ADVANCES IN INORGANIC CHEMISTRY, VOL. 36

DIRECT ELECTROCHEMISTRY OF PROTEINS AND ENZYMES

LIANG-HONG GUO and H. ALLEN O. HILL

Inorganic Chemistry Laboratory, Oxford OX1 3QR, England

- I. Introduction
- II. Electrochemistry of Metalloproteins
  - A. Cytochrome *c*
  - B. Other Proteins
  - C. The Microscopic Model in Protein Electrochemistry
- III. Electrochemistry of Protein–Protein Complexes
- IV. Electrochemistry of Redox Enzymes
  - A. Laccase and Cytochrome *c* Peroxidase
  - B. *p*-Cresol Methylhydroxylase
  - C. Other Enzymes
  - D. General Comments
- V. Applications of Electrochemistry of Proteins and Enzymes
- VI. Concluding Remarks
- References

I. Introduction

Energy conversion and electron transfer are two intimately associated processes of biology. In aerobic organisms, oxidation of fuel molecules by the ultimate electron acceptor, O_2, is coupled to the formation of an energy-rich species, ATP, which is then utilized in many different energy-consuming processes. However, electrons are transferred not directly from fuel molecules to O_2, but by means of an electron transport chain located in the inner membrane of mitochondria. The electron transport chain is composed of a number of electron transfer complexes firmly bound to the biological membrane, and electron-carrying groups in these complexes are flavins, iron–sulfur clusters, heme groups, and copper ions. Therefore, electron transfer reactions within and between proteins play an important role in biological energy transduction, and, not surprisingly, there is a continuing interest (*1*) in the studies of electron transfer processes in biology.

Electrochemical techniques have been recognized (*2*) as powerful means for characterizing electron transfer properties of chemical systems and, more recently, biological systems. The molecules of particular interest in biological electrochemistry are metalloproteins and redox enzymes. The early electrochemical measurements usually involved potentiometric titration wherein small redox-active molecules, often called mediators, were used to shuttle electrons between biomolecules and electrodes. Although information on the thermodynamics and stoichiometry of a number of biological entities can be provided by this technique, serious problems may occur when redox mediators are used to investigate electron transfer processes in membrane bioenergetics, in which components with multiple redox centers are commonly involved and these mediators are inherently indiscriminate with respect to the reactions with different redox groups. Direct electron exchange (i.e., without mediation by small electron carriers) between the redox-active centers of a biomolecule and an electrode offers a possible way to tackle this problem. Moreover, direct electrochemistry can be used to probe the mechanisms and kinetics of biological electron transfer. Consequently, this article will focus on the direct electrochemical study of metalloproteins and redox enzymes.

Until recently, it was believed difficult to achieve direct and reversible electron transfer between redox proteins and electrodes. Among the difficulties are the following factors: (1) The redox sites are generally "insulated" by the protein portion of the molecule to ensure a highly selective route for electrons in and out of the protein. Therefore, electron transfer to an electrode at appreciable rates is prohibited for all but a few orientations of the protein, even when it is in contact with the electrode. (2) The requisite characteristics for macromolecular recognition are not successfully presented on an electrode surface. As a result, the probability for a protein to approach the electrode in "productive" orientations is very low. (3) On metal electrodes proteins tend to undergo rapid and irreversible adsorption coupled with conformational changes and loss of activity. An insulating layer of denatured protein thus formed on the electrode would prevent the native species in the bulk solution from exchanging electrons with the electrode.

II. Electrochemistry of Metalloproteins

In the early investigations of the direct electrochemistry of metalloproteins, polarography was principally employed. The electrode reaction of cytochrome *c* at mercury electrodes has been extensively studied by Anderson, Scheller, Kuznetsov and Haladjian, and has been reviewed by Bowden *et al.* (*3*). Based on their results, several models of the reaction mechanism were proposed, including diffusion of the native cytochrome *c* through the denatured layer, electron mediation by the redox center of the adsorbed protein, and direct electron transfer of weakly adsorbed species on top of the strongly adsorbed molecules. In all the proposals a layer of adsorbed cytochrome *c* was suggested to interact weakly with the species in the bulk solution, indicating the possible role of modified electrode surfaces in promoting the direct electrochemistry of proteins.

The electrochemistry of metalloproteins has developed markedly (*4–6*) over the past 15 years. It has mainly been concerned with the electrochemistry at solid electrodes: gold, upon which are adsorbed redox-inactive promoters, i.e., molecules that bind both to the electrode and the protein; and edge-plane graphite, with or without redox-inert metal ions in solution.

A. CYTOCHROME *c*

Mitochondrial cytochrome *c* is perhaps the most widely studied of all metalloproteins with respect to its electrochemical properties. It is located in the inner-membrane space of mitochondria and transfers electrons between membrane-bound complex III and complex IV. The active site is an iron porphyrin with a redox potential (*7*) of +260 mV vs. NHE. The crystal structures of cytochrome *c* from tuna have been determined (*8, 9*) in both oxidation states at atomic resolution. It is found that the heme group is covalently linked to the protein via two thioether bridges, and part of its edge is exposed at the protein surface. Cytochrome *c* is a very basic protein, with an overall charge of +7/+8 at neutral pH. Furthermore, many of the excess basic lysine residues are clustered around the mouth of the heme crevice, giving rise to a pronounced charge asymmetry.

1. At Modified Gold Electrodes

One of the first reports on the quasireversible electrochemistry of redox proteins appeared in 1977 when Eddowes and Hill demonstrated (*10*) cyclic voltammetry of horse heart cytochrome *c* at a gold electrode in the presence of 4,4′-bipyridyl (Bipy) in solution. In the voltammograms (Fig. 1), the peak-to-peak separations were close to 60 mV and the faradaic currents varied linearly with (scan rate)$^{1/2}$, indicating a quasireversible one-electron transfer process controlled by linear diffusion of redox species to the electrode surface. The midpoint potential

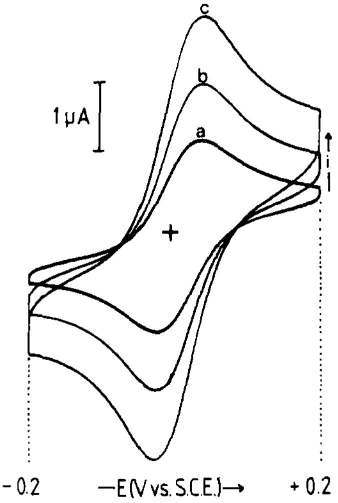

FIG. 1. Cyclic voltammograms of horse heart cytochrome *c* (5 mg/ml) in 0.1 *M* $NaClO_4$/0.02 *M*P_i buffer at pH 7.0, in the presence of 10 m*M* 4,4′-bypyridyl in the potential range +0.20 to −0.20 V vs. SCE, at scan rates of (a) 20, (b) 50, and (c) 100 mV sec^{-1}.

(average of the anodic and cathodic peak potentials), $E_{1/2} = +0.020$ V vs. SCE, is identical with potentiometric values. The reagent, 4,4′-bipyridyl, was believed to adsorb on the gold surface, and its coverage was governed by the Langmuir adsorption isotherm (*11*). Because it is not electroactive in the potential region of interest, it was proposed that the organic adsorbate allowed electron transfer to occur directly by providing the electrode surface with chemical functionalities capable of interacting specifically and reversibly with the protein surface.

A more detailed kinetic investigation of the Au/Bipy/cytochrome *c* system was carried out using the rotating ring–disk technique (*12*). It was found that rate constants for adsorption and desorption of the protein were 3×10^{-2} cm sec^{-1} and 50 sec^{-1}, respectively. The limiting first-order rate constant within the protein–electrode complex was determined as 50 sec^{-1}, a reasonable value as compared to that of long-range electron transfer between or within proteins.

Subsequently, Taniguchi and co-workers found (*13*) that bis(4-pyridyl)disulfide (SS-Bipy) adsorbed so strongly on gold that a "predip" in the SS-Bipy solution for several minutes was sufficient to give excellent electrochemistry of cytochrome *c* in a "promoter-free" solution. However, the adsorption behavior of this promoter was interpretated by the Frumkin isotherm rather than by the Langmuir isotherm. As addition of SS-Bipy to a solution of cytochrome *c* did not show any significant change of absorbance at 697 nm, it was unlikely that the promoter bound to the heme iron as an extrinsic ligand. The importance of such a modified electrode lies in the application to a variety of combined optical measurements, because it does not cause any interference.

Purine and its derivatives were investigated as possible promoters of cytochrome *c* electrochemistry at Au electrodes (*14*). It was suggested that the lone-pair electrons of the N atoms at position 1 were important for promotivity, and that the strong adsorptivity of sulfur-containing purines was due to the favorable interaction between gold and sulfur. From the surface-enhanced Raman scattering (SERS) studies (*15, 16*) a possible model of Bipy and SS-Bipy on a gold electrode was proposed: Bipy adsorbs onto the electrode via one pyridyl nitrogen in a vertical orientation, leaving the other nitrogen directed toward the solution, whereas SS-Bipy adsorbs with a vertical orientation of the pyridine ring as PyS through the S atom with cleavage of the disulfide bond. Furthermore, the SERS spectra of the modified electrode did not change remarkably upon addition of cytochrome *c*. Presumably, the interaction between the protein and modifier was not so strong as to affect the electronic structure and orientation of the absorbates and hence weak hydrogen bonding is very likely important.

These results support the electrode reaction mechanism originally proposed by Hill *et al.* (*17*), i.e., hydrogen bonding between the lysine residues surrounding the exposed heme edge of cytochrome *c* and the pyridyl nitrogens at the electrode surface stabilizes a transient protein–electrode complex oriented so as to allow rapid electron transfer to and from the heme group.

The proposed protein–electrode complex can be compared to protein–protein complexes thought to be involved in biological systems. Electron transfer reactions between proteins are believed to involve formation of kinetically detectable precursor protein–protein complexes that are stabilized and oriented by electrostatic and hydrophobic interactions. The large dipole moment arising from the typically asymmetric charge distribution of a metalloprotein has been suggested (*18*) to be responsible for proper orientation of the protein in the complexes with its physiological partners. The importance of considering this charge distribution is demonstrated (*19, 20*) by studies involving specific lysine modification of cytochrome *c* and its electron transfer reactions with some protein partners. The general results of these studies show that modification of lysines in a small well-defined region surrounding the exposed heme edge affects electron transfer rates, but modification of those outside this region have no effect. Thus, it seems that localized high charge density and resulting dipole moments control the preorientation of the protein reactants as they approach, so that an optimal, rather than a randomly bound, reaction complex is formed.

To understand the structural requirements for a molecule to promote cytochrome *c* electrochemistry at gold electrodes, over 50 bifunctional organic compounds (X ~ Y, where X is a surface-active functional group, Y is the protein-interactive functionality, and ~ is the linking structure) were investigated (*21*) to assess their ability to promote cytochrome *c* electrochemistry at a gold electrode. The results can be summarized as follows:

1. The surface adsorbing groups, X, are most satisfactory when they belong to σ-donor and π-acceptor synergic-type ligands such as 4-pyridyl-N, thio-SH, disulfide "S–S," and phosphine P, which are appropriate for coordination to a Group 11 metal such as Au.
2. The effective Y groups can be divided into two subcategories: (a) neutral groups such as 4-pyridyl-N and an aniline-type amine, Ar-NH_2, which are weakly basic and able to form hydrogen bonds to lysine-NH_3^+; and (b) anionic groups such as carboxylate, sulfonate, phosphonate, or phosphate, which can interact with lysine-NH_3 groups through both hydrogen bonding and salt bridging.
3. The link (~) may be rigid or flexible, aliphatic or aromatic, and of varying length.

One crucial role that promoters may play is to prevent adventitious adsorption on an electrode surface of either impurities in the supporting electrolyte or in the protein itself, which usually undergoes denaturation. Obviously, to be compatible with the polar surface amino acid residues of proteins, the hydrophilicity of the electrode surface is important. However, prevention of direct and degradative adsorption of proteins is not the sole function of promoters. It has been shown that diphenyl disulfide, although adsorbing strongly at gold electrodes, did not act as a promoter for cytochrome *c*. In order that there is a rapid and reversible binding of a protein to the modified electrode surface in a manner that is conducive to electron transfer, it is necessary for promoters to present functional groups at the electrode/electrolyte interface to which protein can bind.

A new group of promoters has recently been employed (*22*) in achieving the direct electrochemistry of cytochrome *c* and other proteins. Several cysteine-containing peptides for example, $(Cys\text{-}Glu)_2$, proved very successful, as they bind tightly to the gold electrode via the sulfur atom and interact favorably with the proteins through the charged amino acids.

2. *At Other Electrodes*

Use of modified gold electrodes is not the only approach to achieve cytochrome *c* electrochemistry. Indeed, a number of studies have been reported on a variety of electrode surfaces. In 1977, Yeh and Kuwana illustrated (*23*) well-behaved voltammetric response of cytochrome *c* at a tin-doped indium oxide electrode: the electrode reaction was found to be diffusion-controlled up to a scan rate of 500 mV sec^{-1}. Metal oxide electrodes were further studied (*24, 25*) independently in Hawkridge and Hill's groups. The electrochemical response of cytochrome *c* at tin-doped indium oxide and fluoride-doped tin oxide was very sensitive to the pretreatment procedures of the electrode surface. At thin-film ruthenium dioxide electrodes, variation of the faradaic current with pH correlating with the acid–base protonation of the electrode surface was observed.

Extensive studies of protein electrochemistry have been carried out at pyrolytic graphite electrodes. The structure of this material provides two highly distinctive types of surface: the parallel (basal plane) face with satisfied carbon valences, and the edge face at which a variety of C–O groups can be generated by various oxidative pretreatments. The cyclic voltammograms of cytochrome *c* at edge electrodes were (*26*) stable and well defined (Fig. 2), and the diffusion coefficient and midpoint potential calculated from the voltammograms were in good agreement with published values. This study showed that a reproducible oxidized surface, as characterized (*27*) by photoelectron spectroscopy, was generated merely upon polishing in air.

3. *Studies of the Genetic Variants*

The involvement of lysine amino acid residues on cytochrome *c* in the heterogeneous reactions with functionalized electrodes seems to have been established. Importantly, it is now thought that the proposed protein–promoter complex is more likely to be dynamic, as revealed by the results of a recent investigation (*28*) of site-specific 4-chloro-3,5-dinitrophenyl (CDNP)-substituted cytochrome *c*. It was found that monosubstitution of either Lys 13 or Lys 72 did not result in any significant change in its electrochemical response, whereas two modifications greatly decreased the heterogeneous rate constant, and complete loss of electrochemical activity was observed upon modification of more lysines. It was proposed that the electrode reaction occurred in numerous rotational conformations. Therefore, for the mono-

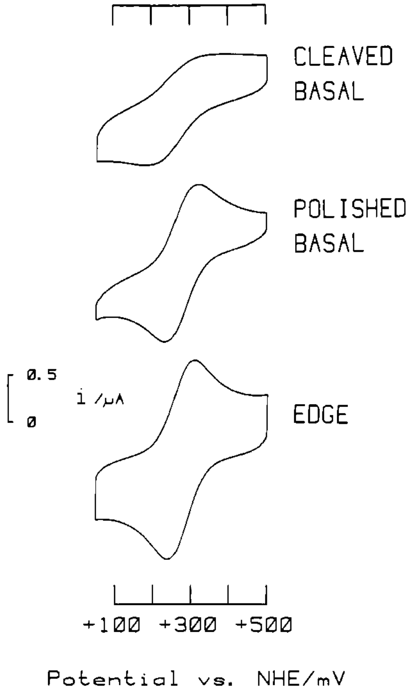

FIG. 2. Cyclic voltammograms of horse heart cytochrome *c* at pyrolytic graphite electrodes: 0.15 m*M* protein in 5 m*M* Tricine/100 m*M* NaCl, pH 8.0. Scan rate: 20 mV sec^{-1}.

substituted proteins, the resulting unfavorable electrostatic repulsion would be reduced by rotation of the molecule into a conformation possessing a similar probability for electron transfer.

Genetic engineering techniques have been proved invaluable in investigating structure–function and stability relationships of proteins. The *iso*-1-cytochrome *c* from bakers' yeast, *Saccharomyces cerevisiae,* is a particularly useful model compound for such studies, because many mutated forms are readily available and the three-dimensional structure of wild-type protein has been determined (*29*) to a resolution of 2.8 Å. One of the disadvantages of chemical modification of cytochrome *c* is the effect of the relatively large size of the chemical group on the protein structure, and genetically engineered variants provide a better alternative.

Several mutants of yeast *iso*-1-cytochrome *c* with single-site internal and surface amino acid replacement have been investigated (*30*) at edge-plane graphite and modified gold electrodes. It is found that midpoint potentials follow the order Lys-13-Ile (+62 ± 5 mV vs. SCE) >

wild type (+40 mV) > Lys-27-Gln (+33 mV) ≈ Lys-72-Asp (+31 mV) > Asn-52-Ala (+15 mV) > Asn-52-Ile (−10 mV). Both the wild-type protein and the mutants show similar voltammetric reversibility at modified gold electrodes, whereas at edge-plane graphite electrodes the reversibility follows the order Asn-52-Ile > wild type > Asn-52-Ala.

The measured potentials offered some insight into the factors that control the redox potential of cytochrome *c*. The change of a surface lysine to a neutral (e.g., Gln 27) or anionic residue (e.g., Asp 72) can either increase or decrease $E^{0\prime}$. These shifts do not support the electrostatic potential model of Rees (*31*), in which a linear correlation between the net charge of proteins and their redox potentials was presented. Moore *et al.* (*32*) have proposed that the key factors influencing redox potentials are the contributions to the Gibbs energy differences between the two redox states, resulting from bonding interactions at the redox center, electrostatic interactions between the redox center charge and polar groups within the protein and solvent, and redox-state conformational changes. The change of the midpoint potentials of cytochrome *c* mutants seems more consistent with this analysis of multiple contributions.

The largest shift in potential (from +40 to −13 mV) is found for the Asn-57-Ile mutant in which one neutral, internal amino acid is replaced by another. The primary result of this change is (*33*) that the larger Ile side chain displaces one water molecule, which is involved in an extensive hydrogen-bonding network including Asn 52, Tyr 67, and Thr 78. It is clear that the presence of bound water near the heme iron does *not* stabilize the oxidized state. In those wild-type cytochromes for which detailed structures exist (horse heart, tuna, yeast, and rice), both the water molecule and the associated amino acids move in a consistent manner when the oxidation state is changed. The replacement, Asn-52-Ile, with loss of this water molecule, results in a side chain configuration that resembles the oxidized conformation more closely than the reduced. This localized conformational change is likely to account for part of the shift in reduction potential.

The studies also revealed some factors that affect the stability of a protein at the electrode/electrolyte interface. On an electrode surface, the native conformation of a protein may be retained or distorted, depending on the extent of the interactions between them. Because in most of the water-soluble proteins the hydrophilic side chains are usually located on the exterior, irreversible adsorption and denaturation of proteins are expected to be considerably less on a hydrophilic electrode surface at which intervening water molecules are more tenaciously bound. Therefore, adsorption at both modified gold and edge-plane graphite electrodes was not observed with either wild-type cytochrome *c* or the mutants, Lys-72-Asp and Lys-27-Gln, in which a surface lysine residue was substituted by a polar group. However, if a lysine was replaced by a nonpolar amino acid (isoleucine), adsorption onto the modified gold occurred with loss of electrochemical activity (Fig. 3). The adsorption of this mutant was found to be more severe on an edge-plane graphite electrode that possesses significant hydrophobic domains and would hence interact favorably with the hydrophobic portion of the protein surface introduced by the isoleucine.

B. Other Proteins

Among the electron transport proteins, there are four known classes that have been isolated and studied by X-ray crystallography. These include cytochromes, iron–sulfur proteins, copper blue proteins, and

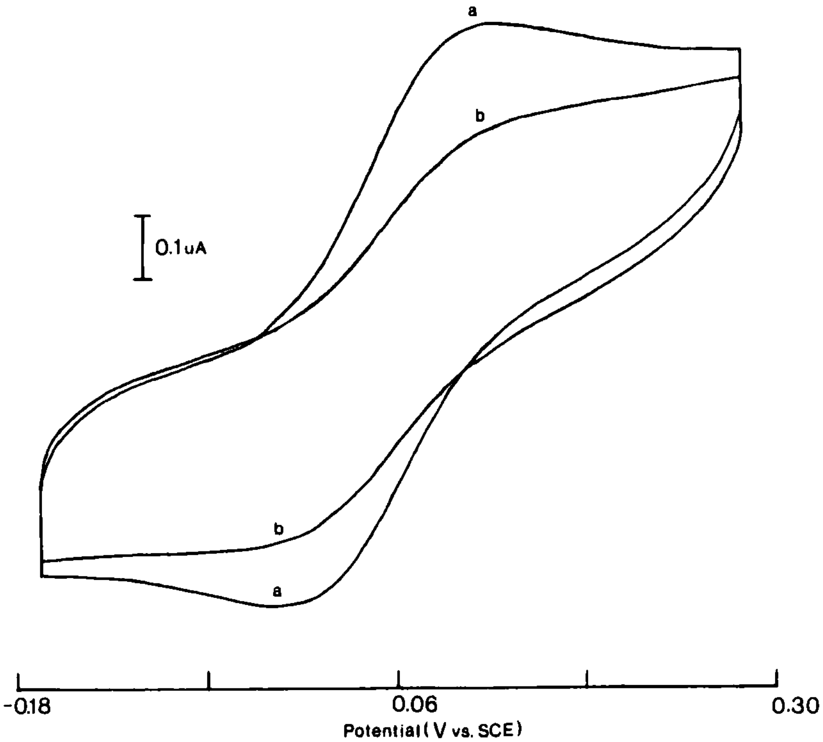

FIG. 3. Cyclic voltammograms of the yeast cytochrome *c* mutant, Ile 13 (180 μM), at a 4,4'-bipyridyl disulfide-modified gold electrode: 20 mV sec^{-1}, 10 m*M* HEPES/100 m*M* KCl, pH 7.4. (a) Initial scan; (b) final scan.

flavin proteins. In contrast to cytochrome *c*, most of these proteins bear an overall negative charge, and studies of their electrochemistry have enabled clarification of the importance of coulombic interactions with electrode surfaces.

A general requirement for protein electrochemistry is that the surface of an electrode is electrostatically compatible with a protein, particularly the binding sites for the biological protein partners. Therefore, to obtain electrochemistry of plastocyanin, a copper blue protein with an overall negative charge and probably acidic binding sites, the promoter should contain either positively charged functional groups or those that can hydrogen bond to the groups that constitute the acidic binding domain on plastocyanin. This has been proved (*34*) true: the compounds, 2-aminoethanethiol and 2,2′-dithiobis(ethanamine), which are protonated at neutral pH, are effective promoters of plastocyanin electrochemistry.

In the same sense, at negatively charged edge-plane graphite electrodes, faradaic response of plastocyanin is normally not obtained due to the repulsive coulombic interactions. However, upon addition of a small amount of a redox-inactive multivalent cation, i.e., Mg^{2+}, stable and well-behaved cyclic voltammetry is observed (*35*). Extensive investigations of anionic proteins revealed (*27, 36*) that the effectiveness of promotion is in the order of $M^{4+} > M^{3+} > M^{2} \gg M^{+}$. These effects have been attributed to the ability of cations to suppress the unfavorable electrostatic interactions. Specific protein–cation associations may also play an important role in the electrochemical reactions.

C. THE MICROSCOPIC MODEL IN PROTEIN ELECTROCHEMISTRY

The opportunity of obtaining direct electrochemistry of cytochrome *c* and other metalloproteins at various electrode materials such as modified gold and pyrolytic graphite has led to numerous reports of heterogeneous electron transfer rates and mechanisms between the protein and the electrode. In all the reports, Nicholson's method (*37*) was employed to calculate rate constants, which were typically within the range of 10^{-4}–10^{-2} cm sec^{-1} with scan rates varying between 1 and 500 mV sec^{-1}. This method is based on a macroscopic model of the electrode surface that assumes that mass transport of redox-active species to and from the electrode occurs via linear diffusion to a planar disk electrode and that the entire surface is uniformly electroactive, i.e., the heterogeneous electron transfer reaction can take place at any area.

Recently, the validity of the assumption of mass transport by linear diffusion has been reassessed (*38–40*) in the case of protein electrochemistry wherein the electroactive sites are usually of microscopic rather than macroscopic dimensions. The central theme of this microscopic model concerns the proteins' selectivity for suitable sites on an electrode. Electron transfer occurs only at some sites and is fast, whereas reactions at other sites are negligible. Accordingly, there are two limiting cases of mode of mass transfer. At an isolated single active site of microscopic dimensions, mass transport is radial and the shape of the associated current–voltage is sigmoidal. In the other limiting case, the density of active sites is high enough to produce a macroscopic active surface to which mass transport is linear. Consequently, the familiar peak-shaped cyclic voltammograms are observed and Nicholson's analysis may be applied (Fig. 4).

By use of the microscopic model, it is concluded (*38, 39*) that differences in shapes of cyclic voltammograms of proteins observed at basal-plane and edge-plane pyrolytic graphite electrodes are a result of different densities of electroactive sites and *not* variations in the electron transfer rate as proposed previously based on the analysis using macroscopic model. The new model assumes that electron transfer takes place at arrays of oxygen-containing functionalized electroactive sites, which are of microscopic dimensions in size, and that no electron transfer occurs at a bare nonfunctionalized carbon electrode. The basal-plane electrode has a low density of oxygen-containing groups, and therefore radial diffusion is the dominant mode of mass transport. Sigmoidal-shaped voltammograms corresponding to a reversible process are observed. In contrast, edge-plane graphite has a much higher density of electroactive sites, at which linear, rather than radial, diffusion becomes the dominant mode, and peak-shaped *i–E* curves indicating a reversible step are obtained (Fig. 2).

The microscopic model has also been used (*40*) to explain the shapes, concentration dependence, and time dependence of cyclic voltammograms observed for the electrochemistry of cytochrome *c* at gold electrodes modified by adsorption of SS-bipy. The electrode can be modified either *in situ* (i.e., with SS-bipy present in solution) or *ex situ* (i.e., by immersion of the electrode into a solution of the modifier). If the modifier solution is dilute, or the immersion time is short, the surface coverage of SS-bipy molecules is low. Electron transfer occurs reversibly only at the modified part of the electrode after mass transport predominantly via radial diffusion, resulting in sigmoidal-shaped voltammograms. In concentrated solutions of the modifier or upon immersion for a longer period of time, higher (and eventually monolayer) coverage of active sites leads to overlaps of the diffusion layers and leaves linear

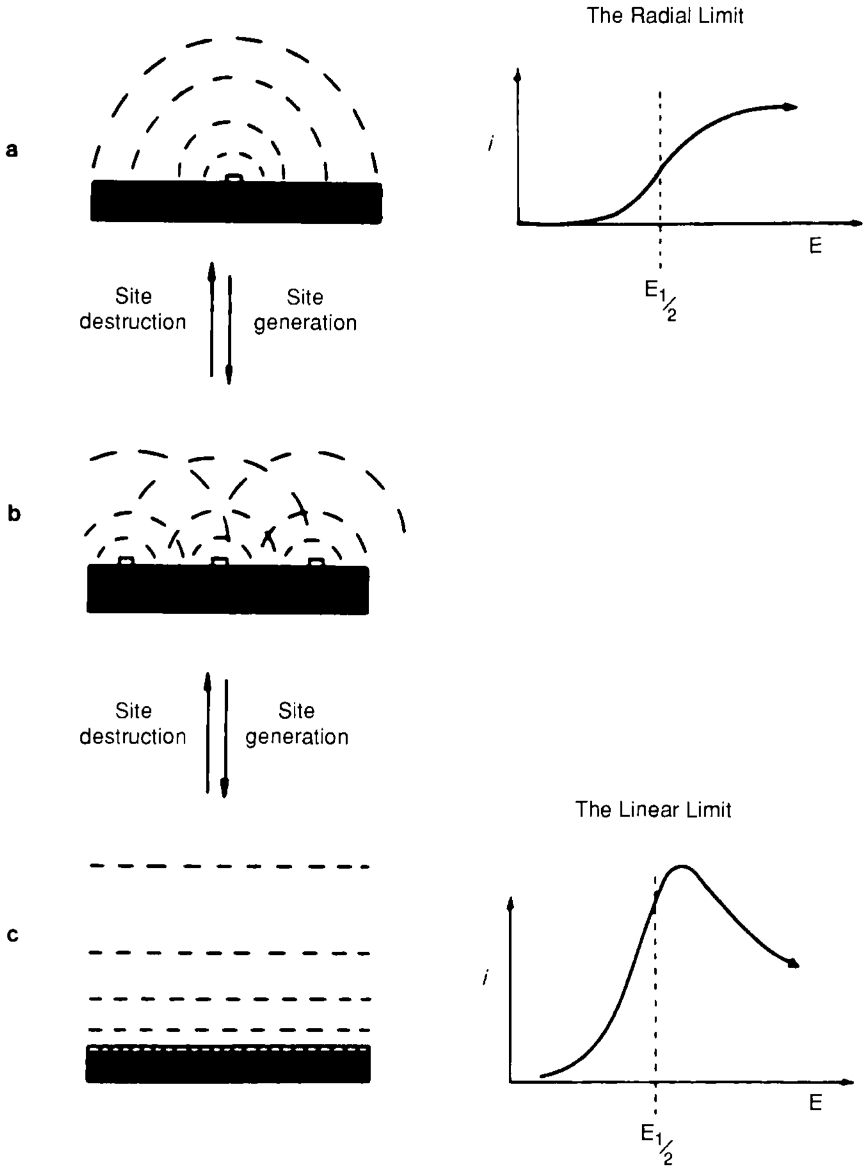

FIG. 4. Schematic representation of the conversion of radial to linear diffusion, as the density of specific electroactive surface sites increases.

diffusion as the dominant mode. Under these conditions, peak-shaped, rather than sigmoidal-shaped, voltammograms are obtained.

The concept of extremely fast electron transfer rates at functionalized electrode surfaces is in accordance with the fact that homogeneous rates of electron transfer for chemical reactions involving redox proteins are known to be fast, particularly among physiological partners.

III. Electrochemistry of Protein–Protein Complexes

As mentioned early in this article, electron transfer reactions in biology usually involve precursor protein–protein complexes, which has been a subject of great interest. Among the complexes, those between cytochrome *c* and cytochrome b_5 and between cytochrome *c* and plastocyanin have been extensively studied, partly due to the availability of their crystal structures. Direct electrochemical studies of redox proteins have furnished new insights into the electron transfer processes and provided information on the thermodynamics, kinetics, and mechanisms of biological electron transfer. Detailed studies suggest that protein–electrode reactions appear to have some similarities to the manner in which the proteins interact with their physiological redox partners. A natural step forward is to extend this biological analogy to the electrochemical study of protein–protein complexes.

The method involves measuring the cyclic voltammetric response of one protein in the presence of another at an electrode that interacts productively with only one component of the complex. Such selectivity is achieved as a result of the different requirements of the two proteins for the electrode surface: at neutral pH, cytochrome *c* is highly positively charged and needs a negatively charged surface, whereas cytochrome b_5 and plastocyanin are highly negatively charged and require positive surfaces. Thus, cytochrome *c* shows a good, quasireversible cyclic voltammetric response at either an edge-plane graphite or (Cys-Glu)$_2$-modified gold electrode (Fig. 5A), at which neither cytochrome b_5 nor plastocyanin gives any faradaic current (Fig. 5B).

However, addition of cytochrome *c* to a solution of either cytochrome b_5 or plastocyanin promoted *(41, 42)* the electrochemistry of the latter at the electrodes with which they do not normally interact (Fig. 5C). That the observed response was direct and not mediated was shown using the redox-inactive protein zinc(II)–cytochrome *c*, instead of iron(III)–cytochrome *c* (Fig. 5D and E). Similarly, direct electrochemical response of cytochrome *c* was obtained *(43)* at an unfavorable electrode by the promotion of either cytochrome b_5 or plastocyanin.

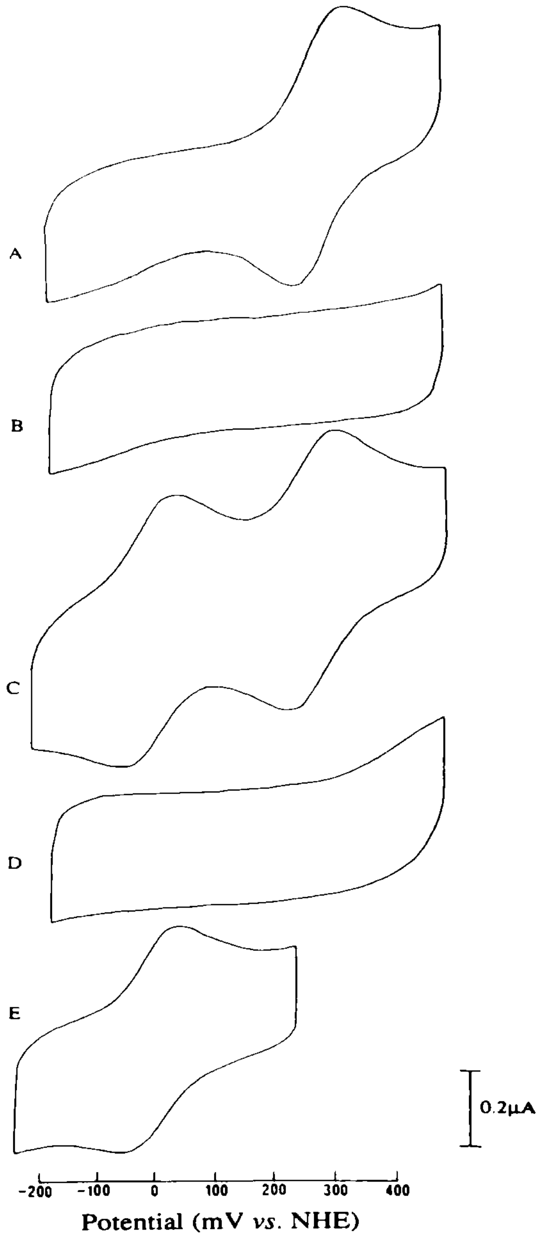

FIG. 5. Cyclic voltammograms of (A) cytochrome *c* alone (95 μ*M*), (B) cytochrome b_5 alone (95 μ*M*), (C) cytochrome *c* with cytochrome b_5 (95 μ*M* each), (D) Zn(II)–cytochrome *c* alone (75 μ*M*), and (E) cytochrome b_5 with Zn(II)–cytochrome *c* (75 μ*M* each) at an edge-plane graphite electrode: 1 m*M* HEPES/20 m*M* KCl, pH 7.0. Scan rate: 10 mV $\sec^{-1}$.

In the study of the direct electrochemistry of metalloproteins, a protein–electrode complex is proposed to be involved in the heterogeneous electron transfer reaction. Accordingly, for electrochemical promotion of one protein by another, the formation of a ternary complex of protein–protein–electrode must be considered. A subsequent question is how one protein can bind to both the electrode and the other protein in such a way as to present its redox center for heterogeneous electron transfer. In Salemme's computer-generated model (*44*) of the cytochrome *c*–cytochrome b_5 complex, lysine residues around the exposed cytochrome *c* heme are matched with the complementary charge groups around the b_5 heme. As a result, both heme faces are masked by each other, so it would be impossible for one protein to bind simultaneously to the other protein and the electrode in an orientation conducive to heterogeneous electron transfer. However, if we suppose multiple (maybe partially overlapping) binding sites on each protein and consider the complex to be dynamic, i.e., the complex undergoes conformational changes from one binding geometry to another without much energy barrier, then its electrochemical behavior may be understood: when approaching a negatively charged electrode surface, the cytochrome *c*–cytochrome b_5 complex may adjust itself to such a conformation that cytochrome *c* can bind to both the electrode and cytochrome b_5 simultaneously.

Because lysine residues 13, 27, 72, and 79 on cytochrome *c* are supposed to be involved in binding to cytochrome b_5, it was interesting to see how modification of these residues would affect the electrochemistry of the complex. Several mutated forms of yeast *iso*-1-cytochrome *c* have been studied electrochemically as described above, and it was now possible to carry out such studies.

When the electrochemistry of the complexes of cytochrome b_5 with wild-type cytochrome *c* and several single-site mutants, e.g., Lys-27-Gln, Lys-72-Asp, and Asn-52-Ile, was examined at edge-plane graphite electrodes, a number of differences were observed (*30*). By comparison with the response in the complex with wild-type cytochrome *c*, faradaic currents of cytochrome b_5 were reduced in the complexes with Lys-27-Gln and Asn-52-Ile (Fig. 6a), and were completely abolished in the complex with Lys-72-Asp (Figure 6b). These results suggest that, depending on the site of modification, the binding domain on cytochrome *c* for the electrode or cytochrome b_5 can be partially or completely destroyed.

The idea of the dynamic nature of protein–protein complexes has also been used (*43*) to interpret the results with the covalently linked

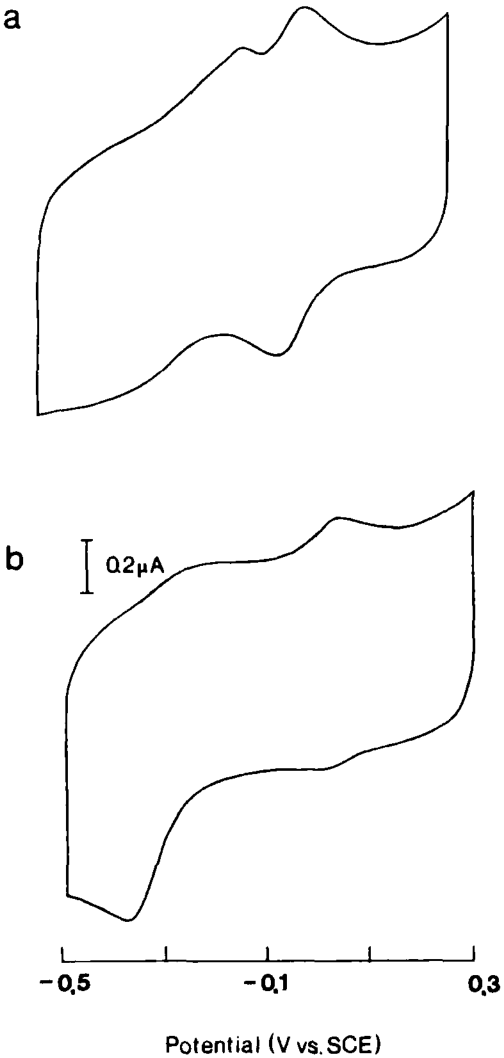

FIG. 6. Cyclic voltammogram of a 1 : 1 mixture of (a) Ile 52/cytochrome b_5 (125 μM) and (b) Asp 72/cytochrome b_5 (115 μM) at an edge-plane graphite electrode. Scan rate: 20 mV $\sec^{-1}$: 1 mM HEPES/20 mM KCl, pH 7.0.

cytochrome *c*–plastocyanin complex. The decrease in electrochemical response of the covalent complex compared to that of the electrostatic complex (Fig. 7) is probably largely due to the limitation of the relative movement of the two proteins within the covalent complex.

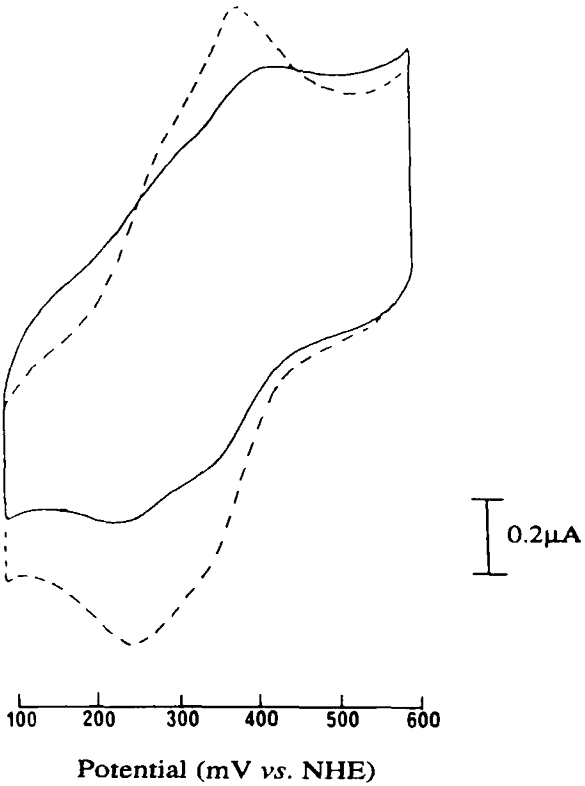

FIG. 7. Cyclic voltammogram of 1 : 1 cross-linked cytochrome *c*/plastocyanin (—) and a 1 : 1 mixture of "free" proteins (---) at an edge-plane graphite electrode: 50 μM protein in 20 mM cacodylate, pH 7.0. Scan rate: 10 mV sec^{-1}.

IV. Electrochemistry of Redox Enzymes

Direct, unmediated electrochemistry of redox enzymes has interested many researchers in several aspects. Understanding of the thermodynamics, kinetics, stoichiometry, and interfacial properties of redox enzymes is obviously important. The most attractive aspect, however, is the use of enzyme electrodes as novel electrochemical biosensors and their applications to bioreactors and biofuel cells. Although the observation of direct electrochemistry of small redox proteins has become almost commonplace as the consequence of extensive research over the past decade, the corresponding study with larger redox enzymes has proved more elusive. The difficulty lies mainly in that the redox centers are located sufficiently far from the outermost surface of enzymes so that electron transfer distance to an electrode is large enough to decrease the rate of electron transfer to a negligibly small value. Because most redox enzymes are much larger and structurally less rigid than metalloproteins, they are more liable to deformation and loss of activity on electrode surfaces. To achieve direct electrochemistry, more careful manipulation of electrodes is required.

There have been a number of reports claiming the achievement of direct electrochemistry of redox enzymes. These studies include peroxidases (*45–47*) laccase (*48, 49*), flavocytochrome b_2 (*50*), amine acid oxidase (*50, 51*), xanthine oxidase (*50, 51*), and glucose oxidase (*51–54*), the last being most extensively investigated for reason of its potential application to glucose sensors. In many of the flavoenzymes, the prosthetic group, either FAD or FMN, is not covalently bound to the protein, and thus has a high tendency of detaching from the protein. The reduction potential of free FAD occurs (*55*) between −150 and −170 mV (vs. NHE) at pH 6.0 on both graphite and dropping mercury electrodes. Because the reduction potentials of some flavoenzymes are close to this region, it is extremely difficult to differentiate the electrochemical response of the free prosthetic group from that of the intact enzyme. In fact, some studies (*52, 53*) failed to demonstrate the catalytic response of glucose oxidase on carbon electrodes by the addition of the substrate, indicating that the observed faradaic current was merely due to the free FAD species.

It was once believed that direct electrochemical oxidation of several flavoenzymes had been achieved (*51*) at electrodes made from the conducting organic salt, tetrathiafulvalinium tetracyanoquinodimethanide. However, as Külys has pointed out (*50*), the oxidation of the active centers of glucose oxidase and xanthine oxidase proceeds in a way of mediation. Mediators are formed in the layer near the electrode surface due to a slight dissolution of the organic metals. Because the organic metal compounds oxidize the reduced flavin enzymes at a high rate, a small amount of dissolved compounds is enough to mediate the reaction with high efficiency.

There appear to be two classes of redox enzymes: intrinsic and extrinsic (*5*). With the former, the catalytic reaction between an enzyme and its substrates takes place within a highly localized assembly of redox-active sites. There need be no electron transfer pathways from these sites to the surface of the enzyme, where, it is presumed, it would interact with an electrode. For such intrinsic redox enzymes, electrode reactions may require (1) that the sites of the catalytic reaction be close to the protein surface, (2) that the enzyme can deform without loss of activity, (3) that the electrode surface projects into the enzyme, or (4) that electron transfer pathways be introduced by modification of the enzyme. With extrinsic redox enzymes, there is usually another protein involved in transporting electrons and therefore an electron transfer pathway exists within the enzyme connecting the active sites to an area on the surface where the ancillary protein binds. If this area could be disposed toward an electrode, it would be possible for the enzyme electrochemistry to be obtained.

A. LACCASE AND CYTOCHROME *c* PEROXIDASE

The first accounts that seemed to give direct enzyme electrochemistry were the reports concerning a soluble "blue" Cu oxidase, laccase, which catalyzed the rapid four-electron reduction of dioxygen to water. An efficient electrocatalysis of O_2 reduction by adsorbed fungal laccase on pyrolytic graphite, glassy carbon, and CO_2-treated carbon black electrodes was first described by Tarasevich and co-workers (*48*). Several control experiments were carried out to verify direct electron transfer from the electrode to the Cu sites of the enzyme.

Electrochemistry of adsorbed laccase on an edge-plane pyrolytic graphite electrode was further studied by cyclic voltammetry and the rotating disk electrode technique (*49*). The resulting Koutecky–Levich plot yielded the number of electrons involved (four) and the limiting rate constant ($1.5 \times 10^4\ M^{-1}\ \text{sec}^{-1}$) corresponding to the reaction between laccase and O_2. Also reported was the electrochemical response of the enzyme in the absence of dioxygen, which could be observed only after addition of the reagents 2,9-dimethylphenanthroline or 4,4′-bypyridyl. This seems puzzling, as the catalytic current in the presence of O_2 was clearly visible without the reagents.

Another example of enzyme electrochemistry is given by cytochrome *c* peroxidase (CCP). This monomer molecule contains a *b*-type heme and catalyzes the two-electron reduction of H_2O_2 to water using cytochrome *c* (II) as the electron donor. In the process, the Fe(III) center of the enzyme reacts rapidly with H_2O_2 to yield a two-electron oxidized species, compound I, which is reduced to the Fe(III) form via another species, compound II.

Direct electron transfer between CCP and an electrode was first reported (*45*) for the nonphysiological one-electron reduction and reoxidation of ferric CCP at fluorine-doped tin oxide. Overpotentials of around 0.5 V were required to drive this electrode reaction in either direction at measurable rates. A more successful approach to direct electroreduction of compound I, described by Armstrong and Lannon (*46*), employed edge-plane graphite electrodes in the presence of aminoglycosides of high positive charges. The electrocatalytic reduction of hydrogen peroxide was achieved by direct electron transfer to compound I at overpotentials as low as 0.4 V, although the nature of the CCP–electrode interface in the work remains unclear. More recently, it has been shown that (*47*) CCP can be immobilized in a stable and active state by irreversible adsorption on unmodified edge-plane graphite electrodes. The bimolecular rate constant for the reaction between hydrogen peroxide and CCP was estimated to be $1 \times 10^6\ M^{-1}$ $\sec^{-1}$, as compared with the homogeneous rate constant, $5 \times 10^7\ M^{-1}$ $\sec^{-1}$. Again, a cathodic overpotential of ~0.4 V was required.

B. *p*-CRESOL METHYLHYDROXYLASE

One of a few cases in which electron transfer of redox enzymes is expressed directly and reversibly at an electrode is concerned with *p*-cresol methylhydroxylase (PCMH). This is a flavocytochrome *c* enzyme of 115 kDa, which catalyzes the oxidative hydroxylation of *p*-cresol to *p*-hydroxybenzyl alcohol and subsequently to *p*-hydroxybenzaldehyde. The structure of PCMH has recently been determined (*56*) at 3 Å resolution. It is an $\alpha_2\beta_2$ tetramer, with one subunit containing a covalently bound FAD and the other containing a *c*-type heme group.

It has been shown (*57*) recently that direct electron transfer between PCMH and an edge-plane graphite electrode is achieved in the presence of a range of electroinactive cationic species (promoters). The results are listed in Table I, with the structures of the promoters depicted in Fig. 8. In the absence of the substrate, *p*-cresol, a quasireversible cyclic voltammogram ascribed to the electrode reaction of the heme group in the intact enzyme is observed (Fig. 9a and b). In the presence of the substrate, the electrochemical response is greatly enhanced by the catalytic reaction between the enzyme and the substrate (Fig. 9c and d). The electrocatalytic currents arise not from mediated reaction via the cleaved cytochrome subunit, but from direct electron exchange between the electrode and the enzyme. One envisages interactions leading to the formation of an enzyme–promoter–electrode assembly. The adsorption of PCMH onto an edge-plane graphite electrode in the presence of a promoter obeys (*58*) the Langmuir isotherm. The nature of this interaction is more likely to be physical, as the estimated standard free energy of adsorption is relatively small. Most probably, electrostatic interactions between a positively charged promoter and the negatively charged C–O functionalities on an edge-plane graphite electrode surface are involved. Similarly, UV–visible absorption spectroscopic studies of the enzyme in the presence of a

TABLE I
RANGE OF COMPOUNDS USED AS POSSIBLE PROMOTERS[a]

Promoter	Catalytic current (μA)	Promoter charge at pH 7.0	Concentration of promoter used (mM)
Aminoglycosides			
Glucosamine (1)	Nil	+1	Up to 10
Dihydroxystreptomycin (2)	2.3	+3	2
Neamine (3)	2.3	+4	2
Neomycin (4)	4.2	+6	0.2
Gentamycin (5)	3.8	+4	2
Ribostomycin (6)	3.0	+4	2
Polyamines			
Spermidine (7)	1.1	+3	10
Spermine (8)	3.8	+4	2
Cyclam (9)	Nil	+2	Up to 20
Diammac (10)	3.5	+4	4
Inorganic cations			
K^+	Nil	+1	Up to 20
Mg^{2+}	3.4	+2	4
$Cr(NH_3)_6^{3+}$	3.5	+3	2

[a] The catalytic current was obtained using a scan rate of 10 mV sec^{-1}, at pH 7.0, in the presence of *p*-cresol methylhydroxylase (1 μM) and *p*-cresol (2 mM) at an edge-plane graphite electrode. Numbers in parentheses refer to structures shown in Fig. 8.

promoter suggest that enzyme–promoter interactions may also be electrostatic in nature.

PCMH can be classified as an extrinsic enzyme. A blue copper protein, azurin, is suspected (*59*) to be the *in vivo* electron acceptor for PCMH. *In vitro,* both horse heart cytochrome *c* and cationic dyes such as phenozine methosulfate, phenozine ethosulfate, and Wurster blue are good reoxidants. These molecules may interact with PCMH in the same region of the enzyme surface that might also be the binding area for the modified electrode surface. Because all the oxidants are positively charged, it is reasonable to assume an interaction domain with negative charges on the enzyme. Based on the structural information of PCMH, the most likely docking site is in the region of the propionic acid groups of the cytochrome subunit, which is not inaccessible because of interaction with the flavoprotein subunit. The results of electrochemical studies support this assumption in that a cationic species is needed to promote appropriate docking of PCMH on the electrode on which it then undergoes direct electron transfer.

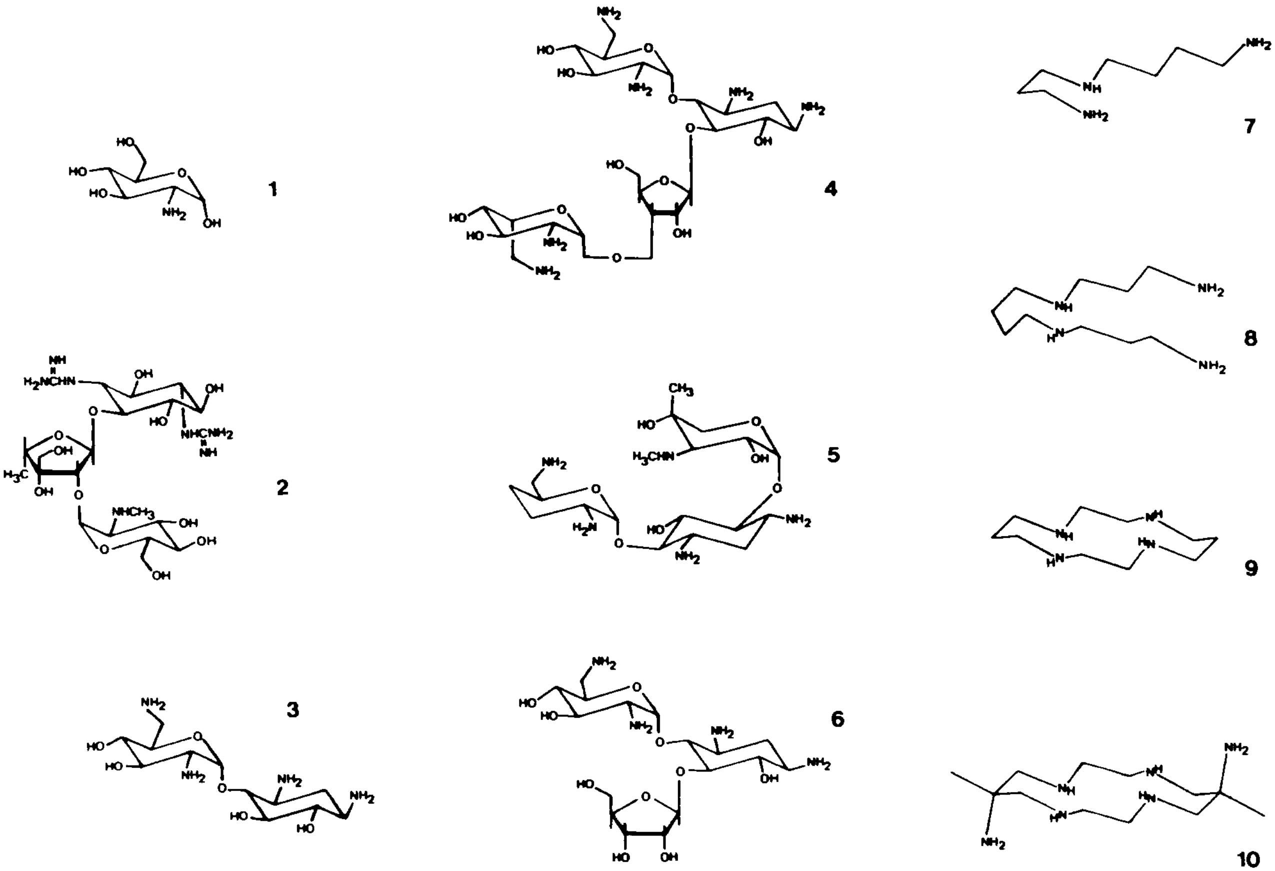

FIG. 8. Structures of some of the cationic molecules used as promoters for *p*-cresol methylhydroxylase electrochemistry.

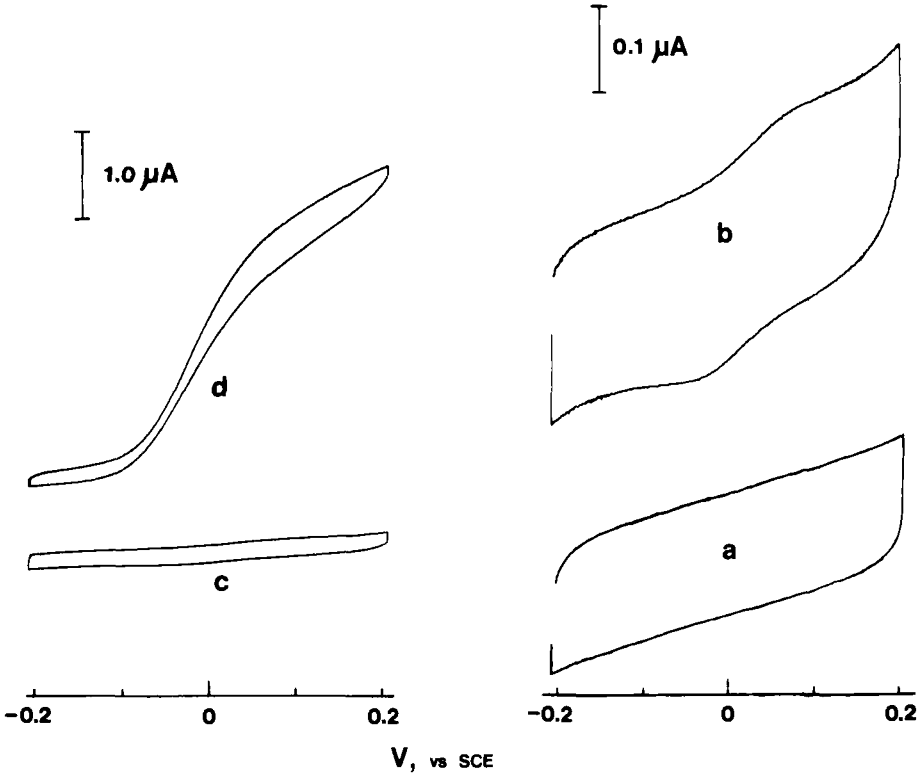

FIG. 9. Direct electrochemistry of *p*-cresol methylhydroxylase. (a) Response at an edge-plane graphite electrode in 10 m*M* KCl/10 m*M* HEPES (pH 7.4) buffer containing 10 m*M* spermine. Scan rate: 5 mV sec^{-1}. (b) Response upon addition of enzyme to ~30 μ*M*. (c) A repeat of (b) at reduced sensitivity. (d) Catalytic response upon addition of *p*-cresol (3 m*M*) to solution.

An important observation for enzyme electrochemistry arising from this study is the capacity of promoters to bridge two anticomplementary surfaces between an edge-plane graphite electrode and PCMH. The trend for simple inorganic cations in terms of their ability to promote electrocatalytic response is $Cr(NH_3)_6^{3+} > Mg^{2+} \gg K^+$ (see Table I), indicative of the importance of charge density. This trend has previously been established (*36*) wherein metal ions modulate the electrochemistry of redox proteins at graphite electrodes. With the larger enzyme, PCMH, the requirement for high charge density is much stronger. For example, the complete range of polyamines employed in this study are *all* effective in promoting cytochrome b_5 electrochemistry at graphite electrodes, whereas cyclam (Table 1 and Fig. 8), for example, failed to promote electrocatalytic response of PCMH. Presumably, promoters with high positive charge density are able to bind more selectively to the sites on PCMH where dense negative charges reside, and which are close to the heme prosthetic group. This may be a simple case of recognition specificity in biological systems.

A possible natural redox partner of PCMH, azurin, is a protein with a rather undefined interaction domain. It features (*60*) a considerably high proportion of nonpolar surface side chains and the spatial distribution of acidic and basic residues permits localized charge pairing. Binding between PCMH and azurin may also involve hydrophobic interactions, apart from complementary hydrogen bonding and salt bridging. In order that direct electrochemistry of PCMH takes place, electrodes with surface characteristics somewhat similar to those of azurin are necessary. The surface of an edge-oriented graphite electrode is heterogeneous; it possesses hydrophilic areas with high densities of C–O functional groups (carboxylic, phenolic, alcoholic, etc.), hydrophobic areas with negligible functionalization, and a range of intermediate domains. Once the negatively charged hydrophilic areas are adsorbed with cationic species, the electrode presents a mixed surface with both positive and negative charges as well as neutral groups. Thus, a system that in some way resembles one of the components of the biological complexes has presumably been formed.

C. OTHER ENZYMES

Following the success with PCMH, electrochemistry of several other extrinsic enzymes has also been achieved (*57, 61, 62*). One of these enzymes, flavocytochrome c_{552}, exhibits sulfide : cytochrome *c* oxidoreductase activity and transfers electrons from sulfide to the main photosynthetic electron transport chain through a *c*-type cytochrome. It has been found (*63*) to form an electrostatic complex with horse heart cytochrome *c* in a 1 : 2 ratio. The stability of this complex and sulfide : cytochrome *c* oxidoreductase activity of the enzyme are both ionic-strength dependent. Further studies by affinity chromatography, cross-linking, chemical modifications, and kinetic analysis suggest that the interaction between flavocytochrome c_{552} and cytochrome *c* involves the positively charged lysine residues around the exposed heme edge of cytochrome *c* and the complementary negative charges on the enzyme located predominantly on its heme subunit. As has been discussed above, studies on the direct electrochemistry of redox proteins show that if the electron transfer reaction between a protein and an electrode is to take place, the electrode surface must represent some features of the binding sites of this protein's physiological redox partner. Because about four carboxylate groups on flavocytochrome c_{552} have been predicted (*64*) to be involved in the interactions with cytochrome *c*, it is not surprising that positive electrode surfaces used for PCMH were (*61*) also effective in promoting direct electron transfer to flavocytochrome c_{552}.

Similar to PCMH, the requirement for a molecule to promote flavocytochrome *c* electrochemistry is adequate charge density and a degree of conformational flexibility. However, unlike PCMH, direct electrochemistry of flavocytochrome c_{552} has proved difficult to examine due to the electroactive nature of the substrate, sulfide, in the potential region under investigation. Nevertheless, the possibility that the observed anodic currents arise from the electrode reaction of sulfide to sulfur is ruled out by the experiment in which both anodic and cathodic waves from the enzyme were observed in the absence of the substrate. Direct, unmediated electron transfer between the enzyme and the electrode is established by the different electrochemical behaviors of the isolated cytochrome subunit and the intact enzyme at SS-Bipy-modified gold or edge-plane graphite electrodes in the absence of any promoter: the former exhibits a quasireversible voltammetric response, whereas the latter does not give any observable faradaic current. If the catalytic currents were obtained via mediation of the dissociated cytochrome subunit, no promoter would be needed at edge-plane graphite electrodes. This is certainly not the case.

Flavocytochrome c_{552} is probably the first enzyme for which direct spectroelectrochemistry has been achieved. This technique has been used previously to determine midpoint potentials of, e.g., cytochrome *c* oxidase, cytochrome *c*, and myoglobin. However, in all these reports, mediators are used to convey electrons between the prosthetic group and an electrode. Such a mediator must have a formal potential close to that of the enzyme of interest and must be capable of fast electron transfer to the enzyme and must exhibit no interference in the spectral region under study. The ability to reduce and oxidize an enzyme directly, as demonstrated with flavocytochrome c_{552}, has greatly simplified the measurement of redox potentials once conditions for direct electron transfer have been established. A collection of spectra obtained is shown in Fig. 10, together with the corresponding Nernst plot of E vs log[O]/[R]. Identical spectra were obtained irrespective of the direction the applied potential was changed, suggesting the reversibility of the electron transfer reaction of the enzyme. The isobestic points, which are an indication of conformational changes of the enzyme during the experiment, were not missing. From the intercept of the Nernst

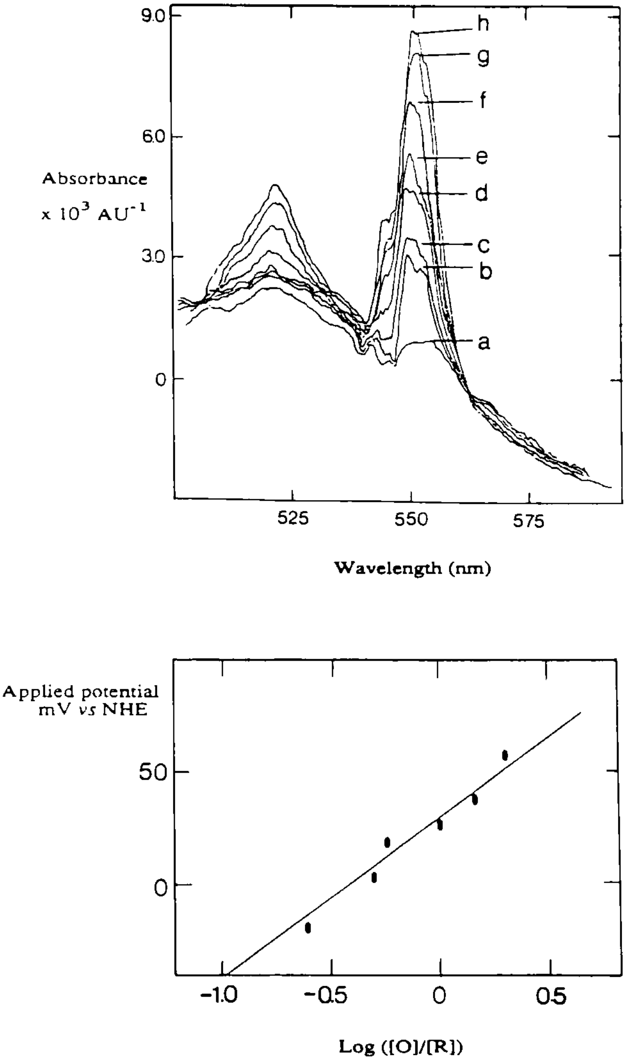

FIG. 10. Spectra of flavocytochrome c_{552} for different values of applied potential. (Top) Flavocytochrome c_{552} (100 μM) and 16 mM neomycin at a -(Cys-Glu-Cys)-modified OTTLE at potentials (a) 100 mV, (b) 59 mV, (c) 41 mV, (e) 20 mV, (f) 1 mV, (g) −20 mV, and (h) −40 mV. (Bottom) The corresponding Nernst plot of E_{app} vs. log[O]/[R].

plot, the formal potential of the enzyme (presumably the heme group) was determined as +34 mV, which is in good agreement with the reported value (*65*) of +32 mV. The slope of the plot, 68 mV, is close to that expected for a reversible one-electron transfer process.

Sulfite oxidase is another enzyme that has been found (*55*) to exchange electrons directly with an electrode. Because it also forms (*66*) an electrostatically stabilized complex with cytochrome *c*, it is not unreasonable to find that a modified electrode surface favorable for PCMH and flavocytochrome c_{552} interacts productively with this enzyme as well.

Methylamine dehydrogenase from bacterium W3A1, which catalyzes the oxidation of methylamine to formaldehyde and ammonia, is a very interesting enzyme. It has (*67*) an $\alpha_2\beta_2$ structure with a new type of redox cofactor in each of the two small subunits. There is a great deal of controversy over the structure of the cofactor. Upon reduction by methylamine, the UV–visible absorption spectra of the enzyme undergo (*68*) a complex biphasic change. Direct voltammetric response of methylamine dehydrogenase has been recently obtained (*62*) at unmodified graphite and modified gold electrodes. Further investigations of the enzyme by means of direct spectroelectrochemistry will help to understand the redox properties of this unique cofactor.

D. GENERAL COMMENTS

The success of direct electrochemistry of these enzymes is by no means accidental. All of them fall into the category of extrinsic enzymes, having metalloproteins such as azurin and cytochrome *c* as the physiological electron donor or acceptor. PCMH, flavocytochrome c_{552}, and sulfite oxidase are multicentered enzymes with a distinct function for each type of redox center. The catalytic center contains, for example, a flavin or a molybdenum cofactor that catalyzes the turnover reaction of the substrate. The electron transfer center contains, for example, a heme group that accepts electrons from the catalytic center and passes them to the redox protein partner. The distance between the two redox groups within the enzyme is short so as to facilitate the intramolecular electron transfer process [the two groups are separated by about 8 Å in PCMH (*56*)], but the pathway may be well shielded from the solvent. However, the electron transfer center of the enzyme is usually not completely inaccessible by the solvent, and there is an interaction domain around the partly exposed group where the protein partner binds. To ensure the high selectivity toward its substrate, the catalytic center is deeply buried in the polypeptides and thus it is almost impossible to exchange electrons directly with an electrode without any structural deformation. However, if the electrode is so designed that it somehow resembles the surface characteristics of the protein partner, the enzyme may bind to the electrode at the interaction domain around the electron transfer center. If the distance between the redox center and the electrode is comparable to that between the center and the prosthetic group of the protein partner, direct electrochemistry of the enzyme may be observed.

Comparing the voltammetric behavior of PCMH, flavocytochrome c_{552}, and sulfite oxidase with that of CCP, two important differences may be noticed. First, the electrochemical response of CCP did not occur at its thermodynamic potential. Instead, an overpotential of at least 0.4 V was required to drive the reduction of the oxidized enzyme at an electrode. No overpotential was observed with the enzymes described in this chapter: electron transfer from the enzyme to the electrode occurs at its thermodynamic potential. As has been suggested by Bowden and Paddock (*47*), the observed overpotential with CCP is probably due to the long-range electron transfer distance in the enzyme. The heme edge in CCP is (*69*) nearly 10 Å from the molecular surface, whereas it is partly exposed in cytochrome *c*. If the distance is the major contribution to the overpotential, then it may be suggested that the heme groups in PCMH, flavocytochrome c_{552}, and sulfite oxidase are quite close to the molecular surface or even partly exposed. This has certainly been confirmed in the case of PCMH: the structural study (*56*) shows that the propionate groups of the heme are quite open to solvent.

The other difference is that, with PCMH and flavocytochrome c_{552}, electron exchange with the modified electrode was achieved in both directions, i.e., oxidation and reduction, whereas electrochemical response of CCP was observed only in the presence of H_2O_2, illustrating that the substrate-oxidized enzyme (compound I) could be electrochemically reduced, but that the enzyme in its normal oxidation state [Fe(II) state] could not be oxidized. What is the structural basis for this unidirectional electron transfer? Poulos and Kraut (*70*) have proposed a hypothetical model for the CCP/cytochrome *c* electron transfer complex based on the observation that certain acidic side chains on the surface of CCP (Asp 33, Asp 34, Asp 37, and Asp 217) are positioned to interact with a complementary set of lysine residues on the surface of cytochrome *c*. In the resulting complex, the closest approach of the two heme edges is approximately 18 Å, and the two irons are separated by 25 Å. Because the distances are quite large, it is suggested that a bridging group between the two heme centers may participate in the electron transfer process. It is a radical site that is formed when the enzyme is oxidized to compound I, and is able to communicate with the surface of the protein via an overlapping system of π orbitals and ionic interactions, thus shortening the electron transfer distance. It must be emphasized here that this bridging group does not exist in CCP originally and is produced by oxidation with the substrate, H_2O_2. As a result, electron transfer is favored in the compound I/cytochrome *c* but not the CCP/cytochrome *c* complex. Similarly, it is understandable now why the enzyme is difficult to be oxidized electrochemically whereas the oxidized form, compound I, is relatively easier to reduce.

Based on the electrochemical studies, it seems that there are two subcategories of extrinsic redox enzymes. In some enzymes such as CCP, which are usually monomers, the prosthetic group is both the catalytic center and the electron transfer center. To secure the high specificity of the catalytic reaction, the prosthetic group must be deeply buried in the polypeptide chains. However, the following electron transfer reaction requires that this group be readily accessible to its electron donor or acceptor. These two apparently contradictory requirements can both be satisfied only if there is a conformational change of the enzyme accompanying the catalytic reaction, which either makes the prosthetic group accessible or somehow creates an electron transfer pathway, as happens in CCP. In other words, the catalytic reaction and electron transfer reaction are coupled, not only sequentially, but also conformationally. In other extrinsic enzymes such as PCMH, flavocytochrome c_{552}, and sulfite oxidase, there are usually two types of redox groups in them: a catalytic center and an electron transfer center. The two centers operate separately and there is no need for the electron transfer center to be highly protected. The two groups are thus said to be conformationally uncoupled, i.e., the environment of the electron transfer group is independent of what is happening to the catalytic center.

It is therefore anticipated that the enzymes studied are not unique in their electrochemical behavior and the experimental approaches adopted should be applicable to other flavocytochromes and even other "uncoupled" extrinsic enzymes.

V. Applications of Electrochemistry of Proteins and Enzymes

An obvious application of direct electrochemistry of proteins is the determination of redox potentials. In cases of thermodynamically inaccessible or kinetically reactive species for which traditional potentiometric methods are inappropriate, direct electrochemistry offers an alternative approach. A good example is the 7Fe ferredoxin from *Azotobacter chroococcum,* which contains one [4Fe–4S] and one [3Fe–4S] cluster. The $[4Fe\text{–}4S]^{2+/+}$ potential obtained (*71*) by cyclic voltammetry is −645 mV vs. NHE, which is by far the lowest ever measured for a ferredoxin system. As a result, the reduced form of the protein has not been generated by chemical reagents.

Because direct electrochemistry is observed only after the problems of interfacial specificity, compatibility, and denaturation have been overcome, it should provide us with a most powerful tool for investigating protein adsorption at surfaces. The studies of genetically engineered cytochrome *c* variants described in Section II serve as an illustration of this application.

Direct electrochemistry has also been used (*72–78*) to couple the electrode reactions to enzymes for which the redox proteins act as cofactors. In the studies, the chemically reduced or oxidized enzyme was turned over through the use of a protein and its electrode reaction as the source or sink of electrons. In the first report (*72, 73*) of such application, the electrochemical reduction of horse heart cytochrome *c* was coupled to the reduction of dioxygen in the presence of *Pseudomonas aeruginosa* nitrite reductase/cytochrome oxidase via the redox proteins, azurin and cytochrome c_{551}. The system corresponded to an "oxygen electrode" in which the four-electron reduction of dioxygen was achieved relatively fast at pH 7.

Another system (*77*) in which the direct electrochemistry of cytochrome *c* has been usefully exploited is in driving the turnover of yeast flavocytochrome b_2 (L-lactate/cytochrome *c* oxidoreductase; E.C. 1.1.2.3). A second-order rate constant of $5 \times 10^6\ M^{-1}\ \text{sec}^{-1}$ was obtained for turnover of the cytochrome *c* by the enzyme, which agreed well with the value from conventional spectrophotometric methods.

The high specificity of enzymes with regard to recognition of substrates has encouraged the development of enzyme electrodes for clinical analysis. An often-quoted example is the glucose oxidase (GOD) enzyme electrode for analysis of glucose, which is based on the following reaction:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2$$

The difficulties in achieving direct electron transfer between GOD and electrodes have led to the use of small electroactive molecules, called mediators, which shuttle electrons efficiently between the enzyme and the electrode. A new design of glucose electrodes depends (*79*) on ferricinium (Fc) ions as the mediator:

$$\text{Glucose} + \text{GOD(ox)} \rightarrow \text{Gluconolactone} + \text{GOD(red)}$$
$$\text{GOD(red)} + 2\text{Fc}^+ \rightarrow \text{GOD(ox)} + 2\text{Fc} + 2\text{H}^+$$
$$2\text{Fc} \rightleftharpoons 2\text{Fc}^+ + 2e^-$$

The system has been developed commercially into a pen-sized meter and has been on the market successfully now for a couple of years. Further progress (*80, 81*) was the incorporation of the mediator into GOD, which resulted in a modified enzyme with high catalytic activity in the absence of mediators in solution. Currently, the possibility of constructing a multimicroelectrode is under investigation in this laboratory. If successful, one single sensor carrying several different enzymes would have the ability of giving a number of analyses.

VI. Concluding Remarks

The direct electrochemistry of redox proteins has developed significantly in the past few years. Conditions now exist that permit the electrochemistry of all the proteins to be expressed at a range of electrodes, and important information about thermodynamic and kinetic properties of these proteins can be obtained. More recently, direct electron transfer between redox enzymes and electrodes has been achieved due to the more careful control of electrode surfaces. The need for biocompatible surfaces in bioelectrochemistry has stimulated the development of electrode surface engineering techniques, and protein electrochemistry has been reported at conducting polymer electrodes (*82*) and in membranes (*83, 84*). Furthermore, combination of direct protein electrochemistry with spectroscopic methods may offer (*85*) a novel way of investigating structure–function relationships in electron transport proteins.

Despite all these advances, there still remains much to be done, e.g., study of the protein/electrode interfacial region and characterization of modified electrode surfaces. The application of a newly developed surface technique, scanning tunnelling microscopy (STM), has been proved (*86*) successful in electrolytic solutions. Studies of modified electrode surfaces by STM should greatly enhance our understanding of electron transfer processes at electrodes. This, coupled with the advancements in biochemistry, would enable us to design more novel electrode surfaces for studying biological molecules and developing new types of electrochemical biosensors.

ACKNOWLEDGMENTS

We thank our colleagues, most of whom are named in the references, for their help and hard work. We are also grateful to the financial support provided by the Science and

Engineering Research Council, MediSense Inc., and the Sino-British Friendship Scholarship Scheme.

REFERENCES

1. Williams, R. J. P., *Mol. Phys.* **68,** 1 (1989).
2. Armstrong, F. A., Hill, H. A. O., and Walton, N. J., *Q. Rev. Biophys.* **18,** 261 (1986).
3. Bowden, E. F., Hawkridge, F. M., and Blount, P. M., *Treatise Electrochem.* **10,** 398 (1982).
4. Eddowes, M. J., and Hill, H. A. O., *in* "Electrochemical and Spectroelectrochemical Studies of Biological Redox Components" (K. M. Kadish, ed.), pp. 173–197. American Chemical Society, Washington, D.C., 1982.
5. Frew, J. E., and Hill, H. A. O., *Eur. J. Biochem.* **172,** 261 (1988).
6. Armstrong, F. A., Hill, H. A. O., and Walton, N. J., *Acc. Chem. Res.* **21,** 407 (1988).
7. Hawkridge, F. M., and Kuwana, T., *Anal. Chem.* **45,** 1021 (1973).
8. Takano, T., and Dickerson, R. E., *J. Mol. Biol.* **153,** 79 (1981).
9. Takano, T., and Dickerson, R. E., *J. Mol. Biol.* **153,** 95 (1981).
10. Eddowes, M. J., and Hill, H. A. O., *J. Chem. Soc., Chem. Commun.* 3154 (1977).
11. Eddowes, M. J., and Hill, H. A. O., *J. Am. Chem. Soc.* **101,** 7113 (1979).
12. Albery, W. J., Eddowes, M. J., Hill, H. A. O., and Hillman, A. R., *J. Am. Chem. Soc.* **103,** 3904 (1981).
13. Taniguchi, I., Toyosawa, H., Yamaguchi, H., and Yasukouchi, K., *J. Electroanal. Chem.* **140,** 187 (1982).
14. Taniguchi, I., Iseki, M., Yamaguchi, H., and Yasukouchi, K., *J. Electroanal. Chem.* **175,** 341 (1984).
15. Taniguchi, I., Iseki, M., Toyosawa, K., Yamaguchi, H., and Yasukouchi, K., *J. Electroanal. Chem.* **164,** 385 (1984).
16. Taniguchi, I., Iseki, M., Yamaguchi, H., and Yasukouchi, K., *J. Electroanal. Chem.* **186,** 299 (1985).
17. Eddowes, M. J., Hill, H. A. O., and Uosaki, K., *J. Am. Chem. Soc.* **101,** 7113 (1979).
18. Koppenol, W. H., and Margoliash, E., *J. Biol. Chem.* **257,** 4426 (1982).
19. Smith, M. B., Stonehuerner, J., Ahmed, A. J., Staudenmeyer, N., and Millett, F., *Biochim. Biophys. Acta* **592,** 303 (1980).
20. Osheroff, N., Brautigan, D. L., and Margoliash, E., *J. Biol. Chem.* **255,** 8245 (1980).
21. Allen, P. M., Hill, H. A. O., and Walton, N. J., *J. Electroanal. Chem.* **178,** 69 (1984).
22. Barker, P. D., Di Gleria, K., Hill, H. A. O., and Lowe, V. J., *Eur. J. Biochem.* **190,** 171 (1990).
23. Yeh, P., and Kuwana, T., *Chem. Lett.* 1145 (1977).
24. Bowden, E. F., Hawkridge, F. M., and Blount, H. N., *J. Electroanal. Chem.* **161,** 355 (1984).
25. Harmer, M. A., and Hill, H. A. O., *J. Electroanal. Chem.* **189,** 229 (1985).
26. Armstrong, F. A., Hill, H. A. O., and Oliver, N. B., *J. Chem. Soc., Chem. Commun.* 976 (1984).
27. Armstrong, F. A., Cox, P. A., Lowe, V. J., and Oliver, B. N., *J. Electroanal. Chem.* **217,** 331 (1987).
28. Hill, H. A. O., and Whitford, D., *J. Electroanal. Chem.* **235,** 153 (1987).
29. Louie, G. V., Hutcheon, W. L. B., and Brayer, G. D., *J. Mol. Biol.* **199,** 295 (1988).
30. Burrows, A. L., Guo, L.-H., H. A. O., McLendon, G., and Sherman, F., *J. Am. Chem. Soc.* (submitted for publication).

31. Rees, D. C., *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3082 (1985).
32. Moore, G., Pettigrew, G., and Rogers, N., *Proc. Natl. Acad. Sci. U.S.A.* **83,** 4998 (1986).
33. Hickey, D. R., McLendon, G., Brayer, G. D., Sherman, F., and Berghuis, A., unpublished results.
34. Hill, H. A. O., Page, D. J., Walton, N. J., and Whitford, D., *J. Electroanal. Chem.* **187,** 315 (1985).
35. Armstrong, F. A., Hill, H. A. O., Oliver, B. N., and Whitford, D., *J. Am. Chem. Soc.* **107,** 1473 (1985).
36. Armstrong, F. A., Hill, H. A. O., Oliver, B. N., and Walton, N. J., *J. Am. Chem. Soc.* **106,** 921 (1984).
37. Nicholson, R. S., *Anal. Chem.* **37,** 1351 (1965).
38. Armstrong, F. A., Bond, A. M., Hill, H. A. O., Psalti, I. S. M., and Zoski, C. G., *Phys. Chem.* **93,** 6485 (1989).
39. Armstrong, F. A., Bond, A. M., Hill, H. A. O., Oliver, B. N., and Psalti, I. S. M., *J. Am. Chem. Soc.* 111, 9185 (1989).
40. Bond, A. M., Hill, H. A. O., McCarthy, M. E., Psalti, I. S. M., and Walton, N. J., *J. Electroanal. Chem.*, submitted.
41. Barker, P. D., and Hill, H. A. O., *in* "Oxidases and Related Redox Systems" (T. E. King, H. S. Marson, and M. Morrison, eds.), p. 419. Alan R. Liss, New York, 1988.
42. Barker, P. D., Guo, L.-H., Hill, H. A. O., and Sanghera, G. S., *Biochem. Soc. Trans.* **16,** 957 (1988).
43. Bagby, S., Barker, P. D., Guo, L.-H., and Hill, H. A. O., *Biochemistry* **29,** 3213 (1990).
44. Salemme, F. R., *J. Mol. Biol.* **102,** 563 (1976).
45. Assefa, H., and Bowden, E. F., *Biochem. Biophys. Res. Commun.* **139,** 1003 (1986).
46. Armstrong, F. A., and Lannon, A. M., *J. Am. Chem. Soc.* **109,** 7211 (1987).
47. Paddock, R. M., and Bowden, E. F., *J. Electroanal. Chem.* **260,** 487 (1989).
48. Tarasevich, M. R., Yaropolov, A. I., Bogdanovskaya, V. A., and Varfolomev, S. D., *Bioelectrochem. Bioenerg.* **6,** 393 (1979).
49. Lee, C.-W., Gray, H. B., Anson, F. C., and Malmström, B. G., *J. Electroanal. Chem.* **172,** 289 (1984).
50. Külys, J., *J. Biosensors* **2,** 3 (1986).
51. Albery, W. J., Bartlett, P. N., Bycroft, M., Craston, D. H., and Driscoll, B. J., *J. Electroanal. Chem.* **218,** 119 (1987).
52. Ianniello, R. M., Lindsay, T. J., and Yacynych, A. M., *Anal. Chem.* **54,** 1098 (1982).
53. Miyawaki, O., and Wingard, L. B., Jr., *Biotechnol. Bioeng.* **26,** 1364 (1984).
54. Szucs, A., Hitchens, G. D., and Bockris, J. O'M., *Bioelectrochem. Bioenerg.* **21,** 133 (1989).
55. Scheller, F., Srtnad, G., Neumann, B., Kuhn, M., and Ostrowski, W., *Bioelectrochem. Bioenerg.* **6,** 117 (1979).
56. Mathews, F. S., Chen, Z., and Bellamy, H. D., *Biochemistry* (submitted for publication).
57. Guo, L.-H., Hill, H. A. O., Hopper, D. J., Lawrance, G. A., and Sanghera, G. S., *J. Electroanal. Chem.* **266,** 379 (1989).
58. Guo, L.-H. Ph.D. Thesis, University of Oxford (1990).
59. Causer, M. J., Hopper, D. J., McIntire, W. S., and Singer, T. P., *Biochem. Soc. Trans.* **12,** 1131 (1984).
60. Adman, E. T., and Jensen, L. H., *Isr. J. Chem.* **21,** 8 (1981).
61. Guo, L.-H., Hill, H. A. O., Hopper, D. J., Lawrance, G. A., and Sanghera, G. S., *J. Biol. Chem.* **265,** 1958 (1990).

62. Burrows, A. L., Hill, H. A. O., Leese, T. A., McIntire, W. S., Nakayama, H., and Sanghera, G. S., *Eur. J. Biochem.*, submitted.
63. Gray, G. O., and Knaff, D. B., *Biochim. Biophys. Acta* **680,** 290 (1982).
64. Bosshard, H. R., Davidson, M. W., Knaff, D. B., and Millett, F., *J. Biol. Chem.* **261,** 190 (1986).
65. Meyer, T. E., Vorkink, W. P., Tollin, G., and Cusanovich, M. A., *Arch. Biochem. Biophys.* **236,** 52 (1985).
66. Kipke, C. A., Cusanovich, M. A., Tollin, G., Sunde, R. A., and Enemark, J. H., *Biochemistry* **27,** 2918 (1988).
67. DeBeer, R., Duine, J. A., Frank, J., and Large, P. J., *Biochim. Biophys. Acta* **622,** 370 (1980).
68. Kenney, K. C., and McIntire, W. S., *Biochemistry* **22,** 3858 (1983).
69. Finzel, B. C., Poulos, T. L., and Kraut, J., *J. Biol. Chem.* **259,** 13027 (1984).
70. Poulos, T. L., and Kraut, J., *J. Biol. Chem.* **255,** 10322 (1980).
71. Armstrong, F. A., George, S. J., Thomson, A. J., and Yates, M. G. *FEBS Lett.* **234,** 107 (1987).
72. Hill, H. A. O., Walton, N. J., and Higgins, I. J., *FEBS Lett.* **126,** 282 (1981).
73. Hill, H. A. O., and Walton, N. J., *J. Am. Chem. Soc.* **104,** 6515 (1982).
74. Coleman, J. O. D., Hill, H. A. O., Walton, N. J., and Whatley, F. R., *FEBS Lett.* **154,** 319 (1983).
75. Davis, G., Hill, H. A. O., Aston, W. J., Higgins, I. J., and Turner, A. P. F., *Enzyme Microb. Technol.* **5,** 383 (1983).
76. Turner, A. P. F., Aston, W. J., Higgins, I. J., Bell, J. M., Colby, J., Davis, G., and Hill, H. A. O., *Anal. Chim. Acta* **163,** 161 (1984).
77. Cass, A. E. G., Davis, G., Hill, H. A. O., and Nancarrow, D. J., *Biochim. Biophys. Acta* **828,** 51 (1985).
78. Hill, H. A. O., Oliver, B. N., Page, D. J., and Hopper, D. J., *J. Chem. Soc., Chem. Commun.* 1469 (1985).
79. Cass, A. E. G., Davis, G., Francis, G. D., Hill, H. A. O., Aston, W. J., Higgins, I. J., Plotkin, E. V., Scott, L. D., and Turner, A. P. F., *Anal. Chem.* **56,** 667 (1984).
80. Hill, H. A. O., Eur. Pat. Appl. 84/303,090.9 (1984).
81. Degani, Y., and Heller, A., *J. Phys. Chem.* **91,** 1285 (1987).
82. Bartlett, P. N., and Farington, J., *J. Electroanal. Chem.* **261,** 471 (1989).
83. Campanella, L., Sammartino, M. P., Stefanoni, P., and Tranchida, G., *Bioelectrochem. Bioenerg.* **21,** 55 (1989).
84. Hahn, C. E. W., Hill, H. A. O., Ritchie, M. D., and Sear, J. W., *J. Chem. Soc., Chem. Commun.* 125 (1990).
85. Moss, D., Nabedryk, E., Breton, J., and Mäntele, W., *Eur. J. Biochem.* **187,** 565 (1990).
86. Cataldi, T. R. I., Blackham, I. G., Briggs, G. A. D., Hill, H. A. O., and Pethica, J. B., *J. Electroanal. Chem.* **290,** 1 (1990).