Interprotein Electron Transfer: An Electrochemical Approach

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Theoretical and experimental aspects of intermolecular and intramolecular electron-transfer kinetics of Ru-modified proteins and metal substituted cytochrome c are briefly discussed in the first part. In the second part, spectroelectrochemical (electroreflectance) studies of horse heart cytochrome c electrostatically immobilized on carboxylic acid-terminated alkanethiol self-assembled monolayers on a well-defined gold electrode are discussed. It was found that intermolecular electron-transfer between cytochrome c and the carboxylic acid terminus is dynamically gated by configurational changes in the cytochrome c at the surface of alkanethiol self-assembled monolayers: transformation from the thermodynamically stable configuration to the electron-transfer complex, which features an efficient electron transfer channel, ultimately limits the rate of electron transfer. Kinetic measurements were carried out on horse heart cytochrome c and a recombinant rat cytochrome c (RC9-K13A), in which lysine 13 was replaced by alanine. The results revealed that the association of lysine 13 with the carboxylic acid terminus forms the electron transfer channel. A potential application of electrochemical techniques to mechanistic studies of biological intra- and intermolecular electron transfer processes is discussed as a concluding remark.

Key Words : Cytochrome c, Mutant, Organic Monolayers, Electron Tunneling, Gated Electron Transfer, Spectroelectrochemistry

1 Introduction

Electron transfer (ET) reactions in biological systems are far more complex than those of small molecules. The investigations of electron flow in biological systems have elucidated many of the factors that are critical for high selectivity and efficiency of processes in the photosynthetic and respiratory chains. Furthermore, ET reactions in biological systems can occur at reasonable rates between prosthetic groups separated by distances greater than 10 Å. Nonuniform charge distributions on protein surfaces create anisotropic interactions between physiological redox partners and form protein/protein complexes prior to electron transfer. Two mechanisms have been proposed to explain the "gating" and directional ET in protein/protein complexes: (1) multiple conformational states in the polypeptide matrix surrounding the redox sites may create the potential for gating and directional ET; (2) the initial step in the formation of the protein complex is a non-specific association between the two proteins, followed by rotational diffusion on the molecular surface to reach the proper configuration for the ET event. When intermolecular ET rate is limited either by a conformational change of the protein complex or rotational diffusion of the molecules, these processes can be viewed as directional electron transfer regulated by a "gating mechanism." The disadvantage of studies of ET kinetics in protein/protein complexes is that they are often limited in scope and are not easily amenable to the systematic changes needed to understand and control intermolecular ET reactions. The advantages of an electrochemical approach to investigate the ET mechanism, on the other hand, are that one can both regulate driving force and the ET path length by using alkanethiol self-assembled monolayers (SAMs) of varying chain lengths. One can also deconvolute the intermolecular ET event of the protein/protein complexes from the intramolecular ET event, provided that one can measure ET rate constants up to 10^8 s^{-1}. The disadvantage of the electrochemical system is that the counter part of protein molecules is not a globular molecule but a two-dimensional well-defined surface with functional groups. In the beginning of the present article a brief discussion of biological electron transfer reactions will be given as background for subsequent discussion of the electrochemical approach.

2 Electron Transfer in Biological Systems

2.1 Semi-classical electron transfer theory

Extensive theoretical work on electron transfers was published in 1985 by Marcus and Sutin, in which they showed that the ET in biological systems could be treated in the same theoretical framework as in the cases of simple chemical systems. A highly successful theoretical model proposed by Marcus describes the ET rates in terms of a small number of experimentally accessible parameters.

Electron transfer between most reactants in biological systems occurs between spatially fixed and oriented sites. In this case the reaction is formally a first-order one. The rate constant $k_0$ can be written as

$$k_0 = \kappa(r) v_0 \exp \left[ - (\lambda + \Delta C)^2 / 4 \lambda RT \right]$$

where $\kappa(r)$ is the transmission coefficient when the reac-
tants are a distance $r$ apart in the prevailing medium, $v_n$ is the nuclear frequency factor, $\lambda$ is the reorganization energy and $\Delta G^*$ is the standard free energy of the reaction.

In the nonadiabatic regime (for long range ET processes) $\kappa (r) \ll 1$, $\kappa (r) v_n$ is actually independent of the nuclear motion along the reaction coordinate and is given by eq. (2).

$$
\kappa (r) v_n = (2\pi / h) H^{n2}(4\pi \lambda RT)^{-1/2}
$$

$$
H^{n2} = H^{g2} \exp \left[ -\beta (r - r_0) \right]
$$

The matrix element $H^{g2}$ decreases exponentially with separation distance for many systems and one can write $\kappa (r) v_n$ as

$$
\kappa (r) v_n = 1 \times 10^{13} \exp \left[ -\beta (r - r_0) \right] \text{ s}^{-1}
$$

When $r = r_0$ and $\Delta G^* = -\lambda$, there is no free energy barrier to the ET reaction, $k_e$ is expected to be $\sim 10^{13}$ s$^{-1}$ and ET rates reach their maximum value. The activationless ET rate constant $k_e(\text{max})$ is given by the following equation.

$$
k_e(\text{max}) = 1 \times 10^{13} \exp \left[ -\beta (r - r_0) \right] \text{ s}^{-1}
$$

2. Intramolecular electron transfer in protein molecules

The tunneling-pathway model suggests, and experimental evidence supports the notion that different protein secondary structures mediate electronic coupling (super-exchange) with different efficiencies. The total coupling (the matrix element) of a single ET pathway, which consists of different intervening bridges, is given as a product of the individual bridges.

$$
H^{g2} \propto \Pi \kappa(\text{covalent}) \Pi \kappa(\text{hydrogen bond}) \Pi \kappa(\text{space jump})
$$

The simple pathway model has been remarkably successful in explaining the role of peptide structure in facilitating electron tunneling through proteins in numerous unimolecular and bimolecular protein ET experiments.

The use of chemically modified proteins to study the distance dependence of ET, notably by Gray and co-workers, has opened a whole new field of activity. Gray and co-workers first measured the ET rate between Ru(NH$_3$)$_5$(His-33)$^3^+$ and the heme center of cyt. c.$^6$ Since then, his group has been investigating extensively intramolecular ET rates between Ru(bipy)$_2$(im)$^2^+$ (bipy = 2, 2′-bipyridine; im = imidazole) coordinated at different sites of the histidine residue on the surface of azurin.$^6$ The ET rates with metal-metal separation from Cu$^+$ to Ru$^{2^+}$ in coupling along β-sheets (strands) in azurin is well described by an exponential factor with a decay constant of 1.1 Å$^{-1}$. In contrast to the extended peptides found in β-sheets, the ET rates along (helices are expressed by an exponential factor with a decay constant of 1.4 Å$^{-1}$. The experimental data provide a remarkably uniform picture of long range ET predicted by eqs. (5) and (6), and the results are shown in Fig. 1.$^{10}$ The ET rates $k_e(\text{max})$ decrease exponentially with increasing donor - acceptor separation. The β-zone in Fig. 1 represents the tunneling through β-sheets ($\beta = 0.9 - 1.15$ Å) and α-zone represents the tunneling through α-helices ($\beta = 1.26 - 1.6$ Å).

2. Intermolecular electron transfer in protein/protein complexes

Intermolecular ET processes between the positively charged and negatively charged sites of electron transfer protein molecules (such as cyt. c/cyt. b$_5$, cyt. c/cyt. c peroxidase, cyt. c/plastocyanin, and cyt. c/cyt. b$_{56}$, b$_{59}$ couples) have been investigated in solutions having different ionic strengths, and pH: ET rates depend strongly on the ionic strength, pH, and temperature of the solution, and are reported to be in the range 10$^9$ - 10$^{10}$ s$^{-1}$. Effects of solution viscosity on the ET rates in the protein/protein complexes have been studied theoretically and experimentally.$^3$ Tezcan et al. measured ET kinetics between native and Zn-substituted tuna cyt. c molecules in crystals of known structure.$^{12}$ The ET reaction took place over a Zn - Fe distance of 24.1 Å and the ET rates match closely those for intraprotein electron tunneling shown in Fig. 1. Their results indicate that van der Waals interactions and water-mediated hydrogen bonds are effective coupling elements for tunneling across a protein/protein interface.

The interaction between cyt. c and its physiological reduction or oxidation partner is specific; there are complementary electrostatic interactions between lysine amino groups on cyt. c and carboxylate groups on the counter proteins. The intermolecular ET processes between proteins involve at least four steps: (1) formation of protein/protein complex(s) (thermodynamically stable conformation) guided by electrostatic interactions; (2) formation of

Fig. 1 Tunneling timetable for ET in Ru-modified protein: (●) azurin; (○) cytochrome c; (△) myoglobin; (□) cytochrome b$_{56}$, HiPIP. Solid lines illustrate tunneling pathway predictions for coupling along β-strands ($\beta = 1.0$ Å$^{-1}$) and α-helices ($\beta = 1.3$ Å$^{-1}$); dashed line illustrates a 1.1 Å$^{-1}$ distance decay. Distance decay for electron tunneling through water is shown as the distance decay constant as a gray wedge ($\beta = 1.6 - 1.75$ Å$^{-1}$). Estimated distance dependence for tunneling through vacuum is shown as the black wedge ($\beta = 3.0 - 4.0$ Å$^{-1}$).
the protein/protein complex, which facilitates an optimum ET reaction rate, from the equilibrium conformation of the complex; (3) electron transfer through the tunneling pathway between the proteins; (4) and formation of the thermodynamically stable complex(s) after the ET event. If specific site(s) or specific area is present for strong coupling the question arises as to whether or not these specific sites are physiologically responsible to control the ET processes in the complexes. Extensive chemical modification studies identified a group of lysine residues on the surface of cyt. c that is responsible for its interaction with cyt. c oxidase.13-16 The electron transfer activities of cyt. c derivatives modified at single lysine residues with its redox partners provide evidence for the importance of lysines 13, 86, and 87 surrounding the heme crevice. In general, the closer the modification is to the enzymatic interaction domain of cyt. c on the "front surface" of the protein, the greater the decrease in the ET rate.

Figure 2 shows a schematic diagram of the binding sites on cyt. c for cyt. c oxidase.15

The electronic coupling associated with an electron tunneling pathway in a protein/protein complex can be written as a product

\[ H_{M}^{2} \propto \Pi \kappa_{\text{protein I}} \Pi \kappa_{\text{protein II}} \kappa_{\text{interface}} \]  

where \( \kappa_{\text{protein I}}, \kappa_{\text{protein II}}, \) and \( \kappa_{\text{interface}} \) represent the transmission coefficients of protein-I, protein -II, and the interface of the (protein-I/protein-II) complex.

The intermolecular ET rate constants of the electrostatic complexes of Zn-cyt. c/plastocyanin (pc) and Sn-cyt. c/pc decrease as the solution viscosity is raised while the ET rate of the covalent complex of Zn-cyt. c/pc is independent of solution viscosity.14b The viscosity effects on the electrostatic complexes are explained in terms of two conformations of the electrostatic protein/protein complexes. The initial state is the thermodynamically stable one and then the rearrangement of the complex occurs to facilitate electron tunneling prior to the ET event. The intermolecular ET reaction is gated by the rearrangement of the protein/protein complex.

3 Electrochemical Approach

3.1 Electrochemical systems

The electrode reaction of cyt. c through \( \epsilon \)-derivatized alkanethiol SAMs is considered to be a simplified model system for biological ET processes (Fig. 3). Desorption of cyt. c (horse heart) immobilized on carboxylic acid-terminated alkanethiol SAMs into supporting electrolyte solutions is negligible at low ionic strengths (<50 mM phosphate buffer solution) with the pH range of 6~9, and cyt. c undergoes a rapid and reversible electron transfer reaction through the alkanethiol films.17-24 The pK\(_a\) value of the carboxylic acid terminus is nearly independent of the alkanethiol chain length and is in the range of 6.0~7.1, which are 1.5~2.5 pH units more basic than those in the solution phase25 (pK\(_a\) values of aspartic and glutamic acids are about 4.4). Electrostatic interactions between the positively charged lysine-amino groups on cyt. c and the negatively charged carboxylate termini of the SAMs enhance the binding of cyt. c, analogous to its complex with other negatively charged proteins such as the cyt. c/cyt. b\(_5\) complex.3b In solutions of low ionic strength, the observed voltammetric peak current is proportional to the potential sweep rate, indicating that there are no diffusion processes involved in the electrochemical redox reaction.19,21,23,24 On the other hand, in solutions having ionic strengths greater than 130 mM (>60 mM phosphate buffer solution) at pH 7.0, cyt. c molecules tend to desorb from the carboxylate surface.19,23,24 The formal potential of cyt. c on the carboxylic acid-terminus is +260 mV (vs. NHE), which is in close agreement with the values for cyt. c in the solution phase. Surface-enhanced Raman studies of cyt. c immobilized on these films have indicated that the heme center of cyt. c is in a low spin, six-coordination state.26a,b These combined findings reveal that cyt. c immobilized on carboxylic acid-terminated alkanethiol SAMs maintains its native structure on a surface monolayer.

3.2 Electrochemical instrumentation

Traditional electrochemical techniques use a current-time (at constant electrode potential, linear potential sweep, or sinusoidal modulation of potential) or potential-time (at constant current) transient to evaluate the ET rates at electrodes. However, the charging current of the
The exponential decay factor $\beta$ has been found to be $1.09 \pm 0.02$ per methylene group ($0.71 \pm 0.01 \text{Å}^{-1}$) regardless of the type of redox species at the terminus of alkanethiol SAMs when $n > 10$. \(^{21-23, 28-33}\)

In our previous study, we proposed that the ET process from the electrode to the cytochrome $c$ through carboxylic acid-terminated alkanethiol SAMs consists of three steps, namely, (1) an intramolecular ET process from the heme edge to the binding site of cytochrome $c$, (2) an intermolecular ET process at the interface between cytochrome $c$ and carboxylic acid terminus, and (3) electron tunneling through the alkyl chain:

$$k_{\text{et}} = k_{\text{m}} \exp \left( -\beta n \right)$$

where $k_{\text{m}}$ is the apparent ET rate constant extrapolated to $n = 0$. The exponential decay factor $\beta$ has been found to be $1.09 \pm 0.02$ per methylene group ($0.71 \pm 0.01 \text{Å}^{-1}$) regardless of the type of redox species at the terminus of alkanethiol SAMs when $n > 10$. \(^{21-23, 28-33}\)

It is assumed that there is a configurational rearrangement of cytochrome $c$, eq. (10), prior to the ET reaction takes place from the binding complex (state I), which is thermodynamically stable form of cytochrome $c$ on the carboxylate terminus of SAM, to the ET complex (state II), which facilitates the most efficient ET reaction. Then, the ET reaction between the electrode and cytochrome $c$ through the carboxylic acid terminated-alkanethiol SAM takes place, eq. (11), followed by a configurational rearrangement reaction represented by eq (12) to form thermodynamically stable complex again. \(^{23}\)

The energy level diagram of cytochrome $c$ (ox) (I) and cytochrome $c$ (red) (II) are assumed to be equal to those of cytochrome $c$ (red) (I) and cytochrome $c$ (red) (II) ($k_1 = k_2$, $k_3 = k_4$). At the formal potential ($E = E^\circ$) of the ET reaction the relation given by eq. (13) can be assumed.

$$
\Gamma_1 + \Gamma_2 = \Gamma_3 + \Gamma_4, \quad \Gamma_1 = \Gamma_2 = \Gamma_3,
$$

where subscripts 1, 2, 3, and 4 denote the amounts of species cytochrome $c$ (ox) (I), cytochrome $c$ (ox) (II), cytochrome $c$ (red) (II), and cytochrome $c$ (red) (I) on the SAM. The ET rate constants of cytochrome $c$ immobilized on the alkanethiol SAMs are evaluated by using the potential modulated UV-visible electroreflectance technique at the formal potential with a small amplitude of $\Delta E_{\text{ac}}$. \(^{20, 27}\)

The ET rate constant of cytochrome $c$ is given by eq. (14) from eq. (8). \(^{25}\)

$$
\frac{1}{k_i} = \frac{1}{k_{\text{m}}} \exp \left( -\beta n \right) \exp \left[ -\beta_i (r - r_0) \right]
$$

where $k_{\text{m}}$ represents the ET rate constant between the heme edge of cytochrome $c$ and the electrode at an alkanethiol film whose $n = 0$ in a solution of zero ionic strength, $r$ represents the distance between the binding site of cytochrome $c$ and carboxylic acid terminus, $r_0$ represents that at zero ionic strength, and $\beta_i$ represents the exponential decay factor at the interface.

When the ET rate is limited by the rearrangement rate of cytochrome $c$ on the SAM surface, $k_i \gg k_2$, the ET rate is

Fig. 4 Logarithmic plot of the ET rate constant $k_{\text{et}}$ of cytochrome $c$ immobilized on HOOC(CH$_2$)$_n$S/Au electrode vs. number of methylene groups $n$. (●) our results obtained by ER spectroscopy and (○) results obtained by ac impedance spectroscopy. \(^{23}\)

The overall matrix element of the system shown in Fig. 3 is given by eq. (9)

$$H_{\text{MM}} \propto k_{\text{protein}} K_{\text{(SAM)}} K_{\text{interface}}$$

The $k_{\text{protein}}$ values of the process (1) shown in Fig. 1 and the $K_{\text{SAM}}$ values of the process (3) have been studied extensively. \(^{21-24, 28-32}\)
given by eq. (15). That is, the ET rate is independent of the alkyl chain length through short alkanethiol chains.

\[ k_{\text{app}} = k_1/2 \] (15)

When the ET rate is limited by the ET through the alkyl chain (through long alkyl chains), \( k_1 \gg k_n \) and therefore

\[ \ln k_{\text{app}} = \ln \left| 1 + 2\left(\frac{k_1}{k_n}\right) \right| - 2 \ln(1 + k_1/k_n) + \ln k_{\text{app}} \mid_{r = r_0} - \beta n - \beta_i (r - r_i) \] (16)

The first two terms are a function of the rearrangement reactions, the third term is constant, the fourth term is a function of the alkanethiol chain length, and the last term is a function of the interfacial distance between cyt. c and carboxylate terminus.

3.4 Electrochemical studies of cytochrome c through carboxylic acid-terminated alkanethiol SAMs

3.4.1 Cytochrome c on carboxylic acid-terminated alkanethiol SAMs The amount of cyt. c (horse heart) immobilized on the carboxylic acid terminated alkanethiol SAMs was evaluated to be \( (12-13) \times 10^{-12} \text{ mol cm}^{-2} \) by potential sweep voltammetry and \( (10.5-13.5) \times 10^{-12} \text{ mol cm}^{-2} \) by ER measurements. These values agree well with the calculated monolayer coverage, which was estimated from the crystallographic data to be \( 13 \times 10^{-12} \text{ mol cm}^{-2} \). In the ER voltammograms the peak potentials of both real and imaginary components were identical, while the band shapes were symmetrical about the peak position in the modulation frequency range used in the present studies. These results suggest that the transfer coefficient of the ET reaction (the redox reaction of cyt. c) is close to 0.5.

3.4.2 Effect of ionic strength on the electron transfer reaction rate of cyt. c The ET rate vs. chain length plot in dilute supporting electrolyte solutions (up to 10 mM phosphate buffer solution at pH 7) are nearly independent of the solution ionic strength. The ET rates decrease linearly with \( \sqrt{I} \) (\( \sqrt{I} > 5 \text{ mM}^{1/2} \)) through longer alkanethiol chains \( (n>7) \) but the plateau value through short chains is independent of the solution ionic strength as shown in Fig. 5. The limiting values of \( k_{\text{app}} \) are independent of both chain length and solution ionic strength. The ET rate through a short alkanethiol is limited by the preceding configurational rearrangement reaction rate \( k_1 \) given by eq. (10), this rate constant \( k_1 \) at the plateau is estimated to be \( 2.6 \times 10^9 \text{ s}^{-1} \). The binding constant of cyt. c to HOOC-terminus decreases with increasing ionic strength of the solution because cyt. c tends to desorb from the surface of the SAM in phosphate buffer solution with concentrations above 60 mM at pH 7. The intermolecular electrostatic interaction between cyt. c and HOOC-terminus may decrease in higher solution ionic strengths by electrostatic shielding, and its spacing may increase.

3.4.3 Effect of pH on the electron transfer reaction rates The pH dependence of the ET reaction rate of cyt. c (horse heart) through alkanethiol SAMs with different chain lengths in the pH range 6.0-9.0 is shown in Fig. 6. The ET rate constant through short-chain alkanethiols is independent of pH (6-9). In solutions of lower pH, the degree of deprotonation of carboxylic acid is less, such that the reverse reaction rate constant \( k_1 \) for the rearrangement reaction of cyt. c may be small; this would thereby decrease the value of \( k_2/k_1 \) in eq. (10) for the rearrangement reaction. The first and second terms of eq. (16) decreases with increasing pH. The fourth term would be affected by the solution pH because the decrease in the surface charge density on the SAM may lead to decrease in the electrostatic interaction between the cyt c/HOOC- complex. We have previously reported that the ET rate of cyt. c through mixed alkanethiol SAMs increases when the carboxylic acid-terminated alkanethiol is diluted by methyl-terminated ones. This dilution effect results in a decrease in the number of negatively charged groups at the surface, thereby reducing \( k_2 \).

3.4.4 Viscosity effects on the electron transfer rate of cyt. c The viscosity of electrolyte solutions was adjusted by the addition of sucrose because it does not affect the solubility of cyt. c in aqueous solutions. The formal potential of cyt. c on the SAMs, which is very sensitive to the folded state of cyt. c, is unaffected by the
addition sucrose.\textsuperscript{24} As a further verification of the conformation of the heme moiety, cyt. c (immobilized on SAM of HOOC(CH\textsubscript{2})\textsubscript{n}-S-Ag) in the presence of sucrose was investigated by SERRS and it was shown that immobilized cyt. c remains in its native form (six-coordinate low-spin state) in the presence of sucrose.\textsuperscript{24}

Figure 7 depicts the relationship between log $k_{\text{app}}$ vs. number of methylene groups in the alkanethiol SAMs in terms of the solution viscosity. A sharp decline in ET reaction rates through shorter chain lengths ($n=2, 4$ and $7$) was noted with an increase in sucrose concentration.

The theoretical explanation for the effect of solvent viscosity on unimolecular rate processes in the condensed phase is provided by Kramers theory, which shows that the rate of a diffusive barrier-crossing process is inversely proportional to the friction.\textsuperscript{40} The theoretical equation was modified by Ansari \textit{et al.} for application to experimental data\textsuperscript{40}

$$k = |C/\alpha + \eta| \exp (-E_0/RT)$$ (17)

where $E_0$ is the average height of the potential energy barrier separating the protein configurations, and $\eta$ is the solvent viscosity. $C$ and $\sigma$, both of which have units of viscosity, can be thought of as contributions of the protein friction to the total friction, and are adjustable parameters. If $E_0$ is independent of the solution composition (viscosity), eq. (17) can be rewritten as

$$1/k = A(\eta/\eta_0) + B$$ (18)

Regression analysis of the ET rates through mercapto-propionic acid (\textit{i.e.} $n=2$ in Fig. 7) SAMs in various sucrose-containing solutions, which correspond to the forward reorganization reaction rate $k_1=2k_{\text{app}}$, was accomplished using eq. (18) and the result is represented by the following equation.

$$1/k_{\text{app}} = 2.83 \times 10^{-4}((\eta/\eta_0) + 4.84 \times 10^{-4}$$ (19)

As can be seen from Fig. 7, the apparent ET rate constants through longer alkanethiol chains were nearly independent of solution viscosity within the range studied.\textsuperscript{24} The rearrangement reaction rate $k_1$ decreases with increasing solution viscosity but the ratio $k_1/k_2$ is expected to be independent of the solution viscosity from eq. (17) because the denominator of eq. (17) depends only on the viscosity (both $E_0$ and $\sigma$ are independent of viscosity). In addition, the $\beta(r-r_0)$ term in eq. (16) is anticipated to be independent of solution viscosity at constant ionic strength. Therefore, it is reasonable to conclude that the ET rates through longer alkanethiols SAMs can be adequately described by eq. (16) regardless whether or not sucrose is present in the solution.

Addition of sucrose to the electrolyte solutions changes not only their viscosity but also their dielectric constant, which may give rise to changes in the ET reaction rate of cyt. c at the carboxylic acid-terminus. This effect has been examined previously by Zhou and Kostic, who measured ET reaction rates of metal-substituted cyt c/plastocyanin couples in various solvent systems having different dielectric constants (such as ethylene glycol/water, glycerol/water and sucrose/water).\textsuperscript{24} They found that the ET rates were dependent only on the solution viscosity, and that the effect of solvent dielectric constant on the intermolecular ET reaction rate could be ignored.

3. 4. 5 The binding site of cyt. c to carboxylate - terminus

It is possible to elucidate the binding site of cyt. c (II) from the extrapolated value $k_{\text{app}}$ from eq. (16) to $n=0$ at low ionic strength, the value of which is $6.3 \times 10^5 \text{ s}^{-1}$, which corresponds to $k_{(n=0,r=\infty)}$ in eq. (14).\textsuperscript{40} The ET rates from the heme edge to various lysine residues on the surface of cyt. c when $\Delta G^e=0$ (at the formal potential) can be estimated from the results of Gray and Winkler.\textsuperscript{4-6} The intramolecular ET rates from the heme edge to lysine 13, which is considered to be one of the important ET sites between protein/protein complexes, is estimated to be $2 \times 10^5 \text{ s}^{-1}$ and from the heme edge to lysine-79 is also $2 \times 10^5 \text{ s}^{-1}$. Lysine-79, however, could be excluded because there is hydrogen bonding between the -NH\textsubscript{2} terminus of lysine-79 and serine-47 and, consequently, lysine-79 could not be an ET site to the carboxylate terminus of the SAM.\textsuperscript{41} The ET pathway to lysine-27 is expected to show the next highest ET rate, which is $50 \text{ s}^{-1}$ (if hydrogen bond ET pathway is taken into account, otherwise it would be 0.006 s\textsuperscript{-1}), which is much smaller than the extrapolated value to $n=0$. Therefore, lysine-13 appears to be the most probable ET site to form an ET complex with the carboxylate-terminus of alkanethiol SAMs and rest of lysine residues could be excluded as an ET pathway.

3. 4. 6 Gated electron transfer at the binding site of cyt. c to the carboxylate terminus

The asymmetric distribution of positive charges (lysine residues) on cyt. c gives rise to a preferential adsorption geometry with a narrow orientation distribution. The rate-limiting ET step through short alkyl chains results from a configurational rearrangement process preceding the ET event. The "gating" process arises due to rearrangement...
of the cyt. c from a thermodynamically stable state (binding complex) on the carboxylic acid-terminus to one (ET - complex) which provides the most efficient ET pathway. The ET reaction takes place whenever lysine 13 directly interacts with the carboxylic acid-terminus of alkane thiols SAMs. The bindings between other lysine residues, which are located in the vicinity of the heme crevice, with the carboxylic acid-termini stabilize the binding complex but are very unlikely to form ET pathways.

3. 5 Electrochemistry of cytochrome c mutant (RC 9-K13A)

We then studied the ET kinetics of a cyt. c mutant (RC9-K13A), in which lysine-13 is replaced by alanine, immobilized on the carboxylic acid-terminated alkane thiols SAMs to confirm that lysine-13 plays an important role to form the efficient ET tunneling pathway of cyt. c to the carboxylate terminus of the alkane thiols SAMs. The ET rate constant of RC9-K13A immobilized on 3-mercaptopropionic acid SAM on gold electrode was measured to be 0.2 ± 0.05 s⁻¹. The ET rate constant of the native cyt. c through 3-mercaptopropionate SAM (n = 2) is estimated to be 8.4 × 10⁵ s⁻¹ from the extrapolated value to n = 2 in Fig. 4 provided that the configurational rearrangement reaction is not involved. One can calculate the difference in the effective number of covalent bonds in the ET path \( \Delta n_{ed} \) between the native cyt. c and RC9-K13A cyt. c by using eq. (14) and the value of \( k_{ed}(\text{app}) = 0.2 \) for RC9-K13A cyt. c.

\[
0.2 = 8.4 \times 10^5 \exp (-0.99 \Delta n_{ed})
\]

where C represents covalent bonding defined by Gray and Winkler.⁷ a, 7 b, 8 c

A potential candidate of the binding site of RC9-K13A could be the Lys-8 because the difference in the effective number of covalent bonds from the heme edge to Lys-13 and that of Lys-8 is 15 C if we ignore the hydrogen bond pathway between Lys-8 and Glu-12, and its ET rate shows a reasonable correspondence with that expected from the native cyt. c. Lys-27 could create an efficient ET pathway for RC 9 K 13 A if hydrogen bond between N of Gly-29 and O of Cys-17 in the ET pathway from Lys-27 to Cys-17 is taken into account. However, Lys-27 could be excluded because the value of \( \Delta n_{ed} \) in this ET pathway is only 8 C, which is much less than 15.4 C, and the ET rate is estimated to be 47.8 s⁻¹ (the ET rate through (- bond is expected to be 0.006 s⁻¹), which is much higher than expected. Other lysine residues such as Lys-25 (\( \Delta n_{ed} = 13 \)), Lys-72 (\( \Delta n_{ed} = 13.6 \)), and Lys-86 (\( \Delta n_{ed} = 16 \)), which are highly conserved lysines surrounding the heme crevice, could form multiple ET pathways.

3. 6 Future direction of electrochemical studies of biological electron transfer

The electrostatic interaction between lysine on the surface of cyt. c and the carboxylate termini of alkane thiols SAMs leads to the formation of hydrogen bonds. Gaigalas and Niauria,⁴⁴ and Ulstrup and his co-workers,⁴⁵ found a new model system for biological ET. The immobilization of azurin on a methyl-terminated alkane thiols SAM is achieved through hydrophobic interaction between the hydrophobic area around the copper atom in azurin and the methyl-head of the alkane thiols SAM to form a monolayer or monolayer. As these bonds can be part of intermolecular electron tunneling pathways these systems could be an interesting electrochemical system to investigate ET reactions of protein/protein complexes and protein binding to membranes. Ulstrup and his co-workers measured the ET rate of azurine immobilized on methyl-terminated alkane thiols SAMs with different chain lengths at well-defined gold electrode by CV technique.⁴⁵ The feature of the ET rate against the alkane thiols chain length is the same as our results shown in Fig. 4.

It has been shown in the foregoing discussion that electrochemical studies of the interfacial ET events between cyt. c and carboxylate terminus of alkane thiols SAMs on gold electrodes could lend insight into the site-specificity of interfacial ET pathways and the ET kinetics of protein/protein complexes. The intermolecular ET rates of protein/protein complexes, however, are too rapid to measure by traditional electrochemical techniques. Most of the earlier kinetics studies of the cyt. c/ HOOC-alkane thiols SAM/Au couple have been limited to long alkane thiols SAMs which attenuate the unimolecular ET rates as high as 10⁵ s⁻¹. The ET rate constants vary exponentially with the number of methylene groups in the alkane thiols.

Smalley et al. measured the ET reaction rate between a gold electrode and ferrocene attached to the alkane thiols monolayers up to 10⁶ s⁻¹ by using a laser-induced temperature jump method with Nd: YAG laser as a power source.⁴⁰ This technique is inappropriate for the study of redox kinetics of heme proteins because the heme moiety is photochemically reduced by laser excitation. Rapid scan dc voltammetry with an ultramicroelectrode (a radius of the gold electrode: 25 ~ 500 µm; the scan rate: ~10 kV s⁻¹) was applied to the ferrocene/ SAM/gold electrode system⁴⁵ and the highest ET rate measured was as high as 10⁶ s⁻¹. This technique would be a powerful electrochemical technique in studies of ET kinetics of protein/protein complexes provided that one can regulate the surface of an ultramicroelectrode. The potential-modulated electoreflectance spectroscopy (ER) technique enables one to measure electrode reaction rates up to 10⁸ s⁻¹ at a well-defined electrode with large area because the effect of double layer charging at the electrode interface can be minimized.⁴⁰, 27 Small perturbation techniques (ER and temperature jump) are appropriate to measure the ET rate of the redox system in the vicinity of its formal potential from which one can evaluate the distance dependence of the ET rate (the electronic coupling constant between the redox protein and electrode). It is also possible to evaluate the reorganization energy of the reaction from the temperature dependence of the ET rate. Dc potential sweep voltammetry and potential step technique have been commonly used in the high overpotential regime to evaluate the reorganization energy of the redox species. In this respect, ER and rapid scan voltammetry would be suitable techniques to
measure rapid ET rates to elucidate the interfacial ET mechanism between redox proteins and (functional groups at the terminus of alkanethiol SAMs on a molecular level.

The important issues in the study of ET at the interface of alkanethiol SAMs are elucidation of the ET channels at the interface and the configurational rearrangement of cyt. c attached to the SAMs. Although we proposed that lysine 13 on cyt. c (horse heart) is a potential candidate to form hydrogen bonds to the carboxylate terminus of alkanethiol SAM to facilitate the most efficient ET pathway, there remain several questions to be solved. The use of cyt. c mutants would give us information about the binding site of cyt. c but we need to keep in mind that the charge distribution on the cyt. c surface will be sensitive to its electrochemical properties (protein stability, dipole moment and interaction with SAM may change). The intramolecular ET rate from the heme to the specific lysine on the surface of cyt. c, in principle, should agree with the ET rate of the cyt. c/SAM/electrode system extrapolated to \( n = 0 \) (the ET site of cyt. c is ideally attached to the electrode surface). There is, however, a significant discrepancy between the measured ET rate and the extrapolated ET rate in our results. The cross-linking between the specified lysine of cyt. c and carboxylate terminus would provide us not only useful information on the intramolecular ET of cyt. c and interfacial ET but also the reorganization energy of cyt. c covalently bonded to the electrode through the alkanethiol SAM.

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References

35) The ET rate from the heme edge of cyt. c to the electrode through carboxylic acid terminated alkanethiol SAM is given from eqs. (3) and (9).

\[
\begin{align*}
k_{\text{et}} &= H_{\text{AD}}^* \exp[-\beta_{\text{SAM}}(r_{\text{SAM}})] \\
& \quad \times \exp[-\beta \text{interface}(r_{-r_0}\text{interface})] \\
& = k_{n=0, r=r_0} \exp(-\beta n) \exp[-\beta_{\text{intra}}(r-r_0)]
\end{align*}
\]

40) \(k_{n=0, r-r_0}\) does not represent the ET rate when cyt. c is ideally attached directly to the electrode surface because there still remain intervening bridges at the cyt. c/ HOO-SAM interface when \(n = 0\).
42) RC9 is a recombinant of rat cyt. c, which has 93% amino acid sequence homology to that of horse heart cyt. c, but the lysine residues surrounding the heme-crevice are well-conserved.
46) There is an excellent review article related to this paper entitled "Dynamic effects of self-assembled monolayers on thermodynamics and kinetics of cytochrome c electron transfer reactions" by M. Fedurco, *Coord. Chem. Rev.*, 209, 263 (2000).