex is in dynamic equilibrium between several different conformations (Eley and Moore, 1983; Burch *et al.*, 1990; Whitford *et al.*, 1990; Wendoloski *et al.*, 1987). For a more extensive review of this aspect of protein/protein interaction see the contribution in this issue by Mauk *et al.*

RATE MEASUREMENTS

Several investigators have reported various aspects of the electron transfer reaction between cytochrome *c* and cytochrome b_5. A small number of these have focused on the reaction rates measured under second-order reaction conditions (Strittmatter, 1964; Eltis *et al.*, 1991; Northrup *et al.* 1993; Guillemette *et al.*, 1994). Millett and coworkers (Ng *et al.*, 1977; Stonehuerner *et al.*, 1979) reported steady-state kinetic studies involving chemically modified cytochrome *c*. Recent studies have focused on reactions performed under conditions which provide more information about electron transfer within the electrostatically stabilized 1:1 protein complex (Mauk *et al.*, 1982).

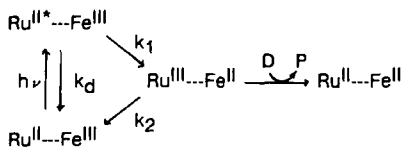
One of the earliest studies of electron transfer in the cytochrome b_5/c complex was reported by McLendon and Miller (1985) using the trypsin-cleaved water-soluble domain of bovine liver microsomal cytochrome b_5. These investigators employed pulse radiolysis to study the reaction. Under low ionic strength conditions the rate constant for electron transfer was reported to be 1600 s^{-1} with horse heart cytochrome *c* and 900 s^{-1} with yeast iso-1-cytochrome *c* (McLendon, 1988). Qin *et al.* (1991) obtained a rate constant of 1700 s^{-1} for the reaction of horse heart cytochrome *c* with synthetic rat liver cytochrome b_5 which contained only the water-soluble domain. In this case the reaction was initiated by flash photolysis of an acridine dye followed by nonselective reduction of cytochromes b_5 and *c*. Qin *et al.* also reported a decline in rate with increased glycerol (i.e., viscosity) and a slight increase in rate when variants specifically modified to alter the electrostatic interaction in the protein complex were used. The later results suggest that the observed rate may be limited by structural changes in the protein complex or complex dissociation rather than electron transfer.

The reaction of cytochrome *c* with synthetic outer membrane cytochrome b_5 has been extensively investigated by Tollin and Hazard using a methodology based on the flash photolysis of flavin derivatives (1991; Meyer *et al.*, 1993). The technique relies on the rapid (i.e., not rate limiting) reduction of cytochrome b_5 by a flavin semiquinone. Flavin semiquinones are nonselective reductants and can react with other reducible species in solution (albeit with different rate constants).

When flash photolysis of a solution containing 5-deazariboflavin was performed under low ionic strength conditions with a 1:1 ratio of cytochromes b_5 and *c*, no transient corresponding to the reduction of cytochrome b_5 was observed (Meyer *et al.*, 1993). Further experimental work indicated that the lack of observed reduction of cytochrome b_5 was a result of very rapid electron transfer from initially reduced cytochrome b_5 to cytochrome *c*. Under these conditions reduction of bound cytochrome b_5 by the semiquinone is not sufficiently rapid to allow resolution of the interprotein electron transfer reaction. In order to monitor the reactions of reduced cytochrome b_5, experiments were performed with 8:1 excess cytochrome b_5. Under these conditions transient absorbance changes were observed which were indicative of electron transfer from free cytochrome b_5 to bound cytochrome *c* in a transient ternary complex. Increasing the ionic strength resulted in an increase in rate up to approximately I = 40 mM after which the rate of reaction rapidly declined. The rate constant for electron transfer within the transient ternary complex was estimated to be greater than 10^4 s^{-1} (with bovine liver cytochrome b_5 which reacts 3 times faster than rat outer membrane cytochrome b_5).

We have also studied the electron transfer reaction in the cytochrome b_5 system extensively (Willie *et al.*, 1992, 1993). Our investigations are built around a new technique for measuring the rates of electron transfer reactions in metalloproteins (Pan *et al.*, 1990). The technique relies on the covalent attachment of derivatives of $Ru(bipyridine)_3^{2+}$ to metalloproteins at locations which do not interfere with protein/protein binding. The method used to covalently attach the ruthenium complex to cytochrome b_5 involves the for-

mation of a thioether link between the sulfur atom of cysteine and 4-bromomethyl-4′-methylbipyridineRu(II)L_2^{2+}. The photochemical properties of the ruthenium complexes used in these studies are very well characterized (Kalyanasundaram, 1992) and provide a means of rapidly generating the reduced state of a metalloprotein.

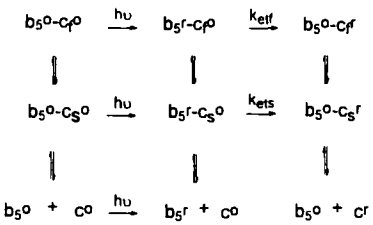


Scheme I.

The photoinitiated reaction sequence is shown in Scheme I. In this reaction sequence, Ru(II)* represents the long-lived excited state of the ruthenium complex (typically 300 nsec), k_1 is the rate constant for oxidative quenching of that state, and k_2 is the rate constant for the ground-state back reaction. The rate constant k_d describes all processes other than electron transfer which return the excited state to the ground state. D is an electron donor such as aniline or EDTA which can react with Ru(III) and essentially eliminates the back reaction, k_2. The measured electron-transfer rate constants k_1 and k_2 span a range from greater than 40 $\times 10^6$ to less than 1×10^5 s^{-1} (Pan *et al.*, 1988; Scott *et al.*, 1993). In the absence of a donor, the reaction sequence is cyclic and no degradation of protein has ever been observed despite extensive recycling. In the presence of a donor, generation of the reduced form of cytochrome b_5 is complete within a few hundred nanoseconds. Any processes slower than the formation of reduced cytochrome b_5 (such as oxidation by cytochrome *c*) can be readily followed using laser flash photolysis techniques.

Transient kinetic behavior observed in the study of electron transfer between horse heart cytochrome *c* and the ruthenium-labeled T65C variant of cytochrome b_5 is shown in Fig. 3 (Willie *et al.*, 1992). This particular derivative was genetically engineered to place the ruthenium complex close to the heme iron but outside of the proposed binding domain as indicated in Fig. 2. The ruthenium complex, in this derivative, is very efficiently coupled to the heme iron through a 12 covalent bond link. Figure 3A shows the transient absorbance changes observed with only the labeled protein. The rise and fall in absorbance correspond to the formation and decay of the reduced heme center with rate constants $k_1 = 12 \times 10^6$ s^{-1} and $k_2 = 8.5 \times 10^6$ s^{-1}. In the presence of aniline but in the absence of cytochrome *c*, about 20% of the ferricytochrome b_5 is reduced to the Fe(II) form which remains constant over a millisecond time frame (Fig. 3B). If cytochrome *c* is also present, the buildup of reduced cytochrome b_5 is followed by a rapid decline which is due to intraprotein electron transfer.

The intracomplex electron-transfer reaction represented by the absorbance decay shown in Fig. 3C is best described by a two-exponential fit containing a fast (4×10^5 s^{-1}) and slow (3.4×10^4 s^{-1}) component. The two rate constants were not a function of the concentration of cytochrome *c* or cytochrome b_5 which were varied independently over the range of 5–30 μM in 1 mM phosphate buffer at pH = 7. The observed rate constants were also independent of the specific donor used to reduce Ru(III). The amplitudes of the two phases, however, were related to the concentration of the protein present in the smaller amount. The results were interpreted as indicating that two forms of cytochrome b_5/*c* complex exist in solution as suggested by Mauk and others (Mauk and Mauk, 1986; Burch *et al.*, 1990; Wendoloski *et al.*, 1987). The fast form reacts very rapidly, indicative of very strong electronic coupling between the two hemes. The slow form may be a weakly coupled form or an inactive form in which electron transfer is limited by conformational change to an active form. The overall reaction is illustrated in Scheme II where the subscripts "f" and "s" signify the respective fast and slow components.



Scheme II.

The rate constants decrease and become monophasic with increasing ionic strength. At sufficiently high ionic strength (>20 mM) the reaction becomes second order, indicative of a weakening of the interprotein electrostatic interactions. The rate of reaction at high ionic strength is slow enough to allow comparison to stopped-flow measurements with native proteins. The ionic strength dependence of the reduction of cytochrome *c* by Ru-65-cyt b_5 is shown in Fig. 4 along with second-order rate data for the native enzymes reported by Eltis *et al.* (1991). The measured rate constants are identical within experimental limits. In further experiments, we have shown that the dissocia-

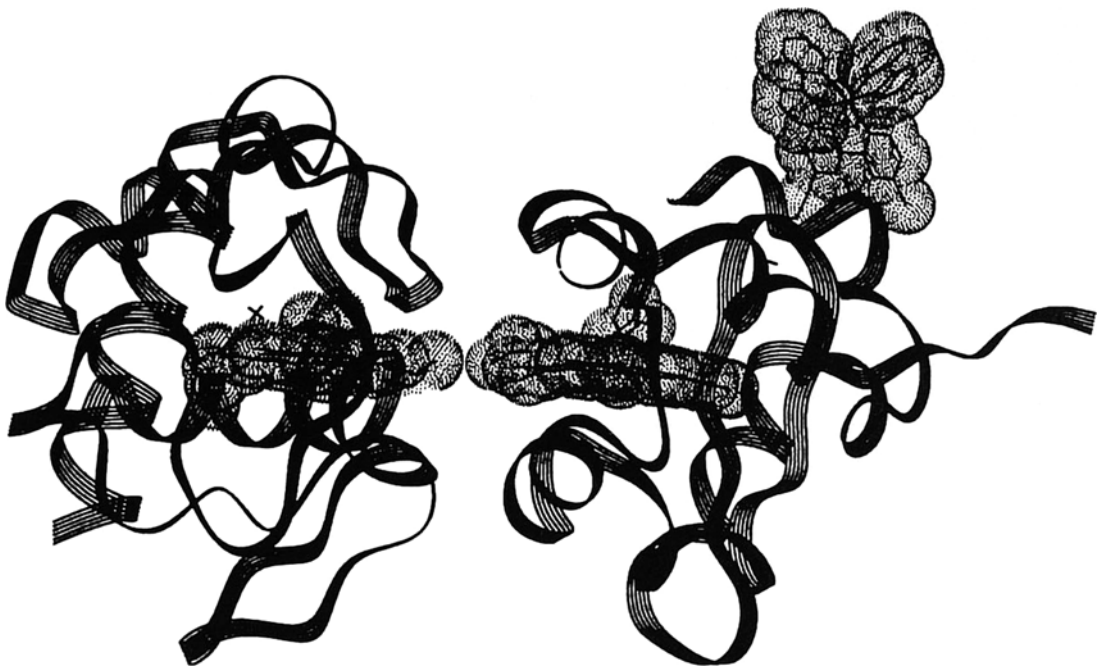


Fig. 2. Molecular model of the interaction of cytochrome c with cytochrome b_5 labeled with 4,4′-dimethylbipyridine(bisbipyridine)ruthenium(II) at Cys65. The protein/protein oreintation is based on the model developed by Salemme (1976).

tion constant is 11 μM at 10 mM ionic strength, which compares very favorably to a value of 12.5 μM reported by Mauk *et al.* (1982) for this ionic strength. Taken together, these data indicate that there are no significant conformational differences between Ru-65-cyt b_5 and native cytochrome b_5 that affect the rates of reaction under second-order conditions at high ionic strengths nor the dissociation constant of the protein complex at low ionic strength.

In an effort to further verify our basic premise that the ruthenium complex does not interfere with protein binding, we carried out kinetic measurements over a wide range of ionic strengths with the labeled T73C variant. The labeling site in this derivative is further removed from the protein/protein binding domain than the T65C derivatives. Figure 5 shows a comparison of the rate constants obtained with the T65C and T73C derivatives as a function of the square root of the ionic strength. The rate constants obtained with both derivatives, including the very large rate constants observed at low ionic strengths, are essentially identical. The overall results show convincingly that the ruthenium complexes do not interfere with protein binding and that the measured rate constants are an accurate indication of the rate of electron transfer between native cytochromes c and b_5 under the conditions used.

Using the methodology described above, we have also tested the idea (Wendoloski *et al.*, 1987; Everest *et al.*, 1991) that the aromatic ring on the invariant residue Phe-82 in cytochrome c acts as an electron-transfer bridge between cytochrome c and cytochrome b_5 (Willie *et al.*, 1993). The idea was tested by examining the electron transfer rate constants for the reaction of Ru-65-cyt b_5 with a number of variants of *Saccharomyces cerevisiae* iso-1-cytochrome c. Variants of iso-1-cytochrome c substituted at Phe-82 with Tyr, Gly, Leu, and Ile were used. A list of the first-order rate constants obtained under low ionic strength conditions and second-order rate constants obtained at high ionic strength is shown in Table I.

While there are numerous small differences noticeable in comparisons of the rate data listed in this table, there is no evidence to support the idea that Phe-82 is needed for rapid electron transfer. For example, comparing the first-order rate constant, k_{etf}, for the reactions involving C102T versus F82G;C102T, we see only a factor of two difference and that difference is in a direction opposite to expectations. The small differences observed are not consistent with the change in electronic coupling expected if a major coupling component were removed. In addition, the second-order rate constants obtained at 106 mM ionic strength for all variants tested were essentially identical.

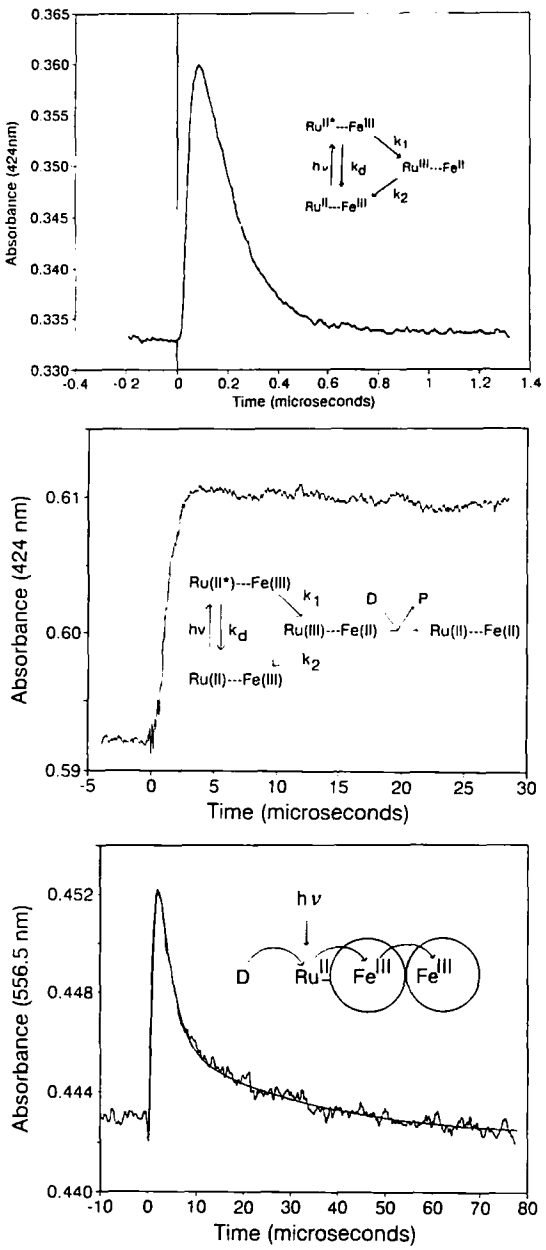


Fig. 3. Transient absorbance changes following laser flash photolysis of (A) solution containing 10 μM Ru-65-cyt b_5 in 1 mM phosphate buffer at pH = 7, (B) solution containing 20 μM Ru-65-cyt b_5 and 10 mM aniline, and (C) solution B containing 26 μM horse heart cytochrome *c*.

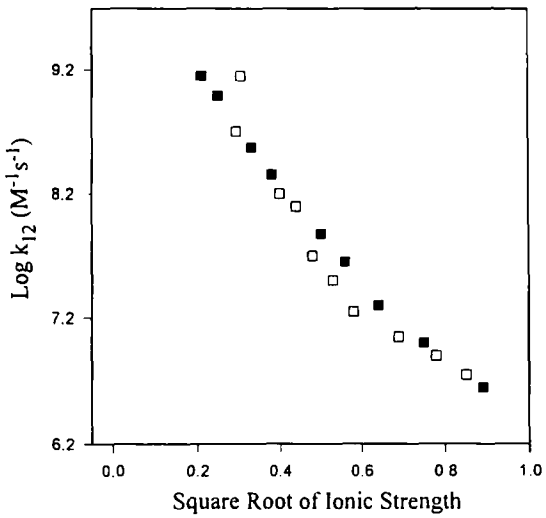


Fig. 4. Comparison of the ionic strength dependence of the second-order rate constants for the reduction of horse heart cytochrome *c* by Ru-65-cyt b_5 (■) and the second-order rate constants for the reduction of cytochrome *c* by native cytochrome b_5 (□) obtained by Eltis *et al.* (1991). The second-order rate constants were determined from dependences of rate on cytochrome *c* concentrations with 5 μM Ru-65-cyt b_5, 10 mM aniline, pH = 7, and sufficient NaCl added to define the ionic strength.

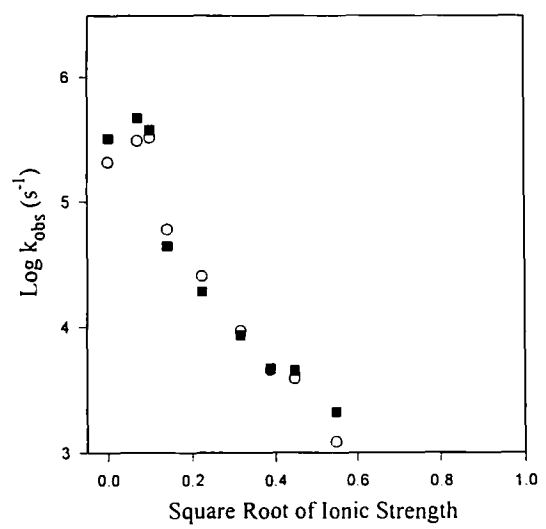


Fig. 5. Plot of logarithm of the rate constant k_{etf} versus ionic strength for the reaction of 9.4 μM Ru-65-cyt b_5 (■) or Ru-73-cyt b_5 (○) with 12 μM horse heart cytochrome *c* in the presence of 10 mM aniline in phosphate buffer at pH = 7. Ionic strength was adjusted by the addition of NaCl.

It is interesting to note that there are some substantial reductions in the rate constants for the slow phase of the reaction with yeast iso-1-cytochromes *c* variants F82L;C102T, F82G;C102T, and F82I;C102T. Reactions of several of these variants also have rate constants for the slow-phase reaction which increase with increasing ionic strength which suggests that the slow phase observed at low ionic strength is rate limited by conformational changes to an active form, i.e., a conformationally gated reaction.

Guillemette *et al.* (1994) have reported an extensive study of the critical residues involved in the bind-

Table I. Rate Constants for Electron Transfer Between Cytochromes b_5 and c (Willie *et al.*, 1993)

Cytochrome c	k_{fast}, s^{-1}	k_{slow}, s^{-1}	k, M^{-1} s^{-1}
Horse	4×10^5	3.0×10^4	3.8×10^8
Iso-1	1×10^5	1.8×10^4	3.3×10^8
C102T	1.1×10^5	2.0×10^4	3.0×10^8
F82Y;C102T	4.2×10^4	5.0×10^3	3.8×10^8
F82G;C102T	1.9×10^5	3.5×10^3	3.2×10^8
F82L,C102T	2.1×10^5	9.4×10^2	
F821,C102T	2.0×10^5	4.4×10^2	

ing and electron transfer in the cytochrome b_5/c system. The investigation utilized a large collection of variants of yeast iso-1-cytochrome c and involved kinetic measurements under high ionic strength or second-order reaction conditions. Several second-order rate constants (at an ionic strength of 190 mM) were reported for the reaction of cytochrome b_5 with yeast iso-1-cytochrome c variants modified at Phe82. Each of the variants examined showed a small increase in rate which was interpreted in terms of improved electronic coupling relative to the native enzyme. Amino acid replacements at position Ile85, which NMR studies have shown to be affected by binding of cytochrome b_5, had no affect on the rate constants. Rate constants were also measured using the variants Lys79Ala and Tml72Ala which are critical in salt bridge formation in modeling studies. The second-order rate constants in these two cases were reduced by a factor of 2 relative to the native enzyme, consistent with their role in modeling studies and earlier chemical modification studies (Ng *et al.*, 1977).

INTRINSIC REORGANIZATIONAL ENERGIES

In an effort to interpret the above data in the context of Marcus electron transfer theory (Marcus, 1956; Marcus and Sutin, 1985), we have investigated the internal electron-transfer reaction between the heme iron and a series of ruthenium complexes located at Cys65 of T65C cytochrome b_5. Labeling at this location is particularly advantageous since the ruthenium complex is coupled to the heme iron through 12 well defined covalent bonds. The robust nature of the protein and availability of an efficient labeling scheme has allowed us to prepare labeled proteins with a variety of different ruthenium complexes. By using a series of ruthenium complexes which spanned a range of redox potentials comparable to the expected reorganization energy, we were able to demonstrate the free-energy dependence of the rate constants predicted for electron transfer reactions by Marcus (1956). The free-energy dependence is summarized by

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

In this equation λ is the reorganizational energy and H_{AB} describes the electronic coupling between the redox centers. All other symbols have their usual meaning. The complexes used included members which fell in the inverted region ($\Delta G > \lambda$) as well as the linear region ($\Delta G < \lambda$) of the free energy curve. The experimental free energy dependence and that predicted by theory are shown in Fig. 6. From these data we were able to establish the reorganizational energy for the reaction (Scott *et al.*, 1993). A value of 1.02 eV was obtained, which agrees well with the magnitude of the barrier predicted from the intrinsic reorganizational barriers of $Ru(bpy)_3^{+2}$ and cytochrome b_5.

We have also looked at the question of whether protein/protein binding alters the intrinsic reorganization energies of the component proteins. This is a

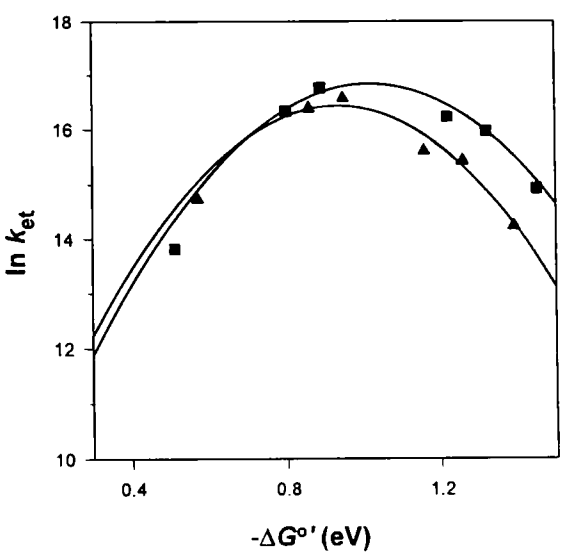


Fig. 6. Plots of the natural logarithm of k_{et} versus the free energy of reaction. The rate constants are for the reactions represented by k_1 and k_2 in Scheme I using different ruthenium complexes attached to Cys65. The solid line is a theoretical prediction based on Eq. (1). The data points shown by (■) were determined in the absence of cytochrome c and those shown by (▲) were determined with an equivalent amount of cytochrome c present. The solid line was drawn assuming λ = 0.93 eV, and the dashed line with λ = 1.02 eV.

fundamental question which must be addressed before a working theoretical description of electron transfer between proteins can be obtained. Gray and Malmström (1989) have suggested that under certain circumstances reductions in reorganizational energies on the order of 1 eV could be observed. The effect derives primarily from a reduction of the dielectric constant of the solvent layer surrounding the redox centers. In the case of two redox proteins, water with a high dielectric constant is displaced by binding the two proteins which presumably lowers the dielectric constant of the medium surrounding the redox sites (i.e., water is replaced by protein).

We addressed this problem by determining the reorganization energies of the intramolecular electron transfer reaction between the ruthenium complex and heme of T65C cytochrome b_5 with and without bound cytochrome *c* (Scott *et al.*, 1993). *In these experiments no electron transfer to or from cytochrome c was expected nor observed since electron transfer to cytochrome c is not competitive with electron transfer between cytochrome b_5 and the ruthenium complex.* This is a good test case since cytochrome b_5 has a large portion of the porphyrin exposed to solvent; thus, exclusion of water by binding to cytochrome *c* should have a large impact.

A plot of natural logarithm of the rate constants for internal electron transfer as a function of free energy with and without bound cytochrome *c* is shown in Fig. 6. As indicated above, this plot allows the determination of the reorganization energy of the reaction. Rigorous statistical analysis indicates that binding of cytochrome *c* lowers the reorganization energy to 0.93 eV, as expected, based on exclusion of water from the binding domain. The magnitude of the effect is obviously small, but it is consistent with crude calculations of reorganizational energies expected if only that part of the heme exposed to the solvent is replaced by protein.

CONCLUSION

Several different methodologies have been used to determine the rate of the intracomplex electron transfer in the cytochrome b_5/*c* system. Early reports seemed to indicate that the rate of electron transfer within the protein complex was slow with a rate constant of about 1600 s^{-1}. Eltis *et al.* pointed out that second-order rate constants obtained at higher ionic strengths were not consistent with such a small intracomplex rate constant. Meyer *et al.* (1993) also indicated that their kinetic studies were best interpreted in terms of a large intracomplex rate constant. Recent measurements using flash photolysis of ruthenium-labeled cytochrome b_5 have provided convincing evidence that the rate constant is much larger: 4×10^5 s^{-1} with horse heart cytochrome *c*. These later measurements where obtained by labeling cytochrome b_5 with photoactive ruthenium complexes. All previous methodologies have relied on diffusion of a strong reducing agent in order to initiate the interprotein electron transfer process. The ruthenium labeling method does not, and it is the only method by which reduced cytochrome b_5 can be produced in the presence of bound cytochrome *c with or without further reaction.* Photoinitiated reduction of cytochrome b_5 is complete within 100 ns and, thus, any process with a rate constant smaller than 10^7 s^{-1} can be followed. Comparisons of the reactivity of labeled cytochrome b_5 with native protein under conditions in which the reactions are substantially slowed indicate that the labeled proteins are native-like in terms of rate constants and binding constants. The native-like reactivity is further evidenced by the fact that derivatives labeled at residues 73 or 65 give identical kinetic results.

An obvious question to ask at this point is how well these rate measurements fit into the pictures described by the modeling studies and current theories of electron transfer (e.g., Marcus, 1956; Marcus and Sutin, 1985). The question has been briefly addressed by Northrup *et al.* (1993) using a simple electronic coupling model in which the preexponential term in Eq. (1) was replaced by $10^{12}\exp(-\beta d)$. Thus, the electronic coupling was assumed to decrease exponentially with increasing distance between the redox centers. This model predicts a rate constant of 8×10^5 s^{-1} for the reaction of cytochrome b_5 with cytochrome *c* using the parameters given by Northrup *et al.* ($\beta = 1$ $Å^{-1}$, $d = 12$ Å, $\lambda = 0.7$ eV). The calculated rate constant is remarkably close to the measured rate constant of 4×10^5 s^{-1} reported by Willie *et al.* (1992). The similarity must be considered with caution since there is considerable uncertainty in the correct choice for the parameters used in the calculation. For example, the reorganizational energy is based in part on earlier work by McLendon and Miller (1985) using what appears to be an incorrect rate constant for the reaction of cytochromes b_5 with *c*. Our studies of the reorganization energy as well as as others seem to indicate that the energy barrier is significantly higher than 0.7 eV. Despite the problems associated with the parameters,

the model calculations consistently give rate constants of 10^5 to 10^8 s^{-1}.

These calculations are of particular interest in light of the results recently reported by Qin and Kostic (1994). These investigators have shown that the reaction between zinc-substituted cytochrome c and ferricytochrome b_5 at low ionic strength appears to be rate limited by configurational fluctuations rather than electron transfer. In this case, the driving force for the reaction is significantly larger than the native system (1.2 vs 0.25 V) and probably in the "inverted region" of the free energy relation expressed by Eq. 1 (Marcus and Sutin, 1985). The first-order rate constant in water is only 3.5×10^5 s^{-1} and decreases as a function of increasing viscosity.

Further developments in this area will require a careful interplay between experiments and theory. Recent experimental work has begun to refine the kinetic picture, but problems associated with solution and protein/protein dynamics are clearly evident. Application of theoretical models of electron transfer will require close attention to experiments designed to determine the reorganizational energies involved in the models. Electronic coupling across the protein interface will have to be addressed from both an experimental and theoretical perspective.

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