The Heterogeneous Electron Transfer Properties of Cytochrome c

EDMOND F. BOWDEN and FRED M. HAWKRIDGE[1]

Virginia Commonwealth University, Department of Chemistry, Richmond, VA 23284

HENRY N. BLOUNT[1]

The University of Delaware, Brown Chemical Laboratory, Newark, DE 19711

The heterogeneous electron transfer kinetic parameters of horse heart cytochrome c were evaluated at pH 7.0. This work was directed at determining the formal heterogeneous electron transfer rate constant, $k^{o'}_{s,h}$, *and the electrochemical transfer coefficient,* α, *at three different electrode surfaces: gold electrodes electrochemically modified with methyl viologen, fluoride-doped tin oxide optically transparent electrodes (OTEs), and tin-doped indium oxide OTEs. Kinetic parameters of cytochrome c were evaluated using samples in the totally oxidized and in the totally reduced forms. Kinetic effects arising from anion binding to cytochrome c were investigated for phosphate and chloride in the presence of the nonbinding buffer tris(hydroxymethyl)aminomethane–cacodylic acid. The kinetic parameters were determined using single potential step chronoabsorptometry at all three electrodes and using rotating disk electrode voltammetry at the methyl viologen-modified gold disk electrode.*

The thermodynamics and homogeneous electron transfer kinetics of cytochrome c have been studied widely. Extensive reviews point to the important questions that remain unanswered regarding the mechanism by which electrons are transferred by cytochrome c in mammalian oxidative phosphorylation (*1–8*). The pathway by which

[1] To whom correspondence should be addressed.

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cytochrome c accepts electrons from the membrane-bound cytochrome c reductase and then donates electrons to cytochrome c oxidase, which is also membrane bound, remains a point of controversy. The impetus for studying the energetics and kinetics of cytochrome c electron transfer reactions derives primarily from the need to understand its electron transfer mechanism(s).

Several mechanisms have been proposed for cytochrome c (*1–6*), all based on indirect evidence. An electron hopping mechanism involving transfer of an electron through various aromatic residues in the protein fabric was proposed (*8*). This mechanism was subsequently abandoned because of its failure to account for structural and energetic factors (*9*). The involvement of a π-cation radical intermediate was also proposed (*10*) but was not experimentally verified. Electron tunneling was proposed for bacterial cytochromes (*11–13*) and this mechanism also may be operative in mammalian cytochrome c. Possibly the most widely accepted mechanism involves electron transfer at the exposed heme edge of cytochrome c. This mechanism was first proposed for cytochrome c (*14*) and later for *Rhodospirillum rubrum* c_2, a photosynthetic cytochrome (*15*). This outer sphere mechanism was widely tested through use of exogenous and endogenous redox reactants and through studies of the effect of solution pH, ionic strength, and ion binding on the homogeneous electron transfer kinetics of cytochrome c (*1–5*). Support for the heme edge electron transfer mechanism was provided by these homogeneous electron transfer kinetic studies. However, the mechanism by which cytochrome c transfers electrons physiologically remains to be established.

The determination of the electron transfer kinetics of direct heterogeneous reactions between cytochrome c and several electrode surfaces was the objective of this study. The reason for pursuing this type of measurement is that cytochrome c physiologically transfers electrons at membrane interfaces. Hence, the physiological electron transfer reactions of cytochrome c may proceed via a mechanism that contains elements of a simple heterogeneous electron transfer model. This work utilized newly developed and previously reported electrode surfaces and methods.

Direct Electrochemical Studies of Cytochrome c

Cytochrome c has been studied extensively by direct voltammetric methods at mercury electrodes (*16–24*). Strong adsorption of cytochrome c on the mercury surface during reduction has been widely reported. The adsorbed layer has been variously described as forming a flattened layer with pores where reduction of diffusing cytochrome c occurs (*23*), a layer at which a self-exchange reaction occurs between the reduced adsorbed molecules and those diffusing to the electrode

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(*20*), and an adsorbed layer of denatured cytochrome c (*24*). Cytochrome c also has been studied directly at gold minigrid electrodes (*25*), indium oxide thin-film optically transparent electrodes (OTEs) (*26*), and at gold electrodes on which 4,4′-bipyridine was adsorbed (*27–31*).

Formal heterogeneous electron transfer kinetic parameters for the reduction of cytochrome c have been reported (*24, 29*). Based on linear sweep voltammetry, the formal heterogeneous electron transfer rate constant ($k^{o\prime}_{s,h}$) and the electrochemical transfer coefficient (α) for the reduction of cytochrome c at mercury were estimated to be 10^{-10} to 10^{-11} cm/s and ca. 0.5, respectively (*24*). At the 4,4′-bipyridine/gold electrode surface, ac impedance methods were used to determine that $k^{o\prime}_{s,h} = 1.6 \times 10^{-2}$ cm/s, with no value given for the electrochemical transfer coefficient (*29*).

Single Potential Step Chronoabsorptometry

The method of single potential step chronoabsorptometry (SPS/CA) permits the determination of heterogeneous electron transfer kinetic parameters for optically absorbing species at OTEs (*32*). The principal advantages of this method compared to other electrochemical methods are its insensitivity to charge consuming processes other than the reaction of interest and the molecular specificity provided by the optical probe. A detailed description of the application of this method, which neglects the effect of the back reaction (irreversible processes), as well as the more recent application of a method that accounts for the back reaction (quasi-reversible processes) was presented (*33*).

The need for the SPS/CA method directly followed the reports of the electroactivity of gold minigrid electrodes, which were electrochemically modified with methyl viologen, toward the direct reduction and oxidation of ferredoxin (*34*) and myoglobin (*35*). The application of the SPS/CA method to the determination of the heterogeneous electron transfer kinetic parameters was reported for the reduction of myoglobin (*36*) and ferredoxin (*37*) at this electrode surface. Recent work extended the application of SPS/CA to cytochrome c, which was studied at the modified gold minigrid surface and at fluoride-doped tin oxide and tin-doped indium oxide OTEs (*38*). The effects of pH and ionic strength on the heterogeneous reductive electron transfer parameters for myoglobin were also described (*38*).

In the present work, SPS/CA was used to evaluate the effects of ion binding to cytochrome c on heterogeneous electron transfer kinetic parameters at fluoride-doped tin oxide OTEs. In addition, initial results from oxidative SPS/CA measurements for cytochrome c were obtained at the modified gold minigrid surface and at the fluoride-

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doped tin oxide OTE surface. These latter experiments were performed to directly measure the rate constants for the back reaction (oxidation) to determine the agreement of the heterogeneous electron transfer reactions of cytochrome c with the simple electron transfer theory used in kinetic analyses.

Experimental

Apparatus. The electrochemical and optical instrumentation was described previously (*36, 38*). The spectroelectrochemical cells were based on a previously reported design and had an optical pathlength of ca. 1 mm (*39*). Rotating disk voltammetry was performed with a Pine Instrument Company Model ASR-2 rotator.

The gold minigrid electrodes were 200 lines per inch, 67% transmittant and 0.1 mil nominal thickness from Buckbee–Mears Co. The gold rotating disk electrode, 7.5-mm diameter, was Model DD20 from Pine Instrument Co. Tin-doped indium oxide and fluoride-doped tin oxide OTEs were ca. 20 ohms/square from PPG Industries.

Chemicals. Methyl viologen (K & K Laboratories) was recrystallized three times from methanol. The phosphate buffer was prepared from Titrisol, pH 7.0 (E. Merck Co.) or from reagent grade salts. Cacodylic acid and tris(hydroxymethyl)aminomethane, reagent grade, were obtained from Sigma Chemical Co. The cacodylic acid was recrystallized twice from ethanol. All other chemicals were reagent grade and solutions were prepared in glass distilled water.

Procedures. Gold electrodes were modified with methyl viologen as previously described (*36*). The semiconductor OTEs were cleaned by successively subjecting them to 5 min of ultrasonic agitation in Alconox, ethanol, and distilled water (twice) after a previously described procedure (*40*).

SPS/CA measurements were performed at 550 or 416 nm and $\Delta\epsilon$ values of 21,100 (*41*) and 57,000 $M^{-1}cm^{-1}$ (*42*), respectively, were used in all calculations. The diffusion coefficient used in all calculations was 1.1×10^{-6} cm^2/s (*28*). This value was experimentally verified (± 0.05) from the slope of plots of absorbance vs. $t^{1/2}$ for 22 diffusion-controlled SPS/CA transients at a fluoride-doped tin oxide OTE. The formal potential for cytochrome c, which was used to determine overpotential step values, was 0.260 V vs. NHE (*2*). All experiments were performed at 25 ± 2°C.

Reduction and Oxidation of Cytochrome c at Various Electrodes

Table I summarizes the electron transfer kinetic behavior seen for horse heart cytochrome c at the three electrode surfaces reported here. These results were all obtained with solutions containing 0.07 *M* phosphate and 0.10 *M* NaCl, pH 7.0, using SPS/CA. Rate parameters obtained from previous reductive potential step experiments with ferricytochrome c (Entries 1, 4, 6) (*38*) are shown in Figure 1. Entry 3 data for cytochrome c reduction at tin oxide resulted from a recent experiment that duplicated the conditions for Entry 4. The consistency between these two sets of data obtained 6 months apart is quite good.

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Table I. Heterogeneous Electron Transfer Kinetic Parameters for Cytochrome c at Various OTEs

Entry	[*Cyt c*], μM[a]	*log* $k^{o'}_{s,h}$, *cm/s*	α	*Electrode*	*Potential steps*
1	77.3	−4.99(±0.12)[b]	0.24(±0.03)	MGM[c]	reductive[d,e]
2	77.3	−5.24(±0.05)	0.74(±0.02)[f]	MGM	oxidative
3	101	−5.20(±0.08)	0.28(±0.02)	SnO_2[g]	reductive
4	103	−5.17(±0.05)	0.32(±0.01)	SnO_2	reductive[e]
5	103	−4.31(±0.07)	0.95(±0.01)[f]	SnO_2	oxidative
6	43.0	−4.51(±0.05)	0.50(±0.04)	In_2O_3[h]	reductive[e]

[a] All solutions contained 0.07 *M* phosphate buffer, pH 7.0 and 0.1 *M* NaCl.
[b] Parentheses contain one standard deviation.
[c] Methyl viologen-modified gold minigrid electrode.
[d] Reductive SPS/CA performed on oxidized sample of cytochrome c.
[e] From Ref. 38.
[f] Oxidative SPS/CA performed on reduced sample of cytochrome c, value is from (1 − α).
[g] Fluoride-doped tin oxide OTE.
[h] Tin-doped indium oxide OTE.

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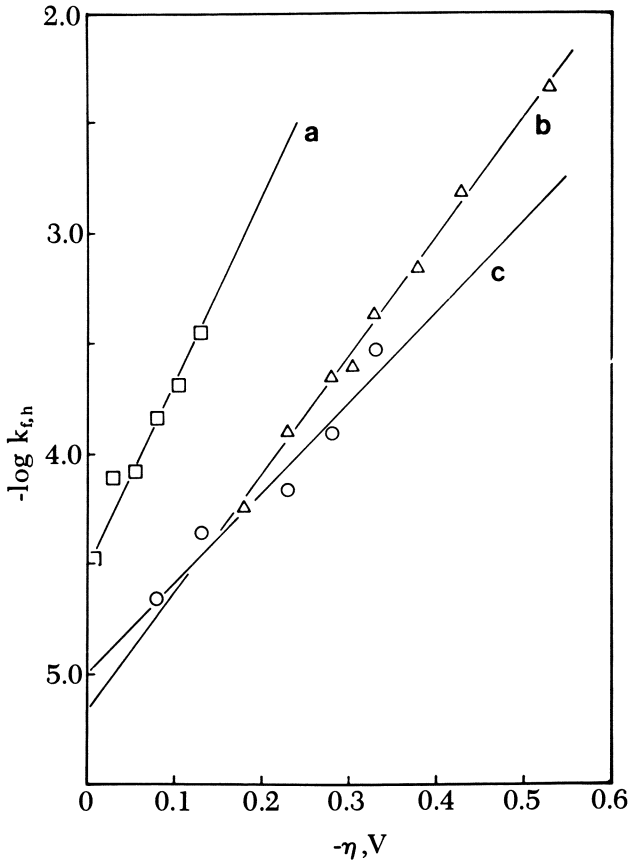

Figure 1. Log $k_{f,h}$ vs. overpotential for the reduction of cytochrome c. Letter designation/electrode material/entry correspondence in Table I: a, tin-doped indium oxide, Entry 6; b, fluoride-doped tin oxide, Entry 5; c, methyl viologen-modified minigrid, Entry 1.

As noted previously (*38*), the sameness of the reductive kinetic results, when compared to those obtained at the 4,4′-bipyridine/gold electrodes (*29*) and at mercury (*24*), argues for the existence of a similar protein/solution interface at these three electrode surfaces. The presence of an adsorbed protein layer at these electrode surfaces is a likely possibility but is not established.

Oxidative SPS/CA experiments were performed in a manner identical to the reductive experiments except that positive overpotential steps were applied to the electrodes exposed to bulk ferrocytochrome c. Analysis of absorbance–time data yields, for each overpotential, a

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$k_{b,h}$ (assuming that $k_{f,h}$ corresponds to reduction). A linear plot of log $k_{b,h}$ vs. overpotential (η) affords $k^{\circ\prime}_{s,h}$ from the intercept and $(1 - \alpha)$ from the slope. According to Butler–Volmer formalism (*43*), a simple heterogeneous electron transfer reaction (with only one physical pathway for both oxidation and reduction) should yield the same value of $k^{\circ\prime}_{s,h}$, independent of whether the measured reaction is a reduction or an oxidation. Furthermore, the values of the transfer coefficient obtained from the separate reductive and oxidative experiments should agree. In the experiments of Table I, these criteria are not met for the fluoride-doped tin oxide OTE (Entries 3 and 4 compared to 5) or the methyl viologen-modified gold minigrid electrode (compare Entries 1 and 2). For the former electrode, the transfer coefficients differ by ca. 0.65, and the $k^{\circ\prime}_{s,h}$ values differ by nearly an order of magnitude in the oxidation and reduction experiments. Similar disparities also exist for the latter electrode, but to a lesser degree. Reasons for this discrepancy may involve ion binding to cytochrome c, a pathway dependence on the reaction direction, and semiconductor surface effects. Work is in progress to determine the reason(s) for these discrepancies.

The heterogeneous electron transfer kinetic parameters for the reduction of cytochrome c were also investigated at a methyl viologen-modified rotating gold disk electrode (RDE) to compare the results of this steady state technique with the results obtained by the SPS/CA transient technique. The gold RDE was first polished successively with 1-, 0.3-, and 0.1-μm alumina slurries followed by an ultrasonic distilled water rinse. The gold RDE was then modified following the procedure described for gold minigrids (*36*). Standard RDE kinetic analysis (*43*) of data obtained for a deoxygenated solution of 166 μM cytochrome c, 0.07 M phosphate buffer, and 0.10 M NaCl, pH 7.0, yielded values for log $k^{\circ\prime}_{s,h}$ of -5.24 (± 0.22) and α of 0.21 (± 0.03). These preliminary results demonstrate that RDE voltammetry at the methyl viologen-modified gold disk electrode can be utilized to measure the heterogeneous electron transfer kinetics of cytochrome c. This result is in agreement with the results of the SPS/CA transient technique (*38*).

Anion Effects on the Heterogeneous Electron Transfer Kinetics of Cytochrome c

Specific cation and anion binding to one or both redox forms of cytochrome c is a well-established phenomenon (*44*, *45*). Changes in homogeneous electron transfer rates between cytochrome c and soluble redox partners also have been observed and attributed to ion binding (*2*, *46*, *47*). This section describes evidence which shows that

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specific anion effects, presumably resulting from binding to cytochrome c, can influence heterogeneous electron transfer rates in a measurable and reproducible fashion.

For these experiments, cytochrome c was dissolved in pH 7.0 tris(hydroxymethyl)aminomethane (0.09 *M*)/cacodylic acid (0.10 *M*) buffer (Buffer A) of calculated ionic strength equal to 0.08 *M*. This buffer system is considered to be nonbinding with respect to cytochrome c (*46*, *48*). Evaluation of reductive electron transfer kinetic parameters was then performed at fluoride-doped tin oxide OTEs both in the presence and absence of added salts. First the results with buffer alone will be presented in some detail followed by results obtained in the presence of chloride and phosphate.

Figure 2 shows typical absorbance–time transients for the reduction of cytochrome c in Buffer A for a number of overpotential steps.

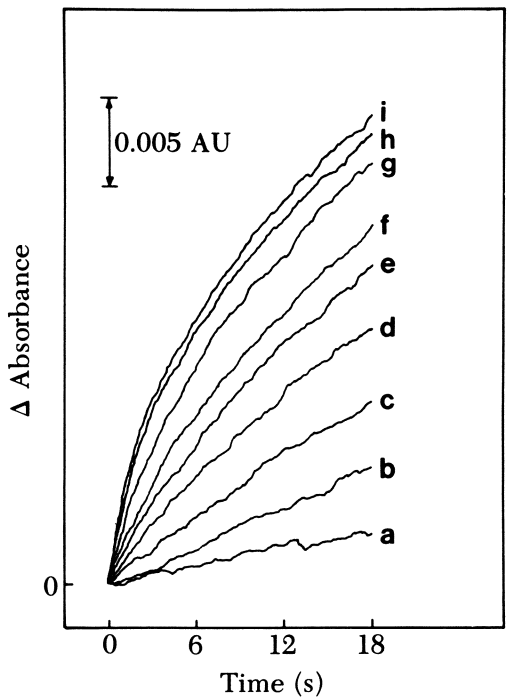

Figure 2. Typical SPS/CA absorbance–time transients for the reduction of cytochrome c. Solution contained 97.6 μM cytochrome c, and Buffer A, pH 7.0 (ionic strength = 0.08 M) at a fluoride-doped tin oxide OTE from Table III, Entry 1. Trace/overpotential in mV/transient number in experimental sequence: a, −78, #10; b, −128, #6; c, −178, #20; d, −228, #14; e, −278, #18; f, −328, #4; g, −428, #1; h, −628, #5; and i, −728, #8.

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The reproducibility was very good and no measurable loss in response was seen during the random acquisition of more than thirty SPS/CA transients. Table II shows the $k_{f,h}$ values calculated for transients a through g of Figure 2. Excellent fit to the SPS/CA theory for irreversible electron transfer is indicated by the small standard deviations. This assertion is further corroborated in Figure 3, which presents the kinetic working curve along with data from transients b, c, d, f, and g. Only these data are shown for clarity. For each transient, experimental normalized absorbance is plotted for five observations (t = 6, 9, 12, 15, and 18 s) vs. log $[(k_{f,h}t^{1/2})/D^{1/2}]$ using the average $k_{f,h}$ values from Table II. If experiment fits theory, all five points for each transient should fall on the working curve and this is indeed the case. Values obtained for t = 3 s were not included in any of the $k^{o\prime}_{s,h}$ and α determinations, because their fit to the working curve was not good in some cases. This deviation is probably a result of the greater relative error inherent in measuring the absorbance–time response at short times.

For the reduction of cytochrome c at fluoride-doped tin oxide OTEs in pH 7.0 Buffer A, averaging the results in Entries 1 and 2 of Table III yields log $k^{o\prime}_{s,h} = -4.75$ and $\alpha = 0.32$. Entries 3 and 4 of the same table indicate that the effect of 10 mM phosphate ($[H_2PO_4^-]/[HPO_4^{2-}] \simeq 0.7$) in this same system is to decrease log $k^{o\prime}_{s,h}$ by ca. 0.4 and α by ca. 0.07. In the electrochemical sense, cytochrome c reduction at tin oxide is more irreversible in the presence of phosphate. Experimentally, this fact is evidenced by a significant reduction in the magnitudes of the absorbance–time transients shown in Figure 2 upon addition of phosphate. That this observed difference is significant is shown by the error limits and the experimental reproducibility indicated in Entries 1–4 of Table III. One experiment performed with 10 mM NaCl added to the Buffer A showed a slight decrease in reversibility as evidenced by a smaller transfer coefficient (*see* Entry 5). However, compared with the phosphate effect, this result is not striking and repetitive experiments will be necessary to establish the validity of this difference. The kinetic results presented in Table III are graphed in Figure 4.

The results just presented indicate that specific ion binding can significantly influence heterogeneous electron transfer rates of cytochrome c. Using values for ion binding constants previously reported (*44*), a 10-mM concentration of phosphate or chloride is sufficient to bind essentially all of the cytochrome c molecules at 100 μM concentration. Evidently, two anions bind to each cytochrome c (*49*). The results presented in Table III and Figure 4 support the view that phosphate and chloride bind at different sites on cytochrome c (*50*) and suggest the involvement of this molecular feature in the reduction of this metalloprotein at tin oxide OTEs.

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Table II. Heterogeneous Electron Transfer Rate Constants for the Reduction of Cytochrome c at a Fluoride-Doped Tin Oxide OTE

η, mV	$k_{f,h}$, cm/s[a]
− 78	$3.10(\pm 0.20) \times 10^{-5}$
−128	$7.49(\pm 0.07) \times 10^{-5}$
−178	$1.51(\pm 0.02) \times 10^{-4}$
−228	$2.68(\pm 0.03) \times 10^{-4}$
−278	$4.53(\pm 0.07) \times 10^{-4}$
−328	$6.21(\pm 0.02) \times 10^{-4}$
−428	$1.28(\pm 0.05) \times 10^{-3}$

[a] Rate constants are mean values of five observations taken at equal increments over the 6- to 18-s time domain. Parentheses contain one standard deviation.
Note: Solution conditions are given in Figure 2.

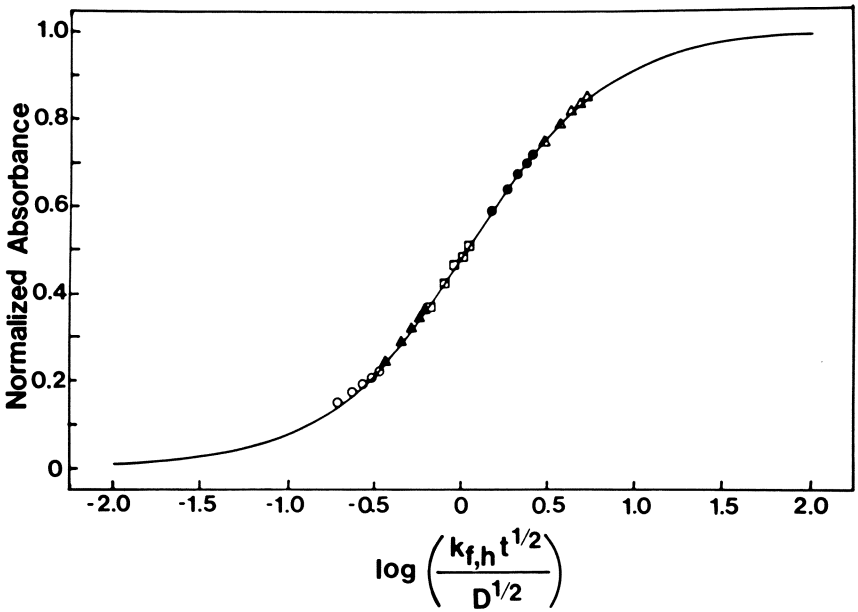

Figure 3. Normalized absorbance vs. log $[(k_{f,h}t^{1/2})/D^{1/2}]$ *working curve with typical data for the reduction of cytochrome c at a fluoride-doped tin oxide OTE. Data shown correspond to appropriate transients in Figure 2 and were calculated using the* $k_{f,h}$ *values from Table II. Key:* ○, $\eta = -128$ *mV;* ▲, $\eta = -178$ *mV;* □, $\eta = -228$ *mV;* ●, $\eta = -328$ *mV; and* △, $\eta = -428$ *mV.*

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Table III. Anion Effects on the Heterogeneous Electron Transfer Kinetic Parameters for Cytochrome c

Entry	*log* $k^{\circ\prime}_{s,h}$, *cm/s*	α	*Electrolyte*
1	−4.79(±0.05)[a]	0.30(±0.01)	Buffer A
2	−4.71(±0.01)	0.34(±0.01)	Buffer A
3	−5.13(±0.05)	0.24(±0.01)	Buffer A + 10 m*M* phosphate
4	−5.14(±0.02)	0.26(±0.01)	Buffer A + 10 m*M* phosphate
5	−4.78(±0.03)	0.28(±0.01)	Buffer A + 10 m*M* NaCl

Note: SPS/CA at SnO_2 OTEs, 96 to 98 μM cytochrome c, all solutions at pH 7.0, and all experiments are reductive.

[a] Parentheses contain one standard deviation.

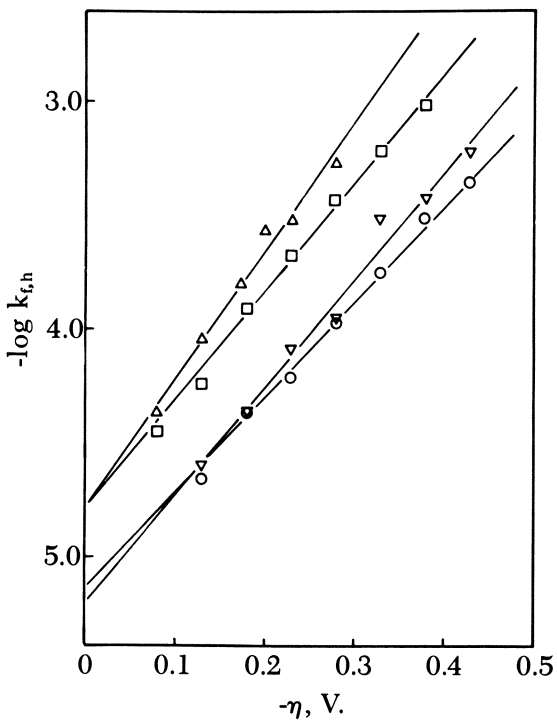

*Figure 4. Anion effect on the SPS/CA reduction kinetics of cytochrome c at fluoride-doped tin oxide OTEs. Key: △, Buffer A, pH 7.0 (Entry 1 of Table III); □, Buffer A + 10 m*M *NaCl (Entry 5 of Table III); ○, Buffer A + 10 m*M *phosphate (Entry 3 of Table III); and ▽, 0.07* M *phosphate, 0.10* M *NaCl, pH 7.0 (Entry 3 of Table I).*

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The results and conclusions just presented are the first reported evidence for specific ion effects on heterogeneous electron transfer kinetics for a biological redox molecule. Although the effect is thought to arise from binding to cytochrome c molecules, the anions may possibly be exerting an important effect on the oxide semiconductor surface. Additional experiments, including variation of anion concentration, will be required to assess these potential causes. A final point that requires clarification concerns the phosphate effect. At pH 7.0, both $H_2PO_4^-$ and HPO_4^{2-} are present at significant concentrations and it is not clear whether there is a difference in their ion binding behavior. Additional experiments performed over a pH range of ca. 6–8 should resolve this question.

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