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The development of bioelectrochemistry[1]

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Contents

Abstract

The development of the interest in bioelectrochemistry, from that of redox proteins and enzymes to the use in analyses, is described. Some of the difficulties that arose during this exercise are described.

Keywords: Bioelectrochemistry; Marketing

1. Introduction

I have to emphasize that our work on what is now known as bioelectrochemistry arose because of an interest in, and involvement with, the structure and function of electron transfer proteins. It did not stem from any interest in the possible applica-

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tions of the subject. In the 1970s, I was working on the structure and function of copper-containing superoxide dismutases and the blue copper proteins and R.J.P. Williams, both of us members of the unfortunately lapsed Oxford Enzyme Group, was working on haem proteins. Having been impressed by the simplicity, yet power, of electrochemical methods in unravelling [1] some aspects of the chemistry of vitamin B_{12}, I wondered why there had been no such investigations of redox proteins other than a good paper in 1971 which had concluded [2] that, at mercury electrodes, the electrochemistry was very short lived. This struck me as odd since these proteins are beautifully arranged for electron transfer. Hence, an investigation seemed to be in order.

2. Protein electrochemistry

2.1. The direct electrochemistry of proteins

We considered that the problem must be connected with the interaction of the protein with the electrode surface and so it turned out to be: when we adsorbed [3] electroinactive substances on the electrode surface such that the latter retained its hydrophilic character, electrochemistry of cytochrome *c* and other proteins resulted. Over the years, a succession of compounds have been employed, by ourselves [4] and others [5], to modify, usually gold, surfaces to enable the electrochemistry of redox proteins to proceed without let or hindrance. These compounds were called promoters (simply because they promoted the electrochemistry) and were always, by those in the know, distinguished from mediators since, while the latter take part in electron transfer mechanisms with the formation of reduced and/or oxidized intermediates, promoters simply aid the electron transfer without, in the potential range chosen, taking part in electron transfer. (Perhaps it is time to consider a new name for such compounds as "promoters" has found ready acceptance, and is much used, in genetic engineering.) What of the compounds that have been successfully used? Although 4,4′-bipyridyl was the first [6] introduced, 4,4′-bipyridyl disulphide is [7] more useful since it binds better to a gold surface. Recently we introduced [8] a series of peptides which contain either positively or negatively charged amino acid residues which allowed negatively or positively charged redox proteins to be transiently adsorbed onto the surface.

Although gold electrodes are very useful, various forms of graphite electrodes have proved [9] to be effective. They were first introduced in this context some years ago when the search was on for electrodes that allowed the electrochemistry of negatively charged proteins to be observed. (Since these form the majority of redox proteins, indeed all proteins, bioelectrochemistry certainly required this problem to be solved.) Pyrolytic graphite proved to be the most versatile of the forms of graphite available and, when the oxidized, edge-plane of pyrolytic graphite was the electrode surface, the simple addition [10] of di-, tri- or even tetravalent cations to the solution allowed the electrochemistry of, for example, plastocyanin and even spinach ferredoxin (which carries a charge of -14 at pH 7) to be observed. More recently [11],

my colleague, F.A. Armstrong, introduced aminoglycosides as positively charged promoters. They have the singular advantage of causing many proteins to be adsorbed and held at the electrode surface. This enables the electrochemistry to be observed on minute amounts of material.

2.2. *The structure of modified electrodes*

Although there were a number of attempts [12] to elucidate the structural form of the promoters on the electrode surface, early interpretations of the scanning tunnelling microscopy (STM) of these modified electrodes were frustratingly impeded. There were many technical reasons for this but the principal problem may be connected with the movement of the promoters on the electrode surface, at least in water. Success has recently been achieved [13] with satisfactory resolution of pyrimidine thiols adsorbed on gold surfaces: it is likely that, although bound to the gold via sulphur, the molecules are in their thione forms with the pyrimidine ring tilted out into solution. It remains to be seen whether or not proteins can be sufficiently immobilized on such a surface to be observed by STM or, more likely, by atomic force microscopy.

2.3. *Enzyme electrochemistry*

There had been few reports of enzyme electrochemistry until recently. Most concerned flavoenzymes but there must be serious doubt as to whether the researchers were observing direct electrochemistry: given that most flavoenzymes readily lose their flavin prosthetic group, electrochemical activity may have been associated with the dissociated flavin. The first report of the electrochemistry of a non-flavoenzyme was of fungal laccase where the electrochemistry associated with the blue copper centre was [14] described. Although the work has frustratingly not been followed up, it provided encouragement that the problems could be overcome. (I should not complain since we reported [3], in 1977, in the first paper on protein electrochemistry, that the electrochemistry of laccase would shortly be reported. With our sample of laccase, the results were sufficiently irreproducible not to warrant publication.) Again, there had been a brief report [15] of the electrochemistry of a lysyl oxidase but no evidence was given that the enzyme was still active under the circumstances of the electrochemistry. Since we reported [16], in 1989, the electrochemistry of *p*-cresolmethylhydroxylase, the door has been opened and there seems now to be no limit to the redox enzyme whose electrochemistry can be observed, with one major exception. In 1988, we had proposed [17] that redox enzymes could be assigned to two classes: intrinsic and extrinsic. The former would be those enzymes without a natural partner with which they exchanged electrons. In these cases, the electron transfer between substrate–enzyme–product took place at the site of the prosthetic group and there was no need for electron transfer "through" the enzyme to the surface. Therefore, if the prosthetic group was "buried" in the centre of the enzyme, there would be a significant distance for the electron to move from the surface of the enzyme to the redox centre. Obviously one can conceive of such intrinsic redox

enzymes where the prosthetic group is not located in the centre of the enzyme but such enzymes may be rare. Extrinsic enzymes, on the contrary, which have a "partner" protein, have to have a "pathway" for electron transfer. If, therefore, the enzyme can position itself on the electrode surface in such a configuration that gives the electron transfer pathway a chance of being disposed towards the electrode surface, then the electrochemistry should be capable of being observed. I say "should" because, although it may be fine to envisage enzymes attached, or attracted, to the electrode surface on the basis of their overall charge, there is no guarantee that the charge on the surface of the protein above the electron transfer pathway will be the same as the overall charge.

The flavocytochrome *p*-cresolmethylhydroxylase, whose structure is now known, expresses its electrochemistry at edge-plane graphite. Only electron transfer to the cytochrome component is observed, at least at this electrode. This could be due to the mode of interaction of the enzyme with the electrode, the cytochrome component lying closest to the surface. Be that as it may, the enzyme is still enzymatically active, the addition of substrate giving a so-called catalytic wave, the height of which is proportional to the amount of substrate added (until a limiting value is reached). The electrochemistry of a number of other enzymes has been described [18–20]. More recently we have concentrated on enzymes whose substrates are nitrogen oxyanions. For example, the copper-containing nitrite reductase [21] expresses electrochemistry at a graphite electrode although this is complicated by inactivation by the product, nitric oxide. It remains to be seen whether or not this can be used as an electrochemical method of detecting this important neurotransmitter.

2.4. *Dynamics within, and between, proteins*

One aspect of the behaviour of all types of molecules but especially, in the context of this article, proteins, concerns dynamics. By this I mean that there is motion within and between proteins. Not only that but there is movement between protein(s) and more static structures, for example electrodes. In solution especially, we can consider proteins as involved in a whole range of movements ranging from vibrations and rotations of individual aminoacid residues to the movement of whole sections of the enzyme structure as in cytochrome P-450. This became apparent to us when studying [22] the nuclear magnetic resonance (NMR) spectroscopy of the complex formed between plastocyanin and cytochrome *c*. It seemed as if the NMR were inconsistent with a "static" structure in which the two proteins were in fixed positions one with respect to the other. However, a dynamic structure was possible in which the two proteins moved, with respect to one another within a complex, over a short pathway, perhaps bringing the haem portion of the cytochrome from the negatively charged site on the surface of the plastocyanin close to Tyr 83 to the site closest to the copper centre near His 87. It is interesting to speculate that, if this could happen with two proteins, then it could occur between proteins or enzymes in membranes and, with the influence of charge and/or dipole on interaction, could control and/or regulate the extent of the electron transfer. These considerations need not, indeed must not, apply only to systems involved in electron transfer but must extend to all

interactions in biological structures. When a supposedly static material (but see the comments earlier about the mobility of some promoters on the electrode surface) is introduced into a solution containing a single protein or a collection of them, one must assume that the same mobility can occur even on the surface of the electrode. One should consider the protein as mobile, perhaps "rolling over" the surface: electron transfer can then occur if and when the appropriate surface of the protein is exposed directly to the electrode surface. It may be that the role of promoters is not only to keep the surface hydrophilic and hence the protein within its environment but to allow the opportunity of the correct motion of the protein on the electrode surface.

3. The use of bioelectrochemical methods in analysis

3.1. Analytical bioelectrochemistry

It was necessity that drove us to find an analytical use for the bioelectrochemical investigations. Of course, ever since the technique of protein electrochemistry had been discovered, we realized that if the electrochemistry of enzymes could be achieved that it would be possible to use that for analytical purposes. However, at the beginning of the last decade, all attempts to achieve the electrochemistry of, say, cytochrome P-450 were frustratingly irreproducible [23]. Professor I.J. Higgins and I, trying to gain support for our research, entered the BP Energy Research Prize. We were lucky enough to be selected among those who were given support for one year's research. Our chosen topic was [24] "biofuel cells". We showed that there is little chance that these could ever be useful as power sources unless the problem of the pathetic current density is drastically improved. However, our work on mediated electron transfer to enzymes, in particular methanol dehydrogenase [25], showed that it could be usefully employed in the analysis of the substrate.

I had been impressed by the versatility of ferrocenes when writing, with Professor P. Day, a chapter [26] in *Physical Methods in Advanced Inorganic Chemistry*, partly because they could be electrochemically exploited using methods I had found valuable when studying the electrochemistry of vitamin B_{12}. I had supervised an undergraduate doing research on the ferrocene derivative to modify proteins [27] and I took up this idea in 1981 when I was engaged in an attempt to modify proteins selectively. During "refreshment" with Dr Tony Cass, we realized that the ferrocenes might act as mediators for the redox enzymes. That indeed turned out to be the case and so the hunt was on for useful applications.

3.2. The ExacTech glucose electrode

We showed quite early on (although the publication of the results was delayed [28] for reasons connected with the patent) that ferrocenes could act as excellent mediators between glucose oxidase and an electrode and that the current produced was linearly related, up to an appropriate level, to the amount of glucose in solution.

It was left to chance to find a suitable partner. Professor Higgins had had contact with a young Englishman, Mr R. Zwanziger, who, having been to Imperial College and at the Harvard Business School, had set up a company in Boston, Genetics International, seeking ideas in which to invest a very small amount of money. When he saw the work that we had done on the ferrocene–glucose oxidase–glucose system, he said that he would support the work for one year and try to raise money to exploit it properly. While we worked on extending the discovery in such a way that it would be suitable for development, Mr Zwanziger raised sufficient monies, from Baxter-Travenol and others, to enable the company to open small premises in Abingdon, near Oxford, in 1984. A key decision had been taken earlier: to attempt to translate this discovery into a device for use by diabetics at home. Initially, thoughts were of competing with the well-known Yellow Springs system but there seemed a good chance of creating an amperometric device capable of giving a linear readout relevant to hypo- and hyperglycaemic patients. Devices which existed at the time were slow, inconvenient, imprecise and/or expensive. Of course, we did not realize just how difficult and expensive the task would prove to be. Eventually it was decided that the devices would be prepared using the technique of screen printing but it was not a trivial matter to acquire the necessary materials and instruments for manufacture but, thanks to the energy of one of the partners, Mr J. McCann, this was achieved.

Eventually, after the company grew in size, the device was at a stage where clinical testing was required. A problem emerged when it was discovered that diabetics appeared to have a very variable rate of blood clotting: the diffusion rate of glucose was difficult to predict and thus a fixed time for the coulometric reading seemed out of the question. That problem was eventually overcome and good results could be obtained within 30 s (20 s in today's device).

The company had a research department, led by an ex-colleague of mine, Dr M.J. Green. Apart from studying a variety of amperometric systems, including a cholesterol assay [28], they did much work extending the use of ferrocenes as mediators. Eventually, after much investigation, a ferrocene (Fig. 1) was chosen which had first been prepared for use as a mediator by Dr K. di Gleria in Oxford.

Having received its appropriate FDA license, the device went on the market in December 1988, just over six years after the discovery had been made and four years after the company had started. If it is necessary for venture capitalists to have some return within two years, then they were correct in repelling the advances of John Higgins and myself. Having said that, my recollection is that the idea was well received by the younger scientists to whom we spoke. However, it seemed to be the

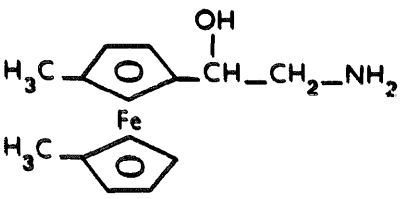

Fig. 1.

case that, in the UK-based industry, all decisions were taken by those remote from day-to-day affairs in the laboratory and who had little or no connection with recent advances.

3.3. *The future of bioelectrochemical devices*

There are a number of problems with current methods: (a) not all enzymes show any electrochemical activity; (b) the sensitivity of the present devices is too poor for many applications especially those involving antibodies or DNA; (c) the present devices are not suitable for use in vivo; (d) it is difficult to configure electrodes such that they are responsive to a number of analytes simultaneously.

(a) The majority of enzymes are not redox active and therefore it might appear that one cannot exploit these in electroanalytical devices. The common way of overcoming such a problem would simply be to couple such an enzyme to one that was redox active such as, for example, in the case of a system [29] which has ATP as the analyte: by coupling hexokinase with glucose oxidase, the system gives an excellent linear dependence on ATP. It is not beyond biochemical ingenuity to couple most enzymes to a redox-active enzyme, although whether problems such as limited analytical range, appropriate speed of response and stability of the system employed can be resolved is another matter. One way forward is to engineer genetically the enzyme to include an electroactive, or for that matter, a fluorescent or spectrophotometrically active group. There are at least two ways of doing so. One method is by genetically engineering the enzyme in such a way that it would contain an unnatural amino acid. The general method has been pioneered [30] by Schultz: the only problem seems to be to find a way of using the methods to make enough enzyme. The other method is to engineer genetically the enzyme thereby introducing a amino acid that can be selectively modified to include the desired group. This has been achieved to incorporate a fluorescent group or an electrochemically active group, the former in the maltase-binding protein [31], the latter in penicillinase [32].

(b) The sensitivity of the present examples employing mediated electron transfer to enzymes is currently rather poor. There are at least two approaches to overcoming this problem: by amplifying the initial response of the system by coupling [33] the reaction to an ancillary enzymatic process or by using direct enzyme electrochemistry at a microelectrode. It is a characteristic of electrodes that, the smaller they are made, the larger the signal-to-noise ratio. It is likely that the most convenient size of such microelectrodes would be about 5 μ: whether the system could be fashioned to make a device suitable for personal use remains to be seen.

(c) The solubility of ferrocenes, or other mediators, makes the present basis of the glucose sensor inappropriate for the design of in vivo sensors. However, in the absence, as it then was, of direct enzyme electrochemistry, it seemed as if the problem could be overcome by modifying [34] the enzyme with ferrocene, hoping that electron transfer would take place from the active site. This did indeed seem to work [35] with both ferrocene and other mediators. With the ability to

position the mediator specifically in the enzyme, or with the use of extrinsic redox enzymes, that aspect of the problem might be solved.

(d) By a combination of many of the possibilities referred to above, a device capable of responding to a number of analytes specifically and reproducibly is theoretically feasible. If one would be satisfied with, say readings for eight analytes, then such a device would be possible provided that the enzyme, with or without a mediator attached, is immobilized on the electrode surface. We have designed [36] devices consisting of eight electrodes, each electrode composed of about 200 microelectrodes (5 μm), each microelectrode being about 100 μm from neighbouring electrodes (to retain the characteristics of radial diffusion).

3.4. The difficulty of translating a research idea into a marketable property

There is now no doubt that the discovery and development of the electrochemical glucose detector has finally resulted in a successful outcome; but why did we have such difficulty finding support for the idea? There were a number of factors that could have been involved. We were not analytical chemists and had no experience in the area. Even for the subject of immediate interest, the detection of glucose, we had not properly reviewed the current market and, although we were sure that the ferrocene–enzyme trick had not been used before for analytical purposes, we had not conducted a proper search of the patent literature. Therefore, although we have been somewhat critical in public of the lack of response from companies that we approached, we had not prepared a really convincing case. Not that that would have made a real difference. In 1982, it was still the case that one thought that one had to approach UK companies (it is hard to believe now that there ever was such a time). However, at that time, the UK was in the midst of recession and it seemed as if the last thing that the companies wanted was new ideas that each required significant investment before they could be marketed. That was at a time that "venture capital" was playing a more prominent part in funding work that might result in marketable products. However, when we were asked how long it would be before the idea resulted in a marketable product (to which we replied, four years), those that were considering the idea lost interest. In the end, the ten concerns who we visited showed no interest. Fortunately, as recounted above, someone did show interest and the work, which was beginning to lose momentum, took life and a successful outcome resulted. It would have been so easy to give up and return to protein electrochemistry.

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