An Electrochemical Approach to Investigate Gated Electron Transfer Using a Physiological Model System: Cytochrome *c* Immobilized on Carboxylic Acid-Terminated Alkanethiol Self-Assembled Monolayers on Gold Electrodes

Albert Avila,[†] Brian W. Gregory,[‡] Katsumi Niki,*[,‡] and Therese M. Cotton[†]

Department of Chemistry, Iowa State University, Ames, Iowa 50011, and Department of Chemistry, Illinois State University, Normal, Illinois 61790

Received: July 26, 1999; In Final Form: November 1, 1999

The electron transfer (ET) scheme of cytochrome *c* (cyt. *c*) coupled to carboxylic acid-terminated alkanethiol self-assembled monolayers (SAMs) on well-defined gold (111) electrodes is a simplified model system to investigate both long range and intermolecular ET processes. The advantages of an electrochemical approach to investigate the ET mechanism are that one can both regulate the ET path length by using alkanethiol SAMs of varying chain lengths and deconvolute the intermolecular ET event at the interface from the intramolecular ET event. It has been shown that the interactions between cyt. *c* and the carboxylate termini are electrostatic in nature, analogous to those between cyt. *c* and negatively charged proteins such as cytochrome *c* peroxidase. In the present work, the effects of alkanethiol chain length, ionic strength, pH, and viscosity of supporting electrolyte on the ET kinetics were studied. The ET rates through long alkanethiol chains were observed to be slow because electron tunneling through the alkyl chain was the rate-limiting step in the process. On the other hand, the ET rate through shorter chain alkanethiols appeared to be independent of chain length, and the effect of ionic strength and pH on the observed ET rates was insignificant. It is proposed that the rate-limiting ET step through short alkyl chains results from a configurational rearrangement process preceding the ET event, and that its rate is 2.6×10^3 s^{-1}. This "gating" process arises from a rearrangement of the cyt. *c* from a stable binding form (binding complex) on the carboxylic acid terminus to a configuration (ET complex) which facilitates the most efficient ET pathway. The rate of the configurational rearrangement reaction that precedes the ET reaction was found to be markedly influenced by solution viscosity, but its equilibrium constant was independent of solution viscosity. The change in the configurational rearrangement reaction rate with solution viscosity follows a modified Kramers equation.

Introduction

Electron transfer (ET) reactions between ET proteins have been extensively studied.[1] The nonuniform charge distributions on the surfaces of protein molecules often lead to multiple configurational states in their coupling. The initial step in the formation of the protein complex is a nonspecific association between the two proteins, followed by rotational diffusion on the molecular surface to reach the proper configuration for the ET event. When the protein couple forms multiple configurations, the intermolecular ET rate is limited by the rotational diffusion of the molecules. This process can be viewed as directional electron-transfer regulated by a "gating mechanism".[2−21] Intermolecular ET rates depend strongly on the ionic strength, pH, and temperature of the solution and are reported to be in the range of $10^3 - 10^5$ s^{-1}.

The electrode reaction of cytochrome *c* (cyt. *c*) through ω-derivatized alkanethiol self-assembled monolayers (SAMs) is considered to be a simplified model system for biological ET processes. Desorption of cyt. *c* immobilized on carboxylic acid-terminated alkanethiol SAMs into supporting electrolyte solutions with low ionic strengths (<50 mM phosphate buffer solution) in the pH range of 6−9 is negligible, and cyt. *c* undergoes a rapid and reversible electron-transfer reaction through the alkanethiol films.[22−28] 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 is 1.5−2.5 pH units more basic than those in the solution phase.[29,30] Electrostatic interactions between the positively charged lysine amino groups on cyt. *c* and the negatively charged carboxylate termini of the SAMs stabilize the binding of cyt. *c*, analogous to its complex with other negatively charged proteins such as the cyt. *c*/cyt. b_5 complex.[1d,15,16,19,21] In solutions of low ionic strength, the observed voltammetric peak current is proportional to the potential sweep rate, indicating that there are no diffusional processes involved in the electrochemical redox reaction.[24,26,27] 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.[24,27] The formal potential of cyt. *c* on the carboxylic acid terminus is + 260 mV (vs. NHE), which is in close agreement with values for cyt. *c* in the solution phase.[33] 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.[34] These combined findings reveal that cyt. *c* immobilized on carboxylic acid-terminated alkanethiol SAMs is present in monolayer quantities and maintains its native structure.

* Corresponding author: Fax: (309) 438-5538, e-mail: kniki@xenon.che.ilstu.edu.
[†] Iowa State University.
[‡] Illinois State University.

10.1021/jp992591p CCC: $19.00
Published on Web 03/07/2000

The advantages of an electrochemical approach to investigate the ET mechanism are that one can both regulate the ET path length by using alkanethiol SAMs of varying chain lengths and deconvolute the intermolecular ET event at the interface from the intramolecular ET event, provided that one can measure ET rate constants up to 10^5 s^{-1}. 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 electrical double layer at the electrode interface limits the measurable ET rate. For well-defined surfaces having geometrical areas of about 1 cm^2, the fastest reliable ET rates that have been reported are around 100 s^{-1}, which are much slower than intermolecular ET rates of protein complexes.[7,17,20,21] The potential-modulated electroreflectance spectroscopy (ER) technique, on the other hand, involves the measurement of a faradaic current as an optical response generated by an ac modulation of the electrode potential.[25] This technique enables one to measure electrode reaction rates up to approximately 10^4 s^{-1} because the effect of double layer charging can be minimized.[25] Advantages of the ER technique over other spectroelectrochemical techniques include the simplicity of the spectroelectrochemical cell configuration and the wide assortment of highly reflective electrode materials available for use. Previous investigations have examined electrode reactions of cyt. *c* on gold electrodes[26−28,35,36] and dyes on glassy carbon or graphite electrodes.[37−42]

It has been shown that the long-range ET rate between metal electrodes and electroactive species decreases with chain length of the SAM.[27,43−48] For these systems, the ET reaction rate constant k_s at the binding site between cyt. *c* and the carboxylate terminus depends on the number of methylene groups n in the alkyl chain:

$$k_s = k_{(n=0)} \exp(-\beta n) \tag{1}$$

where $k_{(n=0)}$ is the apparent ET rate constant extrapolated to $n = 0$.[49] The exponential decay factor β has been found to be 1.09 ± 0.02 per methylene group regardless of the type of redox species at the terminus of alkanethiol SAMs when $n > 10$.[27,43−48] In our previous study, we proposed that the ET process from the electrode to cyt. *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 cyt. *c*, (2) an intermolecular ET process at the interface between cyt. *c* and carboxylic acid terminus, and (3) electron tunneling through the alkanethiol SAM.[27] The ET processes of (1)[50−53] and (3)[27,43−48] have been extensively studied.

Intermolecular ET processes between the positively charged and negatively charged sites of electron transfer protein molecules (such as cyt. *c*/cyt. b_2,[4] cyt. *c*/cyt. *c* peroxidase,[1e,5] cyt. *c*/plastcyanine,[17,20,54,55] and cyt. *c*/cyt. b_5[1d,15,16,19,21] couples) have been investigated in solutions having different ionic strengths, pH, and viscosities. Effects of solution viscosity on the configurational rearrangement reaction (i.e., gated ET reaction) rate between ET protein complexes have been studied theoretically[57,58] and experimentally.[15−17,20,21,54,55,59−61]

The present study has focused on the ET mechanism and kinetics at the interface between cyt. *c* and the carboxylic acid terminus of the SAMs, which were associated through attractive electrostatic interactions. It was expected that the ionic strength and pH of the supporting electrolyte would affect the nature of the electrostatic binding between the protein and the carboxylate moiety. Increasing the concentration of supporting electrolyte leads to weaker electrostatic interactions between cyt. *c* and the carboxylate groups. In addition, the pH of the supporting electrolyte used in this study (between 6 and 9) may affect the stability of the binding of cyt. *c* to the carboxylate terminus of SAM. If a gating process is involved in the ET reaction between cyt. *c* and carboxylate terminus at alkanethiol SAMs, the interfacial electron transfer should also be markedly influenced by changing the solution viscosity.

Theoretical Background

It is assumed that there is a configurational rearrangement of cyt. *c* prior to the ET reaction, from the stable adsorbed structures of cyt. *c* (binding complex) (which are formed upon adsorption of cyt. *c* from the solution phase to the carboxylate termini, and have the lowest free energy of adsorption) to one at which the most efficient ET reaction takes place (ET complex). The electron transfer reaction 3 between the electrode and cyt. *c*, which is immobilized on the carboxylic acid terminated-alkanethiol SAM, follows the preceding configurational rearrangement reaction 2, and in turn is followed by a second configurational rearrangement reaction 4:

$$\text{Cyt. } c \text{ (ox)(I)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Cyt. } c \text{ (ox)(II)} \tag{2}$$

$$\text{Cyt. } c \text{ (ox)(II)} + e^- \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{Cyt. } c \text{ (red)(II)} \tag{3}$$

$$\text{Cyt. } c \text{ (red)(II)} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{Cyt. } c \text{ (red)(I)} \tag{4}$$

where Cyt. *c* (ox)(I) and Cyt. *c* (red)(I) represent the binding complexes, and Cyt. *c* (ox)(II) and Cyt. *c* (red)(II) represent the ET complexes.

We assume that the binding energies of Cyt. *c* (ox)(I) and Cyt. *c* (ox)(II) are equal to those of Cyt. *c* (red)(I) and Cyt. *c* (red)(II), respectively. This assumption is justified on the basis that the binding between the lysine residues and the carboxylate group on the SAM is electrostatic (which is a short-range force) and thus the effect of the oxidation states of cyt. *c* would be insignificant. Therefore, it is reasonable to assume that the equilibria of reactions 2 and 4 are symmetrical and that $k_1 = k_4$ and $k_2 = k_3$.

At the formal potential ($E = E^{\circ\prime}$) of the ET reaction 3,

$$\Gamma_1 + \Gamma_2 = \Gamma_3 + \Gamma_4 \text{ and } \Gamma_1 = \Gamma_4, \Gamma_2 = \Gamma_3 \tag{5}$$

where subscripts 1, 2, 3, and 4 denote the species Cyt. *c* (ox)-(I), Cyt. *c* (ox)(II), Cyt. *c* (red)(II), and Cyt. *c* (red)(I). The electrode potential is modulated at the formal potential by ac with a small amplitude of ΔE_{ac}, and the overpotential is represented by eq 6

$$\eta = E - E^{\circ\prime} = \Delta E_{ac}\, e^{j\omega t} \tag{6}$$

where $j = (-1)^{1/2}$ and ω is angular frequency of modulation. Then, the ac current response is represented by eq 7 as the combination of the real and imaginary components

$$i_{ac}{}^{o}/\Delta E_{ac} = (R_{et} + 1/j\omega C_a)^{-1} \tag{7}$$

where $i_{ac}{}^{o}$ represents the ac current when the electrode potential is modulated by ΔE_{ac} at the formal potential. Consequently, the real and imaginary parts are represented by eqs 8 and 9, respectively:

$$R_{et} = (2RT/\Gamma_t F^2)(1/k_{app}) = 2(RT/\Gamma_t F^2)\{(k_1 + k_2)/k_s k_1\} \{(2k_s + k_1 + k_2)/(k_1 + 2k_2)\} \quad (8)$$

$$1/j\omega C_a = 2(RT/k_s \Gamma_t F^2)\{(k_1 + k_2)/k_s k_1\}\{k_s(k_1 + k_2)/j\omega(k_1 + 2k_2)\} \quad (9)$$

Equation 1 can be modified by taking into account the ET resistance, which occurs at the interface between cyt. *c* and the carboxylate terminus

$$k_s = k_{(n=0,r=r_0)} \exp(-n\beta) \exp\{-(r - r_o)\beta_r\} \quad (10)$$

where $k_{(n=0,r=r_0)}$ represents the ET rate constant between the heme edge of cyt. *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 cyt. *c* and carboxylic acid terminus, r_o represents that at zero ionic strength, and β_r represents the exponential decay factor at the interface.

When the ET rate is independent of the alkyl chain length (through short alkyl chains), $k_s \gg k_1$, k_2 and $k_1 \gg k_2$

$$k_{app} = k_1/2 \quad (11)$$

When the ET rate is limited by the ET resistance through the alkyl chain (through long alkyl chains), k_1, $k_2 \gg k_s$, and therefore

$$\ln k_{app} = \ln\{1 + 2(k_2/k_1)\} - 2\ln(1 + k_2/k_1) + \ln k_{(n=0)} - n\beta - (r - r_o)\beta_r \quad (12)$$

Evaluation of Electron Transfer Kinetic Parameters

The frequency dependence of the ER response from the electrode, on which the redox species is immobilized and at which the redox reaction is taking place, in the frequency domain is represented by eq 13 [25]

$$-\omega \cot\phi = (1 - \omega^2 R_s R_{ct} C_a C_d)/(R_{ct} C_a + R_s C_a + R_s C_d) \quad (13)$$

where $\cot\phi$ is the ratio of the magnitudes of the real and imaginary components of of the ER response, ω is the angular frequency of the potential modulation, R_{ct} and C_a are the charge transfer resistance and capacitance, and R_s and C_d are the solution resistance and double layer capacitance. From eq 13, the values of R_{ct} and C_a for the redox reaction at the electrode surface can be evaluated from the slope and y-intercept at $-\omega^2 = 0$ in the plot of $-\omega \cot\phi$ vs $-\omega^2$.

Ruzgas et al. proposed a different approach to evaluate the kinetic parameters of cyt. *c* immobilized on carboxylic acid-terminated alkanethiol-modified electrodes using the ER technique.[62] The ET rates they obtained together with cyclic voltammetry and ac impedance techniques were too small in comparison to Gray's prediction.[50−53] The rates they evaluated from their ER data according to our theory correspond to the rate of surface diffusion given by eq 2.

Experimental Methods

Chemicals. Horse heart cyt. *c* (type VI) was obtained from Sigma Chemical and purified chromatographically.[63] 3-Mercaptopropionic acid and 11-mercaptoundecanoic acid were purchased from Aldrich Chemical and used as received. 5-Mercaptopentanoic acid, 6-mercaptohexanoic acid, 7-mercaptoheptanoic acid, 8-mercaptooctanoic acid, 10-mercaptodecanoic acid, and 12-mercaptododecanoic acid were synthesized and purified in our laboratory using standard literature methods.[64] Ethanol used as the solvent for monolayer depositions was of punctilious grade. Water was purified with a Milli-Q system (Millipore Corp.) and had a nominal resistivity of 18 MΩ•cm. ACS reagent grade disodium hydrogen phosphate, potassium dihydrogen phosphate, and sucrose were used as received. A 6 M aqueous sodium hydroxide solution was used to adjust the pH of the phosphate buffer solutions in the pH range from 6 to 9 in 10 mM phosphate buffer solution.

Substrate Preparation. Electrodes were prepared by resistive evaporation of 325 nm of Au onto freshly cleaved mica slides. The gold depositions were carried out in an Edwards model E306A coating system with all depositions occurring at a rate of 0.2 nm s^{-1} at a base pressure of 10^{-6} Torr. Upon removal from the coating system, all Au/mica slides were annealed in a muffle furnace at 300−400 °C for 5 h. After cooling, the Au/mica slides were stored in a clean desiccator until use. Monolayers of carboxylic acid-terminated alkanethiols were prepared by immersing the Au/mica electrodes into 1 mM ethanolic solutions for a minimum of 15 h. The SAM-modified electrodes were thoroughly rinsed with ethanol and Milli-Q water upon removal from the alkanethiol deposition solutions to eliminate excess alkanethiol. After positioning the SAM-modified electrodes in the ER cell, cyt. *c* was immobilized on the alkanethiol SAMs by exposing the electrodes to an approximately 50 μM cyt. *c* solution (pH 7, 10 mM phosphate buffer) at open circuit for 45 min at 25 °C. The ER cell was then rinsed with fresh phosphate buffer solution to remove any excess cyt. *c* from the electrode surface. The ER cell was then filled with deaerated phosphate buffer solution of the desired ionic strength or pH to be studied.

Electrochemical Measurements. Electrochemical measurements were made at the SAM-modified Au (111) electrodes in the ER cell using a BAS 100 electrochemical system. Potential sweep voltammetry and the ac impedance method were used to determine the solution resistance R_s and double layer capacitance C_d. A coiled gold wire served as the counter electrode, and a KCl-saturated Ag/AgCl electrode (+ 0.199 V vs NHE) served as the reference. The electrolyte solutions were deaerated with Ar bubbling for >20 min, and all spectroelectrochemical measurements were made at 25 °C.

ER Instrumentation. The instrumentation used for the ER measurement is described elsewhere.[65,66] The potential of the working electrode was controlled by an EG&G Instruments (Princeton Applied Research) model 362 scanning potentiostat. A BK Precision model 4010 2-MHz function generator was used to apply a sinusoidal ac signal to the potentiostat for the required ac modulation. Data processing was achieved using Slidewrite and Spectra Solve software.

Raman Measurements. Roughened noble metal electrodes for surface enhanced resonance Raman scattering (SERRS) spectroscopy were prepared from an Ag rod electrode sheathed in a Teflon rod. The electrodes were rinsed with purified water and absolute ethanol prior to the deposition of the alkanethiol SAMs. The Raman experimental configuration has been detailed elsewhere.[67] A Pyrex flow cell, which allowed the necessary electrolyte solutions to be easily changed while minimizing the absorption of atmospheric oxygen, was employed to collect the SERRS spectra. No external potential was applied to the electrodes for the SERRS measurements.

Experimental Results

Electrochemical Measurements. Cyclic voltammograms (CVs) of cyt. *c* immobilized on carboxylic acid-terminated alkanethiol/Au films were found to be stable and reproducible

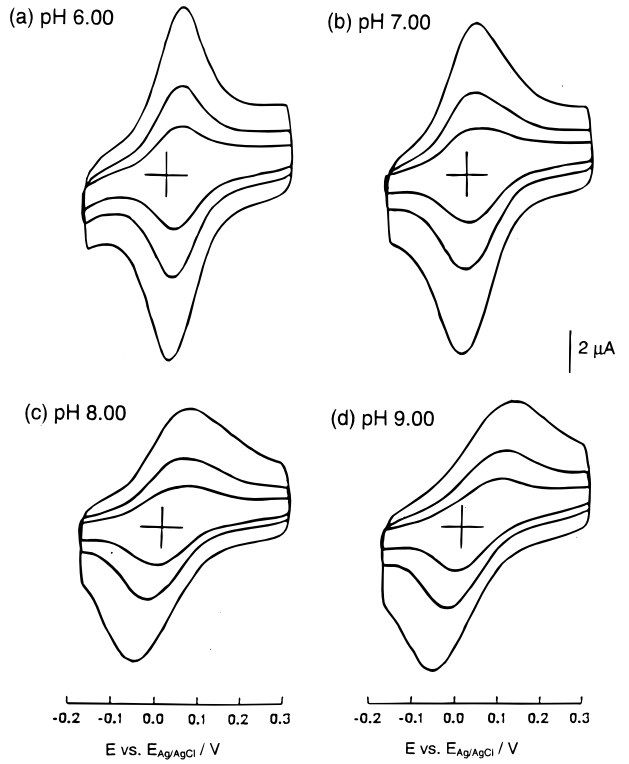

Figure 1. Cyclic voltammograms of cyt. *c* immobilized on an $HO_2C(CH_2)_{11}$−SH modified Au electrode in 10 mM phosphate buffer at various pHs: (a) 6.00; (b) 7.00; (c) 8.00; (d) 9.00. The potential sweep rates are 50, 100, and 200 mV s^{-1}. The electrode potentials are measured against Ag/AgCl in saturated KCl.

over a wide range of carbon chain lengths and phosphate buffer solutions. The formal potential of cyt. *c* did not change in this pH range, but the peak potential separation of the CVs increased with pH when longer chain alkanethiol SAMs were used. The amount of cyt. *c* immobilized on these SAMs was evaluated to be $(12-13) \times 10^{-12}$ mol cm^{-2} by potential sweep voltammetry, $(10-17) \times 10^{-12}$ mol cm^{-2} by ac impedance,[24] and $(10.5-13.5) \times 10^{-12}$ mol cm^{-2} by ER measurements.[26−28] These values agreed well with the calculated monolayer coverage, which was estimated from the crystallographic data to be 13×10^{-12} mol cm^{-2}.[68] As an example, Figure 1 shows CVs of cyt. *c* immobilized on an $HO_2C(CH_2)_{11}SH$-modified Au electrode at various pHs (in 10 mM phosphate buffer).

These CVs are comparable with previously reported literature results.[24,27,28] The double-layer capacity C_d determined from different SAM-modified Au electrodes varied depending on the alkanethiol chain length and were in the range of 3 to 10 μF cm^{-2}. Monolayers of 3-mercaptopropionic acid gave C_d values in the 10 $\mu F \cdot cm^{-2}$ range, while monolayers of longer carboxylic acid terminated alkanethiols yielded C_d values in the 3 to 7 μF cm^{-2} range. These values are consistent with previously reported results.[48]

Electroreflectance Measurements. In electrolyte solutions of lower phosphate concentrations (<60 mM), the ER spectra measured at 425 nm were stable and reproducible over a period of several hours. The lower power densities of the Xe lamp, as compared to a laser, were believed to have contributed to the stability of the ER signal because of lower sample degradation. Examples of typical ER voltammograms are shown in Figure 2. Curve a in Figure 2 shows a background ER voltammogram of a $HO_2C(CH_2)_7$−SH SAM-modified Au electrode in 10 mM phosphate buffer (pH 7) with 20 mV ac modulation (20 Hz) taken at the $E^{\circ\prime}$ of cyt. *c* immobilized on the SAM. The ER

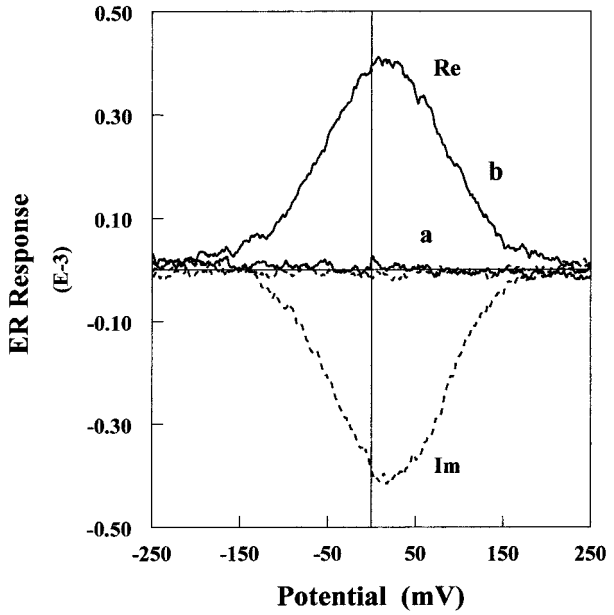

Figure 2. ER voltammograms on $HO_2C(CH_2)_7$−SH modified Au electrodes. Conditions: 10 mM, pH 7 phosphate buffer, 20 mV ac modulation at 20 Hz. (a) SAM modified electrode before cyt. *c* deposition, (b) SAM modified electrode after cyt. *c* deposition. The electrode potentials are measured against Ag/AgCl in saturated KCl.

background was flat, indicating no electrochemical reactions were occurring. Curves b (Re and Im) in Figure 2 show the same modified Au electrode after cyt. *c* adsorption. The optical response peaks seen are the real (Re) and imaginary (Im) components of the reflected light at the $E^{\circ\prime}$ of cyt. *c*. The peak potentials of both components were identical while the band shapes were symmetrical about the peak position in the modulation frequency range used in the present studies, suggesting that the ET reaction is reversible and that its transfer coefficient $\alpha \approx 0.5$.[70]

Effect of Ionic Strength on the Electron Transfer Reaction Rate of Cyt. *c*. The effect of ionic strength of supporting electrolyte on the ET rates of cyt. *c* were investigated in phosphate buffer solutions at pH 7.0; the results are shown in Figure 3 for carboxylic acid-terminated alkanethiols with 2, 5, 7, and 10 methylene units. Two regions appeared in the plot of $\log(k_{app})$ vs square root of ionic strength $\sqrt{I}$. In the first region, the ET rate constants were nearly independent of the concentration of supporting electrolyte up to 10 mM ($\sqrt{I} = 4.7$ $mM^{1/2}$), indicating that electrostatic interaction between the binding sites remained nearly constant in dilute solutions. In the second region, $\log(k_{app})$ decreased proportionally to $\sqrt{I}$ in the solutions having $\sqrt{I} > 5$ $mM^{1/2}$. The plots of $\log k_{app}$ vs $\sqrt{I}$ for 7 and 10 methylene units are nearly parallel when $\sqrt{I} > 8$ $mM^{1/2}$. At phosphate concentrations above 60 mM ($\sqrt{I} = 11.4$ $mM^{1/2}$), cyt. *c* tended to desorb from the surface of the SAM.

Figure 4 displays the plots of $\log k_{app}$ vs number of methylene groups in terms of the concentration of supporting electrolyte. The k_{app} values at the plateau were independent of both chain length and ionic strength; however, the extrapolated values to $n = 0$ depended on the ionic strength of the solution.

Effect of pH on the Electron Transfer Reaction Rates. The pH dependence of the ET reaction rate of cyt. *c* through alkanethiol SAMs with different chain lengths in the pH range 6.0−9.0 is shown in Figure 5, where alkanethiols with $n = 4$, 6, 7, and 11 were used. The ET rate constant through short-chain alkanethiols is independent of pH (6−9) as in the case of ionic strength (Figure 4). It is apparent in Figure 5 that the values

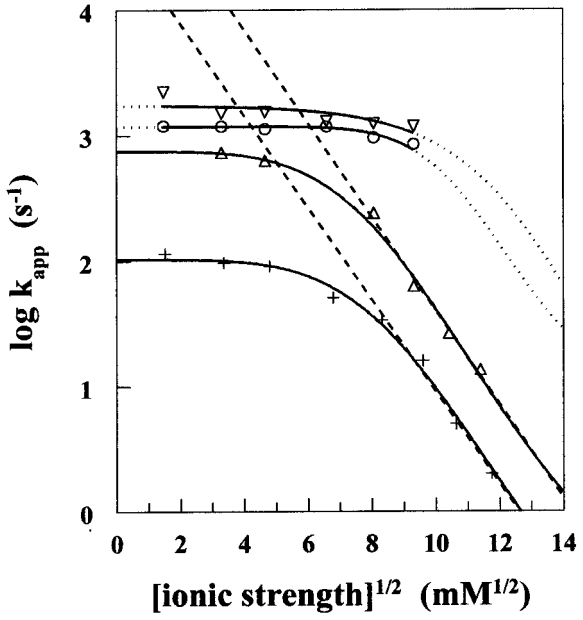

Figure 3. Log k_{app} vs $\sqrt{I}$ of cyt. *c* on $HO_2C(CH_2)_n-SH$ SAMs on Au: (+) $n = 10$, (△) $n = 7$, (○) $n = 5$, (▽) $n = 2$.

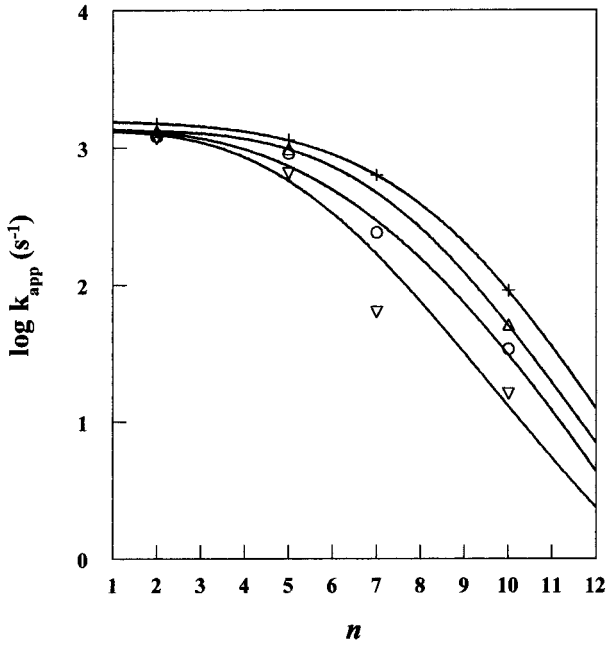

Figure 4. Log k_{app} vs number of methylene groups n of different alkanethiols on Au. Ionic strength of phosphate buffer solutions (at pH 7): (+) 21.6 mM, (△) 43.3 mM, (○) 64.9 mM, (▽) 86.6 mM.

of k_{app} extrapolated to $n = 0$ depended on the solution pH, and that the ET rate decreased with increasing pH as expected from the CV experiments.

Viscosity Effects on the Electron Transfer Rate of Cyt. *c*. The viscosity of electrolyte solutions was adjusted by the addition of sucrose, the use of which is advantageous because it does not affect the solubility of cyt. *c* in aqueous solutions. In addition, the formal potential of cyt. *c*, which is very sensitive to the protein folding of cyt. *c*, is unaffected by the addition of sucrose. As a further verification of the conformation of the heme moiety, cyt. *c* (immobilized on SAM of $HOOC(CH_2)_4-S-Ag$) in the presence of sucrose was investigated by SERRS with 413.1 nm excitation. Previous investigations have indicated that cyt. *c* adsorbed on alkanethiol SAMs maintains its native structure.[34] Exposure of the $HO_2C(CH_2)_4-SH$ SAM-modified

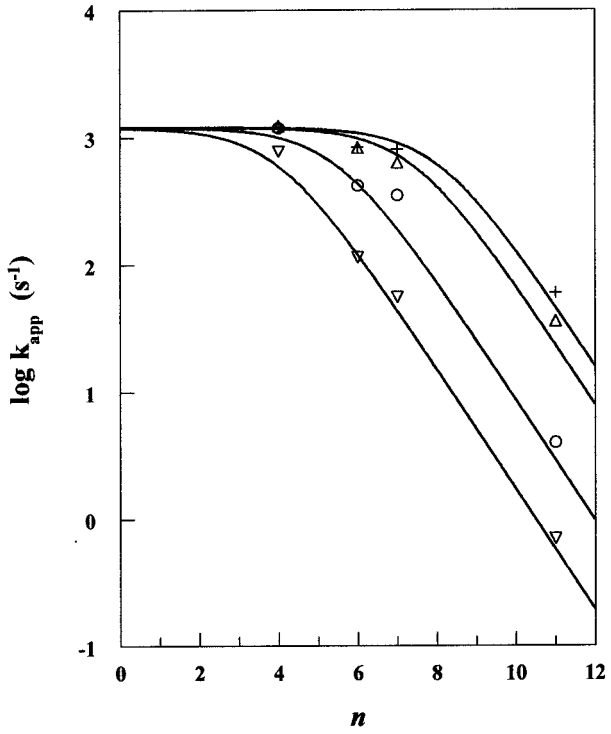

Figure 5. Log k_{app} vs number of methylene groups n in 10 mM phosphate buffer solution at various pHs: (+) pH 6, (△) pH 7, (○) pH 8, (▽) pH 9.

Ag electrode to cyt. *c* in solutions without sucrose yielded spectra exhibiting SERRS bands at 1369 and 1631 cm^{-1}, which are the spin state marker bands for cyt. *c*. These SERRS bands indicated that cyt. *c* existed in a six-coordinate low-spin state. The positions of these bands also indicated that the cyt. *c* existed in a mixed oxidation state with the heme group exhibiting both the Fe(II) and Fe(III) states. The same spin state marker bands were visible in cyt. c solutions containing sucrose (46 wt %), suggesting that the added sucrose did not alter the structures of the heme moiety of cyt. *c* on the SAM-modified silver electrode. These results also clearly imply that the immobilized cyt. *c* retains its native state in the presence of sucrose.

Figure 6 depicts the relationship between log k_{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. These rates tended to level off as the viscosity approached 5 cP, and remained virtually constant up to $\eta = 10.3$ cP (46 wt % of sucrose). For the SAM-modified electrodes having $n = 10$, a rather small ET reaction rate change was observed as the sucrose concentration was increased, and appeared to be nearly independent of viscosity.

Discussion

Effect of Ionic Strength on the Electron Transfer Reaction Rate of Cyt. *c*. The results of the ionic strength on the ET rate suggest that as the phosphate concentration increases, electrostatic interaction between the binding sites is shielded by the formation of compact ionic atmospheres and becomes weaker (i.e., the ET distance between the binding sites may increase). The ET rate (log k_{app}) decreases with the distance between the binding sites r given by the last term of eq 12, which is proportional to $\sqrt{I}$ of the solution. On the other hand, the ET rates through short alkanethiol chains were independent of the ionic strength of the solution. These results suggest that at the

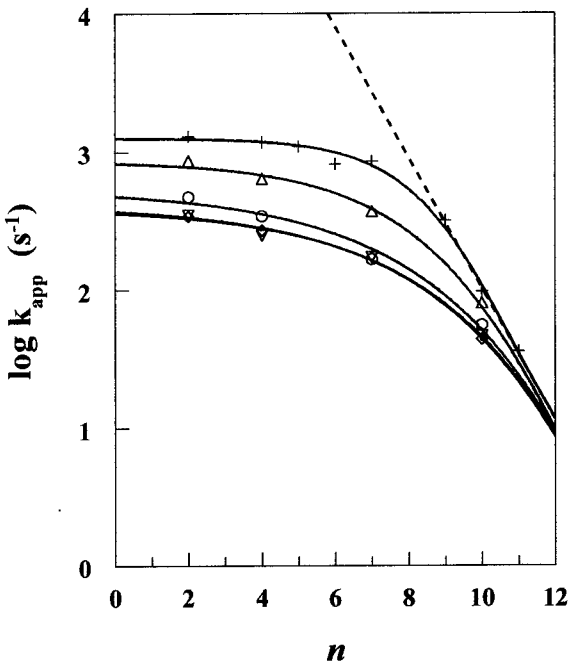

Figure 6. Dependence of ET reaction rate k_{app} on alkanethiol chain length at different solution viscosities: (+) $\eta = 1.002$ cP (no sucrose), (△) $\eta = 1.790$ cP, (○) $\eta = 3.762$ cP, (▽) $\eta = 5.315$ cP, (◇) $\eta = 10.310$ cP. The dashed line represents the ET rate in the absence of sucrose (including extrapolated values to short chain length).

plateau, the ET rate is limited by the forward reaction rate k_1 of the preceding configurational rearrangement reaction represented by eq 2, and that this rate constant at the plateau is estimated to be $k_1 = 2.6 \times 10^3$ s^{-1}. The present results are in accord with intermolecular ET rates of protein complexes.[4]

Effect of pH on the Electron Transfer Reaction Rates. In solutions of lower pH, the degree of deprotonation of carboxylic acid is less, such that the reverse reaction rate constant k_2 for the rearrangement reaction of cyt. *c* may be small; this would thereby decrease the value of k_2/k_1 in eq 12 for the rearrangement reaction. The sum of the first and second terms of eq 12 decreases with increasing pH, whereas the third term is constant, and the fourth term is proportional to the chain length. The fourth term should also be constant with pH if the ionic strength of the solution remains constant. We have previously reported that the ET rate to cyt. *c* through alkanethiol SAMs increases when the carboxylic acid-terminated alkanethiol is diluted by methyl-terminated ones.[28] This dilution effect results in a decrease in the number of negatively charged groups at the surface, thereby reducing k_2.

Viscosity Effects on the Electron Transfer Rate of Cyt. *c*. 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.[57] The theoretical equation was modified by Ansari et al. for application to experimental data[58]

$$k = \{C/(\sigma + \eta)\} \exp(-\boldsymbol{E}_o/RT) \qquad (14)$$

where $\boldsymbol{E}_o$ is the average height of the potential energy barrier separating the protein configurations, and η is the solvent viscosity. C and σ, 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 $\boldsymbol{E}_o$ is independent of the solution composition (viscosity), eq 14 can be rewritten as

$$1/k = \mathrm{A}(\eta/\eta_o) + \mathrm{B} \qquad (15)$$

Regression analysis of the ET rates through mercaptopropionic acid (i.e., $n = 2$ in Figure 6) SAMs in various sucrose-containing solutions, which correspond to the forward reorganization reaction rate $k_1 = 2\ k_{app}$, was accomplished using eq 15, and the result is given by the following equation with a correlation coefficient of 1.000:

$$1/k_{app} = 2.83 \times 10^{-4}(\eta/\eta_o) + 4.84 \times 10^{-4} \qquad (16)$$

For mercaptopropionic acid at viscosities above 5 cP, the change in the ET reaction rate leveled off and the experimental data were not easily fit by eq 16. This may be due to a change in the ET reaction scheme in highly viscous media in comparison to those without sucrose.

As can be seen from Figure 6, the apparent ET rate constant of cyt. *c* through longer alkanethiol chains were nearly independent of solution viscosity within the range studied. Since the effect of viscosity on the forward and backward reaction rate constants of reaction 2 can be expressed by eq 14, the ratio k_1/k_2 would be independent of the solution viscosity provided that the denominator of eq 14 depends only on the viscosity (both $\boldsymbol{E}_o$ and σ are independent of viscosity). In addition, the $(r - r_o)\beta_r$ term in eq 12 is anticipated to be independent of solution viscosity at constant ionic strength. Therefore, it is reasonable to conclude that the ET rates through longer alkanethiol SAMs can be adequately described by eq 12 regardless whether 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).[20] 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.

The Binding Site of Cyt. *c* to Carboxylate Terminus. It has been shown that the interaction between cyt. *c* and the carboxylic acid terminus of the SAM is electrostatic in nature. It is possible to elucidate the binding site of cyt. *c* (II) from the extrapolated value k_s from eq 12 to $n = 0$ at low ionic strength, the value of which is about 5×10^6 s^{-1}. The ET rates from the heme edge to various lysine residues on the surface of cyt. *c* can be estimated from the results of Gray and Winkler[53−56] (the intramolecular ET rates from the heme edge to lysine 13 is estimated to be 2×10^5 s^{-1} and from the heme edge to lysine 79 is also 2×10^5 s^{-1}). It has been reported that there is hydrogen bonding between the $-NH_2$ terminus of lysine 79 and serine 47; consequently, lysine 79 could not be a binding site to the carboxylate terminus of the SAM.[71] Therefore, lysine 13 appears to be the most probable site to form an ET complex with the carboxylate terminus of alkanethiol SAMs.

Gated Electron Transfer at the Binding Site of Cyt. c to the Carboxylate Terminus. Conformational studies of cyt. *c* on carboxylic acid-terminated SAMs by spectroscopic techniques have revealed that a macroscopically ordered film of adsorbed cyt. *c* molecules is formed.[72] The asymmetric distribution of positive charges (lysine residues) on cyt. *c* gives rise to a preferential adsorption geometry with a narrow orientation distribution. It is proposed here that the interaction between cyt. *c* and the carboxylic acid terminus is electrostatic in nature and

that 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 facilitates the most efficient ET pathway. That is, the ET reaction takes place whenever lysine 13 directly interacts with the carboxylic acid terminus of alkanethiol SAMs regardless of their chain length. The bindings between other lysine residues, which are located in the vicinity of the heme crevice, and the carboxylic acid termini are very unlikely to be ET pathways.

Conclusions

The UV−vis electroreflectance spectroscopic technique was applied to elucidate the ET mechanism between cyt. *c* and Au(111) electrodes through carboxylic acid-terminated alkanethiol SAMs. The ET reaction rate depends on the chain length of the alkanethiol as expected from Marcus theory. A "gating mechanism" involving a configurational rearrangement process of cyt. *c* prior to the ET event has been proposed to account for the ET reaction mechanism at the interface between cyt. *c* and carboxylate groups of the SAM on the electrode. The solution ionic strength plays an important role in the ET reaction rate. In lower ionic strength solutions, the apparent ET rate constant remains nearly constant; in contrast, for higher solution ionic strengths, the ET rate constant decreases with the square root of ionic strength. This decrease in ET reaction rate is attributed to screening of electrostatic interactions between binding sites, thereby increasing the distance between the lysine residues of cyt. *c* and the carboxylic acid terminus. The negative charge density on the SAM surface depends on solution pH, and the pH dependence of the ET reaction rate is attributed to changes in the rearrangement reaction rate of cyt. *c* on the SAM. Based on the extrapolated value of $k_{(n=0,r=r_0)}$ from the present work, we conclude that lysine 13 provides the most efficient ET pathway to the carboxylate terminus of alkanethiol SAMs.

ET reaction rates through shorter alkanethiol SAMs are strongly influenced by the solution viscosity and are limited by the configurational rearrangement reaction rates (k_1 and k_2). However, the ratio of the forward and reverse reaction rate constants of the configurational rearrangement reaction (k_1/k_2) is independent of the solution viscosity.

Acknowledgment. This work was supported by National Institutes of Health (Grant GM 35108-15) for A.A. and by the New Technology and Industrial Technology Development Organization (NEDO), Japan for K.N. We thank Professors R. Marcus (California Institute of Technology), M. Newton (Brookhaven National Laboratory), H. Sato (Yokohama National University), and T. Sagara (Nagasaki University) for their valuable discussions. We also thank Prof. M. Porter (Iowa State University) for providing the Edwards coating system.

Supporting Information Available: Derivation of gated electron transfer of cytochrome *c* in a model system. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) (a) Moser, C. C.; Page, C. C.; Farid, R.; Dutton, P. L. *J. Bioenerg. Biomembr.* **1995**, *27*, 263, and references therein. (b) Tollin, G. *J. Bioenerg. Biomembr.* **1995**, *27*, 303, and references therein. (c) Mauk, A. G.; Mauk, M. R.; Moore, G. R.; Northrup, S. H. *J. Bioenerg. Biomembr.* **1995**, *27*, 311, and references therein. (d) Durham, B.; Fairris, J. L.; McLean, M.; Millett, F.; Scott, J. R.; Sligar, S. G.; Willie, A. *J. Bioenerg. Biomembr.* **1995**, *27*, 331, and references therein. (e) Millett, F.; Miller, M. A.; Geren, L.; Durham, B. *J. Bioenerg. Biomembr.* **1995**, *27*, 341, and references therein.

(2) (a) Hoffman B. M.; Ratner M. A. *J. Am. Chem. Soc.* **1987**, *109*, 6237. (b) Hoffman B. M.; Ratner M. A. *J. Am. Chem. Soc.* **1988**, *110*, 8267.

(3) Hazzard, J. T.; Poulos, T. L.; Tollin, G. *Biochemistry* **1987**, *26*, 2836.

(4) McLendon, G.; Pardue, K.; Bak, P. *J. Am. Chem. Soc.* **1987**, *109*, 7540.

(5) Hazzard, J. T.; McLendon, G.; Cusanovich, M.; Tollin, G. *Biochem. Biophys. Res. Commun.* **1988**, *151*, 429.

(6) Moser, C. C.; Dutton, P. L. *Biochemistry* **1988**, *27*, 2450.

(7) McLendon, G. *Acc. Chem. Res.* **1988**, *21*, 160.

(8) Brunschwig, B. S.; Sutin, N. *J. Am. Chem. Soc.* **1989**, *111*, 7454.

(9) Eltis, L. D.; Herberty, R. G.; Barker, P. G.; Mauk, A. G.; Northrup, S. H. *Biochemistry* **1991**, *30*, 3663.

(10) Feitelson, J.; McLendon, G. *Biochemistry* **1991**, *30*, 5051.

(11) Nocek, J. M.; Stemp, E. D. A.; Finnegan, M. G.; Koshy, T. I.; Johnson, M. K.; Margoliash, E.; Mauk, A. G.; Smith, M.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 6822.

(12) Waker, M. C.; Tollin, G. *Biochemistry* **1991**, *30*, 5546.

(13) Sullivan, E. P., Jr.; Hazzard, J. T.; Tollin, G.; Enemark, J. H. *J. Am. Chem. Soc.* **1992**, *114*, 9662.

(14) Waker, M. C.; Tollin, G. *Biochemistry* **1992**, *31*, 2798.

(15) Willie, A.; Stayton, P.; Sligar, S. G.; Durham, B.; Millett, F. *Biochemistry* **1992**, *31*, 7237.

(16) Willie, A.; McLean, M.; Liu, R.-Q.; Hilgen-Willis, S.; Saunders, A. J.; Pielak, G. J.; Sligar, S. G.; Durham, B.; Millett, F. *Biochemistry* **1993**, *32*, 7519.

(17) Zhou, J. S.; Kostić, N. M. *J. Am. Chem. Soc.* **1992**, *114*, 3562.

(18) Andrew, S. M.; Thomasson, K. A.; Northrup, S. H. *J. Am. Chem. Soc.* **1993**, *115*, 5516.

(19) Northrup, S. H.; Thomasson, K. A.; Miller, C. M. *Biochemistry* **1993**, *32*, 6613.

(20) Zhou, J. S.; Kostić, N. M. *J. Am. Chem. Soc.* **1993**, *115*, 10796.

(21) Ling, Q.; Kostić, N. M. *Biochemistry* **1994**, *33*, 12592.

(22) Tarlov, M. J.; Bowden, E. F. *J. Am. Chem. Soc.* **1991**, *113*, 1847.

(23) Collinson, M.; Bowden, E. F.; Tarlov, M. J. *Langmuir* **1992**, *8*, 1247.

(24) Song, S.; Clark, R. A.; Bowden, E. F.; Tarlov, M. J. *J. Phys. Chem.* **1993**, *97*, 6564.

(25) Feng, Z. Q.; Sagara, T.; Niki, K. *Anal. Chem.* **1995**, *67*, 3564.

(26) Feng, Z. Q.; Imabayashi, S.; Kakiuchi, T.; Niki, K. *J. Electroanal. Chem.* **1995**, *394*, 149.

(27) Feng, Z. Q.; Imabayashi, S.; Kakiuchi, T.; Niki, K. *J. Chem. Soc., Faraday Trans.* **1997**, *93*, 1367.

(28) Arnold, S.; Feng, Z. Q.; Kakiuchi, T.; Knoll, W.; Niki, K. *J. Electroanal. Chem.* **1997**, *438*, 91.

(29) Simmons, N. J.; Zak, J.; Wu, C. C.; Walczak, M. M.; Porter, M. D. Presented at the 205th ACS National Meeting in March, 1993 (Denver, CO), private communication.

(30) Sugihara, K.; Shimazu, K. Presented at the Annual Meeting of the Electrochemistry Society of Japan, October, 1998 (Nagaoka, Japan), private communication.

(31) Margoliash, E.; Bosshard, H. R. *Trends Biochem. Soc.* **1983**, *8*, 316.

(32) Pettigrew, G. W.; Moore, G. R. in *Cytochrome c*: *Biological Aspects*, Springer-Verlag: Berlin: 1987; pp 29−111, and references therein.

(33) Heineman, W. R.; Norris, B. J.; Goelz, J. F. *Anal. Chem.* **1975**, *47*, 79.

(34) a) Hobara, D.; Niki, K.; Cotton, T. M. *Denki Kagaku* **1993**, *61*, 776. (b) Hobara, D.; Niki, K.; Zhou, C.-L.; Chumanov, G.; Cotton, T. M. *Colloids Surf. A*: *Physicochem. Eng. Aspects* **1994**, *93*, 241.

(35) Hinnen, C.; Parsons, R.; Niki, K. *J. Electroanal. Chem.* **1983**, *147*, 329.

(36) Hinnen, C.; Niki, K. *J. Electroanal. Chem.* **1989**, *264*, 157.

(37) Sagara, T.; Murakami, H.; Igarashi, S.; Sato, H.; Niki, K. *Langmuir* **1991**, *7*, 3190.

(38) Sagara, T.; Iizuka, J.; Niki, K. *Langmuir* **1992**, *8*, 1018.

(39) Sagara, T.; Takagi, S.; Niki, K. *J. Electroanal. Chem.* **1993**, *349*, 159.

(40) Wang, H.-X.; Sagara, T.; Sato, H.; Niki, K. *J. Electroanal. Chem.* **1992**, *331*, 925.

(41) Sagara, T.; Niki, K. *Langmuir* **1993**, *9*, 831.

(42) Sagara, T.; Wang, H.-X.; Niki, K. *J. Electroanal. Chem.* **1994**, *364*, 285.

(43) Becka, A. M.; Miller, C. J. *J. Phys. Chem.* **1992**, *96*, 2657.

(44) Finklea, H. O.; Hanshew, D. D. *J. Am. Chem. Soc.* **1992**, *114*, 3173.

(45) Xu, J.; Li, H.-L.; Zhang, Y. *J. Phys. Chem.* **1993**, *97*, 11497.

(46) Smalley, J. F.; Feldberg, S. W.; Chidsey, C. E.; Linford, M. R.; Newton, M. D.; Liu, Y. P. *J. Phys. Chem.* **1995**, *99*, 13141.

(47) Finklea, H. O.; Liu, L.; Ravenscroft, M. S.; Punturi, S. *J. Phys. Chem.* **1996**, *100*, 18852.

(48) Finklea, H. O. In *Electroanalytical Chemistry*; Bard, A. J., Rubinstein, I., Eds.; Dekker: New York, 1996, Vol. 19, pp 110−335.

(49) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265, and references therein.

(50) Wuttke, D. S.; Bjerrum, M. J.; Chang, I.-Jy; Winkler, J. R.; Gray H. B. *Biochim. Biophys. Acta* **1992**, *1101*, 168.

(51) (a) Wuttke, D. S.; Bjerrum, M. J.; Winkler, J. R.; Gray H. B. *Science* **1992**, *256*, 1007. (b) Langen, R.; Chang, I.-J.; Germanas, J. P.; Richards, J. H.; Winkler, J. R.; Gray, H. B. *Science* **1995**, *268*, 1733. Langen, R.; Colón, J. L.; Casimiro, D. R.; Karpishin, T. B.; Winkler, J. R.; Gray, H.B. *J. Bioinorg. Chem*. **1996**, *1*, 221. Gray, H. B.; Winkler, J. R. *J. Electroanal. Chem*. **1997**, *438*, 43.

(52) Bjerrum, M. J.; Casimiro, D. R.; Chang, I.-J.; Di Bilio, A. J.; Gray, H. B.; Hill, M. G.; Langen, R.; Mines, G. A.; Slov, L. K.; Winkler, J. R.; Wuttke, D. S. *Bioenerg. Biomembr*. **1995**, *27*, 295, and references therein.

(53) (a) Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369, and references therein. (b) Gray, H. B.; Winkler, J. R. *Annu. Rev. Biochem*. **1996**, *65*, 537, and references therein.

(54) Zhou, J. S.; Kostic, N. M. *Biochemistry* **1993**, *32*, 4539.

(55) Crnogorac, M. M.; Shen, C.-Y.; Young, S.; Hansson, O.; Kostic, N. M. *Biochemistry* **1996**, *35*, 16465.

(56) Harris, M. R.; Davis, D. J.; Durham, B.; Millett, F. *Biochim. Biophys. Acta* **1997**, *1319*, 147.

(57) Kramers, H. A. *Physica (Utrecht)* **1940**, *7*, 284.

(58) Ansari, A.; Jones, C. M.; Henry, E. R.; Hofrichter, J.; Eaton, W. A. *Science* **1992**, *256*, 1796.

(59) Zhou, J. S.; Kostić, N. M. *Biochemistry* **1992**, *31*, 7543.

(60) Qin, L.; Kostić, N. M. *Biochemistry* **1994**, *33*, 7543.

(61) Ivković-Jensen, M. M.; Kostić, N. M. *Biochemistry* **1997**, *36*, 8135.

(62) Ruzgas, T.; Wong, L.; Gaigalas, A. K.; Vilker, V. L. *Langmuir* **1998**, *14*, 7298.

(63) Brautigan, D. L.; Fergason-Miller, S.; Margoliash, E. *Methods Enzymol*. **1978**, *53*, 128.

(64) Troughton, E. B.; Bain, C. D.; Whitesides, G. H.; Nuzzo, R. G.; Allara, D. L.; Porter, M. D. *Langmuir* **1988**, *4*, 365.

(65) (a) Kolb, D. M. In *Spectroelectrochemistry: Theory and Practice*; Gale, R. J., Ed.; Plenum: New York, 1988; pp 78−188. (b) Plieth, W. In *Spectroscopic and Diffraction Techniques in Interfacial Electrochemistry*; Gutierrez, C., Melendres, C., Eds.; Kluwer Academic Publishers: The Netherlands, 1990; pp 223−260. (c) Plieth, W.; Kozlowski, W.; Twomey, T. In *Adsorption of Molecules at Metal Electrodes*; Lipkowski, J., Ross, P. N., Eds.; VCH: New York, 1992; pp 239−284.

(66) (a) Sagara, T.; Igarashi, S.; Sato, H.; Niki, K. *Langmuir* **1991**, *7*, 1005. (b) Sagara, T. *Recent Res. Devel. Phys. Chem*. **1998**, *2*, 159, and references therein.

(67) Sibbald, M. S.; Chumanov, G.; Cotton, T. M. *J. Electroanal. Chem*. **1997**, *438*, 179.

(68) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol*. **1990**, *214*, 585.

(69) Ulman, A. *Ultrathin Organic Films*; Academic Press: San Diego, 1991; pp 237−338.

(70) The peak potentials of the real and imaginary parts shown in Figure 2 are almost identical suggesting that $\alpha \approx 0.5$ in the frequency range up to 1 kHz.[66a]

(71) Brayer, G. D.; Murphy, M. E. P. In *Cytochrome c*: *Multidisciplinary Approach*; Scott, R. A., Mauk, A. G., Eds.; University Science Books: Sousalito, CA, 1996; pp 103−166.

(72) Edmiston, P. L.; Lee, J. E.; Cheng, S.-S.; Saavedra, S. S. *J. Am. Chem. Soc.* **1997**, *119*, 560.