ESR spectroscopy of $RR'CN_2^{\bullet+}$ and, after photolysis, the derived carbene cation radical, although the absence of resolved hyperfine coupling means that the assignment of structure is largely circumstantial.

Synthetic application of this chemistry seems unlikely at present because of the multiplicity of reaction channels available both to $RR'CN_2^{\bullet+/-}$ and to $RR'C^{\bullet+/-}$. However, as further information on a wider spectrum of structures of diazo compounds becomes available, this preliminary assessment may have to undergo revision. Indeed, this chemistry will need to be borne in mind if recent interest in the cycloaddition of alkene cation radicals to diazo compounds[51] is to be extended.

We gratefully acknowledge NTNF (Norway), NAVF (Norway), SERC (UK), and NATO, which has made our collaboration possible. Professors R. N. McDonald and M. D. Hawley kindly made results available prior to publication.

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Direct Electrochemistry of Redox Proteins

FRASER A. ARMSTRONG, H. ALLEN O. HILL,* and NICHOLAS J. WALTON

Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, England

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Among studies of electron-transfer reactions of proteins, the area of direct (i.e., unmediated) electrochemistry has interested us for several reasons: First, from an innovatory viewpoint, a marriage between electrode materials and biological macromolecules that bear highly specific modes of catalytic or sensory action is highly desirable. Second, as with electrochemical reactions of simpler molecules, we are able to obtain important information about intrinsic thermodynamic and kinetic properties. Third, since direct electrochemistry is observed only after the problems of interfacial specificity, compatability, and denaturation have been overcome, we stand to learn much about the dynamic operation of macromolecular interfaces, and this has far-reaching implications. Studies undertaken in this laboratory, as well as in several others, have sought to explore this "new" field and to define the relationships between electrochemistry, colloid and interface science, and biochemistry. A "thought tree" illustrating our view of the connectivities in this area is shown in Figure 1.

The simplest redox proteins are those whose only established role is to convey electrons. They are comparatively small, with molecular masses in the range 5–20 kDa, and, as a class, have been characterized extensively with regard to structure and function. It may be said that the cytochromes, the "blue" copper proteins, and the iron–sulfur proteins are today as familiar to chemists as they are to biochemists. Thus the idea of an electron-transfer protein displaying reversible or quasi-reversible cyclic voltammetry analogous to that of a small redox reagent such as ferrocene may seem straightforward. Yet it embraces a contradiction: from various studies of electron-transfer reactions between protein molecules, the emerging consensus emphasizes the requirement for fast and reversible formation of an intimate, precursory, donor–acceptor complex docked so as to maximize the rate of electron transfer between redox centers. By contrast, in outer-sphere electron transfer between small inorganic molecules precursor complex formation is generally undetectable and of much less mechanistic significance.

If we consider the electron-transfer reactivity of a protein at an *electrode*, then the requirement for intimate precursory interaction merges uncomfortably with the well-known tendency of proteins to undergo irreversible adsorption coupled with conformational changes and loss of activity. Electrode "fouling" by biological material is indeed a familiar problem in bioelectrochemistry, yet the now-routine measurements of protein voltammetry, with standards of reversibility comparable to those seen with the redox couples of small molecules, demonstrate clearly that this can be avoided under appropriate conditions. We believe that this reflects provision, at the electrode interface, of the essential elements of macromolecular recognition, i.e., complementary hydrogen bonding, salt bridging, and hydrophobic contacts, that are important in a protein's interactions with its physiological partners. Our own approach has been to evaluate systematically the interactive characteristics of electron-transfer proteins and thenceforth venture upward in complexity toward the achievement of clear and well-defined electrochemistry of larger enzymes. In this Account we discuss some of the more salient findings and their implications.

Fraser A. Armstrong was born in Cambridge, England, in 1951. He received his B.Sc. (1975) and Ph.D. (1978) from Leeds University. He held a Royal Society European Exchange Fellowship at the University of Konstanz, West Germany, followed by postdoctoral research assistantships at New Mexico State University and the Institute for Enzyme Research, Madison. In 1981 he was a Postdoctoral Fellow at the Inorganic Chemistry Laboratory, Oxford, and in 1983 he became a Royal Society University Research Fellow. His interest is in bioinorganic chemistry, particularly the development and exploitation of techniques such as electrochemistry and cryokinetics for the investigation of kinetic and thermodynamic properties of metal-containing active sites in biomolecules.

H. Allen O. Hill was born in 1937 in Belfast, Northern Ireland, and attended Queen's University, where he received his B.Sc. and Ph.D. He then went to Oxford, where he was appointed a Fellow of Queen's College in 1965. He received a D.Sc. from Oxford University in 1985 and, apart from his electrochemical interests, is working on phagocytosis and on the NMR spectroscopy of metalloproteins.

Nicholas J. Walton was born in Birmingham, England, in 1958 and received his B.A. (1981) and D.Phil. (1984) at the University of Oxford. Since 1984 he has been Departmental Demonstrator in the Inorganic Chemistry Laboratory, Oxford. His main research interests include the study of chemisorbed monolayers by electrochemical and spectroscopic techniques together with the application of relevant methodology in analysis.

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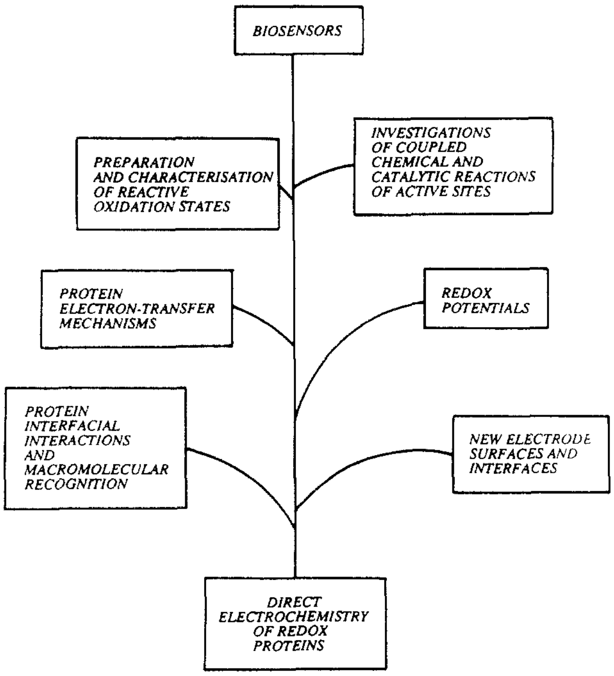

Figure 1. A "thought tree" for protein direct electrochemistry, indicating the topics and objectives associated with the area.

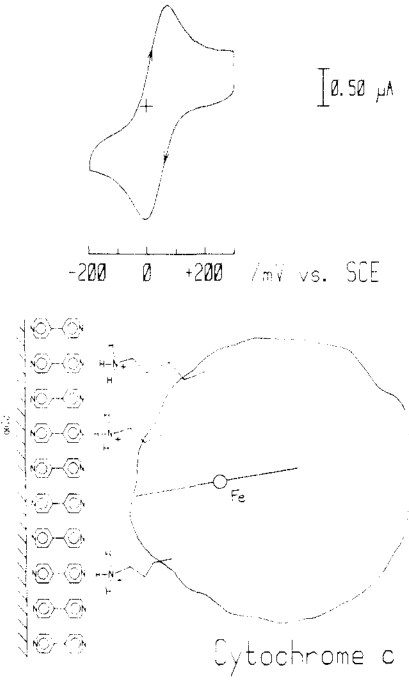

Figure 2. Typical cyclic voltammogram of horse heart cytochrome *c* at a gold electrode, modified by adsorption with 4,4′-bipyridyl, together with an illustration of the electrode–protein complex envisaged for the idealized limiting case of a planar surface.

Direct Electrochemistry of Cytochrome *c*

The first clear demonstrations of quasi-reversible and nondegradative direct electrochemistry of a protein appeared in 1977. Yeh and Kuwana studied[1] horse heart cytochrome *c* and showed that a good cyclic voltammetric response could be obtained at a tin-doped indium oxide electrode. At about the same time Eddowes and Hill demonstrated[2] quasi-reversible cyclic voltammetry of cytochrome *c* at a gold electrode onto which was adsorbed, from the same solution, a monolayer of the reagent 4,4′-bipyridyl. This result is shown in Figure 2. Direct current cyclic voltammetry peak separations were close to 60 mV and faradaic currents varied linearly with (scan rate)$^{1/2}$. Such criteria indicate electrochemical reversibility and show that electron transport is effectively dominated by planar diffusion of cytochrome *c* to and from the electrode. The half-wave potential, $E_{1/2}$ = 260 mV vs NHE, is identical with potentiometric values, and the response is stable, showing no marked attenuation with time.

The important point about each of these experiments appeared to be that both types of electrode surface possess chemical functionalities capable of interacting reversibly and specifically with the protein surface. The structure of cytochrome *c* (MW = 12 400) is[3] well characterized. The Fe porphyrin (heme) center is buried within the molecule; its solvent-exposed surface corresponds[4] to a very small proportion (0.06%) of the total molecular surface. A large overall positive charge (+9 at pH 7 for the oxidized state) stems from an excess of basic residues, and a significant dipole moment (324 D for the oxidized form) arises[5] from an imbalance in the spatial distribution of acidic ($-CO_2^-$) side chains. The surface region surrounding the exposed heme edge (the mechanistically important interaction domain) contains a number of uncompensated lysines ($-NH_3^+$). Eddowes and Hill proposed[6,7] that hydrogen bonding between these lysines and pyridyl nitrogens at the modified electrode surface stabilized a transient protein–electrode complex oriented so as to allow rapid electron transfer to and from the heme group (Figure 2). In support of this, it had been shown[8] that electrochemical activity disappeared as the pH was lowered from 7 to 4. The effective pK_a (5.1) was consistent with protonation of one of the two pyridyl nitrogens of 4,4′-bipyridyl leading to loss of hydrogen-bond acceptor capability and its replacement by (repulsive) positive charge. Complementary salt bridging and hydrogen-bonding interactions between lysine groups on cytochrome *c* and optimally positioned carboxylates on a reaction partner are, notably, important features of the physiological situation.[9]

A more detailed study of the cytochrome *c*/4,4′-bipyridyl/Au system was carried out[10] with rotating-disk and rotating-ring–disk techniques. With strict hydrodynamic control, it was possible to isolate individual kinetic steps. Rate constants for adsorption and desorption of cytochrome *c* from the modified electrode surface were found to be, respectively, 3×10^{-2} cm s^{-1} and 50 s^{-1} and essentially equal for oxidized and reduced forms. The first-order standard rate constant for the elementary electron-transfer event itself (at zero overpotential) was estimated to be 50 s^{-1}, certainly a respectable figure as compared to measured values[11] of

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long-range electron transfer between or within proteins. The resulting free-energy profile involving adsorption, electron transfer, and desorption is accordingly symmetrical. Ellipsometric data obtained for 4,4′-bipyridyl and 1,2-bis(4-pyridyl)ethene adsorbed on gold[12] indicated clearly that they bind in an end-on fashion; i.e., one pyridyl nitrogen coordinates to the gold surface, leaving the remaining nitrogen directed "into" the solution (Figure 2). For these two reagents, both functions, i.e., that of surface "anchor" and that of "bait", are performed by pyridyl nitrogens. Subsequently, Taniguchi et al.[13] noted that sulfur-containing nitrogen bases such as bis(4-pyridyl) disulfide were excellent promoters of cytochrome *c* electrochemistry with the attraction that they were adsorbed so strongly on gold that a "predip" in the reagent solution was sufficient for a stable response.

In an extensive investigation[14] of the necessary functional requirements of a molecule to render it active as a promoter of the direct electrochemistry of horse heart cytochrome *c*, we sought to define more generally the principles involved. The majority of the molecules studied were bifunctional, i.e., X $\sim$ Y, where X is a surface-active functional group that will interact with the metal to give quasi-irreversible adsorption, Y is the protein interactive functionality, and ($\sim$) is the linking structure. The surface-adsorbing functional groups, X, found to be most satisfactory were 4-pyridyl-N, thiol-SH, disulfide "S–S", and phosphine P. All are σ-donor, π-acceptor synergic-type ligands, appropriate for coordination to a group 11 metal like gold. Those functional groups, Y, that were found to be effective divide roughly into two subcategories: (a) neutral groups such as 4-pyridyl-N and aniline-type amines, $Ar\text{-}NH_2$, which are weakly basic and able to form hydrogen bonds to lysine-NH_3^+; (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.

The link ($\sim$) may be rigid or flexible (see below), aliphatic or aromatic, and of varying length. We have found no significant difference between aliphatic and aromatic links in the effectiveness of the compounds as promoters of cytochrome *c* electrochemistry. The question of link flexibility is interesting. If this allows both X *and* Y to interact with the surface of the metal, as with 1,2-bis(4-pyridyl)ethane, then clearly Y is unavailable for constructive interaction with the protein surface. Consider, however, an aromatic molecule like 4-mercaptopyridine, for which there are *no* conformational options: adsorption of the thiol group orients the pyridine nitrogen outwardly at the extended interface, thus positioning it correctly for hydrogen bonding to cytochrome *c*. In the same way, success would be ensured when using conformationally rigid but nonaromatic adsorbates. We believe that it is the rigidity and *not* the conjugation per se of delocalized systems that may make them superior to analogous aliphatic systems.

It is worth emphasizing that these effects cannot arise through isolated single molecules but must involve collective interactions between the protein surface and *ensembles* of adsorbed promoters. For example, progressive substitution of bis(4-pyridyl) disulfide by the "inert" analogue thiophenol, in effect a dilution of the functional monolayer, results in more than just a parallel loss of cytochrome *c* electrochemical activity: the observed current density drops much faster than expected on the basis of slower kinetics alone.[15] The conclusion is that there must be some minimum number of active promoters required, in any given ensemble of molecules on the surface, to permit cytochrome *c* electrochemistry. Indeed the very nature of the surface, i.e., its topography, the crystal plane(s) revealed, and the type of defects exposed, will markedly influence both the modified electrode surface and its interaction with the protein. Furthermore, both the promoter on the surface *and* the protein are best considered to be *dynamic*. There is no reason to suppose that, during the lifetime of an electrode–protein complex, the protein must remain in the same place on the modified surface: lateral diffusion across the surface may be relatively facile, at least on surfaces where the Y functional group distribution is homogeneous. What is more important, however, for diffusion-dominated electrochemistry, is that the protein *must* dissociate from the electrode surface *after* electron transfer.

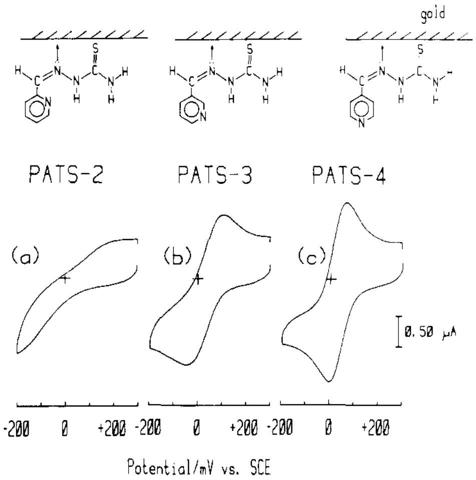

Figure 3. Systematic improvement in cytochrome *c* electrochemistry observed as the isomer of the surface promoter pyridine-*n*-carboxaldehyde thiosemicarbazone is changed from (a) $n = 2$ to (b) $n = 3$ to (c) $n = 4$.

Subtle alterations of the structure of promoters give us important information concerning the importance of close-range polar interactions. The pyridine-*n*-carboxyaldehyde thiosemicarbazones (PATS) illustrate[16] such chemical and stereochemical requirements. Some results are shown in Figure 3. The position in which the ring is attached is indicated by "*n*" in the acronym PATS-*n*. There is a systematic change in the electrochemistry of cytochrome *c* at a gold surface modified by PATS-*n* as *n* is changed progressively from 2 to 3 to 4. This matches the change in ring orientation, and consequently the position of the pyridine nitrogen, with respect to the metal–solution vector. For PATS-2, furthermore, hydrogen bonding between the interactive nitrogen and the thioamide N–H adjacent to the thio-

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carbonyl group may be possible, thereby taking the hydrogen-bond acceptor out of operation. Interestingly, the electrochemistry of cytochrome *c*, even that so poorly expressed at a PATS-2 modified gold surface, is improved[17] through addition of lyotropic anions such as perchlorate, perrhenate, iodide, and thiocyanate. Ease of interfacial desolvation may thus be an important factor in allowing protein molecules to "dock" effectively and exchange electrons with the modified electrode surface.

Another crucial role that promoters may play is to prevent adventitious adsorption of other molecules on the electrode surface. This far-from-trivial problem is particularly relevant since the proteins themselves usually adsorb strongly, often with denaturation, on a "clean" electrode surface. In some special cases, however, clean surfaces can result in electron transfer. This is the case for cytochrome *c*, which is[18] electrochemically active, albeit transiently, at a Au electrode that has been specially "cleaned" in a hydrogen flame. There is also a small class of proteins, the cytochromes c_3, that contain four heme groups in a relatively small protein; these are[19] electrochemically active at an unmodified electrode.

The important requirement for interactive functionalities at the electrode has been echoed in a number of studies with cytochrome *c* at electrode materials that feature *intrinsic* surface groups. Hawkridge and coworkers studied[18] the electrochemistry at tin-doped In_2O_3 and fluorine-doped SnO_2. Two aspects are particularly significant: first, the electrochemical response (as measured at SnO_2) is dependent upon the history and pretreatment of the electrode. The authors suggest that this reflects variations in surface hydration, loosely defined, whereby the fully hydrated surface at equilibrium is least active. Again we may view strongly bound vicinal water molecules as a hindrance. Second, the response is attenuated sharply upon acidification from pH 8 to pH 6. Cytochrome *c* does not undergo a kinetically relevant protonation within this region; thus the pH effect is associated with the electrode surface or vicinal water molecules. A similar effect is observed[20] at RuO_2 electrodes.

Pyrolytic graphite (PG) and glassy carbon, polished in air, possess a surface layer of various carbon oxide functionalities, which can be modified further by selective reagents. The large potential "window" encompasses satisfactorily the wide spectrum of redox potentials exhibited by aqueous biological systems. Furthermore, for PG, the crystalline anisotropy permits comparisons of electrochemical response at surface orientations having very different chemical properties. As illustrated in Figure 4, cytochrome *c* gives[21,22] a small response at a freshly cleaved basal plane PG electrode, which is largely hydrophobic and devoid of surface oxides (O/C ratio estimated by X-ray photoelectron spectroscopy (XPS)). If this surface is polished in air, then the response alters dramatically. If the polished "edge plane" is used, the cyclic voltammetry is stable and quasi-reversible. The successive change in behavior parallels the increase in surface O/C ratio; this is about 0.33 at the polished edge but ~0.02 at cleaved basal. At the "edge", there is once again a pH effect. Titrations of the responses of cytochrome *c* and $Fe(CN)_6^{3-}$ revealed a common surface "p*K*" of 5.6. The protonated surface is inactive toward cytochrome *c* but active toward $Fe(CN)_6^{3-}$.

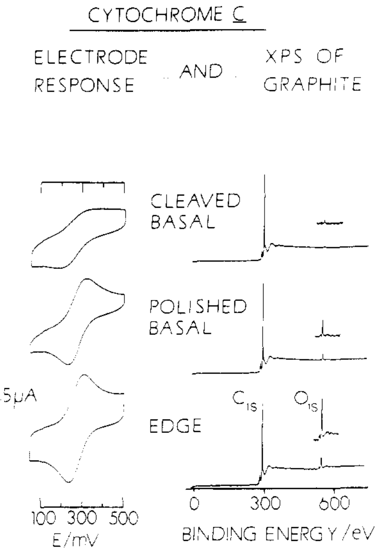

Figure 4. dc cyclic voltammetry of cytochrome *c* at various surfaces of pyrolytic graphite characterized by X-ray photoelectron spectroscopy (XPS). For the voltammetry, measured on the fourth scan at 20 mV s^{-1}, cytochrome *c* is 0.15 mM in 5 mM Tricine, 0.10 M NaCl, pH 8.0, temperature 20 °C. The potential scale is referenced against the normal hydrogen electrode. For XPS, a scale enlargement (×3) is shown for the O_{1s} peak.

Direct Electrochemistry of Other Redox Proteins

Studies of other proteins have enabled us to clarify the importance of Coulombic interactions. Unlike cytochrome *c*, most electron-transfer proteins bear overall *negative* charges. Examples include the bacterial flavodoxins and [4Fe–4S] ferredoxins, which operate as electron carriers in nitrogen fixation, the chloroplast [2Fe–2S] ferredoxins, which convey electrons out of the Photosystem I complex, and the "blue" copper protein plastocyanin, which is the essential electron donor to Photosystem I in higher plants. Hawkridge and his group reported[23] in 1977 that spinach chloroplast ferredoxin displayed cyclic voltammetry at a gold electrode modified with a polymeric coating of methylviologen. This protein, MW = 10 000, which contains a single [2Fe–2S] cluster with a low redox potential (−420 mV vs NHE), carries a very large negative charge (−17 for the oxidized form). We ourselves searched for conditions that would permit electrochemistry of the small ferredoxin from the nitrogen-fixing anaerobe *Clostridium pasteurianum*. This protein contains two [4Fe–4S] clusters (each transferring one electron at similar potentials, ca. −380 mV vs NHE) and, like spinach ferredoxin, has a large negative charge (ca. −12 for the oxidized form). We were able[24] to promote stable and

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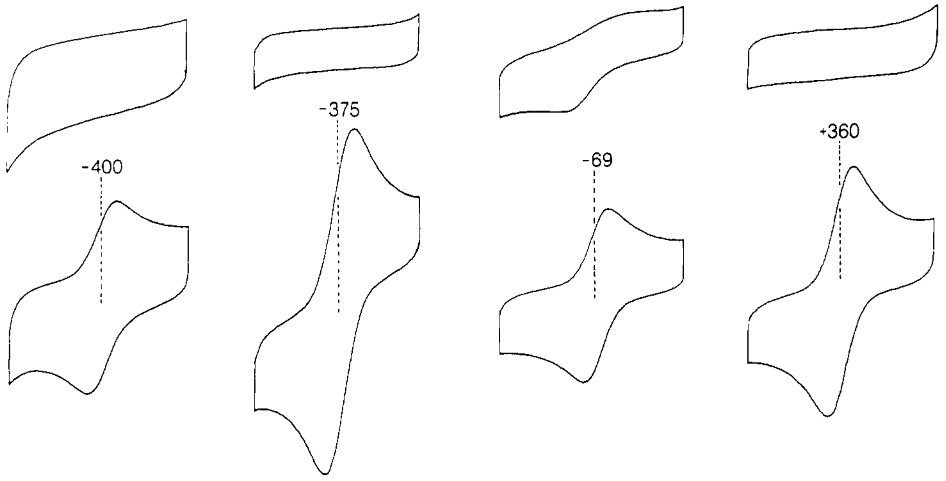

Spinach ferredoxin $[2Fe\text{-}2S]^{2+/1+}$

Clostridium ferredoxin $2[4Fe\text{-}4S]^{2+/1+}$

Clostridium rubredoxin $Fe^{3+/2+}$

Spinach plastocyanin $Cu^{2+/1+}$

Figure 5. dc cyclic voltammetry of a range of small negatively charged redox proteins at pyrolytic graphite "edge" electrodes, showing the promotion of direct electrochemistry by multivalent cations. Scan rate is 20 mV s^{-1}. Electrodes polished with 0.3-μm alumina slurry and sonicated. Half-wave potentials in millivolts vs NHE are indicated in each case. Spinach [2Fe–2S]ferredoxin: (Top) Protein 65 μM in 5 mM Tricine, 1.0 M NaCl, pH 8.0, temperature 25 °C. Note the absence of response in the 1:1 electrolyte even at this high concentration. Range is −145 to −680 mV vs NHE. (Bottom) Cyclic voltammetry of the same solution after inclusion of 1.5 mM $Cr(NH_3)_6^{3+}$. The latter is *not* redox-active in this potential range. *Clostridium pasteurianum* 2[4Fe–4S] ferredoxin: (Top) Protein 150 μM in 5 mM Tricine, 1 mM NaCl, pH 8.0, temperature 25 °C. Range is −106 to −606 mV vs NHE. (Bottom) Cyclic voltammetry of the same solution after inclusion of 15 mM decamethonium (DM^{2+}) bromide; $DM^{2+} = (CH_3)_3N(CH_2)_{10}N(CH_3)_3^{2+}$. *Clostridium pasteurianum* rubredoxin: (Top) Protein 67 μM in 5 mM Tricine, 0.10 M NaCl, pH 8.0, temperature 25 °C. Range is +194 to −361 mV vs NHE. Note that this protein gives a poor yet discernible response in this medium (a similar observation is made for *Clostridium pasteurianum* 2[4Fe–4S] ferredoxin in 0.1 M NaCl). (Bottom) Cyclic voltammetry of the same solution after inclusion of 4.4 mM $Cr(NH_3)_6^{3+}$. Spinach plastocyanin: (Top) Protein 200 μM in 10 mM MES, 1 mM KCl, pH 6.0, temperature 25 °C. Range +619 to −175 mV vs NHE. (Bottom) Cyclic voltammetry of the same solution after inclusion of 0.5 mM $Pt(NH_3)_6^{4+}$.

well-behaved cyclic voltammetry at PG electrodes simply by adding a little $MgCl_2$. Our action was prompted by various reports of the effects of intracellular Mg^{2+} in screening membrane potentials and promoting close interaction between negatively charged surfaces. We found[25] that $Cr(NH_3)_6^{3+}$ was even more effective.

We have since made[21,25,26] extensive investigations of the effects of cations on protein electrochemistry. We have now found that for all of these small negatively charged proteins, electrochemistry at the deprotonated "edge" surface of PG (i.e., pH >5.6) is promoted by multivalent cations. Some of our results are depicted in Figure 5. Since electrochemistry of cytochrome *c* at the protonated "edge" (i.e., pH <5.6) is not promoted, in corresponding fashion, by multivalent *anions*, we proposed[21] that the surface p*K* of 5.6 relates to a transition from negative charge ($-O^-$) to neutrality (–OH). Plastocyanin, by contrast with cytochrome *c*, shows[26] quasi-reversible electrochemistry, without multivalent cations, at the protonated "edge". We believe that this reflects suppression of Coulombic repulsion and transformation of the electrode surface into a hydrogen-bond donor that can interact with a focal area of carboxylates on the protein surface. Our experiments indicated that promotion by cations is rather more complicated than is accountable for by Gouy–Chapman screening alone. All titrations of faradaic response with cation promoters have shown saturation behavior with the optimal cation concentrations dependent also upon protein concentration. Furthermore, the stability, or persistence, of response with different proteins is dependent on the cation used. Thus Mg^{2+} *or* $Cr(NH_3)_6^{3+}$ will promote stable electrochemistry of the *Clostridium* ferredoxin, but for plastocyanin Mg^{2+} is effective only at lowered temperatures; $Cr(NH_3)_6^{3+}$ and particularly $Pt(NH_3)_6^{4+}$ give excellent voltammetry at ambient temperatures. For spinach [2Fe–2S] ferredoxin, Mg^{2+} is virtually ineffective, whereas $Cr(NH_3)_6^{3+}$ promotes well.

How can these observations be rationalized? How do multivalent cations both promote *and* stabilize reversible interactions between negatively charged surfaces? Are localized and/or overall charge effects of importance? The Coulombic interactions must be at least ternary in order, involving electrode surface, protein, and one or more cations. At low ionic strengths, and without multivalent cations, there is no observed interaction at all; the electrode surface clearly repels the protein molecule. We proposed[21] that a useful starting point for visualizing the overall interaction is to construct a hypothetical "docking" configuration for the native protein constrained to adsorb at an area of electrode surface *without* participation of cations. Stabilization can occur by reconformation and adoption of a new nonnative "flattened" structure that minimizes any localized accumulation of charge in the low-dielectric space. The large and more conformationally labile a protein is, the more facile this degradative process will be. Such behavior is[27] in accordance with a recent description of irreversible protein adsorption

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at charged latex particles. Reconformation is averted if local Coulombic requirements are met by correctly placed counter charges. Multivalent ions fulfill this role by binding in areas of severe charge accumulation, best termed as "cavities". The extent to which M^{2+} is less effective than M^{3+} is dependent upon the magnitude of this charge accumulation. The size, shape, and distribution of "cavities" will be reflected also in the required geometrical complexity of the cation promoter. We thus approach macromolecular recognition at an electrode. For larger macromolecules the complexities of interfacial electrostatics will dictate increasingly discriminatory demands upon the spatial properties of the promoter. Indeed we have found that, in the presence of aminoglycosides, a wide range of iron–sulfur proteins give excellent electrochemistry at an "edge" PG electrode, thereby permitting detailed quantitative studies of their properties[28] (see below). It is most likely that, e.g., gentamycin adsorbs on to the electrode surface from which it projects spatially defined networks of $-NH_3^+$ groups toward the solution. With variation of the nature of the oligosaccharide, optimized arrangements might eventually be found for many redox proteins.

An important extension of this theme is to confine complex interactive structures *permanently* to electrode surfaces. We could, for example, covalently attach such groups to PG using a variety of methods. In a simple demonstration of this strategy we generated[29] coordinated cationic centers at PG "edge" electrodes by electrochemical reduction of $Cr(NH_3)_6^{3+}$ in aqueous ammonia. The resulting electrode gave electrochemistry of plastocyanin at pH 7 *without* free multivalent cations in solution. The scope for elaborate modification of metal electrodes by multifunctional $X \sim Y$ type adsorbates now appears wide, and a variety of proteins have now been shown[30] to exhibit well-behaved electrochemistry at surfaces derivatized by cysteine-containing peptides.

We thus find ourselves with a versatile armory of systems with which to demonstrate and study direct electrochemistry of proteins, and there is every reason now to believe that we shall, in the future, be able to tailor a biological electrode interface *exactly* to our requirements. For example, from within our armory, we can choose interfaces that select for one protein in the presence of other oppositely charged proteins. This is particularly interesting when the proteins involved form physiologically relevant electron-transfer complexes. Direct electron transfer to, and from, cytochrome b_5 can be promoted[31] by ternary complex formation with cytochrome *c* at an electrode surface that binds the latter, but *not* the former, protein. That this is due to cytochrome b_5 associating in a dynamic fashion with cytochrome *c* already bound to the electrode and *not* coupled homogeneous electron transer is demonstrated by the observation, shown in Figure 6, that zinc-substituted cytochrome c, which is redox inactive, also promotes b_5 electrochemistry.

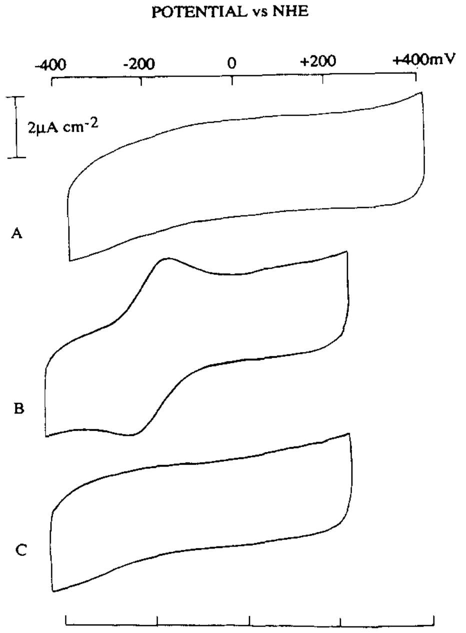

Figure 6. Promotion of cytochrome b_5 electrochemistry by zinc(II) cytochrome *c* at PG edge electrode in 1 mM KCl, 1 mM K-HEPES at pH 7.0 with a scan rate of 20 mV s^{-1}: (A) zinc(II) cytochrome *c* (75 μM); (B) zinc(II) cytochrome *c* (95 μM) and cytochrome b_5 (95 μM); (C) cytochrome b_5 (95 μM).

Some Applications of Direct Electrochemistry

Throughout the preceding discussions we have included several of the divisions depicted in our "thought tree". The comparison with biological systems is, we believe, a very relevant area. In addition to purely protein–protein interactions, many reactions of electron-transfer proteins occur at or in the vicinity of charged membranes and are thus subject to extensive electric field effects similar to the situation close to an electrode surface. Furthermore, our conclusions concerning the importance of Coulombic interactions in electrochemistry are unlikely to be undermined in physiological activity. However, one aspect merits particular mention: since the transferable electron is an excellent reporter for interfacial dynamics and chemical integrity of the protein at the electrode, direct electrochemistry should provide us with a most powerful tool for investigating protein adsorption at surfaces.

Direct electrochemistry has already been useful in identifying and clarifying various idiosyncratic properties of electron-transfer proteins. The determination of redox potentials is an obvious application, particularly where these relate to thermodynamically inaccessible or kinetically reactive species for which potentiometric methods are inappropriate. An example of interest is the 7-Fe ferredoxin from *Azotobacter chroococcum*[32] (a nitrogen-fixing organism), which contains one [4Fe–4S] and one [3Fe–4S] cluster. In the presence of the aminoglycoside tobramycin, this protein gives,[28] at pH 8.3, at an "edge" PG electrode, two stable quasi-reversible waves with E (vs NHE) = −460 mV ($[3Fe\text{–}4S]^{+/0}$, and E = −645 mV ($[4Fe\text{–}4S]^{2+/+}$). The

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(32) This protein is spectroscopically indistinguishable from *Azotobacter vinelandii* ferredoxin I, for which extensive structural data are available. See, for example: Stout, G. H.; Turley, S.; Sieker, L. C.; Jensen, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1020.

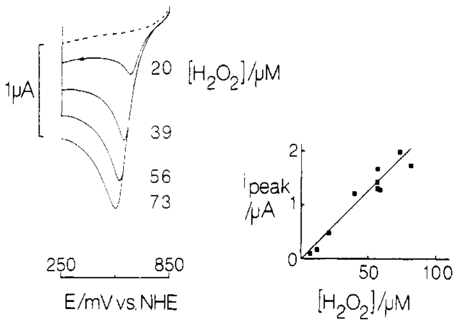

Figure 7. (Left) dc voltammograms showing the catalytic response toward H_2O_2 reduction exhibited by a PG edge electrode upon which are laid molecules of yeast cytochrome *c* peroxidase (adsorbed from a 0.2 μM solution of the enzyme in the presence of 5 mM gentamycin C_{1a}). Other conditions are as follows: 5 mM HEPES, 0.1 M KCl, pH 7.0, temperature 0 °C, scan rate 10 mV s^{-1}, electrode surface area 0.17 cm^2. (Right) A plot of peak currents (i_{peak}) vs H_2O_2 concentration demonstrates the possibilities for developing novel biosensors based upon direct electron transfer to a native enzyme bound at an electrode surface.

[3Fe–4S] system shows an interesting pH dependence, indicative ($-dE/d(pH) = 55 mV$; pK = 7.8) of single H^+ binding associated with one-electron cluster reduction. The $[4Fe–4S]^{2+/+}$ potential is by far the lowest ever measured for a ferredoxin system. As a consequence, the reduced form has previously defied generation by chemical agents. With direct electrochemistry, bulk preparation of this state and its characterization by EPR spectroscopy have been straightforward. Transient unstable oxidation levels of Fe–S clusters can also be generated and characterized by direct electrochemistry. It is now believed that many [4Fe–4S] clusters are subject to degradation under aerobic or oxidizing conditions. We were able to demonstrate[33] the transient formation of a superoxidized state of *Clostridium pasteurianum* 2[4Fe–4S] ferredoxin generated at potentials around +800 mV vs NHE. The lifetime of this species, most likely the $[4Fe–4S]^{3+}$ oxidation level, was estimated to be <4 ms.

Direct electrochemistry may also project alternative descriptions of protein electron-transfer reactivity that can modify or enhance our understanding of in vivo redox systems. One example concerns the electrochemistry of the photosynthetic "blue" copper protein plastocyanin. As viewed through stopped-flow studies using simple inorganic oxidants,[34] the Cu(I) form is inactive toward oxidation under acidic conditions. From elaborate X-ray studies,[35] the kinetic pK of around 5 can be linked with dissociation of a ligating imidazole N atom (presumably coupled with its protonation) to yield a 3-coordinate Cu(I) that is unsuited for facile oxidation to 4-coordinate Cu(II). However, as viewed through cyclic voltammetry,[26] well-behaved electrochemistry with peak separations close to 60 mV are observed at pH 4, and there is no evidence for kinetic complexity even at scan rates of 1 V s^{-1}. Here, protonation of the Cu(I) form is manifested merely as the expected increase in redox potential. The conformational switch between the protonated and deprotonated forms is clearly very fast, $t_{1/2} < 1$ ms, and may be an important factor in the physiological function of plastocyanin where the pH conditions in the intrathyalkoid space under illumination may favor the "inactive" Cu(I) form. We have recently found[36] that cytochrome *c* peroxidase (relative molecular mass 34 000) is adsorbed strongly at "edge" plane pyrolytic graphite in a fully active configuration. As shown in Figure 7 the cytochrome *c* peroxidase/PG edge system constitutes a novel sensor for H_2O_2 (which is reduced rapidly even at potentials >600 mV vs NHE).

Concluding Remarks

We have seen how the investigation of the electrochemistry of redox proteins leads to consideration of the nature of the interface formed between electrode and protein, and most attention has centered upon relatively simple electron-transfer proteins. Now much more effort can be expected on the more complex enzymes. Many of these, e.g., cytochrome *c* peroxidase, may be expected to show fast rates of heterogeneous electron transfer and hence might be termed "extrinsic" redox enzymes. On the other hand, enzymes such as glucose oxidase lack a "natural" long-range electron-transfer pathway; the catalyzed reaction occurs entirely within the locus of the active site. To arrange electrode reactions of glucose oxidase and other "intrinsic" redox enzymes may require (a) that the site of the catalytic reaction be close to the protein surface, (b) that the enzyme can deform without losing activity, (c) that the electrode surface (with or without a promoter) projects into the enzyme, or (d) that electron-transfer pathways be introduced by modification.

What of the reactions catalyzed by these enzymes? Can much use be made of them? Although a number of synthetically useful reactions may be driven electrochemically, e.g., the conversion of *p*-cresol to *p*-hydroxybenzaldehyde[37], the emphasis has been placed on mediated electron transfer. The subject received much encouragement with the discovery[38] of the ferricinium-coupled glucose/glucose oxidase electrode and its recent development into a commerical product. Others might be expected to follow soon thereafter. With the development of protein (and enzyme) electrochemistry and its application in analytical devices, the subject can be said to have finally arrived.

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Registry No. Cytochrome *c*, 9007-43-6.

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