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The Direct Electron Transfer Reactions of Cytochrome *c* at Electrode Surfaces

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Examples of facile electron transfer between electrode surfaces and electron transfer proteins were first reported nearly twenty years ago. Substantial progress has been achieved in understanding the fundamental requirements that must be met in order to observe such reactions. Despite the large body of work that has now been published on a host of examples of such reactions, there continues to be a lack of understanding over just what conditions must be met in order to use direct electrochemical methods to characterize the electron transfer thermodynamics and kinetics of electron transfer proteins. Part of the problem lies in the inherent difficulties associated with using solid electrodes. Equally as important is the wide range of attention that has been paid to the purity of the electron transfer protein solutions being studied. The goal here is to bring together those aspects of this problem that enjoy some degree of agreement among scientists in this field and to reflect upon those issues that remain to be resolved. The widely studied electron transfer protein, cytochrome *c*, will be the focus of this paper.

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INTRODUCTION

This review aims to focus on those factors that have been shown to affect the application of direct electrochemical methods to the study of protein electron transfer reactions. Although this review will not be comprehensive in presenting all factors that have been reported to be of importance in this area, the discussion will focus on those factors that clearly must be considered important in a most universal sense. Work published from the authors' laboratories will largely be used to develop a picture of what is known about the electrode/solution interface in studies of protein electron transfer reactions. Using these examples some of the problems in past work, and even in current work, will be made clear. In evaluating the work published in this area it must be recognized that, upon reflection, much has been reported that may be presently understood as artifactual due to the condition of the electrode surface, the protein sample solution or both. This requires that the reader and anyone starting work in this field understand this history and be cognizant of the current status of this field in order to critically evaluate publications in this area. Some current work continues based upon the status that existed in this field a decade ago.

Before beginning to review the field of protein electrochemistry it is very important to define initially what is meant by the term "facile" heterogeneous electron transfer. There are numerous terms used in this literature to describe rates of heterogeneous electron transfer between an electrode surface and a protein in solution. Here "facile" means a redox couple that exhibits reversible to quasi-reversible heterogeneous electron transfer kinetics as originally defined by Matsuda and Ayabe.[1,2] When using cyclic voltammetry, the most commonly applied electrochemical method in this area, this means that the redox couple exhibits heterogeneous electron transfer kinetics that permit acquisition of interpretable cyclic voltammograms at what can be defined as "X–Y recorder" potential scan rates, i.e., less than ca. 500 mV/s. At these scan rates this corresponds to differences in cyclic voltammetric peak potentials that are less than about 200 mV

for an $n = 1$ electron transfer protein with a nominal diffusion coefficient of 1×10^{-6} cm^2/s. Another useful definition is that the difference in cyclic voltammetric peak potentials falls within the classic Nicholson treatment for the determination of heterogeneous electron transfer rate constants using cyclic voltammetry.[3] Nicholson's table for peak potential difference versus the dimensionless kinetic parameter for determining heterogeneous electron transfer rate constants is reliable between values of about 65 and 200 mV. For any redox couple falling outside of this range, use of cyclic voltammetry to determine heterogeneous electron transfer kinetic parameters becomes problematic. Values of peak potential separation smaller than ca. 65 mV, i.e., reversible kinetics, are difficult to measure reliably, and for values greater than ca. 200 mV, i.e., irreversible kinetics, heterogeneous electron transfer rate constants are difficult to evaluate. In the former case caution must be used as higher potential scan rates are probed to obtain larger values of peak potential separation due to errors arising from uncompensated solution resistance. Although this is rarely a problem with protein heterogeneous electron transfer kinetics because the rate constants are not that large, the advent of fast scan microelectrode voltammetry has provided a means of reliably attacking such systems.[4] In the latter case irreversible heterogeneous kinetics exhibit cyclic voltammograms in which both reductive and oxidative peak currents can become indistinguishable from the background charging currents. Potential step methods are applicable to the irreversible kinetic regime, but these experiments are far more laborious to conduct than is cyclic voltammetry.[5,6]

Although space does not permit a detailed discussion of the caveats that must be considered in acquiring reliable cyclic voltammograms of proteins, there are occasions when careful consideration of the shape of the entire cyclic voltammogram can reveal subtleties that arise from unexpected reaction mechanisms. However, this will require acquisition of buffer/electrolyte background cyclic voltammograms that can be subtracted from the cyclic voltammograms of the protein sample. In a recent work cyclic voltammetry was used to study a ligand binding reaction of myoglobin accompanying electron transfer.[7] Careful comparison of cyclic voltammograms with digitally simulated cyclic voltammograms showed evidence of a ligand association/dissociation reaction, i.e., water, that had not been observable spectroscopically. The requirement for doing background

subtraction of buffer/electrolyte voltammograms from cyclic voltammograms of protein solutions is now easily assessable with computer data acquisition systems. One must be careful to consider any time-dependent effects that might occur during these experiments that could alter the background cyclic voltammograms. It is useful to obtain buffer/electrolyte cyclic voltammograms, both prior to experiments with the protein sample and after these experiments, to know if such changes have occurred. While the use of digital simulation methods to model the thermodynamics and kinetics of electrode reaction mechanisms has largely been restricted to the electrochemical community via locally written programs, a general digital simulation package has just become commercially available so that this barrier to its use should be removed.[8] Even line on line agreement between background subtracted cyclic voltammograms and digitally simulated voltammograms should not be solely used with full confidence for electrode reaction mechanism elucidation involving coupled chemical reactions, as is clearly demonstrated in this recent paper.

With this background in hand the foundations of direct protein electrochemistry will now be considered. The early development of this field was sporadic due to experimental pitfalls, a substantial body of literature that had only observed highly irreversible direct electron transfer between an electrode and a redox protein (i.e., reactions were only observed at large overpotentials), and a reluctance to accept early results as other than artifacts, which in some cases proved to be accurate. The litany at that time was that electrodes *could not* serve as electron transfer partners with redox proteins, i.e., the distance between the electrode surface and the redox site was too great; strong adsorption of protein on electrode surfaces would block access to solution resident protein; and electrode surfaces do not possess the relevant surface chemistry of *in vivo* reaction partners.

The initial reports of facile direct electron transfer reactions of redox proteins, as defined earlier, can be summarized with some ease due to the paucity of examples. Certainly a substantial body of literature on this subject preceded these reports, but this work involved what would be defined as redox reactions exhibiting irreversible heterogeneous electron transfer kinetics, systems not readily examined by cyclic voltammetry and kinetics that required substantial

overpotentials in order to observe Faradaic electron transfer reactions. An earlier review attempted to cover this work comprehensively.[9]

Perhaps the first report of an electron transfer protein exhibiting facile heterogeneous electron transfer kinetics is that of Niki's group at the start of their pioneering work on the protein, cytochrome c_3.[10] This cytochrome is special because it possesses four heme iron moieties within a protein of quite small size, ca. 10,000 Daltons. Although the detailed picture of how this protein undergoes electron transfer is highly complex and is believed to involve reaction cooperativity, its facile electron transfer kinetics at numerous electrode surfaces has been largely ascribed to the accessibility of the hemes to the surface of the protein affording a minimal distance for electron transfer at an electrode surface. For this reason cytochrome c_3 is not representative of the more typical single heme iron cytochromes and it will not be considered further here. However, work from this group has continued to probe the highly complex nature of the reactions of this particular electron transfer protein as well as that of cytochrome *c* (vide infra).

The next example of facile electron transfer kinetics of a redox protein was that of the iron–sulfur protein, spinach ferredoxin, at a modified gold minigrid electrode surface by Landrum *et al.*[11] The gold minigrid electrode was modified by the electrochemical polymerization of methyl viologen at negative potentials. Later studies indicated that this polymerization reaction was driven by the formation of adsorbed hydrogen atoms that irreversibly produce an electroinactive polymeric film on the gold surface.[12] This modified electrode did not function to transfer electrons with ferredoxin through a redox mediator type mechanism but rather through modification of the electrode/solution interface. Spinach ferredoxin is a one-electron transfer protein with a size of ca. 11,000 Daltons. It is a rather fragile protein in that its two-iron, two-sulfur bridged redox center readily decomposes upon exposure to oxygen as evidenced by loss of its 420 nm absorption maximum. Although the heterogeneous electron transfer kinetics of ferredoxin at this modified gold minigrid surface were further studied by several different electrochemical and spectroelectrochemical methods,[13] more recent work suggests that greater success can be achieved by using better electrode-modifying reagents.[14–16]

The first reports of facile heterogeneous electron transfer kinetics for cytochrome *c* were those of Eddowes and Hill[17] and Yeh and Kuwana.[18] The former group used 4,4′-bipyridyl in solution at a gold electrode to observe a facile electron transfer reaction of cytochrome *c*. In this work 4,4′-bipyridyl is proposed to adsorb on the gold surface, displacing or preventing adsorption by cytochrome *c*, so that direct electron transfer can occur. Moreover, interaction of the nitrogen lone pairs with positively charged lysine residues on the reaction surface of cytochrome *c* was proposed as a mechanism that enhanced this reaction, although that interpretation has been questioned.[19] The latter group used clean indium oxide electrodes alone and were able to observe direct cyclic voltammetric responses for cytochrome *c* at this surface.[18] Also described in that paper was the less reversible reaction of cytochrome *c* at tin oxide electrodes.

These examples of facile electron transfer for electron transfer proteins at electrode surfaces served to stimulate work in this area when the prospects for using direct electrochemical methods for such measurements were seen as quite dim at that time.

STUDIES OF CYTOCHROME *c* ELECTRON TRANSFER REACTIONS AT UNMODIFIED ELECTRODE SURFACES

Following the initial reports of success in observing facile electron transfer of cytochrome c_3 at mercury,[10] ferredoxin at methyl viologen modified gold,[11] and cytochrome *c* at 4,4′-bipyridyl modified gold[17] and at indium oxide,[18] interest grew in finding other electrode surfaces at which protein electron transfer could be observed. This period was marked by a substantial degree of irreproducibility between laboratories in obtaining particular results. An effort to observe facile cyclic voltammetry for cytochrome *c* at the methyl viologen modified gold minigrid electrode failed, although electron transfer could be observed in an optically transparent thin-layer electrochemical cell using large overpotential steps.[20] Similar results were observed for reactions of cytochrome *c* at indium oxide and tin oxide optically transparent electrodes. Several spectroelectrochemical methods for the determination of heterogeneous electron transfer kinetic parameters had been or were under development at that time in Blount's group.[21–24] These methods permitted the determination of heteroge-

neous electron transfer kinetic parameters for proteins at these optically transparent electrode surfaces when the magnitude of the rate constants was too small, i.e., irreversible, to permit use of cyclic voltammetry, vide supra.

The result from one kind of spectroelectrochemical experiment, derivative cyclic voltabsorptometry (DCVA), is shown for the reaction of cytochrome *c* at an optically transparent indium oxide electrode in Fig. 1.[25] This method is an optical analogue of cyclic voltammetry which is free from the interferences due to background currents. As is clearly evident, the experimental result agrees well with results calculated using single potential step chronoabsorptome-

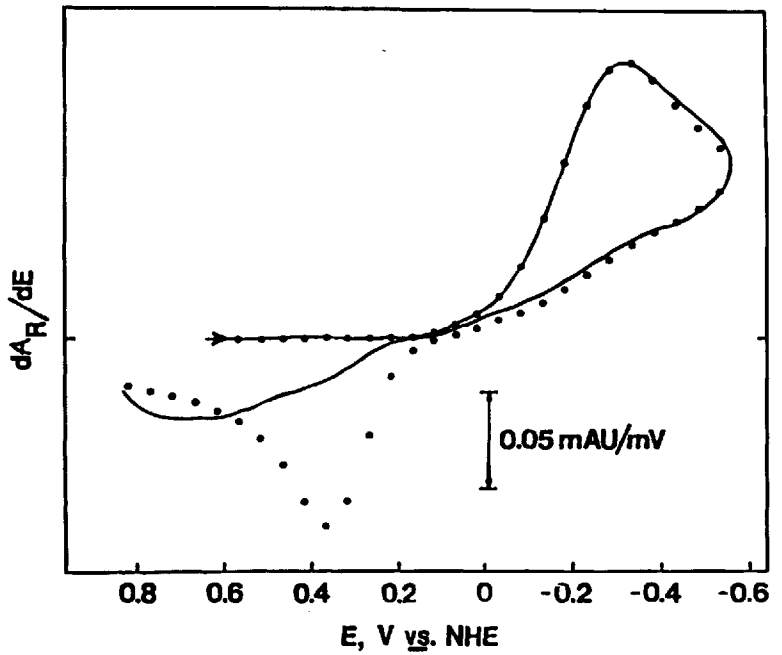

FIGURE 1 Derivative cyclic voltabsorptometry of unpurified cytochrome *c* at tin oxide optically transparent electrodes. [cyt *c*] = 89 μM: pH 7.0 phosphate buffer (0.07 M), scan rate 4.0 mV/s, monitored wavelength 416 nm. Line: experimental result, circles: digitally simulated response taken from SPS/CA results with $k' = 2.2 \times 10^{-6}$ cm/s, alpha = 0.31, $D = 1.1 \times 10^{-6}$ cm²/s. (Reproduced from Ref. 23 with permission, Academic Press.)

try (SPS/CA) kinetic data for the reductive potential sweep, but poor agreement is evident in the oxidative sweep. A potential liability in conducting single potential step experiments, either chronocoulometry or chronoabsorptometry, is the lack of direct information regarding the reverse electron transfer reaction, either oxidative or reductive. Of course cyclic voltammetry and DCVA provide information on both the forward and the reverse electron transfer reactions. The method of asymmetric double potential step chronoabsorptometry (ADPS/CA)[24] was developed with this problem in mind, namely being able to probe the heterogeneous electron transfer kinetics of an electrode reaction in both directions. Results from ADPS/CA experiments, in concert with SPS/CA results, showed the same discrepancy in that the transfer coefficients determined from reductive and oxidative potential steps did not sum to unity.[6]

These data ultimately led to concerns regarding the integrity of the cytochrome *c* sample being studied and to the finding that chromatographically purified samples produced facile electron transfer at both indium and tin oxide electrodes.[26] Upon following long-established procedures for preparing purified cytochrome *c*[27] and storing these samples in lyophilized form, the cyclic voltammetric responses obtained at preconditioned, hydrophilic indium oxide electrodes were facile, reproducible and stable for a long period of time, e.g., a day. Later, the major contaminants of the commercial samples of cytochrome *c*, oligomeric and deamidated forms, were shown to be responsible for the time-dependent heterogeneous electron transfer kinetics, with the former being far more deleterious than the latter.[28] As will be discussed later, promoter modified electrodes have now been shown to be more robust in their electron transfer communication with even these denatured forms of cytochrome *c* compared with unmodified electrode surfaces. It is clear that the future use of electrochemical methods in the study of protein electron transfer reactions will benefit from the use of such electrode modification strategies.

Subsequent studies of various electrode surface cleaning procedures demonstrated the need to have an electrode surface that is hydrophilic in order to observe facile electron transfer with cytochrome *c*.[29] For instance, gold electrodes cleaned in a soft hydrogen flame produce a hydrophilic surface that exhibits facile electron transfer with these samples of cytochrome *c*. However, at that time

the cyclic voltammetry was not stable and the reaction became irreversible with time (later this instability was eliminated by using freshly purified samples, vide infra). This observation offered direction for subsequent studies and it led to a particularly remarkable observation. If indium oxide electrodes were cleaned and then placed in a vacuum oven overnight to dry the surface, no electrochemistry of cytochrome *c* was observed when these hydrophobic electrodes were introduced into sample solutions. However, upon leaving these electrodes in an assembled cell overnight, quasi-reversible cyclic voltammograms were obtained that were stable over time. Consequently indium oxide and tin oxide electrodes have always been equilibrated in buffer by continuous cyclic voltammetric scanning until a stable response is obtained prior to the introduction of cytochrome *c* solutions. The resultant cyclic voltammetry is very stable at indium oxide electrodes. The sensitivity of the spectroelectrochemical probes to Faradaic processes coupled with their insensitivity to non-Faradaic charging currents served to guide the progress described above. Commercial lyophilized cytochrome *c* samples produced solutions with active adsorbents that rendered the heterogeneous electron transfer kinetics irreversible over time.

Another result obtained during work aimed at finding conditions that would permit application of surface enhanced resonance Raman spectroscopy to the electron transfer reactions of cytochrome *c* at silver electrodes proved to be very important. As described above the use of a spectroelectrochemical method also solved this problem. The application of derivative cyclic voltabsorptometry (DCVA)[23] to the study of cytochrome *c* reactions[24] was described earlier. The DCVA method produces a signal that has the same shape as cyclic voltammetry. Unlike cyclic voltammetry, DCVA is not affected by the non-Faradic background signal that corrupts cyclic voltammetry when the optically monitored wavelength is judiciously chosen. Moreover, the DCVA peaks grow in *indirect* proportion to the potential scan rate while, of course, in cyclic voltammetry peak currents grow in *direct* proportion to potential scan rate. This means that quasi-reversible redox couples, which do not produce improved cyclic voltammograms upon scanning potential faster (as is realized for reversible redox couples), do show increased DCVA peaks upon scanning potential slower *and* peak separations decrease.

DCVA results obtained for previously purified and lyophilized cytochrome *c* samples were not stable and rapidly led to irreversible heterogeneous electron transfer kinetics at clean silver electrode surfaces as shown in Fig. 2.[30] This result was reminiscent of the earlier results obtained with as-received cytochrome *c* samples at indium oxide electrodes as shown in Fig. 1 and therefore suggested a surface active adsorbing impurity. When a sample of the purified and then lyophilized cytochrome *c* sample was again subjected to ion exchange chromatography, a band was visually obvious with a retention time consistent with oligomeric forms. This result suggested that some oligomeric forms of cytochrome *c* had been formed on lyophilization. When freshly purified cytochrome *c* samples were collected directly from the chromatography column and then immediately subjected to cyclic voltammetry at clean silver electrodes, facile heterogeneous electron transfer kinetics, stable for many hours, were obtained as

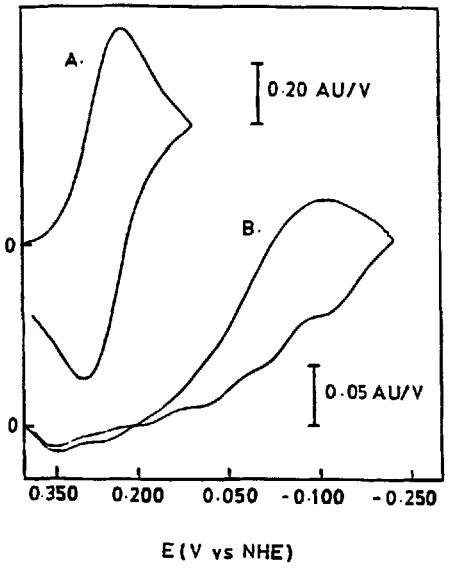

FIGURE 2 Derivative cyclic voltabsorptometry of purified and then lyophilized cytochrome *c* at a clean silver electrode in 0.05 M Na_2SO_4. (A) 199 μM chromatographically purified cytochrome *c;* (B) 98 μM cytomatographically purified and then lyophilized cytochrome *c* sample. (Reproduced from Ref. 30 with permission, American Chemical Society.)

shown in Fig. 3. Clearly, clean metal electrodes do exhibit facile and stable cyclic voltammetry with pure samples of cytochrome *c*. Excellent agreement between background subtracted cyclic voltammograms and digitally simulated results are evident in Fig. 3. Freshly purified solutions of cytochrome *c* must be used in studies with unmodified metal electrodes, whereas the highly hydrophilic surface of indium oxide shows less tendency to adsorb small concentrations of cytochrome *c* impurities. To further demonstrate the stability of cytochrome *c* voltammetry at clean metal electrode surfaces, a study using platinum, gold and mercury was conducted (Fig. 4).[31] Again, excellent agreement between background subtracted cyclic voltammograms and digitally simulated results was obtained.[31] The cyclic voltammetric results are stable for hours.

The point to be drawn from this section is that the thermodynamics and kinetics of heterogeneous electron transfer by cytochrome *c* can be studied by direct electrochemical methods using unmodified metal

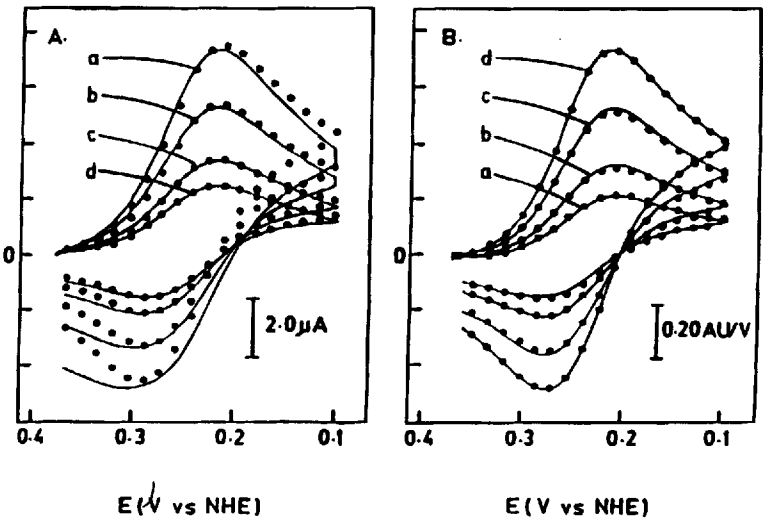

FIGURE 3 Cyclic voltammetry and derivative cyclic voltabsorptometry of freshly purified 199 μM cytochrome *c* at a clean silver electrode. (A) Background subtracted CV; (B) derivative cyclic voltabsorptometry. Circles are simulated responses for $k^{o'} = 1.5 \times 10^{-3}$ cm/s, alpha = 0.55 and working electrode area = 1.23 cm^2. Potential scan rates in mV/s are as follows: (a) 10.40; (b) 5.20; (c) 2.04; (d) 1.04. (Reproduced from Ref. 30 with permission, American Chemical Society.)

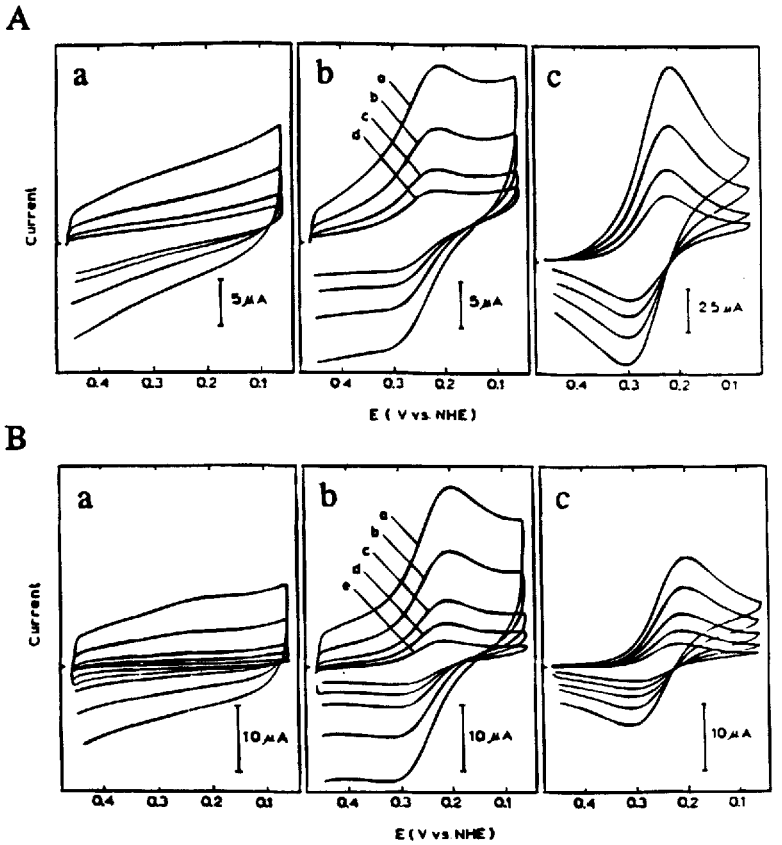

FIGURE 4 Cyclic voltammetry of cytochrome *c* at clean platinum and gold electrodes. (A) Platinum electrode: (a) 0.20 M tris/cacodylic acid buffer, pH 7.0; (b) same as in (a) after the addition of 85 μM cytochrome *c;* (c) background subtracted CV, (a) − (b). Scan rates in mV/s: (a) 64; (b) 32; (c) 16; and (d) 8. Electrode area 1.23 cm^2. (B) Gold electrode, same as in (A) except 134 μM cytochrome *c*. Scan rates in mV/s: (a) 100; (b) 50; (c) 20; (d) 10; and (e) 4.0. (Reproduced from Ref. (31) with permission, Springer-Verlag.)

and metal oxide electrode surfaces. There may be advantages in using unmodified electrode surfaces in such studies if the aim is to elucidate features of cytochrome *c* that are involved in its electron transfer reactions. Although a clean electrode surface is not without its own chemical properties, it may afford a simpler model when compared with an electrode that has its surface modified with a compound that can bring its own chemical properties to bear upon any electrode reaction mechanism. At minimum, comparison of voltammetric data for redox proteins taken at unmodified and at modified electrode surfaces can be used to strengthen interpretations of properties affecting thermodynamics and kinetics as being due to the protein itself and not to the electrode surface.

The robustness of the cyclic voltammetry of cytochrome *c* can be seen in studies of the temperature dependence of the formal potential, and the heterogeneous electron transfer rate constant were being determined.[32,33] Figure 5 shows data illustrating the quality of the data that can be obtained during a series of cyclic voltammetric experiments requiring many hours that permitted the determination of entropic factors affecting electron transfer. The point made above regarding the utility of comparing results obtained at unmodified and modified electrode surfaces is demonstrated by just such a comparison[34] that drew from diverse data with respect to electrode conditions, e.g., Refs. 32, 35 and 36.

STUDIES OF CYTOCHROME *c* ELECTRON TRANSFER REACTIONS AT MODIFIED ELECTRODE SURFACES

As already described in the Introduction and in the previous section, various modified electrodes have been used to obtain cyclic voltammetry for the direct electron transfer reactions of cytochrome *c*. Although the previous section focussed on the use of unmodified electrodes to study protein redox reactions, the utility of modified surfaces has been shown to be especially valuable for many more situations, as will be described below.

The first example of a modified electrode in the study of cytochrome *c*, again, was the use of 4,4′-bipyridyl adsorbed on gold by Hill's group.[17,37] This compound is not electroactive in the potential region used to study cytochrome *c* and therefore it does not serve

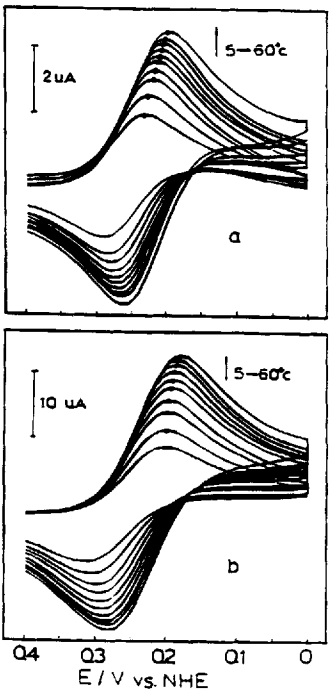

FIGURE 5 Temperature dependence of the cyclic voltammetry of freshly purified cytochrome *c* samples at indium oxide electrodes. [Cytochrome *c*] = 120 μM from tuna heart in tris/cacodylic acid buffer, pH 7.0, ionic strength 0.2 M; scan rates (a) 20 mV/s and (b) 500 mV/s. (Reproduced from Ref. 33 with permission, Elsevier Sequoia S. A.)

as an electron transfer mediator. Mediators are themselves electron transfer redox systems that couple the electron transfer reactions of a biological redox molecule to an electrode. In subsequent work it was shown that the electrode reactions of cytochrome *c* were not affected by the presence of 4,4′-bipyridyl unless its solution concentration was maintained above about 1 mM at gold and above about 5 mM at platinum.[38] This result is consonant with the view that the function of 4,4′-bipyridyl is to displace those forms of cytochrome *c* that readily adsorb to electrode surfaces (two forms have already been mentioned, oligomeric and deamidated forms, but various denatured forms also adsorb more strongly than native cytochrome *c*)

blocking electron transfer reactions with solution resident cytochrome *c*. This type of surface modifier certainly has an important historical place in the literature on the direct electrochemistry of cytochrome *c*, but for reasons that should be evident, superior systems have been found and used extensively by numerous groups.

A different scheme for modifying electrode surfaces was initially reported by one of our groups that involves the formation of a robust covalent bond between the modifying reagent and the gold surface.[39,40] This reagent, bis(4-pyridyl)disulfide, produces gold/thiol covalent bonds upon cleavage of the disulfide bond that creates a modified interface that functions similarly to the 4,4′-bipyridyl modified gold surface with respect to enhancing electron transfer with cytochrome *c*. Electrode modifiers such as 4,4′-bipyridyl and bis(4-pyridyl)disulfide have been appropriately described as "promoters."[39,40] (Current literature can be found that incorrectly uses the terms promoter and mediator interchangeably.) A model for this modified gold electrode surface is shown in Fig. 6. Although there are variations in the interpretation of the mechanism by which this and other promoters function, a number of surface Raman spectroscopic studies suggests that this is a plausible picture of this modified interface.[41,42]

This modified gold surface is especially efficacious in exhibiting facile heterogeneous electron transfer kinetics with cytochrome *c* and with its various denatured forms. A host of adsorption based and gold/thiol bond-forming promoters have been studied with varying degrees of efficacy with respect to facilitating cytochrome *c* electron

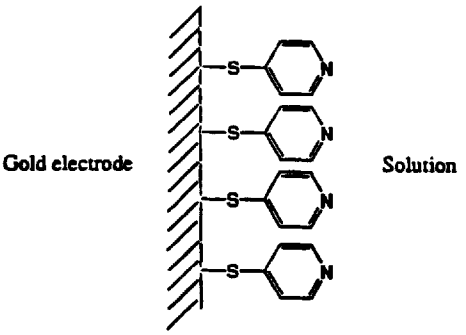

FIGURE 6 Model for the modification of gold by bis(4-pyridyl)disulfide.

transfer reactions.[43–46] However, results of surface enhanced Raman spectroscopy studies of this and other thiol/gold modifiers, shown in Fig. 7, reveal some essential characteristics for facile electron transfer with cytochrome *c*.[47] Each modifier studied in this series showed complete cleavage of the disulfide bond, and no evidence for the S–S stretching vibration at 550 cm^{-1} as noted in the spectra of the these compounds in solid form, indicating that the structures shown in the inset of Fig. 7 are being formed. Gold electrodes modified with bis(4-pyridyl)disulfide (trace (b)) produced effective promoter surfaces for cytochrome *c* electron transfer. Modification of gold with 4-mercaptopyridine gave the same result. Diminished cyctochrome *c* electron transfer activity was seen on gold modified by bis(2-pyridyl)disulfide. Clearly, the presence of the nitrogen para to the gold/thiol bond is sensitive to the electrochemistry that is observed with cytochrome *c*.

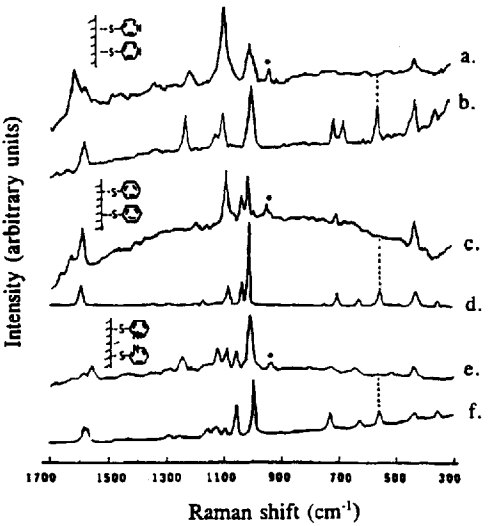

FIGURE 7 Surface enhanced Raman spectra of gold electrodes modified with (a) bis(4-pyridyl)disulfide, (c) bis(pyridyl)disulfide and (e) bis(2-pyridyl)disulfide in pH 7.0 phosphate buffer containing 0.1 M $NaClO_4$ at 0.0 V vs. SCE. Powder Raman spectra for samples of these modifiers are shown in traces (b), (d) and (f). A He–Ne laser (632.8 nm, 30 mW) was used. *: due to perchlorate. (Reproduced with permission from Ref. 47, Plenum Press.)

An important advantage of the electrode modification scheme using the bis(4-pyridyl)disulfide is the ease of electrode modification. A clean gold electrode is simply immersed in a solution of the reagent for a few minutes, followed by rinsing. Hence, no promoter is present in the cytochrome *c* solution. In the absence of mechanical abrasion, extremes of applied potential or similar abuses, these modified surfaces remain robust for periods of over a week. The practical utility of these chemically modified gold/thiolate surfaces can be illustrated by their response to the native, oligomeric and deamidated forms of cytochrome *c* as described earlier.[28] These results are shown in Fig. 8. At indium oxide the cyclic voltammetry is sensitive to the form

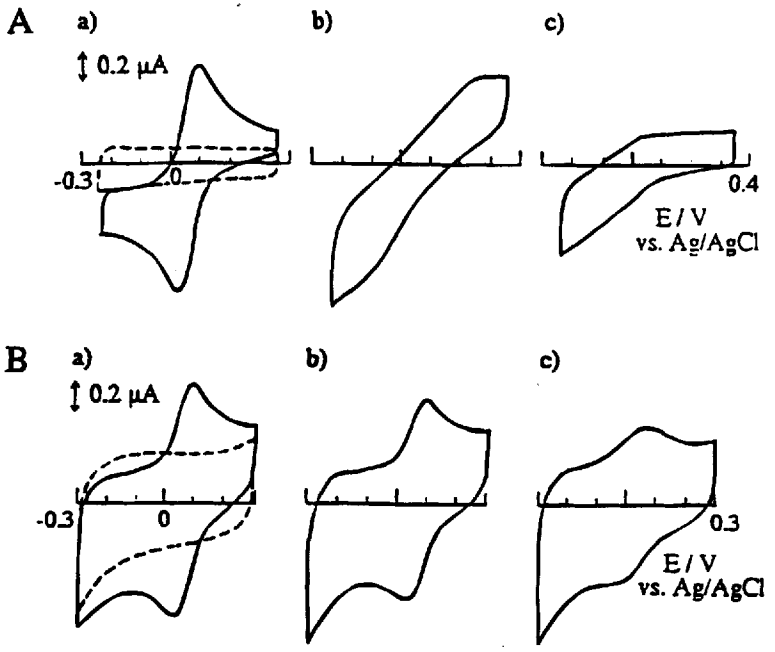

FIGURE 8 Cyclic voltammetry of cytochrome *c* components at indium oxide and bis(4-pyridyl)disulfide modified gold electrodes. (A) Clean indium oxide electrodes: (a) ca. 0.1 mM purified cytochrome *c;* (b) ca. 0.1 mM deamidated cytochrome *c;* (c) ca. 0.05 mM oligomeric cytochrome *c;* in phosphate buffer pH 7.0, 0.1 M NaCl, scan rate 20 mV/s, (-----) background of buffer alone. (B) Bis(4-pyridyl)disulfide modified gold electrodes: (a), (b) and (c) as in (A). (Reproduced from Ref. 28 with permssion, The Electrochemical Society of Japan.)

of the cytochrome *c*, whereas at the gold electrode modified with bis(4-pyridyl)disulfide there are minimal differences in the cyclic voltammetry for the native, deamidated and the oligomeric forms of cytochrome *c*. Clearly, an important function of this modification is to prevent the adsorption of denatured forms of cytochrome *c* on this surface, thereby enabling facile electron transfer to occur between solution resident cytochrome *c* molecules that diffuse to this electrode surface. In the case of adsorptive promoters such as 4,4′-bipyridyl, their function is based on the strength of competitive adsorption between the cytochrome *c* components and the promoter itself, which can require the presence of high solution concentrations of the promoter. This mechanism has been spectroscopically studied in some detail.[19] However, this study did not show that the protein was exclusively excluded from adsorption on the electrode, suggesting that the picture may actually be more complex.

The field of self-assembled monolayers has become an especially broad and intense field of chemistry and materials chemistry over the past decade.[48,49] Much of this work is related to the gold/thiolate chemistry that was reported earlier in the work described above, although this earlier work is not often recognized. It is noteworthy that surface hydrophilicity has been found to be important in a large part of this recent literature as well as in the work described here involving cytochrome *c* direct electron transfer reactions. In work on other electron transfer proteins, this synthetic approach affords a means of imposing anionic, cationic or neutral charge character to the electrode surface and of varying the density of these functionalities on an electrode.[16] It is possible to use either the gold/thiol surface modification strategy described above or to use silylation methods with indium and tin oxide surfaces in a similar manner. Some examples of surface modifiers that have been used and shown to be effective promoters with other electron transfer proteins are illustrated in Fig. 9.[16]

MECHANISTIC IMPLICATIONS FOR THE ELECTRON TRANSFER REACTIONS OF CYTOCHROME *c* AT UNMODIFIED AND MODIFIED ELECTRODE SURFACES

An appealing model for the reaction of cytochrome *c* at both unmodified and modified electrode surfaces involves the requirement for

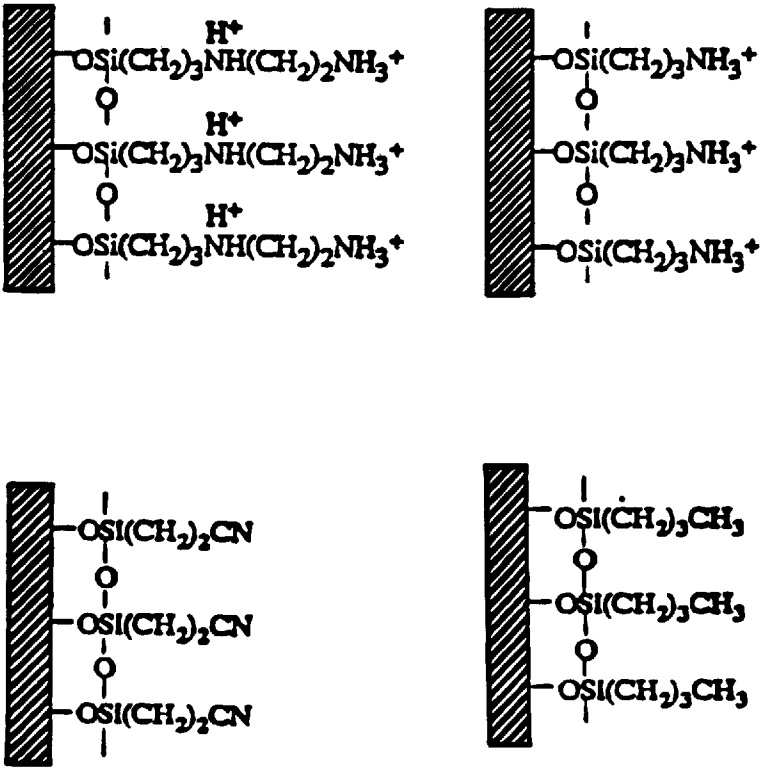

FIGURE 9 Models for the covalent modification of electrodes.

optimal orientation of the molecule at the electrode so that the electrode surface/heme edge distance is minimized.[40] In order for this orientation to occur, favorable electrostatic interactions between the positively charged lysine residues that surround the molecular surface about the heme cleft and chemical features of the electrode surface are invoked. This model draws precedence from the work of Poulos and Kraut on the docking of cytochrome *c* and cytochrome *c* peroxidase[50] and the work of Margoliash[51,52] that probed the effects of anion binding upon the rates of electron transfer between solution resident cytochrome *c* and cytochrome *c* oxidase. A related model has also been used to describe the interactions that occur between the reaction

surface of cytochrome *c* and an electrode's surface charges to produce electroactive adsorbed systems.[53-55] This model results in very good agreement between expected electron transfer kinetic parameters and those that are theoretically predicted for these adsorbed cytochrome *c*/electrode systems. However, the picture of cytochrome *c* and its interaction with an electrode surface that results in facile electron transfer may well involve a number of additional factors as described earlier.[17] There is clearly a need for direct evidence, especially spectroscopic, that can help elucidate the mechanisms that are relevant to these different electrode surfaces.

The facile heterogeneous electron transfer kinetics that have been observed for cytochrome *c* reacting at a number of different electrode surfaces prompts questions about the existence of or the need for a particular orientation of cytochrome *c* at an electrode. There exists a wide range of points of zero charge at these diverse electrode/solution interfaces, and this would seem to argue against a preferred electrostatic orientation. There is a reasonably uniform distribution of lysine residues, nineteen in all for horse heart cytochrome *c*, over the molecular surface.[56] However, a cluster of acidic residues about the backside of the molecule gives rise to the positive dipole moment that exits the molecule near the phenylalanine-82 residue. This is the surface area that is seen as offering the distance of closest approach to the heme edge and therefore the closest distance between the electrode surface and the heme edge. In order for favorable electrostatic interactions to produce this optimum orientation, the charge on the electrode surface must necessarily be negative. Table I gives literature values for points of zero charge for some of the metal electrodes used thus far with cytochrome *c*. In the case of metal oxide electrodes, such as tin oxide and indium oxide, the case is complicated by

TABLE I

The potential of zero charge at different electrodes

Electrode Material	Solution	E_{pzc}, V vs. NHE
Gold	0.02 N Na_2SO_4	+0.23[57]
Platinum	0.003 N $HClO_4$	+0.41[57]
Silver	0.02 N Na_2SO_4	−0.70[57]
Mercury	0.1 N NaCl	−0.23[58]

surface acid/base reactions. However, for both of these electrodes the point of zero charge at pH 7.0 is indeed very negative, ca −1.0 V.[29] Specific adsorption of anions affects all of these values, but the point of zero charge at these electrodes includes positive and negative potentials. This has led to the speculation that the voltammetry that is observed under some conditions of electrode/solution interfacial chemistry reflects a distribution of orientations of cytochrome *c* relative to the electrode surface.[31] Clearly there is a need to probe this question with new methods that can yield orientationally sensitive information about this problem as described above.

Another mechanism for the electron transfer reactions of cytochrome *c* at electrodes has been proposed. In this mechanism the electron transfer reactions actually occur at arrays of microelectrodes rather than at all areas of the planar electrode surface.[59,60] Simulations that incorporate the microelectrode array model indicate that the heterogeneous electron transfer rate constant is much larger, ca. 1 cm/s, than values that have been reported using the model of semi-infinite diffusion to a planar electrode surface. Other experiments have been conducted, and the microelectrode array model was used to evaluate these results. The best fits between experimental results and digitally simulated results were obtained using values for the heterogeneous electron transfer rate constant that are much smaller than the value suggested above.[61] It is difficult to separate the effect of the heterogeneous electron transfer rate constant from the effect of mass transfer mode on an electrode reaction using cyclic voltammetry alone. The effect of the heterogeneous electron transfer rate constant on an electrode reaction can be eliminated using large overpotential potential step experiments. Using a large electrode driving force overcomes the heterogeneous electron transfer kinetic barrier so that the electrode reaction becomes strictly mass transfer controlled.[19,20] Such experiments have been conducted at indium oxide electrodes with no evidence of mixed modes of mass transport.[62] Of some concern in this work is the time dependence of the voltammetry that has been observed and the differences seen between microelectrodes and conventional dimension electrodes.[60] At present it is not clear whether the microelectrode array model applies generally to cytochrome *c* electrode reactions or to certain types of modified electrode surfaces. Careful experiments that probe this question are needed. Cyclic voltammetry experiments, where the morphology of the volta-

mmetric response can depend on electrode geometry and size, mass transfer modes and heterogeneous electron transfer kinetics, should be combined with other methods as described above in order to resolve this issue.

SUMMARY

The use of electrodes to study the electron transfer reactions of proteins and an understanding of the fundamental requirements for achieving facile electron transfer in these systems is becoming a mature field. Central to future work is establishing the integrity of the electron transfer protein sample being studied and the architecture of the electrode surface, be it a clean unmodified surface or a surface that has been chemically tailored. The use of direct surface tension measurements[63] and quartz crystal microbalance data[16] are examples of methods that may prove to be particularly useful in this field.

Enzyme-modified electrodes have been constructed for several decades. Redox mediators have usually been used with the enzyme because of the lack of direct electron transfer activity observed between electrodes and enzymes. A new approach, in which mediator "wires" are covalently assembled within the fabric of the enzyme enabling efficient electron transfer communication between an electrode and an enzyme, has been reviewed.[64] Additional schemes for achieving direct communication between enzymes and electrodes have been reported using electrode/bilayer membrane architectures.[65–67] Apart from the challenge to better understand fundamental aspects of how these electron transfer systems function, the applications that have been demonstrated and that can be imagined in the areas of biosensors, biomass conversion and bioremediation processes are exciting to consider.

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