Kinetic Parameters for Cytochrome *c* via Insulated Electrode Voltammetry

Samuel Terrettaz, Jun Cheng, and Cary J. Miller*
Department of Chemistry and Biochemistry
University of Maryland, College Park, Maryland 20742

R. D. Guiles
Department of Pharmaceutical Sciences
University of Maryland at Baltimore, Maryland 21201
Received March 18, 1996
Revised Manuscript Received May 14, 1996

The cytochromes are a family of electron transfer proteins that play essential roles in bioenergetics and drug metabolism.[1] Cytochrome *c* has been the most extensively studied of this class of proteins[2] and has served as the primary experimental standard for studying long range electron transfer reactions in biological systems.[3] Although much effort has been expended to investigate the electron transfer reactivity of cytochrome *c*, definitive kinetic parameters have yet to be reported. Using insulated electrodes, we are able to determine kinetic parameters for cytochrome *c* with remarkable precision and experimental ease. In spite of considerable amino acid sequence differences, the cytochromes *c* from horse, tuna, and yeast have identical reorganization energies (0.61 eV) and electronic coupling terms.

Electrochemical studies of the cytochromes have been hampered by the poor electroactivity of the redox protein at electrode surfaces. Recent work has indicated that this electroinactivity of the solution cytochrome is due to protein adsorption on the electrode surface.[4] By control of the purity of the cytochrome *c* preparation and/or the solution conditions, stable voltammetry at bare electrode surfaces has been demonstrated.[5] An alternate strategy has been to modify the electrode surface in order to limit this passivation.[6] These surface modifying layers may also serve to "promote" or "hinder" the electron transfer by increasing or decreasing the binding of the active site heme edge to the electrode surface. For self-assembled monolayers of carboxyl-terminated thiols at low ionic strengths, strong surface interactions have been shown to result in a stable monolayer coverage of cytochrome *c*.[7] While strongly adsorbed, the cytochrome reactivity is not drastically altered, allowing quantitative characterizations of the heterogeneous electron transfer properties of the bound cytochrome.[8]

We have taken an alternate strategy in controlling the cytochrome reactivity using thiol monolayers which do not specifically orient the protein. Self-assembled ω-hydroxythiol monolayers are used to limit the closest approach of the solution species to the electrode surface.[9] By increase of the number of methylene groups within the thiol used to form the monolayer, the electron transfer reaction from the electrode to the redox center is forced to proceed at a larger distance, slowing the overall rate. Electron transfer reactions which are too fast to be measured at a bare electrode can be slowed so that heterogeneous electron transfer rates can be measured over a wide range of electrode potentials. The driving force dependence of the electron transfer rate can then be used to determine reorganization energies and electronic coupling parameters characteristic of the solution species.[10]

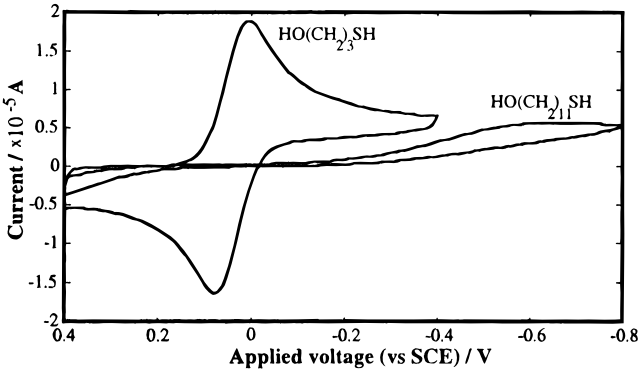

Figure 1. Cyclic voltammograms of cytochrome *c* from yeast. The voltammograms were taken using electrodes coated with 3-hydroxy-1-propanethiol and 11-hydroxy-1-undecanethiol. The solution conditions were 1.1 mM yeast cytochrome *c*, 1.0 M KCl, 2 mM pH 7.1 phosphate buffer. The scan rates were 0.5 V/s, electrode area (0.13 cm^2), and the solution temperature was 0 °C.

Table 1

cytochrome source	E_0' [a] (V vs SCE)	D_0 [b] ($\times 10^7$ cm^2/s)	z_{eff} [c]	λ [d] (eV)	k_{max} [e] ($\times 10^3$ cm/s)
horse	0.023	4.7 (0.3)	2.4 (0.3)	0.58 (0.03)	1.4 (0.2)
tuna	0.018	3.6 (0.7)	1.7 (0.7)	0.62 (0.04)	1.0 (0.3)
yeast	0.039	4.1 (0.3)	2.8 (0.3)	0.61 (0.03)	1.3 (0.4)

[a] Formal potential vs SCE. [b] Diffusion coefficient. [c] Effective charge of the cytochrome determined from the ionic strength dependence of the heterogeneous electron transfer rate. [d] Reorganization energy. [e] Maximum kinetic electron transfer rate extrapolated from the rate/voltage curves. Values in parentheses correspond to the standard deviation of at least three independent measurements. All data were collected at 0.0 °C in solutions which were 1−2 mM cytochrome, 1.0 M KCl, 1 mM pH 7.1 phosphate buffer.

The use of ω-hydroxyalkanethiol-coated Au electrodes greatly simplifies the electrochemical characterization of the cytochromes. The passivation of the electrode surface by the cytochrome is not observed at these insulated electrodes. Figure 1 displays representative voltammograms for a solution of yeast cytochrome *c* measured at electrodes coated with $HO(CH_2)_3SH$ and $HO(CH_2)_{11}SH$ monolayers. When the length of the thiol used to insulate the electrode is short (i.e., $HO(CH_2)_nSH$, were $n = 2-5$), the insulated electrodes give reversible to quasi-reversible voltammetric waves for the cytochromes *c*. From these voltammograms we can readily obtain both the cytochrome *c* redox potentials and the diffusion constants (see Table 1).

The voltammograms for the cytochromes obtained at the $HO(CH_2)_{11}SH$-coated electrodes are corrected for diffusion limitations and double-layer influences in order to extract the potential dependence of the intrinsic heterogeneous electron transfer rate as described previously.[9−11] The derivative of the heterogeneous electron transfer rate constant versus potential is proportional to the density of electronic states distribution and is shown in Figure 2. The density of electronic states distribution for the oxidized cytochromes closely follows the expectations of the Marcus theory.[12] The peak of the density of electronic states plot gives a direct measure of the reorganization energy of the redox molecule. As the electrode potential is swept more

* To whom correspondence should be addressed.

(1) Dickerson, R. E.; Timkovich, R. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. XIa, pp 397−547.
(2) Mathews, F. S. *Prog. Biophys. Mol. Biol*. **1985**, *45*, 1−56.
(3) (a) Moore, G. R.; Huang, Z.-X.; Crispin, G. S. E.; Barker, H. A.; Williams, G.; Robinson, M. N.; Williams, R. J. P. *Faraday Discuss. Chem. Soc*. **1982**, *74*, 311−329. (b) Winkler, J. R.; Gray, H. B. *Chem. Rev*. **1992**, *92*, 369−379.
(4) (a) Maeda, Y.; Yamamoto, H.; Kitano, H. *J. Phys. Chem*. **1995**, *99*, 4837−4841. (b) Sagara, T.; Niwa, K.;Sone, A.; Hinnen, C.; Niki, K. *Langmuir* **1990**, *6*, 254−262.
(5) a) Reed, D. E.; Hawkridge, F. M. *Anal. Chem*. **1987**, *59*, 2334−2339. (b) Szucs, A.; Novak, M. *J. Electroanal. Chem*. **1995**, *383*, 75−84.
(6) Eddowes, M. J.; Hill, H. A. O. *Faraday Discuss. Chem. Soc*. **1982**, *74*, 331−341.
(7) Tarlov, M. J.; Bowden, E. F *J. Am. Chem. Soc*. **1991**, *113*, 1847−1849.
(8) (a) Song, S.; Clark, R. A.; Bowden, E. F.; Tarlov, M. J. *J. Phys. Chem*. **1993**, *97*, 6564−6572. (b) Feng, Z. Q.; Imabayashi, S.; Kakiuchi, T.; Niki, K. *J. Electroanal. Chem*. **1995**, 149−154. (c) Lu, T.; Yu, X.; Dong, S.; Zhou, C.; Ye, S.; Cotton, T. M. *J. Electroanal. Chem*. **1994**, *369*, 79−86.
(9) Becka, A. M.; Miller, C. J. *J. Phys. Chem.* **1992**, *96*, 2657−2668.
(10) Terrettaz, S.; Becka, A. M.; Traub, M. J.; Fettinger, J. C.; Miller, C. J. *J. Phys. Chem.* **1995**, *99*, 11216−11224.
(11) Becka, A.; Miller, C. J. *J. Phys. Chem.* **1993**, *97*, 6233−6239.
(12) Marcus, R. A. *J. Chem. Phys*. **1965**, *43*, 679−701.

S0002-7863(96)00866-9 CCC: $12.00

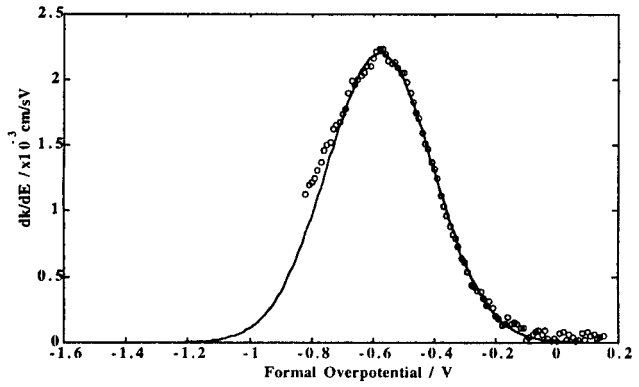

Figure 2. Density of electronic states plot for tuna cytochrome *c*. The density of states distribution is the first derivative of the diffusion and double-layer-corrected rate constant and is shown as open circles. The solid curve is the best fit Gaussian to the data. The data used to produce this plot was taken from voltammograms of 11-hydroxyundecanethiol-coated electrode under similar experimental conditions as those reported for Figure 1.

negative, the heterogeneous electron transfer rate approaches a limiting value, k_{max}, which gives a measure of the electronic coupling efficiency of the redox molecule.[13] Values of the reorganization energies and k_{max} values for the three cytochromes studied are collected in Table 1. There is a remarkable similarity between the kinetic parameters obtained from the three cytochromes. Although there is an 80% homology between the amino acid sequences of the tuna and horse heart structures and only a 60% homology in the structures of the yeast and horse cytochromes, the reorganization energies and k_{max} values are indistinguishable.[14] A conclusion from this work is that the reactivity of these cytochromes is controlled by the local heme structure which is highly conserved in these structures. For redox molecules similar in size to the axially coordinated heme such as the tris(bipyridyl) complexes of Fe, Ru, and Os, we find nearly the same reorganization energies, 0.56 eV.[10] The measured reorganization energy of the cytochromes is therefore consistent with the size of the axially coordinated heme, suggesting only a minimal role of the protein matrix in controlling the activation energy of the cytochromes *c*.

The k_{max} values determined here represent a distinctly new characterization of cytochrome *c* reactivity. In a sense, the monolayer-coated electrode acts as a completely nonspecific reaction partner for the cytochrome allowing all possible orientations of the cytochrome to interact with the electrode. Because of the asymmetric placement of the redox active heme in the cytochrome, only a small percentage of the possible orientations of the protein should position the heme close enough to the metal surface to allow electron transfer at an appreciable rate. The k_{max} values determined at these insulated electrodes will therefore be characteristic of these optimal electron transfer geometries. The close agreement between the k_{max} values suggests a close analogy in the electronic structure and distance of closest approach to the monolayer surface between these structures. The 40% spread between the k_{max} values of these cytochromes is within the experimental uncertainty and can be compared with the 300% decrease in the electronic coupling caused by increasing the thickness of the monolayer by a single methylene group.[9]

Similar kinetic parameters have been reported for cytochrome *c* electrostatically immobilized on carboxyl-terminated alkanethiol monolayer-coated Au electrodes. From the temperature dependence of the electron transfer rate, Song *et al*. have determined a reorganization energy of 0.35 ± 15 eV for horse heart cytochrome *c* electrostatically immobilized at a $HS(CH_2)_{15}$-COOH-coated Au electrode.[8a] The decrease in the reorganization energy of the surface attached compared to the solution species is surprising and is inconsistent with our determination. Comparing the electronic coupling of the surface-attached cytochromes to the solution species is a bit less straightforward. From the standard heterogeneous electron transfer rate constant of 4×10^{-6} cm/s measured at the $HS(CH_2)_{11}OH$-coated electrode, we can calculate a reorganization-corrected first-order rate of 8000 s^{-1}.[15] This rate for the solution cytochrome can be compared to a value of 72 s^{-1} determined by Feng *et al*. for horse cytochrome *c* electrostatically immobilized on a HS-$(CH_2)_{10}$COOH-coated Au electrode.[8b] Our solution rates are about 2 orders of magnitude faster than that of the surface-attached cytochrome. This high electron transfer rate for the solution species is observed even though the cytochrome in solution is not specifically oriented with its heme edge next to the monolayer surface.[16] If one were to incorporate this factor into the analysis,[17] the solution cytochrome would be about 4 orders of magnitude more reactive than the electrostatically bound cytochrome. The large difference in the reactivity of the solution and electrostatically bound cytochromes reflects a difference in the electronic coupling in the two cases. The electrostatically immobilized cytochromes are much more poorly electronically coupled to the electrode when compared to the solution species. These carboxyl-terminated monolayer films are likely to rigidly bind cytochrome *c* in an orientation which is significantly different from the optimal electronic coupling geometry accessible at the hydroxylated surface.

A major strength of this insulated electrode voltammetry for the characterization of redox active molecules is its ability to separate relative electronic coupling from activation effects. Such detailed kinetic information allows one to more clearly understand how the structure of a redox center controls its electron transfer reactivity. Although our monolayer-coated electrodes are a completely unnatural reaction partner for redox proteins, they nevertheless allow us to indirectly probe the basis of protein biofunction. For example, Ho *et al*. have reported that the electron transfer rate between horse heart cytochrome *c* and yeast cytochrome *c* peroxidase is 10 times slower than that between yeast cytochrome *c* and the yeast cytochrome *c* peroxidase.[18] Because the activation energies and optimal electronic coupling contacts of the two cytochromes are the same as those determined from our study, this order of magnitude difference in reactivity has to be explained by a specific molecular recognition of the yeast cytochrome *c* which more strongly electronically couples the cytochrome to the enzyme.

Acknowledgment. This research has been supported through grants from the Petroleum Research Fund, the National Science Foundation (CHE-9417357), and the National Institutes of Health (DK 46510).

Supporting Information Available: Experimental procedures and a more detailed explanation of the rate comparison between the solution and surface bound cytochrome *c* (5 pages). See any current masthead page for ordering and Internet access instructions.

JA960866Y

(13) Miller, C. J. In *Physical Electrochemistry*; Rubinstein, I., Ed.; Marcel-Dekker: New York, 1995; pp 27−79.

(14) (a) Bushnell, G. W.; Louie, G. V; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585−595. (b) Louie G. V.; Brayer G. D. *J. Mol. Biol.* **1990**, *214*, 527−555. (c) Takano, T.; Dickerson, R. E. *J. Mol. Biol.* **1981**, *153*, 79−115.

(15) Here we assume a reaction layer thickness of 10^{-8} cm and a reorganization energy of 0.35 eV for the bound cytochrome and 0.61 eV for the solution species. The heterogeneous electron transfer rate constant is divided by the reaction layer thickness and corrected for the difference in the activation energies to obtain the corrected first-order rate estimate. See the supporting information for a more detailed explanation of this comparison.

(16) This assumption of minimal interactions between the hydroxylated monolayer and the solution cytochrome is supported by the absence of any measurable preconcentration of the cytochrome at the interface as determined from chronocoulometric studies and the independence of the kinetic properties with the solution concentration of the cytochrome. Previous studies strongly suggest that there are minimal interactions between these hydroxlated surfaces and a range of redox molecules.

(17) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985,** *811*, 265−322.

(18) Ho, P. S.; Sutoris, C.; Liang, N.; Margoliash, E.; Hoffman, B. M. *J. Am. Chem. Soc.* **1985**, *107*, 1070−1071.