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Bio-electrochemistry

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Abstract - Direct electron transfer reactions of redox proteins at a variety of electrodes, including modified gold, pyrolytic graphite and ruthenium oxide, are discussed. It is stressed that the electron transfer occurs without mediation. For many redox proteins, the rate of the electron transfer depends critically on the presence in solution or, to be more correct, at the electrode surface, of di-, tri- or even tetravalent metal ions or complexes. The dependence on the type of promoter used to modify the gold surface, the nature of complexes formed with added metal salts and the structures of the adsorbed redox protein are examined. In instances where direct electron transfer is very slow, as is the case at present with many potentially useful enzymes, indirect mediated electron transfer is used. Of particular value as mediators are ferrocenes, not only because they take part in rapid, reversible electrochemistry but because of the wide variety of compounds that can be prepared. Their use in a variety of sensors is illustrated in electrochemical assays for glucose, ATP, creatine kinase, hydrogen peroxide, cholesterol and their application in electrochemical immunoassays is illustrated by the analysis of the drug, lidocaine.

INTRODUCTION

Electrochemical studies played a negligible part in the investigation of electron transfer with redox proteins until relatively recently. Indeed, until 1977, there had essentially only been one paper, (ref. 1), which considered thoroughly electron transfer to cytochrome c, observing fleeting electrochemistry at a mercury electrode. In 1977, there were two reports which dealt with the well-defined electrochemistry of cytochrome c and, in a sense, set the scene for an increasingly active field of research in the eighties. Yeh and Kuwana reported (ref. 2) the electrochemistry of horse heart cytochrome c at the unmodified surface of a tin-doped indium oxide electrode. The other report, (ref. 3), on the promotion of electron transfer of cytochrome c at a gold electrode upon which was adsorbed 4,4′-bipyridyl, has been more productive in the sense that there are now many promoters of such electrochemical events.

There have been many developments towards understanding and achieving conditions of the electrode-solution interface which permit specific, productive and reversible interactions with a variety of proteins. The resulting direct electronic communication with the protein prosthetic group has been exploited to investigate intrinsic redox behaviour. Although direct electrochemistry now presents itself as another means of measuring protein thermodynamic redox potentials, the most important aspect has been the extent to which dynamic properties (i.e., the reversible binding characteristics of proteins at specific types of electrode surface, the orientation requirements of protein-electrode electron transfer, and the global behaviour of protein-mediated electron transport systems) are revealed. Ideally, we distinguish (ref. 4) between electrochemical responses which are persistent, i.e., do not show attenuation with time (due for example to irreversible adsorption and degradation of protein) and those for which activity is transient, or impersistent. Nevertheless, there is good reason to believe that, where observed, heterogeneous electron transfer of freely diffusing protein molecules is proceeding via rapid and reversible binding of protein to the electrode surface in orientations which, like physiological protein-protein electron-transfer, are favourable for the elementary electron-transfer step.

DIRECT ELECTRON TRANSFER TO REDOX PROTEINS AT MODIFIED GOLD ELECTRODES

Cytochrome c had been studied extensively (refs. 6 and 7) at mercury electrodes and found to adsorb increasingly strongly at its surface at higher protein concentrations. Although there were early reports of rapid electron-transfer from the mercury electrode to ferredoxins, more recent work (ref. 8) has suggested that very strong adsorption of these proteins takes place at the electrode surface. Eddowes and Hill (ref. 3 and 9) described how a gold electrode, modified through adsorption from solution of 4,4′-bipyridyl, gave a fast quasi-reversible electrode reaction with cytochrome c. Yeh and

Kuwana (ref. 2) achieved a similar dramatic improvement in electrode kinetics over conventional voltammetric electrodes through the use of a tin-doped indium oxide surface. Both these electrodes are hydrophilic and present chemical functional groups, weakly basic pyridyl groups and oxide groups respectively, at the interface. Such observations run through the remainder of this review as applying in a broad sense to all successful systems which have been used for redox protein electrochemistry. The steps envisaged as important in the overall electron transfer event at the electrode are (ref. 10):

a) Diffusion of reactant protein to the electrode surface.
b) Association of protein with the electrode surface in an orientation suitable for electron-transfer.
c) Electron-transfer.
d) Dissociation of protein from the electrode surface.
e) Diffusion of product protein away from the electrode surface.

It is our belief that, as a general principle, it is necessary for there to be such functional groups at the electrode-solution interface to which the redox protein can bind, either directly or indirectly. The protein is then held at the electrode surface sufficiently long for electron transfer to occur. In order for the complete electrode reaction to be diffusion dominated, not only must the association step described proceed rapidly but equally, the dissociation which liberates freely diffusing product must be similarly fast. It is important to emphasise that, in order to achieve a near-reversible electrode reaction, both "on" and "off" rates must both be fast. The adsorption process must be transient and never irreversible. In this way the electrode surface acts in a mode similar to an enzyme which is turning-over a substrate: it is no use producing dead-end complexes. Instead, fully-reversible binding of both substrate and product are required for efficient functioning. The same can be said for all electrode surfaces which realise the electron-transfer reactions of redox proteins. Surface surface modifiers may have an additional rôle to play, not only providing suitable functional groups at the interface, but preventing also irreversible and degradative adsorption of the protein at the bare electrode.

It was suggested (refs. 11-14) that the surface of the modified gold electrode did indeed resemble the binding domain of the enzyme, cytochrome oxidase, to which cytochrome c physiologically donates electrons. If chemical modification of the lysine residues of cytochrome c retained their positive charge, then the protein was fully active at the modified electrode just as it was enzymatically. Conversely, if chemical modification resulted in a change of charge to neutral or negative then the resulting protein displayed no electrochemistry, while being similarly inactive with the enzyme. Clearly such results suggested a binding interaction taking place between electrode and protein, just as is known to take place between enzyme and protein as a pre-requisite for electron-transfer. The complex formation is envisaged to be a many-to-one interaction consisting of salt bridge or, in this case, hydrogen bond formation from several pyridyl nitrogens at the interface to one of several lysine residues on one cytochrome c molecule. There may be concomitant orientation of the bound cytochrome c with its exposed heme edge disposed towards the electrode. It should be noted that there may need to be a dipolar pre-orientation of the cytochrome before binding through lysine side chains takes place. In this way it will be those lysine residues which "surround the heme edge" which will indeed bind the molecule prior to electron transfer.

The mechanism of the electron-transfer reactions of cytochrome c at a 4,4'-bipyridyl modified gold electrode was investigated in detail (ref. 10) using rotating disc and ring-disc electrode techniques. The conclusion was that binding of the protein to the electrode prior to electron-transfer was an essential feature of the mechanism of the electrode reaction.

Taniguchi et al (ref. 15 and 16) have reported the use of bis(4-pyridyl)bisulphide as a strongly adsorbing and highly effective promoter of the electrochemistry of cytochrome c at gold electrodes. Recent work (ref. 17 and 18), using Surface Enhanced Raman Scattering at modified gold and silver electrodes, has confirmed that adsorption of the promoter indeed takes place through the sulphur atoms of the disulphide part of the molecule.

By now, there are over twenty compounds (ref. 19) that bind to gold (and other metal electrodes) in such a way that the electrode reactions of cytochrome c proceed rapidly and efficiently. They all fall into the same class: they are of the type, $X_n{\sim}Y_n$, where X represents a portion of the molecule which binds to the electrode surface in such an orientation that the group Y is oriented perpendicularly to the electrode surface and is disposed such that it interacts with the protein. More recently, compounds have been synthesized that contain more than one group X or Y, binding to the electrode with the former and the protein with the latter. For example, the so-called PATS promoter, 2-(3- or 4-)pyridinealdehydethiosemicarbazide, binds to gold through the sulphur. The electrochemistry, at gold electrodes modified by these molecules, of cytochrome c,

plastocyanin and a multi-substituted carboxydinitrophenyl (CDNP) derivative of cytochrome c, was investigated (refs. 20-22). The polyfunctional nature of these surface modifiers clearly provides at least two types of functional group at the electrode/solution interface - a pyridyl nitrogen and thioamide N-H groups. The former binds to the lysine groups while the latter, being weakly acid, are ideal for hydrogen bonding to carboxylate groups. Clearly PATS might nearly be described as a "universal promoter" in that it has the capability, when bound to an electrode surface, to hydrogen-bond, not only to positively-charged lysine residues, but equally to negatively-charged aspartate and glutamate residues. Indeed, it promotes direct electrochemistry of not only cytochrome c but also plastocyanin and the multi-CDNP cytochrome c.

It has been shown (ref. 23) that it is possible to modify electrode surfaces with cysteine and its derivatives such that electron transfer to cytochrome c occurs. Various dipeptides containing cystine have been synthesized (ref. 24); $(cys\text{-}lys)_2$ and $(cys\text{-}glu)_2$ are particularly useful promoters as they bind very tightly to the gold yet the positive and negative groups introduced allow proteins with negatively- and positively-charged patches respectively to be attracted to the electrode surface and electron transfer to ensue.

Note that 4,4´-bipyridyl, and all the other compounds which are effective, act as promoters of electron transfer, **not** as mediators. Not only is 4,4´-bipyridyl, for example, electroinactive in the potential region studied, but subsequent work showed that compounds such as pyridine-4-sulphonic acid, which could not conceivably be redox active in this potential region, are effective promoters. No, the roles of the promoters are to bind the redox protein to the surface of the electrode in such a manner that encourage its productive and transient attachment of the protein as the prelude of the electron transfer event itself. How is it known that the promoters bind to the electrode in this way? It is obvious that they bind to the electrode from the marked change they make on the capitance of the electrode but how exactly are they bound to the surface? Firm evidence of their mode of adsorption comes from ellipsometry studies (ref. 25) which show, e.g. that 4,4´-bipyridyl is bound perpindicularly to the surface. The same technique is more difficult to apply if the group **X** binds through a sulphur atom, which is the case for the most recently introduced promoters.

DIRECT ELECTRON TRANSFER AT OTHER ELECTRODES

The simplest electrode, graphite, proved to be remarkably successful. Pyrolytic graphite, with the basal plane oriented towards the solution, provides an ideal hydrophobic surface at which the energy of surface carbons differs little from that in the bulk. On the other hand, with the ´edge´ plane exposed and hence with the rupture of strong C-C bonds, these may react with oxygen to yield various (C-O) functionalities. The effect, on the electrochemistry of a variety of redox proteins, of using the hydrophobic "basal" plane or hydrophilic "edge" orientations of pyrolytic graphite has been investigated (refs. 4, 5, 26-31). At a freshly cleaved basal plane, the electrochemistry of cytochrome c is rather poor. Upon simple polishing with an alumina slurry the response is markedly improved. Use of the polished "edge" surface produces a better response still. It is an interesting notion that these types of group present to the electrolyte a surface that chemically resembles biological surfaces such as membranes or proteins. As with metal oxides, acid-base behaviour, originating at the (C-O)-rich surface, is an important feature in modulating the protein-electrode interaction.

An important feature, of relevance to protein-protein and protein-interfacial interactions in general, has emerged vividly from recent studies of protein direct electrochemistry. This is the ability for small free ions to modulate protein-electrode interactions. Most profoundly, this has been demonstrated by the promotion, by multivalent cations (including the ubiquitous Mg^{2+} ion), of the electrochemistry of proteins possessing negatively-charged interaction domains, at negatively-charged electrode surfaces. This phenomenon has been observed at pyrolytic graphite and at metal oxide electrodes (refs. 32 and 33). It has been studied in most detail for processes occuring at the pyrolytic graphite "edge" surface, at pH values above 6, conditions under which the surface carries an apparent negative charge. Promotion of faradaic response by cations ($M^{4+} > M^{3+} > M^{2+} >> M^{+}$ in order of effectiveness) is observed for bacterial and chloroplast ferredoxins, rubredoxin, flavodoxin and plastocyanin. With plastocyanin, it was even possible to promote its electrochemistry at a graphite electrode by having present in solution $Pt(NH_3)_6^{4+}$ which presumably cross-linked plastocyanin via the negatively charged ´east´ site to the carboxylates (or phenolates) of the surface of the graphite. Similar behaviour was observed at the metallic oxide electrodes, RuO_2 (and IrO_2).

ELECTROCHEMISTRY OF ENZYMES

There is much interest in electron transfer reactions of enzymes, both academically and commercially. Unfortunately there have been few reports of direct electron transfer with enzymes. Most concern flavo-enzymes but, in our hands, under the conditions reported, the electrochemistry was that of the free co-factor. It seems as if, at the surface of the electrode, the cofactor, becomes adsorbed on the electrode surface and it takes part in

the electrode reaction. Recently, there have been two reports of direct electron transfer with enzymes which are worth further investigation. The first (ref. 34) concerns the reported cyclic voltammogram of the copper enzyme, lysyl oxidase. Its half-wave potential is distinctly different from free copper and the apo-enzyme showed no such behaviour. It is a pity that the authors did not describe its electrochemistry with the substrate(s) present. The other report (ref. 35) concerns the electrochemistry of cytochrome c peroxidase at a tin-doped indium oxide electrode. Although the authors did not report the direct electrochemistry of the enzyme, it must obviously have been occuring since the electrochemical reduction of the substrate, H_2O_2, proceeded much more easily when the enzyme was present. It is likely that these reports will be followed up and extended to other enzymes. Even so, it is possible that it will always be difficult to achieve the direct electrochemistry of some enzymes. If the active site of the enzyme is not on the surface, it is going to be difficult to arrange for electron transfer unless such a route already exists. Many enzymes are essentially self-sufficient in that the substrate(s) bind within the environment of the active site and no contact with the surface of the protein (and hence with other proteins or the electrode surface) is made or required. For other proteins, such as cytochrome c peroxidase, this is not the case and, indeed, a structure has been proposed (ref. 36) for the cytochrome c-cytochrome c peroxidase complex. It is relatively easy to imagine the side of cytochrome c peroxidase that binds to the cytochrome c binding instead to the electrode surface and electron transfer ensuing.

There have been number of reports (refs. 37-39) that the so-called organic metals permit electron transfer to a wide number of enzymes. Whilst this is undoubtedly true, the question remains: how direct is the electron transfer? Does in take place with, or without, the intervention of components derived from the organic metal? The original papers of Kulys et al quite clearly state that the electrode is able to act both as a conductor and as a source of either TTF or TCNQ which act as mediators to the enzymes. Thus, as a 'mediator incorporated electrode' it is able to respond rapidly to the substrate via the enzyme. This view was disputed when it was claimed (ref. 38) that the electrode was reacting directly with the enzyme and that no low-molecular weight intermediates intervened. Apart from the difficulty of envisaging the electron pathway to or from the enzyme, it was hard to reconcile the presumed mechanism with the absence of any evidence of electrochemistry of the enzyme.

APPLICATIONS OF BIO-ELECTROCHEMISTRY

Having achieved the electrochemistry of the redox proteins, what use can be of it? These proteins exercise very good control over the electron transfer event, both with regard to other components present in the solution and especially in the directional nature of the event. It may be that it will be possible to make use (refs. 40-42) of these features, particularly the latter, since it is a quality lacking in small redox molecules. The latter are usually distinctly better electron transfer reagents performing the traditional role of mediators efficiently, but, as stressed above, without the control of electron transfer either spatially or with respect to partners. Where the latter requirements are crucial, the investigation of the electrochemistry of the protein will still be preferred. Thus in an attempt (refs. 43 and 44) to explore the relationship between electron transfer and proton pumping in mitochondria, it was important to use the cytochrome c as the mediator. However, when attempting to exploit enzymes to carry-out useful reactions, whether for synthesis or analysis, small molecules are more convenient to use. For example, in the electroenzymological conversion (ref. 45) of p-cresol to p-hydroxybenzaldehyde, a derivative of ferrocene acting as a small redox mediator, gave a more efficient synthesis than the natural mediator, azurin.

Such electrodes must not be confused with the so-called enzyme electrodes which, to date, are characterised by indirect electron transfer to, or from, the enzyme by mediators. These may be the normal participants in the enzymatic reaction, e.g., dioxygen or hydrogen peroxide, or they may be artificial electron donors or acceptors, such as ferrocenes. The former are well-known having been first proposed by Clark and Lyons (ref. 46) over twenty years ago. A new type of amperoteric enzyme electrode has been designed which exploits the organometallic componds, ferrocene, as a mediator between enzyme and electrode just as described above in the electroenzymological synthesis. Ferrocenes are suitable analogues of cytochrome c with which they have several properties in common: the two accessible oxidation states are iron(II) and iron(III), both of which are low-spin; the low oxidation state forms are only slowly autoxidised; the intermolecular electron exchange rates are fast. The use of ferrocenes is also favoured by a number of additional characteristiscs: most are heat stable; they can be polymerised; they can be used to modify other molecules, including proteins; and, most importantly, there is the possiblity of introducing substituents on either of both of the cyclopentadienyl rings whilst retaining the properties of a simple one-electron redox couple. The formal potential is responsive to the substituent(s), but the electron transfer reactions retain their desirable characteristics of rapidity and reversibility.

The first electrode (ref. 47) of this type had, as substrate the clinically important species, glucose. Given the amazing variety of ferrocenes and other organometallics available, and the considerable number of enzymes (ref. 48) to which they act as mediators, there seems to be little doubt that electrodes capable of taking part in simple analyses for many materials, clinical and industrial, will be forthcoming. For example, an electrochemical method for the detection of ATP and creatine kinase, coupled through hexokinase to an amperometric glucose enzyme electrode was used (ref. 49) to monitor creatine kinase activity and formed the basis of a single-use biosensor. Also, the development of a sensitive electrochemical assay for low levels of hydrogen peroxide has been described (ref. 50). The system is based on the enzyme reduction of H_2O_2 by peroxidase and subsequent electron transfer from a gold, or a pyrolytic graphite, electrode to the enzyme, via e.g., a ferrocene derivative as a redox mediator. In another device, the clinically important compound, cholesterol, was the object of three analytical procedures. Ferricinium ion used acts (ref. 51) as an electron acceptor from the flavoprotein, cholesterol oxidase, and the ferrocene, thus formed, was reoxidised electrochemically. The second assay utilised cholesterol dehydrogenase to oxidase diaphorase thereby reoxidising the NADH. The oxidised form of the diaphorase is regenerated by electron transfer to the ferricinium ion which is itself recycled at the electrode. The final assay involved the detection of hydrogen peroxide generated as a product of the oxidation of cholesterol by cholesterol oxidase in the presence of dioxygen. The system is based of the enzymic reduction of the hydrogen peroxide by horseradish peroxidase, and the subsequent electron transfer from the electrode to the enzyme, via the ferrocene mediator. The considerable synthetic variation possible with organometallics means that they can be used as electrochemically-active analogues of, for example, drugs. for example, by attaching ferrocene lidocaine, it was possible (ref. 52) to develop an assay for the un-modified compound based on the use of antibodies. In this case, a modified ferrocene was employed to act as a mediator to, e.g., glucose oxidase. However, in the presence of the antibody to the drug, which also binds the drug-ferrocene complex, the diffusion properties of the modified ferrocene are greatly affected and it is no longer able to act as a mediator to the flavoproteins. The immunoassay can then be configured in which a fixed amount of antibody, ferrocene-labelled drug, enzyme and substrate are used; the only variable is the unknown concentration of the drug. In the absence of any drug, the ferrocene-drug conjugate will bind to the antibody and no ferrocene-drug conjugate will be available to mediate to the flavoprotein. In the presence of the drug, howover, a competition will occur between ferrocene-drug complex and drug for the antibody. The more drug is present in the sample, the less ferrocene-drug complex will be bound and the greater the catalytic current will become. Ferrocene derivatives of lidocaine and theophylline have been prepared and both derivatives act as electron acceptors from glucose oxidase. Dose response curves have been obtained. Of course, there have been a number of publications concerned with the development of electrochemical immunoassays where the novelty has been in the immunological aspect, with the electrochemistry providing the final method of detection; sometimes relatively standard, sometimes quite novel. However, the more intimately associated the electrochemistry is with the immunology, the more likely it is that these methods will yield effective immunosensors. Provided problems associated with the large-scale manufacture of these devices are overcome, it should be possible to marry the specificity and selectivity inherent in the biological processes to the accuracy and utility of electrochemical methods.

CONCLUSION

The investigation of the electrochemistry of biological materials, whether they be proteins, enzymes or components of cells, has begun to gain a secure foundation. Much remains to be done; the nature of the electrochemical interface must be established; the reason for the apparent reluctance of redox enzymes to partake in electron transfer at the electrode must be ascertained; the best configurations for more complex experiments involving electrochemistry used in conjunction with another physical method of investigation, should be determined. The application of these methods in synthetic, or, more likely, analytical chemistry, must be extended. As the second decade of enquiry begins, let us hope that the answers to these questions may not be long delayed.

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