Cyclic Voltammetry and Derivative Cyclic Voltabsorptometry of Purified Horse Heart Cytochrome *c* at Tin-Doped Indium Oxide Optically Transparent Electrodes

Edmond F. Bowden,[†] Fred M. Hawkridge,*[†] Jan F. Chlebowski,[‡] Eric E. Bancroft,[§] Colin Thorpe,[§] and Henry N. Blount*[§]

Contribution from the Departments of Chemistry and Biochemistry, Virginia Commonwealth University, Richmond, Virginia 23284, and Center for Catalytic Science and Technology and Brown Chemical Laboratory, The University of Delaware, Newark, Delaware 19711.
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Abstract: Purified horse heart cytochrome *c* exhibits highly reproducible voltammetric and derivative cyclic voltabsorptometric responses that agree with Butler–Volmer theory for a quasi-reversible one-electron redox couple at tin-doped indium oxide optically transparent electrodes.

The heterogeneous electron-transfer reactions of cytochrome *c* at a variety of electrodes have been widely studied and are currently the subject of intense research focus. An early report described the direct reduction of cytochrome *c* at a platinum electrode.[1] Overpotentials in excess of 0.5 V were required to reduce cytochrome *c*, and this reduction most probably proceeded via electrochemically generated hydrogen.[1,2] The direct reduction of cytochrome *c* at a dropping mercury electrode (DME) was subsequently reported[3] and was also found to exhibit irreversible heterogeneous electron-transfer kinetics. Numerous investigations of the heterogeneous electron-transfer reactions of cytochrome *c* at mercury electrodes have followed, and a wide range of electrochemical reversibility has been evident in these investigations.[4-20] Models for the interaction of cytochrome *c* at the mercury–solution interface have been proposed that seek to explain the observed electrochemical responses. These proposals involve the adsorption of either native or denatured cytochrome *c* molecules in one or more layers of varying porosity and are dependent upon the bulk concentration of cytochrome *c*, the binding of ions present in the electrolyte, and the pH of the solution.[10,11,14,15,17]

The electrochemical responses of cytochrome *c* at gold,[2,21-25] platinum,[1,2,4,25] nickel,[16] silver,[26] and illuminated p-type silicon semiconductor[25] electrodes have been reported to be irreversible. Quasi-reversible cyclic voltammetric (CV) responses have been reported for cytochrome *c* at indium oxide optically transparent electrodes (OTEs)[27] and mark the first time that this molecule has exhibited a measurable and well-defined voltammetric response at a solid electrode. The diffusion coefficient for ferricytochrome *c* calculated from these CV data was, however, low by a factor of ca. 2 (5×10^{-7} cm^2/s). A less reversible CV response was obtained at tin oxide OTEs.[27]

Quite interesting results are contained in several reports of the electrochemical response of cytochrome *c* at gold electrodes with either 4,4′-bipyridine or 1,2-bis(4-pyridinyl)ethylene adsorbed on the surface.[22-24,28-31] The formal heterogeneous electron-transfer rate constant for cytochrome *c* at the former adsorbed layer on gold was determined by ac impedance and rotated disk voltammetry measurements to be 1.5×10^{-2} cm/s.[28,31] A detailed study of the reaction of cytochrome *c* at this surface pointed to a mechanism in which adsorption of both reactant and product was involved.[31] Gold, platinum, and p-type silicon electrodes modified by the covalent attachment of bipyridinium mediators were shown to electrocatalyze the reduction of cytochrome *c* at a mass transfer controlled rate at the redox potential of the surface immobilized mediator (ca. 0.59 V more negative than that of cytochrome *c*).[25] The heterogeneous electron-transfer kinetics for the reduction of cytochrome *c* at methyl viologen modified gold, fluoride-doped tin oxide, and tin-doped indium oxide OTEs have also been evaluated by spectroelectrochemical techniques and have been

[†] Department of Chemistry, Virginia Commonwealth University.
[‡] Department of Biochemistry, Virginia Commonwealth University.
[§] The University of Delaware.

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found to be quasi-reversible.[32,33]

In the reports described, attention to the effect of sample purity on the observed electrochemical responses has been sparse. Purification of cytochrome *c* by Sephadex chromatography did not markedly affect the differential pulse polarographic response obtained at the DME.[19] However, effects of sample purity may not manifest themselves at the DME during the brief drop life. At solid electrodes, the purity of cytochrome *c* can be expected to be a more important determinant of electrochemical response. Hill and co-workers have used chromatographically purified cytochrome *c* in their work with 4,4′-bipyridine/gold electrodes,[23,31] but the effect of sample purity on electrochemical response has not been addressed. Yeh and Kuwana[27] used a purified, noncommercial sample of cytochrome *c* in their work with indium oxide OTEs.

In this report the effect of the purity of cytochrome *c* on the CV responses observed at indium oxide and tin oxide OTEs is described. It is clear that the impurities that are present even in high quality commercial samples of cytochrome *c*[34] profoundly affect the observed voltammetric responses at these solid electrodes. Following purification of commercial samples of cytochrome *c*, highly reproducible, quasi-reversible CV responses are observed. Derivative cyclic voltabsorptometry (DCVA)[33,35] reponses obtained for purified cytochrome *c* samples at indium oxide OTEs demonstrate that under the conditions of this study, the electroreduction and electrooxidation of cytochrome *c* proceed in accordance with simple electron-transfer (Butler–Volmer) theory.[36] The results of this investigation strongly suggest that a major contributor to the variance in the heterogeneous electron-transfer kinetic behavior of cytochrome *c* that has been reported by different laboratories is the *purity* of the biological sample.

Experimental Section

Horse heart ferricytochrome *c*, type VI, was obtained from Sigma Chemical Co. Samples of this material were purified by chromatography on carboxymethylcellulose (CM-52, Whatman) after a published procedure.[34] A linear elution gradient, 40–90 mM phosphate buffer, pH 7.0, was used first and followed by final elution with the 90 mM buffer. The elution profile was monitored spectrophotometrically (ISCO), and found to be essentially the same as previously reported.[34] Fractions corresponding to a conservative central portion of the native cytochrome *c* band were combined and dialyzed (Spectrapor 6, MWCO 3500) against deionized distilled water for a minimum of 3 days with water changed every 12 h. The volume of larger pooled fractions was reduced prior to dialysis using a stirred ultrafiltration cell (Amicon Model 52) with either a UM2 or YM5 filter. All operations up to and including dialysis were carried out at 4 °C. The dialyzed samples were lyophilized and stored at −4 °C. Two separate commercial samples were purified and gave identical electrochemical responses.

Dithionite-reduced purified cytochrome *c* exhibited a molar absorptivitiy of 923 M^{-1} cm^{-1} at 695 nm. In a 1.00-mm cuvette, the ratio of the absorbance of purified ferrocytochrome *c* at 550 nm to the absorbance of purified ferricytochrome *c* at 280 nm was 1.23 (lit.[34] 1.25). Cytochrome *c* concentrations were determined spectrophotometrically at 550 nm by use of either the molar absorptivity of the reduced form, ϵ_{red} = 29 500 M^{-1} cm^{-1},[37] or the reduced minus oxidized difference molar absorptivity, $\Delta\epsilon$ = 21 100 M^{-1} cm^{-1}.[37]

Tris(hydroxymethyl)aminomethane was used as received from Sigma Chemical Co. (Trizma Base, reagent grade). Cacodylic acid (Sigma, 98% pure) was recrystallized twice from 2-propanol. Water used in this work was purified with a Milli RO-4/Milli-Q system (Millipore Corp.) and exhibited a resistivity of 18 MΩ on delivery.

Table I. Heterogeneous Electron-Transfer Kinetic Parameters for the Reduction of Purified Horse Heart Cytochrome *c* at Tin-Doped Indium Oxide Optically Transparent Electrodes[a]

cytochrome *c*, μM	$k^{\circ\prime}_{s,h}$, cm/s	α	technique
38	4.9 (±0.3)[b] × 10^{-3}	0.5[c]	CV[d]
73	1.7 (±0.3) × 10^{-3}	0.5	CV
300	8.1 (±0.4) × 10^{-4}	0.5	CV
73	1.0 (±0.4) × 10^{-4}	0.5	DCVA[e]

[a] All samples in 0.24 M cacodylic acid, 0.21 M Tris, pH 7.0, ionic strength 0.20 M. [b] Parentheses contain standard deviation. [c] The electrochemical transfer coefficient is assumed to be 0.5. [d] Rate constants calculated from CV;[45] scan rates ranged from 5.0 to 200 mV/s; ten individual CVs were used to calculate rate constants in the first entry, eight individual CVs were used in the second and third entries. [e] Rate constant determined by best fit of digitally simulated results to experimental DCVA responses shown in Figure 2B. Error limit is estimated from digital simulation results.

Tin-doped indium oxide and fluoride-doped tin oxide OTE materials were obtained from PPG Industries. Electrodes were cleaned immediately before use by successive 5-min sonications in Alconox solution, in 95% ethanol, and twice in purified water.[38]

For experiments requiring the in situ addition of a solid cytochrome *c* sample to deoxygenated electrolyte, a two-chamber voltammetry cell of Lucite construction was used.[39] The platinum auxiliary electrode was isolated from the working electrode chamber by porous Vycor glass (Corning Glass Works). The OTE was mounted on the side of the cell by using a retainer plate and an O-ring seal. A cell cover plate accommodated a nitrogen blanket purge tube, a sample addition port, and an Ag/AgCl (1.00 M KCl) reference electrode. All potentials in this paper are described vs. the normal hydrogen electrode (NHE).

DCVA experiments utilized an OTE cell[40] incorporating a quartz lightpipe.[41] This cell was filled via glass syringe transfer of a deoxygenated cytochrome *c* solution from a sealed serum bottle. The cytochrome *c* solution was prepared by addition of solid cytochrome *c* to nitrogen-deoxygenated electrolyte in the serum bottle and then the bottle was sealed under nitrogen. CVs of the reversible methyl viologen dication/cation radical redox couple were used to determine the uncompensated resistance of the cells used in this work. The CV peak potentials of cytochrome *c* were corrected for this effect. Tris/cacodylate buffer was used in both the methyl viologen and cytochrome *c* CV experiments. This medium has been shown to be devoid of any significant anion or cation binding to cytochrome *c*.[42,43]

CV and DCVA responses were obtained by using either a Model 174 polarographic analyzer (Princeton Applied Research) or a potentiostat of previously reported design.[35] Digital simulation of DCVA responses has been reported elsewhere.[35,44] All experiments were performed at room temperature, 22 ± 2 °C.

Results and Discussion

When a solid sample of commercial cytochrome *c* was added to deoxygenated buffer, allowed to dissolve in quiet solution, and then stirred for a few seconds, the cyclic voltammetric behavior shown in Figure 1 was obtained at indium oxide OTEs. Analogous responses were observed at tin oxide OTEs but exhibited a lower degree of reversibility, as noted previously by Yeh and Kuwana.[27] When these experiments were repeated with purified cytochrome *c*, the CV responses shown in Figure 2A were obtained at indium oxide OTEs. These responses were reproducible for introduction of cytochrome *c* into the cell in either solid or solution form. The CV responses shown in Figure 2A illustrate the reproducibility

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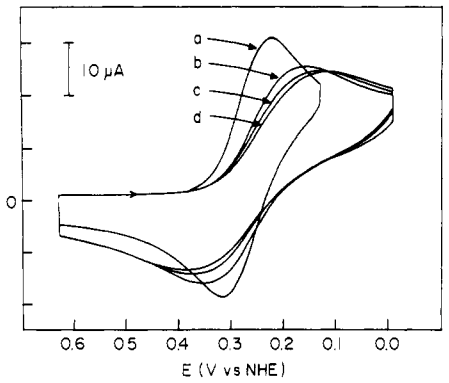

Figure 1. Cyclic voltammetry of cytochrome *c* at tin-doped indium oxide optically transparent electrodes before purification. Solution contained 100 μM cytochrome *c* (Sigma type VI), 0.21 M Tris, and 0.24 M cacodylic acid, pH 7.0, 0.20 M ionic strength. Electrode area = 1.23 cm^2, ν = 10 mV/s. (a) Initial CV taken immediately upon dissolution of solid cytochrome *c* sample; (b) CV taken 10 min after (a); (c) CV taken 20 min after (a); (d) CV taken 40 min after (a); CVs taken more than 40 min after (d) were identical with (d).

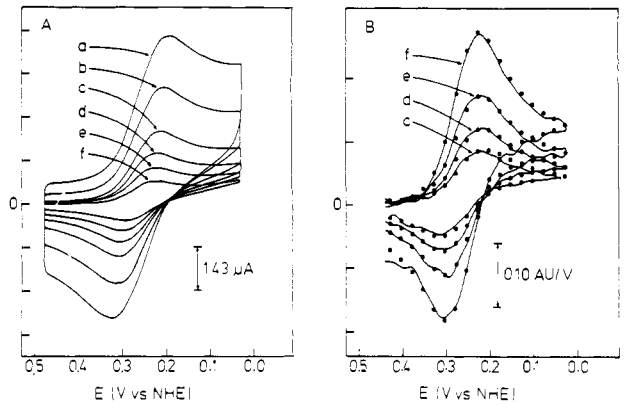

Figure 2. (A) Cyclic voltammetry of cytochrome *c* at tin-doped indium oxide optically transparent electrodes after purification. Solution contained 73 μM cytochrome *c*; other solution conditions are the same as in Figure 1. Electrode area = 0.71 cm^2. Potential scan rates in mV/s are as follows: (a) 100; (b) 50; (c) 20; (d) 10; (e) 5.0; (f) 2.0. (B) Derivative cyclic voltabsorptometry of cytochrome *c* at tin-doped indium oxide optically transparent electrode after purification. Experimental conditions are given in Figure 2A. Circles are calculated DCVA responses for 73 μM cytochrome *c*, $E^{\circ\prime}$ = 0.260 V vs. NHE, n = 1.0, diffusion coefficient of ferri- and ferrocytochrome *c* = 1.2×10^{-6} cm^2/s, difference molar absorptivity at 416 nm = 57 000 M^{-1} cm^{-1},[37] $k^{\circ\prime}_{s,h}$ = 1.0×10^{-3} cm/s, α = 0.5.

of these results with time. As many as 30–40 CVs could be acquired at a single OTE in random order of potential scan rate with only minor changes in the response. No effort was made to evaluate a useable upper limit for repetitive experiments.

DCVA experiments were performed with one sample of purified cytochrome *c* and typical results are shown in Figure 2B together with digitally simulated best-fit responses based on Butler–Volmer theory.[35,44]

CV experiments were also performed with purified cytochrome *c* at tin oxide OTEs under the same conditions used in the indium oxide OTE experiments. The CV responses were reproducible for repetitive experiments, but the electrode reaction was less reversible than that observed at indium oxide OTEs, again in agreement with Yeh and Kuwana.[27]

The heterogeneous electron-transfer kinetic parameters for the reaction of purified cytochrome *c* at indium oxide OTEs were evaluated from the CV and DCVA responses. In CV experiments these kinetic parameters were determined from the scan rate dependence of the separation in peak potentials after the manner of Nicholson.[45] The kinetic parameters used to best fit the

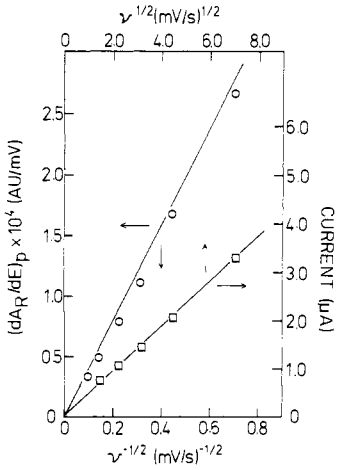

Figure 3. Potential scan rate dependence of CV and DCVA peak responses: (○) DCVA results; (□) CV results. Data taken from Figure 2A,B. Solid lines are theoretical responses calculated using parameters given in Figure 2A,B except the electrode reaction is assumed to be reversible.

digitally simulated DCVA responses to the experimental responses shown in Figure 2B together with the CV kinetic results are given in Table I. Figure 3 shows the scan rate dependencies of the CV and DCVA peak responses from the experiments described in Figure 2A,B. Since DCVA is insensitive to nonfaradaic charge consuming processes,[35] it is unnecessary to correct the experimental peak responses for this effect. However, the CV peak responses are affected by both faradaic and nonfaradaic processes, and subtraction of the latter component is necessary for comparison of experimental and theoretical CV peak responses. In this work CV experiments were performed on buffer alone at the same scan rates employed in the presence of cytochrome *c*. These background CVs were used together with the results shown in Figure 2A to determine the CV peak currents shown in Figure 3. Also shown in Figure 3 are the theoretical CV[46,47] and DCVA (35) peak responses for a *reversible* electrode reaction. Comparison of the experimentally observed CV and DCVA peak responses with those expected for a reversible electrode reaction indicates that these experimental responses are nearly mass transfer controlled.

It is clear from the results shown in Figure 3 that the reaction of purified cytochrome *c* at indium oxide OTEs is quasi-reversible for the experimental conditions used in this work. Bearing in mind that the diffusion coefficient of cytochrome *c* used to calculate the theoretical responses shown in Figure 3 agrees with the accepted nonelectrochemical literature value (1.2×10^{-6} cm^2/s[48]), the definition of the present work as quasi-reversible is well within the criteria set forth by Matsuda and Ayabe.[49]

The data shown in Table I exhibit a concentration dependence of the formal heterogeneous electron-transfer rate constant ($k^{\circ\prime}_{s,h}$). The origin of this effect is not presently known. It may be due to trace impurities present even after the procedure used in this work for cytochrome *c* purification. An experimental delineation of the seminal aspects of this effect is in progress.

The results presented here indicate that the heterogeneous electron-transfer kinetics of cytochrome *c* at solid electrodes are markedly affected by further purification of high quality commercial samples. It is clear that the issue of sample purity must be considered in any studies directed at characterizing the electrochemical responses of cytochrome *c*. This work also suggests that simple CV experiments at solid electrodes may be a powerful complementary tool in establishing the purity of native cytochrome *c* samples.

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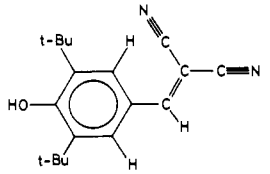
Restricted Intramolecular Rotation of the Potent Uncoupler of Oxidative Phosphorylation of SF 6847 ((3,5-Di-*tert*-butyl-4-hydroxybenzylidene)malononitrile): Enhanced Motional Freedom of SF 6847 Anion by Formation of a 1:1:1 Complex with Valinomycin and K^+

Kenichi Yoshikawa† and Hiroshi Terada*

Contribution from the College of General Education and the Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima 770, Japan. *Received January 27, 1982*

Abstract: The dynamic structure of a potent uncoupler of oxidative phosphorylation, (3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile (SF 6847), was studied by ^1H NMR under various conditions. It was found that the degree of intramolecular motional freedom around the C–C bond between the benzene ring and the malononitrile moiety changes greatly depending on the environment. The freedom of the intramolecular motion (restricted rotation) was greatly reduced by change from the neutral to the anionic form. The freedom of the intramolecular motion in the SF 6847 anion increased on formation of a ternary complex with valinomycin and potassium ion. This unique dynamic structure of SF 6847 is discussed in connection with the acidity and potent biological activity of this uncoupler.

Uncouplers of oxidative phosphorylation abolish the link between substrate oxidation and ATP synthesis in energy-transducing membranes. Various kinds of organic molecules of synthetic fungicides, acaricides, and herbicides exhibit very potent uncoupling activity.[1] The most potent uncoupler known to date is SF 6847 ((3,5-di-*tert*-butyl-4-hydroxybenzylidene)malononitrile),[2,3] which is effective in mitochondria under usual experimental conditions at about 10 nM, while the well-known "classical" uncoupler 2,4-dinitrophenol is effective at about 50 μM. The molecule of SF 6847



is characterized by hydrophobic *tert*-butyl groups, a strong electron-withdrawing malononitrile group, and an acid-dissociable phenolic hydroxyl group.[1] Other potent uncouplers, such as FCCP,[4,5] TTFB,[6] and S-13,[7] have similar structural features. The structural requirements of potent uncouplers for uncoupling activity are still unclear, although extensive studies have been done on this problem from various points of view.[8-15]

These strong uncouplers are thought to be carriers of H^+; the uncoupler cycles in the membrane alone or in the form of a complex with a hypothetical cation translocator, the uncoupler anion picking up H^+ on one side of the membrane–water interface and releasing it on the other side.[1,16-19] Valinomycin is sometimes taken as a model of cation translocators of the latter type.[20,21]

It has been generally thought[19] that the anionic form of potent uncouplers has a planar structure and so is stable in a hydrophobic environment owing to delocalization of the negative charge. Thus, the conformation of the SF 6847 anion is also expected to be planar: the phenol ring and the malononitrile group are thought to be coplanar. However, we found[22,23] that the planar form is quite unstable and that the malononitrile moiety tumbles over the

* Address correspondence to the Faculty of Pharmaceutical Sciences.
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