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ELECTRON TRANSFER MECHANISMS IN HEME PROTEINS

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ABSTRACT

Recent years have seen substantial progress in our understanding of biological electron-transfer mechanisms. Of particular value have been soluble c-type cytochromes, due to the large structural base available. Using structurally homologous families of simple redox proteins, the contribution of driving force, electrostatics, and sterics to the kinetics of electron transfer has been quantified. Importantly, because Marcus' theory for outer-sphere electron transfer is applicable, we have been able to develop an approach termed "kinetic taxonomy." That is, based on the correlations obtained with a large number of redox proteins in different structural families, we can predict structural features from the kinetic properties of redox proteins of unknown structure. More recently, we have been able to establish a role for dynamics, orientation, and intervening media in intracomplex electron transfer when two redox proteins form a long-lived complex.

INTRODUCTION

Studies on the mechanism of electron transfer by heme proteins have focused on the soluble c-type cytochromes because of the availability of a substantial body of structural information. Of particular interest are the Class I c-type cytochromes, which are homologous to mitochondrial cyto-

chrome c (cyt. c). These cytochromes contain covalently bound heme with His, Met ligation at the out-of-plane positions. In terms of three-dimensional structure, this is a structurally homologous family of redox proteins with a variety of physical chemical properties, including a wide range of redox potentials [1]. Importantly, it is well established that electron transfer takes place at the exposed heme edge of Class I c-type cytochromes (see Refs. 1-3 for recent reviews), as shown in Fig. 1. Thus, the kinetics of electron transfer by c-type cytochromes are controlled to a large extent by an interaction domain defined by the specific amino acid side chains in the region of the exposed heme edge.

It is the goal of our studies to understand biological specificity in electron transfer, that is, how do two redox proteins recognize each other such that electron transfer is optimized through the physiologically relevant pathway and not diverted to nonproductive pathways. The nature of this problem is best illustrated in an organism like *Rhodopseudomonas sphaeroides,* where at least five soluble c-type cytochromes and one b-type cytochrome are present, presumably all localized in the periplasmic space [4]. On the basis of redox potentials, a large variety of thermodynamically favorable reactions could occur. However, it is expected that the physiologically relevant pathways function with high efficiency because the contribution of factors other than driving force control the relative rates of electron transfer between reactants. We would like to understand in molecular terms all of the factors controlling the kinetics of electron transfer between redox proteins (topography, electrostatics, orientation, intervening media, and protein dynamics), with the ultimate goal of being able to predict rate constants based on three-dimensional structures.

Our approach is to study electron transfer kinetics with structurally homologous families of redox proteins (Class I c-type cytochromes for this discussion) taking advantage of natural amino acid substitutions and the relevant physical chemical differences (redox potential, topography, electrostatics) and well-characterized electron donors and acceptors (e.g., flavin semiquinones, flavodoxin semiquinone and cytochrome c peroxidase). The principal kinetic techniques used are laser flash photolysis to generate flavin semiquinones as exogenous reducing agents, and stopped-flow spectrophotometry to mix redox proteins rapidly.

RESULTS AND DISCUSSION

Inherent in our analysis of heme protein redox kinetics is the fact that when reacted with an electrostatically neutral reductant (e.g., lumiflavin semiquinone,

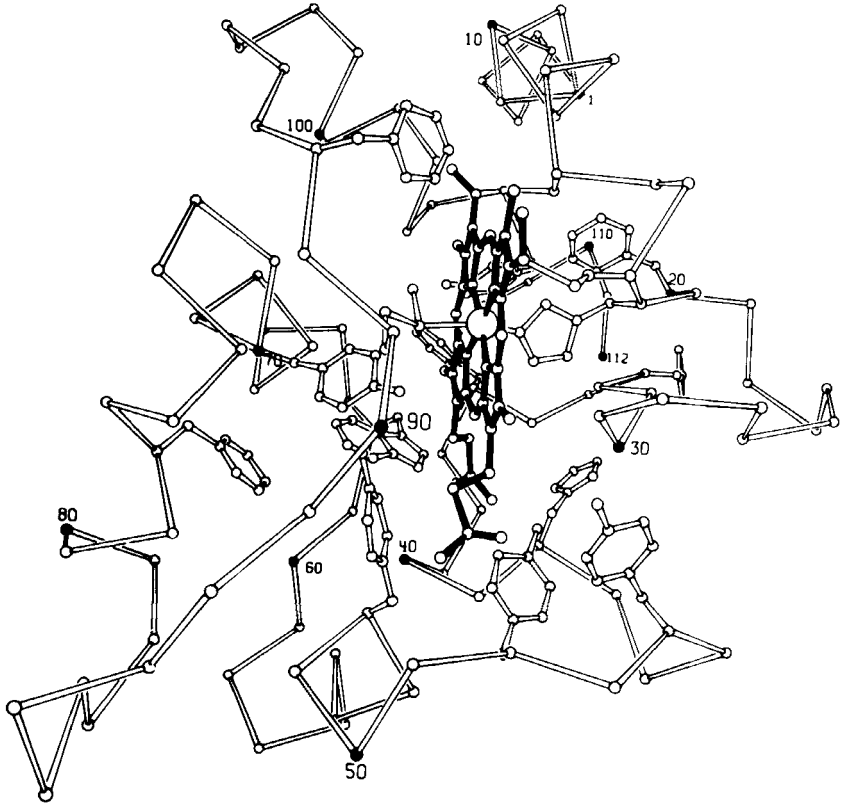

FIG. 1. Structure of *Rhodospirillum rubrum* cytochrome c_2.

LFH·), a structurally homologous family of redox proteins obeys Marcus' theory [1, 2]. This is shown in Fig. 2 for the reaction of LFH· and riboflavin semiquinone (RFH·) with a series of Class I c-type cytochromes with different redox potentials [5]. The solid line is the theoretical curve from Marcus' theory [2] and establishes that there is a relationship between the driving force (difference in redox potential) and the observed rate constant. Two conclusions are important to stress: 1) by modifying the redox potential through specific amino acid replacements, nature can modulate the kinetics of electron transfer; and 2) deviations from Marcus' theory for pro-

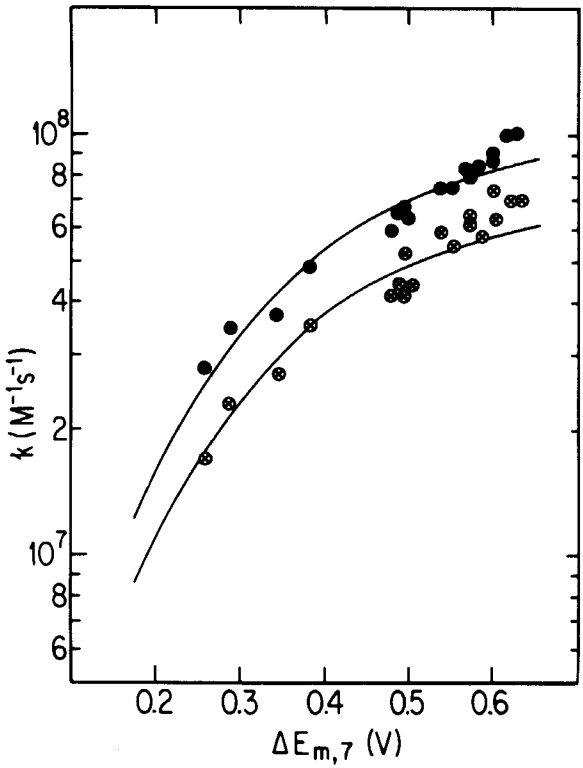

FIG. 2. Plot of the second-order rate constant for reduction of Class I c-type cytochromes vs difference in redox potential. Solid lines are theoretical curves by Marcus' theory [5], with the fitting parameters ν_{ET}, the limiting rate constant when the free energy of activation is zero, 1×10^8 and 0.7×10^8 $L \cdot mol^{-1} \cdot s^{-1}$ for LFH· and RFH· reduction, respectively, and λ the reorganization energy, 11.0 kcal/mol for both reductants. The cytochromes used had a range of midpoint potentials from 390 to 28 mV.

teins within a specific structural family result from structural differences at the interaction domain [6, 7]. In the example given (Fig. 2), there is a threefold variation in the rate constant over the range of cytochrome c redox potentials studied (28-450 mV). However, when flavodoxin semiquinone is used as the reducing agent, the range of rate constants is approximately 1000-fold with the same cytochromes [7]. This results from the substantially larger reorganizational energy required for the formation of productive

transient complexes between the two proteins [7]. Thus, the effect of driving force is greatly magnified in a protein-protein electron transfer system.

Electrostatics also play an important role in the interaction of soluble cytochromes and oxidants and reductants. We find generally that there is no correlation between net cytochrome charge and the charge on the interaction domain as determined from the effect of ionic strength on the kinetics of electron transfer [6, 7]. For example, *Paracoccus denitrificans* cytochrome c_2 has a net protein charge of -7, yet in its interaction with FMN semiquinone and flavodoxin semiquinone it behaves as a cation with a charge of +3 to +4. With a wide variety of Class I c-type cytochromes, the interaction domain charge correlates with the charge distribution about the exposed heme edge [6, 7] and calculated electrostatic potential surfaces [8]. Importantly, for the reaction of flavodoxin semiquinone with Class I c-type cytochromes, we find that the rate constant for electron transfer can vary up to 1000-fold due to variations in the charged amino acid side chains in the interaction domain, thus providing a means to control specificity by optimizing electrostatic interactions for the physiologically relevant pathways.

In some cases no substantial electrostatic field exists in the vicinity of the exposed heme edge, and the situation becomes more complicated. To illustrate this, Table 1 compares the electrostatic interaction free energy (V_{II}) of two similar but not identical flavodoxin semiquinones with tuna cytochrome c and *Pseudomonas aeruginosa* cytochrome c-551 [9]. In the case of tuna cytochrome c where the interaction domain charge is large, the two flavodoxins give very similar results. However, in the case of *Ps. aeruginosa* cytochrome c-551, where the interaction domain charge is small, the results are quite different, with *C. pasteurianum* flavodoxin "seeing" cytochrome c-551 as an anion and *Azotobacter* flavodoxin "viewing" it as a cation. These re-

TABLE 1. Flavodoxin-Cytochrome c Electrostatic Interaction

Oxidant/flavodoxin	V_{II}, kcal/mol
Tuna cytochrome c:	
C. pasteurianum	-16.1
Azotobacter	-13.4
Ps. aeruginosa cytochrome c-551:	
C. pasteurianum	+3.3
Azotobacter	-3.6

sults suggest that, when the interaction domain charge is small, other factors have a dominating influence on the way the two proteins come together. Presumably, the steric differences in the two flavodoxins [9] result in interaction at two different regions on the cytochrome c-551 surface, where different electrostatic fields are present. Both binding sites could be near the heme edge but sufficiently different to sense different electrostatic fields.

In general, electrostatics play a positive role in that strong complementary charged interactions lead to greatly increased rate constants for diffusion-controlled electron transfer. However, this is not always the case. In collaboration with Dr. G. McLendon, University of Rochester, we have investigated the kinetics of electron transfer between reduced cytochrome c and H_2O_2 oxidized cytochrome c peroxidase (CCP(IV,$R^{+}\cdot$)). In these studies the first-order rate constants for electron transfer following formation of the cytochrome c-CCP(IV,$R^{+}\cdot$) complex were measured. At low ionic strength ($\mu < 10$ mmol/L), where electrostatic attraction and stability of the complex is maximal, the first-order electron transfer rate constant (intracomplex) is approximately 200 s^{-1} for the oxidation of yeast iso-1-cytochrome c by yeast CCP(IV,$R^{+}\cdot$). However, at high ionic strength (μ = 260 mmol/L), where the electrostatic interactions should be largely masked and the complex less stable, the first-order electron transfer rate constant is approximately 2000 s^{-1}. These results suggest that, at least in this case, the formation of a tight, electrostatically stabilized complex actually inhibits electron transfer. Thus, electrostatics can lead to a less favorable situation, possibly by inhibiting motions important for electron transfer or by dictating an unfavorable orientation.

In summary, electrostatics can play an important role in specificity, modulating rate constants up to 1000-fold. However, in some cases, electrostatically directed complex formation can lead to structures which are not optimum for electron transfer. It is important to note that in most cases physiological electron transfer takes place at moderate or relatively high ionic strength ($\mu \approx 200$ mmol/L). Hence, it is the high ionic strength complexes which are most relevant.

Substantial data indicate that surface topography in the region of the interaction domain can also play an important role in the electron-transfer kinetics [6, 10]. In Table 2 the second-order rate constants for electron transfer from FMN and flavodoxin semiquinones to different cytochromes c are compared. *Chlorobium* cytochrome c-555 is a Class I c-type cytochrome with a midpoint potential of ~150 mV. *Alcaligenes* cytochrome c′ has the same midpoint potential but is a Class II c-type cytochrome not structurally related to the Class I cytochromes. As can be seen, the reactions of the two cytochromes with FMN semiquinone give similar rate constants. (The rate constants given are

TABLE 2. Steric Effects

Reductant/oxidant	k_∞, $L \cdot mol^{-1} \cdot s^{-1}$
FMN semiquinone:	
Chlorobium c-555	1.3×10^7
Alcaligenes cytochrome c′	2.7×10^7
Flavodoxin semiquinone:	
Chlorobium c-555	3×10^3
Alcaligenes cytochrome c′	4
FMN semiquinone:	
Plastocyanin	1.6×10^7
Stellacyanin	2.2×10^7
Flavodoxin semiquinone:	
Plastocyanin	1.3×10^5
Stellacyanin	1.3×10^3

electrostatically corrected to infinite ionic strength (k_∞); hence, the differences are not due to variations in the interaction domain charges.) However, for reaction with flavodoxin semiquinones, the rate constants at infinite ionic strength are different by three orders of magnitude. These results can be explained by a comparison of the structures of Class I and Class II cytochromes. In Class I c-type cytochromes, the heme edge is solvent accessible at the protein surface, as already discussed. However, in the Class II c-type cytochromes the heme face rather than the edge is solvent exposed at the bottom of a crevice approximately 15 Å deep and 15 Å wide [10]. When reacted with FMN semiquinone, the heme of cytochrome c′ is essentially solvent exposed, since FMN is smaller than the crevice. However, when it is reacted with the much larger flavodoxin semiquinone, close contact of the heme and flavin prosthetic groups is sterically hindered with Class II cytochromes, while steric restriction with the Class I c-type cytochromes is not as important. Thus steric effects, the example cited here being an extreme case, can result in up to a 1000-fold modulation of the kinetics of electron transfer. It is important to note that these types of steric effects are not restricted to cytochromes. As shown in

TABLE 3. Chromophore Accessibility in Protein-Protein Complexes

System	Component reduced	k_{bound}/k_{free}
Flavodoxin-tuna c	Flavodoxin	1.0
Flavodoxin-tuna c	Tuna c	0.4
CCP(III)-tuna c	Tuna c	0.26
CCP(III)-yeast iso-1 c	Iso-1 c	<0.10
CCP(IV,$R^{+\cdot}$)-yeast iso-1 c	Iso-1 c	0.68

Table 2, a very similar effect can be obtained by comparing the copper-containing proteins stellacyanin and plastocyanin [11].

In recent years, putative complexes between redox proteins (e.g., flavodoxin-cytochrome c [12] and cytochrome c-cytochrome c peroxidase [13]) have been modeled by using molecular graphics to obtain the structure of the complexes (no x-ray crystal structures are available). The flavodoxin-cytochrome c and cytochrome c-cytochrome c peroxidase (CCP) computer-generated complexes suggest that solvent (hence small molecule) access to the prosthetic groups should be severely sterically hindered by the amino acid side chains at the interaction domains [14, 15]. We have tested this prediction by investigating the reduction of one or both the prosthetic groups in a preformed (low ionic strength) complex by exogenous flavin semiquinones. As shown in Table 3, the amount of steric hindrance is very dependent on the nature of the reactants (when $k_{bound}/k_{free} = 1.0$, there is no steric hindrance). However, with the possible exception of the ferric-CCP-yeast iso-1 cytochrome c complex, severe steric hindrance is not observed [14, 15]. Assuming the computer-generated complexes are generally correct [14, 15], these results suggest that the complexes are dynamic, subject to motions which alter the accessibility of the prosthetic groups [14, 15]. Interestingly, in the case of the CCP-cytochrome c complex, the solvent accessibility of the cytochrome c heme is quite sensitive to the specific cytochrome c used (yeast iso-1 vs tuna) and the oxidation state of the CCP (Fe III vs Fe IV,$R^{+\cdot}$). Thus, the possibility of different interaction domains (tuna and yeast iso-1 have similar charge distributions in the vicinity of the exposed heme edge and react very similarly with free flavin semiquinones when not complexed) and a conformational change in yeast CCP on oxidation to the IV,$R^{+\cdot}$ state is suggested. These observations demonstrate that extrapolation from the computer-generated complex (tuna cyto-

TABLE 4. Intracomplex Electron Transfer

Reactants	$\Delta E_{m,7}$, V	Estimated distance between prosthetic groups, Å	k, s^{-1}
Tuna c → CCP(IV,$R^+\cdot$)	0.75	18	950
Iso-1 c → CCP(IV,$R^+\cdot$)	0.75	18	250
FNR· → *Paracoccus* c_2	0.57	<15	700
FNR· → HiPIP	0.44	<10	23
Flavodoxin → tuna c	0.40	3.5	85
FNR· → rubredoxin	0.26	<10	2100
Ferredoxin → FNR	0.05	<10	>4000
Horse c → cytochrome a	-0.04	?	>700

chrome c-CCP(III)) to other possible complexes (using yeast cytochrome c or CCP(IV,$R^+\cdot$) is difficult at best.

Although we can describe in some detail the role of driving force, electrostatics, and topography in the kinetics of electron transfer for diffusion-controlled reactions (second-order kinetics), the situation is much more complex in the preformed complexes. Table 4 summarizes the results from several systems we have studied to date, with the electron flow in the direction given by the arrows. Given in this table are the rate constant for intracomplex electron transfer (k), the difference in redox potential between the two reactants ($\Delta E_{m,7}$), and the distance between prosthetic groups, estimated from the computer-generated complex or from the molecular dimensions in the case of ferredoxin $NADP^+$:reductase (FNR) assuming the flavin moiety is at the molecular surface of the FNR. As can be seen, no obvious correlation exists between the kinetics and driving force ($\Delta E_{m,7}$). Although distance cannot be totally excluded as a parameter because of the uncertainty in the actual distances, it does not appear to correlate with rate constant. These results suggest that other factors, for example, intervening media (that is, the amino acid side chains and solvent between prosthetic groups) and the relative prosthetic group orientations may play an important role in determining the kinetics of intracomplex electron transfer.

SUMMARY

Based on our studies to date, we can quantify within reasonable limits the contribution of driving force, electrostatics, and topography to the kinetics of electron transfer in systems where formation of long-lived complexes does not occur (i.e., diffusion-controlled reactions). Importantly, based on the kinetic properties (i.e., the reaction with lumiflavin, FMN, and flavodoxin semiquinone) and midpoint potential, we can predict the structural family to which a redox protein belongs, and make reasonable estimates of prosthetic group exposure to solvent and of the topography and electrostatics of the interaction domain. We term this approach kinetic taxonomy, and find that it is an excellent way to characterize redox proteins of unknown structure.

It is clear that modulation through evolution of the redox potential, interaction domain charge (electrostatics), and topography makes available rate constant adjustments on the order of 10^9 for optimizing electron-transfer kinetics in specific systems. This modulation of rate constants represents the basis of biological specificity in electron-transfer reactions and provides the rationale for understanding physiological function in an environment of thermodynamically favorable, but physiologically irrelevant, possibilities.

Finally, studies to date suggest a role for dynamics, orientation, and intervening media in the modulation of biological electron-transfer kinetics. However, much work remains to establish the contribution of these factors, in molecular terms, to the electron-transfer kinetics.

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