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The electrochemistry of metalloproteins

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Abstract - There is now little difficulty obtaining the direct electrochemistry of metalloproteins, whether at modified electrodes, with additional metal ions in solution or even at normal electrode surfaces. Recently, we have examined the electrochemistry at microelectrodes, whether deliberately designed electrodes or those adventitious in size. From the electrochemistry of protein-protein complexes it appears that mobile, dynamic structures are more compatible with those determined in solution. Indirect electron transfer to various enzymes was achieved using ferrocenes as mediators and led to the maufacture of a glucose sensor for home use by diabetics. Recently, we have achieved the direct electrochemistry of metalloenzymes, i.e., without mediation.

INTRODUCTION

The electrochemistry of metalloproteins has developed (refs.1-4) significantly in the past few years. Conditions now exist that permit the electrochemistry of all redox proteins to be expressed at a range of electrodes, modified or not. For example, it has proved possible to modify metal surfaces, particularly gold, so that the electrochemistry at these electrodes, having adsorbed so-called promoters, proceeds rapidly and reversibly. Thus, whether the protein is cytochrome c, plastocyanin, ferredoxin or azurin, there is now no difficulty in choosing a promoter at gold, or conditions under which e.g., a pyrolytic graphite electrode, can result in excellent electrochemistry. However, there were many circumstances when these electrodes did not, apparently, behave so well. There were a number of occasions, which naturally remained un-reported, where e.g., a promoter, which worked well under other conditions, did not appear to function properly. Traditionally, this could be overcome by, e.g., re-polishing the electrode, adding more promoter or perhaps re-purifying it. There were, of course, conditions under which the promoter or electrode surface did not work at all with a given protein but, almost always, they were explicable in terms of the charge on the protein, the solution conditions, e.g. ionic strength or pH, or the absence of the required metal ion. No, the results obtained were unlike those in that they were characterised by a certain apparent randomness. Thus, the electrochemistry at basal-plane pyrolytic graphite was usually quite `poor´, almost absent, but occasionally it would give reasonable electrochemistry of e.g., cytochrome c which was hard to rationalise. Similarly, the addition of cysteine-containing peptides, or even the normally reliable promoter, 4,4´-bipyridyl disulphide, to gold would give the appearance of `poor´ electrochemistry. Depending on conditions, the electrochemistry of e.g., the ferredoxins, would be `time-dependent´; there would be an `impersistence´ in its behaviour that would be ascribed to "the denaturation of the protein on the electrode surface".

All these results are now interpretable (refs. 5-7) in terms of electron transfer reactions of such redox proteins at **microelectrodes**. The principal difference between say, the cyclic voltammetric response of e.g., a protein at a macroelectrode, as opposed to a microelectrode, is that the former is characterised by a `peak-shaped´ curve, the latter by a `sigmoidal´ one. Depending on the exact shape of the microelectrode, i.e, whether it is a disc, a cylinder, etc., the cyclic voltammogram will result from a particular mode of diffusion of the protein to the electrode surface. If the dominant mode is that of radial motion, as it is when a microelectrode is used, then the resulting cyclic voltammogram at a disc electrode will be sigmoidal in shape: if its movement is linearly towards the electrode, as at a macroelectrode, then a peak-shaped cyclic voltammogram will result. There is no difficulty at all rationalising the behaviour of uniformly covered macro- or microelectrodes: they correspond **exactly** to the expected cyclic voltammograms. However, what about a macroelectrode **incompletely** covered with a promoter? If the promoter is arranged on the surface, e.g., on particular crystal faces, so that some areas are occupied, others aren´t, then, depending on the sizes of the local areas of adsorbed promoter, the supposedly macroelectrode will, in effect, behave as a multi-microelectrode. Consequently, the cyclic voltammograms that result will conform more to those expected from microelectrodes, with a consequent increase in sensitivity, depending on the amount of effective promoter present. The inactive electrode surface may be the metal itself or it

may have adsorbed, deliberately or not, an atom or molecule which provides no resting place for a protein. Similarly, the condition of a freshly-cleaved surface of pyrolytic graphite, where one would expect it to have, ideally, no exposed groups to which a protein might transiently attach itself, might have adventitiously disposed small areas of the edge-plane, either formed in the preparation of the crystal or accidentally made during the preparation of the electrode, which can cause the transient binding of the protein through the oxygen-containing groups which routinely result at such surfaces. The time-dependence of the electrochemistry of proteins at promoted surfaces could either result from a re-distribution of promoter on the surface from small `clumps' to a more even distribution or vice versa or the protein could itself adsorb on to the electrode surface, perhaps with concomitant denaturation, thus creating from a macroelectrode, a multi-microelectrode.

ELECTROCHEMISTRY OF PROTEIN-PROTEIN COMPLEXES

With that major revision of protein electrochemistry accomplished, we can direct our attention to the roles of such proteins in biological systems. Obviously some are co-factors for enzymes (see later) but many have the role of `simply' acting as electron transfer proteins. Under both these conditions, the interaction between proteins, i.e., the formation of protein-protein complexes, is crucial to their effective operation. How then, do the electrochemistries of such systems behave? How does the cyclic voltammogram of the complex formed between the heme protein, cytochrome c and the copper protein, plastocyanin look? The short answer is that it depends on the electrode surface. At gold surfaces (ref. 8), where one has either a promoter that gives good electrochemistry of cytochrome c and none of plastocyanin or the reverse, the electrochemistry is consistent with some form of mediation between the electrode and the non-active protein by the effective one. The situation appears to be different (ref. 9) at an edge-plane pyrolytic graphite electrode. The electrochemistry of cytochrome b_5 is not normally apparent, nor is that of plastocyanin, at an edge-plane electrode but becomes readily apparent in the presence of cytochrome c. So what? they are just behaving exactly as did the promoted gold surface. The strangest result (ref. 9) was when the situation was repeated except that, instead of cytochrome c, zinc cytochrome c was used, i.e, where the iron in the cytochrome c had been replaced by zinc. In this case, whereas, as expected, the zinc cytochrome c was obviously electro-inactive, in the the complex formed between it and plastocyanin (or cytochrome b_5), the electrochemistry of plastocyanin (or cytochrome b_5) was readily apparent. It therefore appears that, in the complex, the cytochrome c acts, in a way, like a co-promoter, i.e., it, when bound to the electrode surface, holds the other partner proteins at the electrode surface in such a way that electron transfer can take place. (Otherwise, one would have to accept that the zinc cytochrome c can provide a path for the electron even though it provides no resting place for the electron itself). Recent work (ref. 10) on the NMR spectra of proteins adds another feature to this story. It appears that, not only must we consider dynamics within proteins, but we must consider them between proteins, i.e., in protein-protein complexes. If the same factors are at work at the electrode surfaces, then we have both to consider the movement of a single protein on the electrode surface and also of the protein-protein complex, i.e., the dynamics of a termolecular complex (treating the electrode surface as a quasi-molecule). It may be that we have to consider the promoter as providing a surface suitable for the protein to diffuse across with a trajectory conducive to electron transfer. The situation with protein-protein complexes is much more complicated since we have to consider, not only the dynamics of the proteins' movement with respect to the surface but with respect to each other.

ANALYTICAL ELECTROCHEMISTRY

The development of the ferrocene-based technology was essentially based on the disappointment that, in 1981, we were unable to achieve the **direct** electrochemistry of enzymes and dissatisfaction with the organic mediators traditionally used to ferry electrons between electrode and enzyme. Their main defect lay in the sensitivity of the reduced form of the mediator to molecular oxygen. What was required was a mediator whose oxidation was slow, like cytochrome c. Ferrocenes have many features in common with cytochromes but the main one was the relative stability with respect to reaction with dioxygen. They gave excellent electrochemistry and they reacted readily with the appropriate states of the enzymes. It was soon shown that ferrocenes can be obtained which react with a wide range of enzymes though, of the latter, the one which attracted most attention was glucose oxidase (ref. 11) since it was soon realised that it offered the chance of developing a sensor which, provided the development of the device encountered no new problems, could be used in air. After much work, both in Oxford and Cranfield, the system was passed over to a company in late 1984 for further development and production. In 1987, following excellent work, the ExacTech device was marketed for home-use by diabetics. It is expected that other devices, based on similar technology, will follow soon.

Since the initial work, there have been a number of significant developments. The first concerned the modification of proteins, e.g., glucose oxidase (ref. 12,13), with ferrocene, i.e., a mediator-incorporated enzyme. The second (ref. 14) was the adaptation to so-called immunoelectrodes, as in the assay for lidocaine, making use of the facility of ferrocene to be attached to other molecules without losing the ability to take part in electrode reactions. The problem that beset these methods was the lack of sensitivity which was particularly relevant to immunoelectrodes since many of the analytes to which, in theory, it was applicable, were present in blood, or other biological fluids, at very low concentrations. One attempt to overcome this has been (ref. 15) by associating the ferrocene-based system with an enzymatic cascade. The improvement in sensitivity observed with the model system, avidin-biotin, is likely to be of value in a system of more analytical importance. Certainly if such improvements can be made, it may well be possible to devise sensors similar to the ExacTech but capable of the analysis of a wide range of clinically important substances.

ENZYME ELECTROCHEMISTRY

The success with protein electrochemistry naturally would lead one to expect that there should be little difficulty achieving the same type of behaviour with enzymes. Of course, there will be additional factors to take into consideration such as the much greater size of most enzymes, how they are reputed to be much more `flexible´ in their structures or the needs of many for co-factors. However, it soon became apparent that there were two classes of redox enzymes: intrinsic and extrinsic. With the former, no co-factor was required (though obviously general or specific ions might affect the rate); the catalytic reaction took place at the active site and any electron transfer between substrate(s) and enzyme took place over a short distance. The latter did have a co-factor or another protein involved in transporting electrons (i.e., an oxidation or reduction reaction) and therefore a pathway for such electron transfer existed within the protein, connecting the active site to an area on the surface where, for example, another protein bound. If this site could be disposed towards an electrode, it would be possible to imagine that the electrochemistry of the enzyme could be observed, perhaps even without the substrate(s) being present. Intrinsic enzymes, on the other hand, need not take part in such electron transfer reactions unless the active site was sufficiently close to the surface (and therefore to an electrode surface) or could deform without its activity being perturbed.

There had been a number of reports of direct electrochemistry of enzymes but, in most cases, these involved flavoenzymes in which the prosthetic group was not covalently attached to the protein. Since it was frequently observed to dissociate from the enzyme, particularly on to the electrode surface, it was difficult to be sure that it was not simply acting as a mediator to the remaining intact enzyme. The use of organic conductors as electrodes appeared (ref. 16), for a time, to give such direct electrochemistry but, as Külys had first suggested (ref. 17), it was likely that one component of the organic conductor was sufficiently soluble to act, over a very short range, as a mediator to the enzyme. The first account which seemed to give enzyme electrochemistry was the report (ref. 18) concerning lysyl oxidase but, unfortunately, no mention of the effect of added substrate was given. There then followed two reports (ref. 19,20) of electrochemistry involving cytochrome c peroxidase where, undoubtedly, electron transfer to the enzyme was taking place though the effects could only be seen in the presence of the substrate. The first authentic example of direct enzyme electrochemistry was reported (ref. 21) with p-cresolmethylhydroxylase (which had previously been investigated (ref. 22) using, as mediators to the protein, azurin or a ferrocene). The flavocytochrome gave both direct electrochemistry of the enzyme in the absence of the substrate but a marked catalytic current when the substrate was added. A number of promoters were successful and the system provided a useful method for the analysis of p-cresol, not, unfortunately, a much-sought-after practice! However, the methodology was capable of being utilised with other extrinsic enzymes and both lactate dehydrogenase (ref. 23) and cytochrome c_{552} (a hydrosulphide oxidoreductase) have proved (ref. 24) successful. It will not be long before this first phase of enzyme electrochemistry is over. Whether it will result in analytical devices or, perhaps, in those more useful to molecular electronics, remains to be seen.

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