THE DIRECT ELECTROCHEMICAL INVESTIGATION OF REDOX ENZYMES

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ABSTRACT : *Even though the use of enzymes as useful analytical reagents when immobilised onto a transducer such as an electrochemical device has been reported in the literature for nearly two decades, the direct, i.e. unmediated electrochemistry of enzymes is, nevertheless, a rather recent development. Efforts will be made to introduce the fundamental basis for protein- surface interactions with emphasis on the powerful technique of cyclic voltammetry. The experimental schemes and ample examples as performed in our laboratory will also be presented to illustrate the kinetic and thermodynamic information acquired based on such enzymatic systems. It is believed that further acquisition of knowledge in the field combined with findings from other complementary disciplines such as modelling and simulation, instrumental computerisation and miniaturisation, and on-line adaptability will ultimately pave the way toward the universal acceptance of biosensors and actuators for chemical analysis.*

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1.0. INTRODUCTION :

The use of electrochemical techniques has, for many years, been standard in fields ranging from solid state physics to organic chemistry. Their widespread use is a result of their versatility in the study of numerous thermodynamic and kinetic problems. For instance, the comparatively simple technique of D.C. cyclic voltammetry can yield a range of information, such as: *a)* the formal reduction potentials of electroactive species; *b)* the number of electrons transferred in electrode reactions, and the rate constants of such reactions; *c)* whether or not the electrode reaction is diffusion-controlled, or involves adsorbed species; *d)* the chemical stability (or fate) of transformed species; and *e)* mechanistic information such as the detection of catalytic processes.

It is not surprising therefore that, for many years, electrochemical techniques have been employed in the study of redox acitve biological molecules. Many of the fundamental processes in nature rely upon the redox processes of constituent biomolecules. For example, cell respiration involves the stepwise oxidation of organic substrates via a cascade of redox reactions, while photosynthetic organisms participate in light- induced electron transport via chains of electron carriers such as cytochromes and iron- sulphur proteins [1].

The aim of this review is to survey the techniques commonly used, and the results obtained, in the electrochemical studies of proteins and enzymes, with emphasis on direct electron transfer, as well as the use of oxidoreductase enzymes in the development of biosensors.

2.0. Protein Electrochemistry

Prior to 1977 it was believed that the direct electrochemistry of redox proteins was not possible [2]. Studies of this kind were rate, electrochemical work being confined to indirect electrochemistry involving the use of small electron carriers as electrochemical mediators. Three reasons were given for this failure:

(a) The slow rates of electron transfer to, or within, proteins.
(b) Adsorption at the electrode surface.
(c) Slow diffusion of the protein.

2.1. Electron Transfer to,or within,the Protein

Proteins (and especially enzymes) are much larger, and more anisotropic than inorganic complexes. The distance between an electrode surface and the protein active centre will be consequently greater for many relative orientations of electrode and protein, and electron transfer rates may consequently be reduced.

Nevertheless, rates of electron transfer between many redox proteins are often comparatively fast. The origin of this effect lies in the amino acid residues which are present around the active sites of electron tranfer proteins. In many cases, these result in charged patches on or near the surface of the protein. These are arranged in such a way as to promote favourable relative orientations of the two proteins: the redox sites within the proteins are brought as close as possible together. *Dutton* et al [3], among others, have shown that the rates of electron transfer between protein redox sites are essentially distance dependent. Hence, due to the recognition of a protein by its redox partner, nearly every collision between the two proteins is a productive encounter.

This effect, however, causes electrochemistry to be critically dependant upon how the protein interacts with the electric field at the electrode-solution interface. The orientation of the protein with respect to the electrode surface, and so the ease of electron transfer, will depend upon the favourability of this interaction.

2.2. Adsorption at Electrode Surfaces

It is well known that proteins display a tendency to adsorb strongly at electrode surfaces [4,5]. This may block any electron transfer to and from proteins in solution, whether or not

the adsorbed protein retains its activity. The development of electrode surface modifiers has, however,led to the possibility of the design of electrode surfaces in such a way as prevent such adsorption.

2.3. Proteins Have Small Diffusion Coefficients

The electrochemistry of species in solution is limited ultimately by the mass transport of reactant and product to and from the electrode. Proteins will usually be slower to diffuse in aqueous solution relative to smaller molecules. This is due both to their greater physical size and their possession, usually, of polar side chains that interact strongly with solvent molecules and ions. In the case of reversible (i.e. diffusion controlled) electron transfer, the electrochemistry of proteins will therefore be limited by their inherently low rates of diffusion.

As will be seen in the following section, conventional "clean" metal electrodes generally provide very poor surfaces for the direct oxidation or reduction of redox proteins. This is not surprising in view of the generally accepted requirement [6], for electron transfer between protein molecules, of the fast and reversible formation of a precursor donor- acceptor complex. A plain metal electrode will present to the protein few of the molecular features required for the formation of such a precursor complex. Not only will this drastically reduce the rate of protein- electrode electron transfer, but may also lead to irreversible adsorption of the protein onto the electrode surface, coupled with conformational changes and subsequent loss of protein activity.

Perhaps it is not surprising, therefore, that it was not until 1977 that the first reports of direct reversible type electrochemistry for proteins were made. In these pioneering studies on horse heart cytochrome *c*, *Eddowes* and *Hill* [7] described how a gold electrode modified by 4,4'-bipyridyl enabled a well- defined one- electron Fe(III)/Fe(II) process to be observed. *Yeh* and *Kuwana* [8] also reported direct electron transfer of the same protein at a tin- doped indium oxide surface.

3.0. The Nature of the Electrode Surface

It would be desirable, therefore, to produce at the electrode surface the essential elements of macromolecular recognition i.e. the complementary hydrogen bonding, salt bridging and hydrophobic contacts that are important in a protein's interaction with its physiological partners. The electrode could, therefore, be tailored to the requirements of the chosen biological system. Such "tailoring" is now possible for most redox proteins via the use of a range of surface modifiers.

3.1. Plain Metal Surfaces

3.1.1. Mercury Electrodes

The first detailed polarographic study of cytochrome *c* was reported by *Betso, Klapper* and *Anderson* [9]. Since that report the reaction of cytochrome *c* at mercury electrodes has been extensively reviewed by *Bowden* et al [10]. Several models for the electron transfer of cytochrome *c* at mercury electrodes have been proposed, but it is clear that the most important feature is that of fast irreversible adsorption of the protein onto the electrode surface. This would explain the concentration dependence of the electron transfer kinetics of cytochrome *c* at mercury electrodes, which ranges from polarographically reversible at low concentrations to quasi- reversible and irreversible at higher concentrations [9,11].

3.1.2. Gold Electrodes

a bare gold electrode placed in aqueous solution does not provide a bare gold surface at which electron transfer can take place. Rather, depending on the applied potential, pH and other conditions, gold oxides may be formed on the electrode surface. Some electrochemical redox processes can be completely inhibited by even a small coverage of such an oxide [12].

Trace impurities adsorbed from solution or from the protein, or absorption of the protein itself can also partially block an electrode. It is now clear, for example from insitu STM studies [13], that roughening, annealing, gold oxide formation and certain dynamic events can widely influence the interfacial electrochemical properties of the gold surface [14].

Again, the most extensively studied redox protein at gold electrodes is cytochrome *c*. It is apparent that well- defined electrochemistry for reduction of cytochrome *c* at the thermodynamic potential does in fact occur at bare metal electrodes including gold [15,16], provided the protein, buffer, and electrolyte are highly purified and scrupulously clean conditions are maintained. However, under many experimental conditions a modified electrode is required in order to observe voltammetry at the thermodynamic potential at a gold electrode (i.e. passivation or blocking of the surface rapidly occurs). Ellipsommetric studies have shown that irreversibly adsorbed cytochrome *c* can mediate to cytochrome *c* in the bulk solution, although in such a way as to produce only a reductive, not an oxidative, response [17].

3.1.3. Other Plain Metal Electrodes

Platinum and silver,and to some extent nickel, have also been used in the study of redox proteins. Irreversible reduction of cytochrome *c* has been shown to occur at platinum [9], silver [18] and nickel electrodes [19], although at an even slower rate than at plain gold [20]. It has been suggested that reduction of cytochrome *c* at platinum may be occurring via electrogenerated hydrogen [21]. Surface enhanced resonance Raman spectroscopy has been used by *Cotton* et al [18] to demonstrate the effects of absorption at silver surfaces on the electrochemistry of cytochrome *c*. Cytochrome *c* has also been shown to adsorb strongly on to platinum electrodes [22].

A number of metallic- type oxide conductors and semi- conductors have been shown to present suitable surfaces for redox protein electron transfer. Indeed, as mentioned earlier, *Yeh* and *Kuwana* [8] were among the first to report direct, reversible redox protein electron transfer. Here well- behaved cyclic voltammetry (and differential pulse voltammetry) of cytochrome *c* was obtained at a tin- doped indium oxide electrode. For such electrodes the amphoteric nature of the surface hydroxyl groups leads to a surface charge due to unequal adsorption of H^+ and OH^- ions [23]. Hence quasi- reversible voltammetric responses have been obtained for cytochrome *c*, azurin, ferrodoxin and plastocyanin at thin film ruthenium dioxide electrodes [24] under suitable solution conditions (i.e. pH, ionic strength etc.).

3.2. Graphite Electrodes

Rapid and reversible electrochemistry is observed at certain forms of graphite more often, and for more redox proteins, than at any other unmodified electrode surface. The characteristics of graphite electrodes may be most easily understood in terms of those of pyrolytic graphite. This material is formed by the deposition of carbon from the vapour phase and has a crystalline structure most closely approximating that of the ideal graphite crystal [25]. Due to its highly ordered structure, pyrolytic graphite is anisotropic and provides two highly distinctive types of surface depending upon the plane along which it is cleaved [26]. The parallel or basal plane provides a hydrophobic surface, while the edge face (formed by cutting across the aromatic rings) displays additional surface structure (i.e. various C-O functionalities). Comparison of the direct electrochemistry of cytochrome *c* (Fig. 1), ferrodoxin and rubredoxin at the edge and basal planes of a pyrolytic graphite electrode [27] revealed the catalysis of heterogeneous electron transfer at the edge surface. The surface oxidized functional groups (negatively charged at pH 7.0) localised at the edge plane can be seen to impart considerable hydrophilicity and ionic character to the surface which aids binding of the protein at the electrode prior to electron transfer.

3.3. Organic Salt Electrodes

It was once believed that direct electrochemical oxidation of several flavoenzymes had been achieved [28] at electrodes made from the conducting organic salt, tetrathiafulvalinium tetracyanoquinodimethianide (or TTF:TCNQ). However, as *Kulys* has pointed out [29], the oxidation of the active centres of glucose oxidase and xanthine oxidase proceeds in a way of mediation. Mediators are formed in the layer near the electrode durface due to a slight dissolution of the organic metal. Because the organic metal compounds oxidise the reduced flavin enzymes at a high rate, a small amount of dissolved compounds is enough to mediate the reaction with high efficiency.

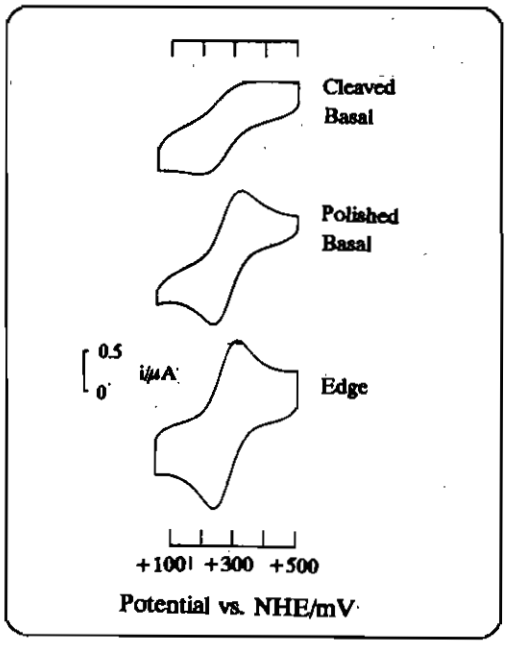

Fig. 1 : Cyclic voltammograms (CV's) of a 200μM solution of cytochrome c in 0.10M phosphate buffer/0.1M KCl at EPG and BPG electrodes (scan rate 50mVs^{-1})

3.4. Modified Electrode Surfaces

The realisation that suitably modified (i.e. functionalised) electrode surfaces could allow stable and reversible direct electrochemistry (without problems such as irreversible protein adsorption) came about in the late 1970's. As stated earlier, the first such report came from *Eddowes* and *Hill* [7] in 1977, who found that essentially reversible cyclic voltammetry of horse mitochondrial cytochrome c could be achieved at a gold electrode onto which was adsorbed the reagent 4,4'- bipyridyl. The result is shown in Fig. 2. The results indicate a one- electron process controlled by linear diffusion, the value of E°(+0.255V), given by $(E_{pc}+E_{pa})/2$, being in good agreement with values determined by potentiommetry (+0.260V). It was proposed [30] that the organic adsorbate allowed electron transfter to occur directly by providing at the electrode surface functionalities with which the protein could interact specifically and reversibly and thereupon donate or accept electrons rapidly. It was thus termed a promoter as opposed to a mediator, in which the latter is considered to convey electrons in bulk solution.

Since these early studies, the field of modified electrodes has developed to such an extent

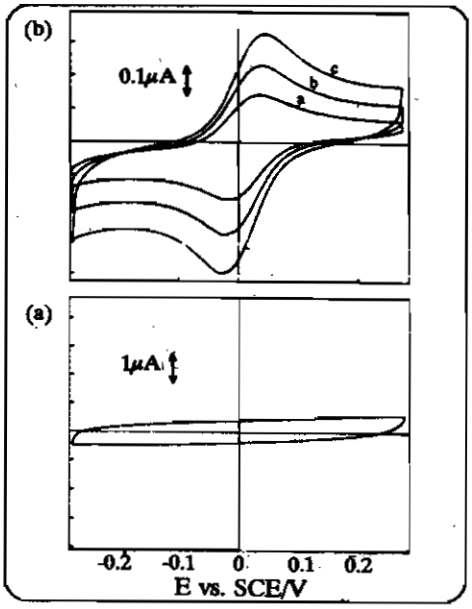

Fig. 2 : (a) A typical background CV profile for a 0.10M phosphate buffer/0.1μM KCl solution at a gold electrode. Scan rate 20mVs^{-1}.

(b) The DV of a 100μM cytochrome c in phosphate buffer at a modified gold electrode, at scan rates: (a) 5mVs^{-1}, (b) 10mVs^{-1}, and (c) 20mVs^{-1}.

that it is now possible to begin to design the electrode/ electrolyte interface. A variety of approaches to the modification of electrode surfaces have been pursued; some of these are presented in Fig. 3. These methods have been reviewed [6, 14, 27] and so will only be summarised here.

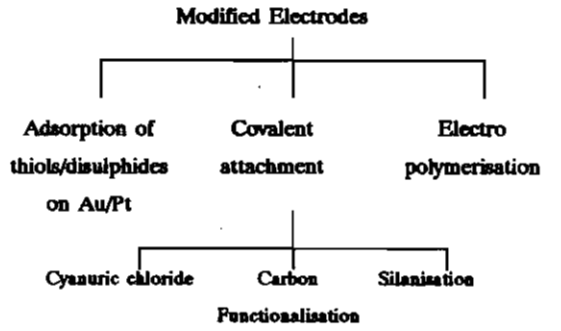

Fig. 3 : Schematic representation of the various approaches to electrode modification.

In order to facilitate binding of the protein to the electrode surface in a manner that is conducive to electron transfer, there must be functional groups at the electrode- solution interface to which the protein can bind, either directly or indirectly. To achieve a near- reversible, diffusion- dominated electrode reaction, both the association and dissociation rates must be fast.

A more clearly defined picture of the structural requirements for successful promotion of electrochemical activity was provided by *Allen* et al [31], who assessed a series of organic compounds in terms of their ability to promote the direct electrochemistry of cytochrome *c* at a modified gold electrode (Fig. 4). They are all bi- (or multi-) functional molecules of the type X~Y, such that X is a substituent which allows binding to the metal electrode surface (for example a pyridyl, phosphine, sulphydryl or thioether) and Y is a functional group (for example a carboxyl or pyridyl) which interacts transiently with some part of the protein surface.

With such a large selection of promoters, it is possible to achieve the electrochemistry of most

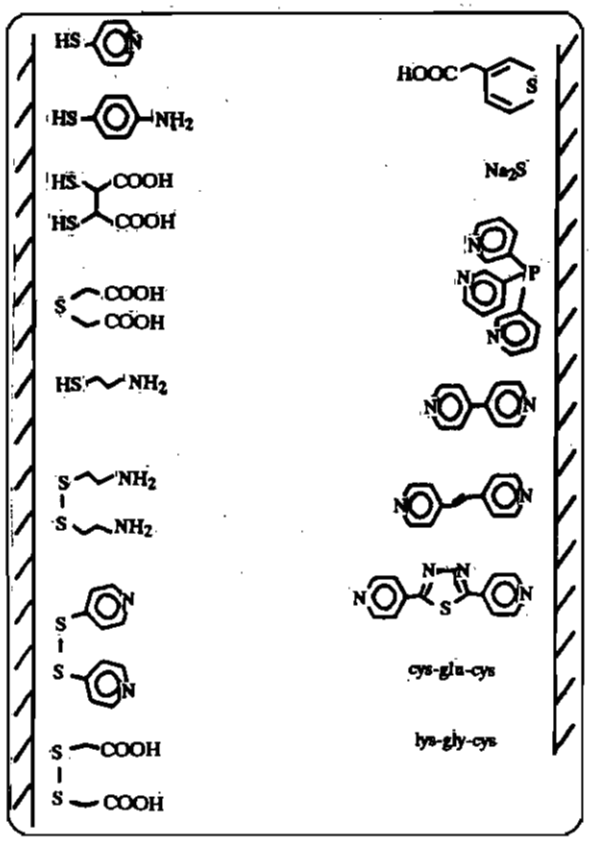

Fig. 4 : Structures and proposed surface conformations of surface modifiers for the promotion of protein electrochemistry.

redox proteins. Few small redox proteins fail to give acceptable electrochemistry, and results are often such that the half- wave potential corresponds to the thermodynamic potential of the protein. However, one must bear in mind that the experimental value reported is for protein-promoter- electrode interaction; if this is significant, for example, when a metal ion or its complex is employed as an ancillary promoter, then a difference may be observed. Such redox potentials are not without interest: it may be that the redox potential of, for example, the complex [32] of magnesium with plastocyanin, may provide a more meaningful description of the complex of the protein in the photosynthetic unit than the redox potential of the "free" protein.

Modification of the electrode by adsorption

of modifier form solution generally yields monolayer coverages. Similar coverages are achieved by the covalent attachment methods mentioned (i.e. cyanuric chloride [33], direct reaction with acidic and carbonyl functionalities on graphite electrodes [34], and silanisation [35]). The above studies depended upon surface modifiers which were irreversibly adsorbed onto the electrode surface. However, surface modification can also be achieved by species in solution which are only reversibly adsorbed. Such behaviour is exhibited principally by two types of cationic species: multivalent cations (such as Mg^{2+}) [31] and aminoglycosides such as neomycin and gentamycin [35].

Multivalent cations, such as Mg^{2+} and $Cr((NH_3)_6^{3+}$, have been shown to be capable of promoting the electrochemistry of proteins possessing negatively charged interaction domains at negatively charged surfaces. This phenomenon has been observed at both metal oxide and pyrolytic graphite electrodes, but has

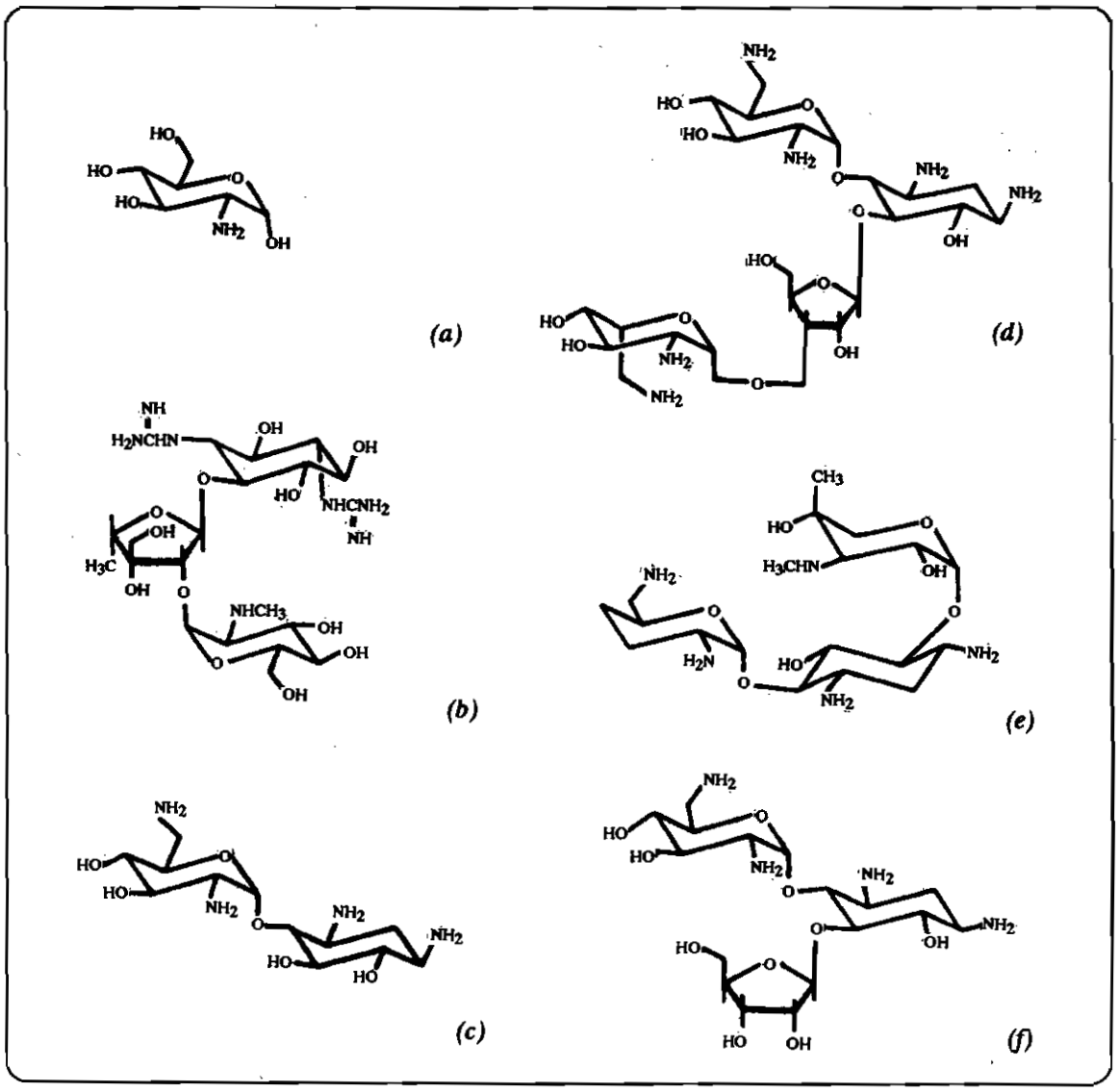

Fig. 5 : Part of the aminoglycoside family: (a) Glucosamine; (b) Dihydroxystreptomycin; (c) Neamine; (d) Neomycin; (e) Gentamycin; (f) Ribostamycin.

been most extensively studied at the latter [32]. The approach of the proteins, which carry anexcess of negatively charged residues, to the negatively charged electrode surface is assistedby the high positive charge density generated by multivalent cations adjacent to the electrode.

Such a mechanism has also been proposed to explain the action of the amino glycosides mentioned. These molecules, as can be seen from Fig. 5, contain spatial arrangements of $-NH_3^+$ groups on a quasi-rigid framework. These act to promote the docking of, for example, cytochrome *c* peroxidase [36] at a pyrolytic graphite edge surface.

4.0. STM and AFM of Electrode Surfaces

It would be misleading to give the impression that these results were straightforward. Of course there were, and still are, problems concerning the exact nature of the electrode surface. Attempts have been made to examinethe detailed nature of these surfaces, e.g. by ellipsometry [17], and more recently by *Scanning Tunnelling Microscopy* (STM) [37]. An exact description in atomic terms still eludes us.

The discovery that STM could be operated under aqueous media [38,39] opened further the possibility that biological substances could be examined in a near- native environment. This, however, did not result in a dramatic breakthrough, mainly due to poor resolution and the fact that most biological substances are not sufficiently conducting. Atomic Force Microscopy (AFM) could overcome many of the STM shortcomings, especially when employed in a non- contanct mode so as to not disturb the soft proteins adsorbed on an electrode surface. Employing AFM, polymerisation of the blood protein fibrin from an aqueous solution onto a net that is the molecular fabric of blood clots has been demonstrated [40]. Physical parameters such as height, stiffness, and viscosity of live platelets that have undergone full activation before AFM imaging has also been demonstrated [41].

5.0. The Microscopic Model

Many early interpretations of the data from direct electrochemical studies of proteins were based on a macroscopic model of the electrode surface. This assumed that transport of redox active species to and from the electrode occurs via linear diffusion to a planar disc surface; the entire surface is uniformly electroactive, and the heterogeneous electron transfer reaction can take place at any point on that surface.

However, under a host of experimental conditions, peak- shaped electrochemical responses could not be obtained. Often, when a promoter was used, rather poor electrochemistry resulted: when the electrode surface was modified in a variety of ways, the electrochemical results indicated poor electrochemistry when essentially no electrochemistry would have been expected. When the results were reassessed [42-45] in terms of the behaviour of the electroactive species at a microelectrode, i.e., one having dimensions of the order of 1mm, they appeared consistent with rapid electron transfer rates. The difference was due to the effect of radial diffusion (Fig. 6) of the electroactive species to the microelectrode, as compared to the normally observed linear diffusion at macro- electrodes. The cyclic voltammogram of proteins whose diffusion to a microelectrode is radial in a manner, albeit superficially, poor electrochemistry at a macroelectrode. Thus it was suggested that due to the non- uniformity of an electrode surface, only an array of microscopically- sized sites are actually electroactive to protein molecules, such that diffusion to these sites occurs in a radial manner. Detailed analysis of the experimental results showed that the electrochemistry of the proteins corresponded, under all conditions, to situations in which the heterogeneous electron transfer rate was very fast.

Another phenomenon which was elucidated by consideration of the radial diffusion was the time dependence of the electrochemistry, whether in situations corresponding to small amounts of promoter on the electrode surface,

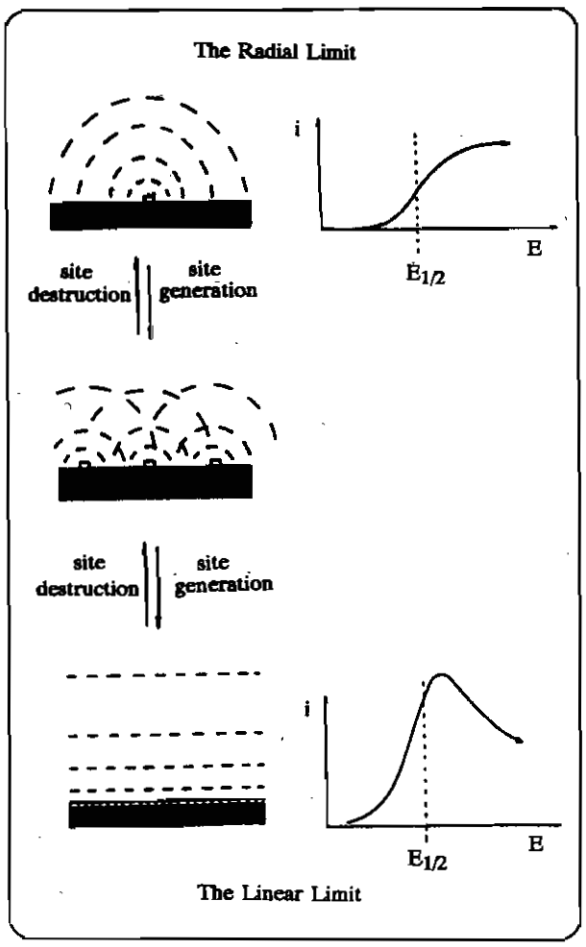

Fig. 6 : Schematic representation of an electrode surface depicting the conversion of radial to linear diffusion as the density of the surface electroactive sites increase.

or with mixtures of promoters, or indeed of the mixture of a promoter with an inactive adsorbate. Even the gradual addition of increasing amounts of an adjunct promoter, for example $Cr(NH_3)_6^{3+}$, to a ferredoxin solution produced a time dependent electrochemistry. Obviously this could be associated to the rearrangement of, for example, the $Cr(NH_3)_6^{3+}$/protein complex, but many of the changes are now thought to be due either the rearrangement of the promoters on the electrode durface, or due to dissolution of the promoter followed by re-adsorption. In most cases, it is relatively easy to envisage how a mixture of promoter and inhibitor could, with time, rearrange to a set of islands, corresponding to an array of micro- electrodes on the electrode surface. There are a few examples of situations where the reverse happens [46], i.e., where two different promoters rearrange such that a protein's electrochemistry alters from a response corresponding to an electrode consisting of an array of microelectrodes to that characteristic of linear diffusion to a macroelectrode.

6.0. Protein- Protein Complexes

The majority of the so called "redox proteins" have no biological function alone, but rather they are associated with other redox proteins, present in membranes in an electron transport assembly in pursuit of some metabolic process. It therefore appeared sensible to attempt to electrochemically investigate protein- protein complexes. The complexes formed between cytochrome *c* and plastocyanin, and cytochrome *c* and cytochrome b_5 have been studied [47].

The availability of electrodes which were selective towards the electrochemistry of the given proteins, for example, edge- plane pyrolytic graphite (EPG) for cytochrome *c*, or gold coated with the tri- peptide cys- lys- cys for plastocyanin, enabled the behaviour of the complexes to be understood. It appears that the protein which binds to the electrode surface, for example cytochrome *c*, at edge- plane pyrolytic graphite, acts as an adjunct promoter, holding plastocyanin or cytochrome b_5 in such an orientation that electron transfer to the latter can occur. This is consistent with zinc cytochrome *c* or indeed many proteins which have an overall charge opposite to that of plastocyanin, acting in the same manner.

There is one important point that emerged from these and related studies. The structures of the complexes must be considered as dynamic: they move with respect not only to each other, but to the electrode surface. The initial description of electron transfer at the electrode envisaged a static arrangement of the protein, or proteins, at the surface but now one must consider a more mobile array of the protein(s)

essentially moving in a lateral manner over the electrode surface until a configuration is reached where electron transfer to, or from, the surface is rapid. Such a dynamic view of the electrode/ protein structure must involve motions within the protein. Indeed, recent studies on the electrochemistry of genetically engineered [48] variants of cytochrome *c*, and of its complexes with cytochrome b_5, indicate that subtle structural changes within cytochrome *c* affect the electrochemistry.

7.0. Direct Enzyme Electrochemistry

The procedures described so far, have not, however, proved as successful in the probing of metalloenzymes. This is essentially due to the same problems encountered in studying protein electrochemistry, i.e. low diffusion coefficients of the biological macromolecule in aqueous solution as result of the size of the molecules, rapid and often irreversible adsorption of the molecule at the electrode surface; and a redox centre buried within the protein and thus shielded for all but a few electrode- protein orientations. The electrochemical study of enzymes has proven more difficult since they are generally substantially larger and more flexible than proteins, and therefore each of the above problems is more apparent. In addition many enzymes are closely associated with membranes, and possess highly lipophilic surfaces, which further hinders aqueous studies.

Redox enzymes may be classified as being intrinsic or extrinsic in nature [49]. An intrinsic redox enzyme is one in which electron transfer associated with the catalysed event is contained within the confines of the active site. An extrinsic redox enzyme requires an associated redox protein (cofactor) as part of the electron transfer process, and therefore there must exist one or more sites for its binding at the enzyme surface (and correspondingly one or more electron transfer pathways through the enzyme between redox centre and binding site). It might be assumed such a binding site could allow interaction with an electrode surface and thus facilitate the heterogeneous electron transfer.

The first reports of the direct electrochemistry of enzymes involved studies with flavoenzymes, but since, in these instances, the prosthetic group is not covalently bound to the protein, and is often observed to dissociate, especially at an electrode surface, it is likely the prosthetic group was acting as a mediator in the electron transfer process. Similarly, some reports suggested that direct electron transfer of a number of enzymes had been observed at conducting organic salt electrodes, such as tetrathiafulvalinium tetracyanoquinodimethanide (TTF-TCNQ) [28]. It has now been generally accepted that the organic groups of the electrode possess sufficient solubility for slight dissolution to occur away from the surface in aqueous solution, and the resulting enzyme electrochemical reaction tion takes place via a mechanism involving heterogeneous catalysis by the organic salt [29,50].

Probably the first genuine study of the direct electrical communication with an enzyme involved the copper- containing laccase [51,52]. *Lee* et al adsorbed fungal laccase A from Polyporous versicolor directly onto edge- oriented plane pyrolytic graphite electrodes, and observed the direct electroreduction of dioxygen, catalysed by the enzyme. A reversible electrochemical response for the adsorbed enzyme, in the absence of dioxygen, was produced in a solution saturated with 2,9- dimethylphenanthroline or 4,4'- bipyridine, which acted as promoters.

Extrinsic redox enzymes require an electron donating or accepting centre to bind to the enzyme, usually at a site not immediately close to the active site. It is presumed that an electron transfer pathway exists between the site at which the ancillary protein binds and this active site region. Thus there is a part of the enzyme surface, no matter how small, across which the electron transfer can occur between electrode and enzyme.One example of such a system is p-cresol methylhydroxylase(PCMH).Electron transfer has

been exhibited directly and reversibly at an electrode by PCMH [53]. The enzyme is a flavocytochrome *c* of 115kDa;it catalyses the oxidative hydroxylation of p- cresol to p- hydroxybenzyl alcohol and subsequently to p- hydroxybenzaldehyde. It is an $\alpha_2\beta_2$ tetramer, with two subunits containing covalently bound FAD's the remaining pair containing c- type heme groups. A blue copper protein is suspected [54] to be its in vivo electron acceptor. It has been shown that direct electron transfer between PCMH and an edge plane graphite electrode can be achieved in the presence of a range of electroinactive cationic species (Table 1). Based on the structural information on PCMH [55], the most likely interaction site between enzyme and electrode is the negatively charged region of propionoic acid groups on the cytochrome subunit [56].

Table 1 : Range of compounds used as promotors for the electrochemistry of PCMH

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

** The catalytic current was obtained using a scan rate of $10mVs^{-1}$, at pH 7.0, in the presence of p- cresol methylhydroxylase(1μM)and p- cresol (2mM) at an edge plane graphite electrode.*

The direct electrochemistry of methylamine dehydrogenase (MADH) from the bacterium Methylbacterium extorquens AM1 has recently been investigated in our laboratory [57]. MADH exhibits a pI value of 5.2, and is thus negatively charged at neutral pH. A quasi- reversible (Ep 58mV) electrochemical response due to direct electron transfer with the enzyme was obtained at an EPG electrode, as depicted in Fig. 7. However, on addition of an aminoglycoside such as neomycin, ribostamycin, streptomycin or tobramycin, much greater faradaic current were obtained. The increase in current was further enhanced when the substrate (methylamine) was added to the working solution to a concentration of 80μM; this was due to the catalytic turnover of the enzyme.

The direct electrochemistry of more and more enzymes is now being reported [58]. For a recent review, see *Hill* and *Hunt* [59].

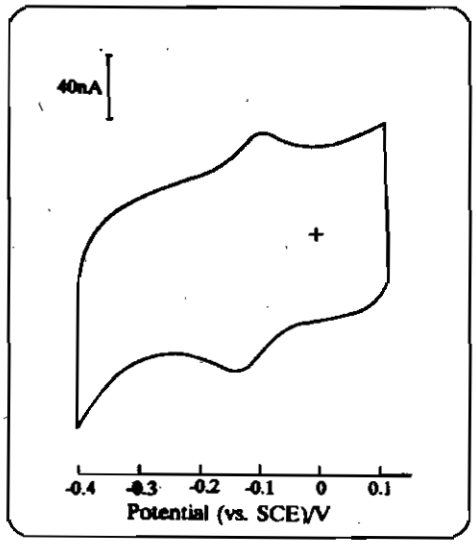

Fig. 7 : A CV of a 40μM solution of MADH at an EPG electrode (scan rate $5mVs^{-1}$).

8.0. Biosensors

Thirty years have elapsed since the concept of enzyme immobilisation for analytical purposes was first introduced by *Clark* and *Lyons* [60]. Biosensor research has since become a very active area. A recent review of electrochemical based biosensor research [61] cited over 75

examples of such research for clinical analysis during the years 1991 and 1992. However, the widespread acceptance and application of such devices has yet to be fully realised. This is due to in part to stability, ease of adaptability, and inconsistency issues in the mass fabrication of such devices.

Electrochemical methods based on enzyme electrodes frequently involve monitoring either the consumption of oxygen using a Clark electrode, or the formation of hydrogen peroxide using a platinum electrode. The latter requires the application of a cathodic potential, typically +650mV vs. Ag/AgCl, at which species such as ascorbic acid, acetaminophen, uric acid, and glutathione, are also electroactive [62]. The effect of such potential interferences, present in considerable concentrations in physiological fluids, renders this approach to the assay of clinical samples difficult without any pretreatment.

The coimmobilisation of glucose oxidase and horseradish peroxidase has been useful for eliminating major interferences [63,64]. Glucose oxidase- substituted polypyrrole coated electrodes offered a greatly improved stability [65]. The coupling of a microdialysis probe and a glucose biosensor for in vivo monitoring of glucose has recently been reported [66]. Implantable glucose sensors have also been evaluated in vivo [67], and in vitro [68,69], with good stability over several days. The fluctuation of dioxygen concentration as the physiological electron acceptor for oxidases can be circumvented by choosing an alternative biological (e.g., cytochrome *c*) or non- biological electron acceptors (e.g., ferrocene monocarboxylic acid). The interest in developing micro- sensing devices for in vivo applications and other health care is also growing rapidly.

8.1. Glucose Analysis of Whole Blood

The transformation of a ferrocene- based enzyme electrode for glucose from laboratory bench to a hand- held commercial device has been achieved and the instrument marketed world wide as the ExacTech glucose meter [70]. The meter comprises a pensized potentiostat with an LCD display for direct glucose reading. The electrode, a disposable test strip, incorporates an immobilised layer of glucose oxidase and 1,1'- dimethyl-3- (1- hydroxy- 2- aminoethyl) ferrocene, coated with a hydrophilic membrane to attract a drop of blood for analysis. Each strip also contains its own reference electrode. A glucose reading is produced in 20 sec.

8.2. Other Examples

The physical principles behind the glucose biosensor may be extended to other enzymes which interact with a mediators. Two possible examples would be creatine kinase assay and cholesterol sensing.

Creatine kinase (ATP: creatine N- phosphotransferase, EC 2.7.3.2) catalyses the reversible transfer of a phosphate residue from adenosine- 5'- triphosphate (ATP) to creatine. Elevated levels of creatine kinase (CK) in blood are linked with acute myocardial infarction. Consequently, the measurement of CK activity is of vital importance in postmyocardial infarction patients. Assays based on the strategy outlined in Fig.8 have been developed for CK. Here, the rate of current decrease at the electrode will be proportional to the rate of creatine phosphate consumption, from which CK activity may be estimated [71]. A typical calibration curve for creatine kinase vs. glucose consumption is shown in Fig. 9.

Three possible ferrocene mediated electrochemical schemes for the determination of cholesterol have been proposed [72], as outlined in Fig. 10. Each of these may be converted to a simple biosensor by immobilisation of the enzymes and mediators involved. The dehydrogenase- based assay exhibited extreme sensitivity to inhibition by surfactants, which are required to free cholesterol esters from lipoprotein complexes in whole sera. The COD assay was limited by the very narrow pH range of the enzyme (pH

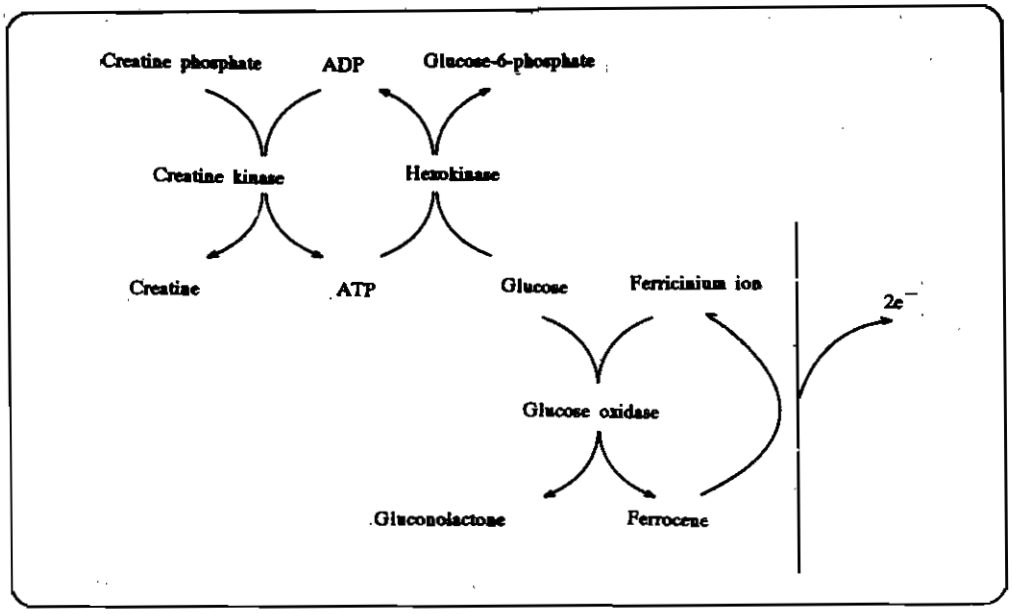

Fig. 8 : Reaction scheme depicting the creatine kinase assay via ferrocene mediated glucose oxidase.

4.5-5.5); this is unsuitable for clinical samples (~pH 7.4). The peroxidase- linked assay proved to be the most reliable [72], exhibiting linearity over the range 0-10mM cholesterol.
Results for cholesterol in serum as compared against a standard clinical method yielded a correlation coefficient of 0.993.

However, so far neither of the creatine kinase or cholesterol systems have resulted in commercial devices.

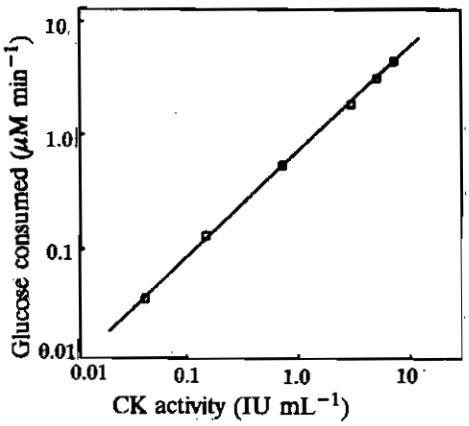

Fig. 9 : Calibration curve for creatine kinase vs glucose consumption.

9.0. CONCLUSION :

The techniques which have been developed over the past fifteen years in the study of protein electrochemistry are now being applied in the study of enzyme systems. Although electrochemical communication with over twenty metalloenzymes has been achieved, most studies in this field involve the development of biosensors, where the enzyme is immobilised at the electrode surface and provides a substrate-dependent current response. The application of electrochemical techniques towards providing kinetic information on enzyme systems is only slowly developing towards the degree with which it is applied in protein systems. In light of structural, conformational, and kinetic similarities between proteins and enzymes on one hand, and monoclonal antibodies, we expect the latter to be the new focus of electrochemical investigations within the next decade.

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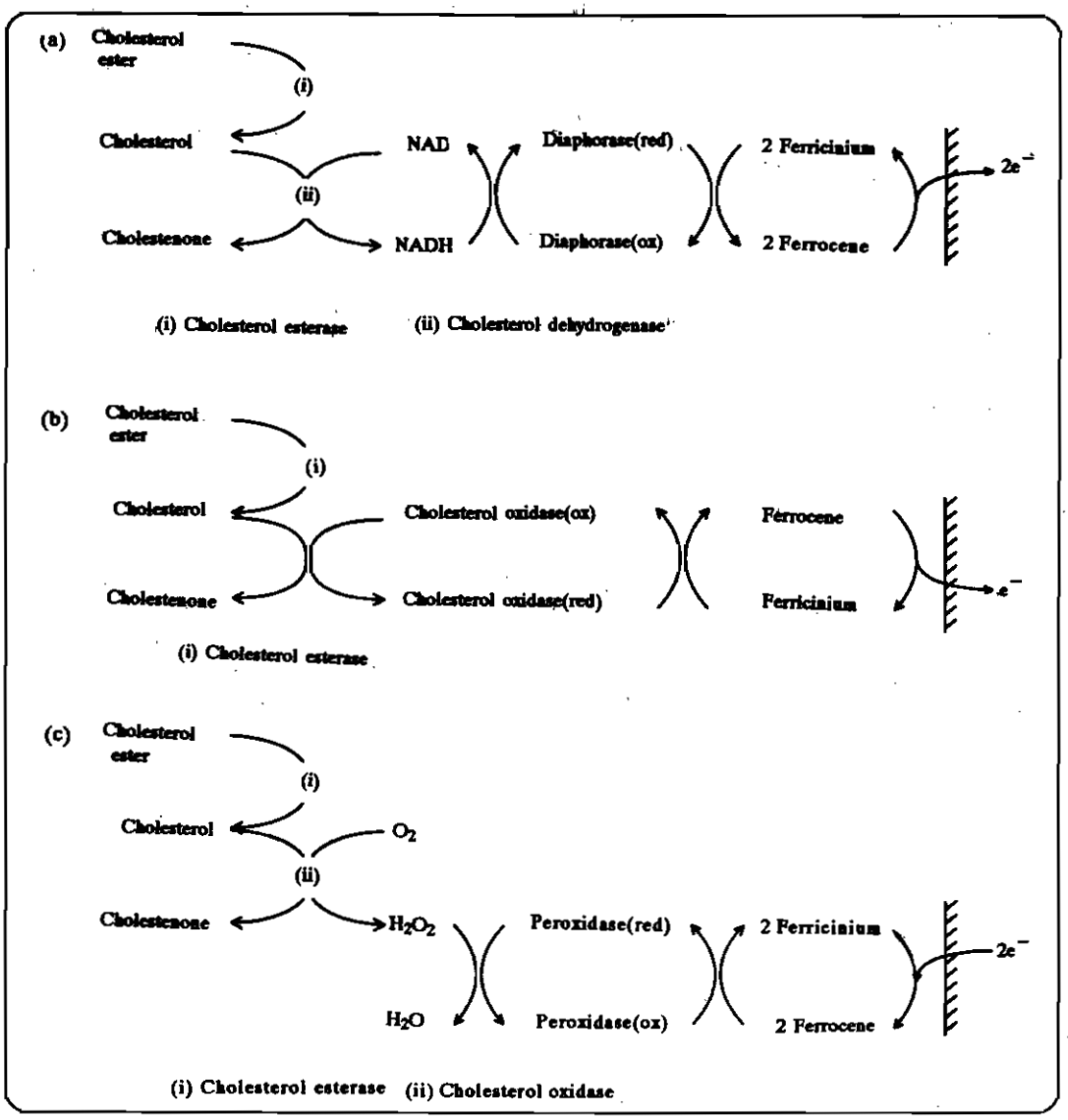

Fig. 10 : Three routes for the design of a ferrocene mediated cholesterol biosensor. (a) Dehydrogenase assay; (b) Oxidase assay; (c) Peroxidase/ oxidase assay.

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