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Electrochemical characteristics of platinum electrodes coated with cytochrome b_5-phospholipid monolayers

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Platinum electrodes can be coated with cytochrome b_5-phospholipid monolayers by the Langmuir–Blodgett technique. Cyclic voltammetry of a series of dyes shows that the coated electrodes become selective for certain electroactive species. The electron transfer reactions of negatively charged species are inhibited at the modified electrode, whereas positively charged species show enhanced reactivity compared with that at a bare metal electrode.

Cytochrome b_5; Phospholipid monolayer; Electrochemistry; Cyclic voltammetry

1. INTRODUCTION

A feature of many of the proteins whose biological function is in electron transport reactions is that, in the living cell, they are located in phospholipid membranes. The proteins are effectively bound to the membrane by hydrophobic interactions [1], and further stabilised by electrostatic forces between the charged phospholipid head-group and specific residues in the protein structure (e.g. [2]). It is possible that use can be made of these interactions to immobilise electron-transfer proteins in amphipathic phospholipid monolayers at an air-water interface and subsequently to transfer the proteolipid film by the Langmuir-Blodgett technique to a metal electrode capable of acting as an electron donor or acceptor (e.g. [3]). The resulting coated electrode might be expected to take on the redox specificity of the protein.

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Various membrane proteins, including examples as complex as photosynthetic reaction centres, have been incorporated into films at the air-water interface and their electrical and structural characteristics have been studied (review [4]). We have chosen the microsomal protein cytochrome b_5 as a model redox protein to test the practicability of this immobilisation technique. In vivo this protein is one of a chain of electron transferring enzymes in the endoplasmic reticulum membrane system of the mammalian cell and spans half of the bilayer. In a previous paper [5], we have described the properties of mixed cytochrome b_5-phospholipid monolayers formed at the air-water interface and have shown that these proteolipid monolayers can be transferred to solid supports by the Langmuir-Blodgett technique. Here we report on the electrochemical properties of such transferred layers on platinum electrodes.

2. MATERIALS AND METHODS

Cytochrome b_5 was prepared from beef liver according to the scheme described by Strittmatter et al. [6]. For some experiments ^{125}I-labelled

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cytochrome was prepared according to Salacinski et al. [7]. The phospholipid used was dipalmitoylphosphatidylcholine (DPPC) of the highest available purity from Sigma. Thin layer chromatography indicated that further purification was not necessary.

Proteolipid monolayers were prepared as described [5] in a constant perimeter 3 l Langmuir trough by injecting 0.2 ml (35 nmol) of an aqueous solution of cytochrome b_5 just below the surface of a previously spread phospholipid monolayer at a number of points. Experiments using ^{125}I-labelled protein (see below) had indicated that cytochrome injected in this way formed a macroscopically homogeneous monolayer which produced pressure-area characteristics identical to those where cytochrome was fully dispersed in the subphase by mechanical stirring before spreading the phospholipid monolayer [5]. This method yields a film which is saturated with protein, but suffers from the disadvantage that it does not allow control of the phospholipid:protein ratio [4].

The proteolipid monolayer was transferred to a 0.5 mm diameter platinum wire electrode by the Langmuir-Blodgett technique at a dipping speed of 6 mm·min^{-1}. Deposition was carried out at a constant surface pressure of 40 mN·m^{-1}, at room temperature, and with a subphase pH of 6.0. Prior to dipping, the platinum wire was thoroughly cleaned by flaming in a bunsen burner flame and by ultrasonication in a 50:50 chloroform/methanol mixture. All solvents were of Aristar grade. The electrodes were dipped to a depth of 30–40 mm. Little deposition occurred when the platinum wire was dipped through the monolayer into the subphase, but on subsequent withdrawal a deposition ratio greater than 0.95 was obtained. In experiments where radiolabelled protein was used the coated electrode was counted for radioactivity in a gamma counter immediately after dipping.

The electrochemical behaviour of the coated and uncoated electrodes was studied by cyclic voltammetry [8] using various dyes as electron acceptors. The platinum wire electrode was mounted as the working electrode of a three terminal cell with a platinum mesh counter electrode and a saturated calomel electrode (SCE) as a reference. The working electrode was dipped to a depth of 20 mm into the cell electrolyte giving a working area of 31 mm^2, and its potential was controlled by a scanning potentiostat (EG&G Model 273). The cell contained 40 ml of a 0.1 M KCl solution together with an appropriate electron acceptor dye (see below) and was housed in a light-tight box to prevent photooxidation of the dye acceptors.

Prior to the voltammetry experiments these solutions were routinely flushed with nitrogen for at least 5 min. The various electron acceptor dyes (Sigma) are listed in table 1 and were used at concentrations of between 0.25 and 1.0 mM. These dyes have been used previously for potentiometric measurements as redox mediators between metal electrodes and cytochromes and other redox proteins in bulk solution [9]. Dye reduction and reoxidation by the working electrode was monitored by recording the currents passed as a function of the working electrode potential, i.e. by recording the voltammogram.

3. RESULTS

Our previous studies with radiolabelled cytochrome b_5 [5], showed that after dipping the electrode as described above the deposited layer contained cytochrome at a concentration of ~6 pmol·cm^{-2}. The cytochrome appears to be tightly bound to the electrode. Even ultrasonication in high salt buffers and/or in the presence of detergents removed only about 40% of the deposited cytochrome. The material which was recovered into aqueous buffers retained its redox activity as indicated by characteristic spectral changes (increase in $E_{422}-E_{413}$) produced on addition of sodium dithionite reducing agent. The magnitude of these spectral changes were consistent with the amount of cytochrome recovered from the electrode by the treatment described above.

Figs 1 and 2 show typical cyclic voltammograms obtained for bare and proteolipid coated platinum electrodes with two different electron acceptor dyes in solution. Fig.1 (curve a), shows the electrochemical reactions of potassium ferricyanide at a bare platinum electrode. The usual reduction (cathodic) and oxidation (anodic) current peaks are clearly visible. The peaks occur at potentials of 135 mV (cathodic) and 205 mV (anodic). The peak separation of 70 mV indicates a quasi-reversible electrochemical system with a standard potential

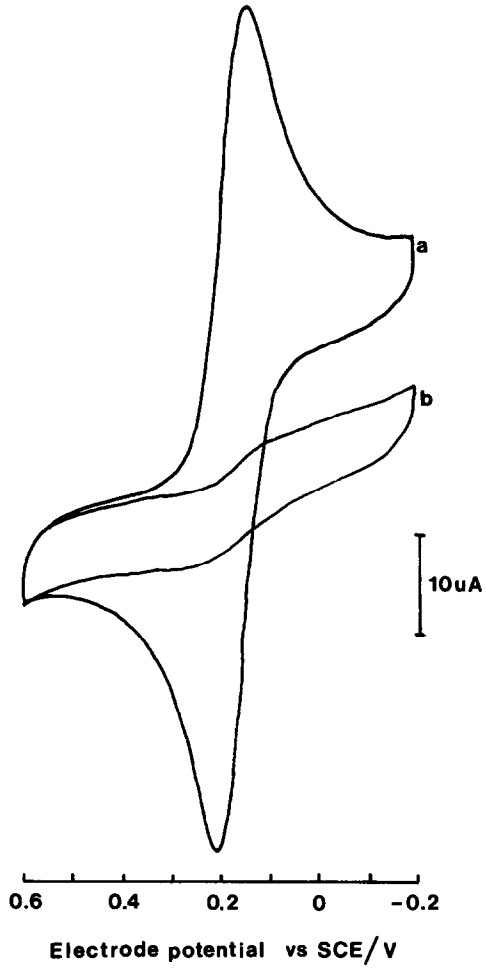

Fig.1. Cyclic voltammograms for a platinum working electrode in a 1 mM potassium ferricyanide, 0.1 M KCl solution. (a) Bare platinum, (b) platinum coated with a two layer cytochrome b_5-DPPC Langmuir-Blodgett film. Scan rate, 100 $mV \cdot s^{-1}$; temperature, 20°C; pH, 6.0.

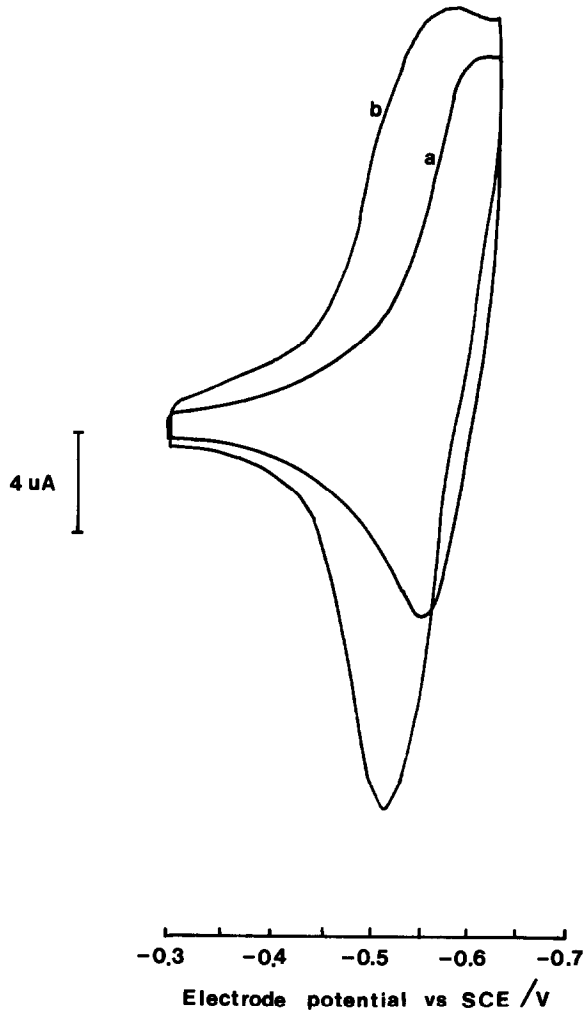

Fig.2. Cyclic voltammograms as for fig.1, but with 1 mM benzyl viologen replacing potassium ferricyanide.

of 170 mV, in good agreement with previously reported values [10]. When the working electrode was coated with the two layers of the proteolipid film (fig.1, curve b), there is a dramatic elimination of both anodic and cathodic peaks.

Fig.2 shows the same experiments with benzyl viologen replacing potassium ferricyanide as the electron acceptor in solution. The electrochemical reactions of benzyl viologen at a bare platinum electrode (curve a) appear well behaved with the usual cathodic and anodic peaks at −595 and −540 mV vs SCE, respectively, giving an estimated standard potential of −568 mV; again in good agreement with published values [10]. Coating the electrode with the proteolipid layers now results in peak shifts of approx. 70 mV to more positive potentials and there is a significant enhancement of the anodic peak suggesting an increase in the rate constant for the reaction. The contrast between figs 1b and 2b is most marked. Analysis of scan rate dependence of peak height and peak separation (in the range 20–250 $mV \cdot s^{-1}$) for the coated electrode reactions, suggested quasi-reversible behaviour with values for kinetic con-

stants approaching those required for a Nernstian system.

The results shown in figs 1 and 2 were very reproducible. Films containing cytochrome were stable at the air-water interface in the Langmuir trough for periods of at least 1 week and electrodes coated with proteolipid films gave identical cyclic voltammograms for at least 48 h. No irreversible effects on the coating are produced by the interaction with the dye solution. For example, after the coated electrode had been in contact with the potassium ferricyanide solution (in which a voltammogram such as in fig.1b, indicating very low rates of electron transfer, was recorded), it was possible to observe high electron transfer rates when this same electrode was transferred to a benzyl viologen solution (when a voltammogram as in fig.2b was observed). Thus no permanent effect on the coating is produced by interaction with ferricyanide, although of course we cannot exclude subtle, reversible interactions.

These results suggested that coating the electrode with a cytochrome b_5 containing layer made the electrode selective for electron transfer to certain acceptor dyes. To examine this selectivity further, voltammograms were compared for bare and coated platinum electrodes for a series of other redox dyes covering a wide range of standard potentials. The results are presented in table 1. It is seen that for certain dyes (e.g. triphenyl tetrazolium) voltammogram peaks are enhanced, as they were for benzyl viologen, whereas for others (e.g. anthraquinone 2-sulphonic acid) they are diminished, as they were for potassium ferricyanide. With a number of dyes there is a shift of the peaks along the potential axis. The effects reported are less marked if only one layer of proteolipid is deposited but are increased if more than two are deposited. However the increase becomes less as the number of layers increases and reaches a limit at about six layers.

4. DISCUSSION

Using the methods described above, it is clearly possible to incorporate an amphipathic protein in-

Table 1

Effect of cytochrome b_5-DPPC coating on platinum electrode redox reactions of various dyes

Dye	Ionic charge	$E^{\circ\prime}$ (mV vs SCE)	i_{pc}/i_{pco}	i_{pa}/i_{pao}	Cathodic peak shift (mV)
Potassium ferricyanide	−3	+167	0.06	0.05	0
Dichlorophenol indophenol	−1	−458	0.80	0.67	−150
Anthraquinone 2-sulphonate	−1	−466	0.71	0.38	0
Tetramethyl phenylenediamine*	0	+35	0	0.78	0
Methylene blue*	0	−230	1.3	1.25	0
Neutral red*	0	−570	1.22	1.20	+40
Triphenyl tetrazolium*	+1	−321	1.20	2.80	−130
Benzyl viologen	+2	−239	1.23	2.15	+75

Cyclic voltammograms were recorded for dyes having the range of standard potentials, $E^{\circ\prime}$, given above under the same conditions as those shown in figs 1 and 2. Peak cathodic and anodic currents (i_{pc} and i_{pa}) were measured for platinum electrodes coated with two layers of the cytochrome b_5-DPPC Langmuir-Blodgett film and are expressed as ratios of the corresponding values (i_{pco} and i_{pao}) for a bare platinum electrode. Also shown are the shifts in cathodic peak potential. For those dyes (marked *) having multiple peaks, the measurements were made on the major peaks

to a phospholipid monolayer which can then be deposited onto a metal electrode. The layers produced appear to be macroscopically homogeneous and a protein : phospholipid ratio of 1 : 33 has been found [5]. It is possible that at the microscopic level the films are heterogeneous with areas of high and low cytochrome concentration. (The diffusion coefficients of macromolecules in phospholipid layers are reported to be low [11].) It is notable, however, that the ratio of 1 : 33 for cytochrome b_5 : DPPC is very close to values obtained by other workers in experiments using phospholipid vesicles [12].

Experiments in which cytochrome was recovered from the electrode showed that deterioration of the redox activity of the protein did not occur in spite of the immobilisation procedure. The films containing cytochrome were also very stable electrochemically over periods of days whether at the air-water interface or on the platinum electrode. The resistance to severe washing procedures also indicated a high degree of mechanical stability for the proteolipid layers. The stability of the DPPC component in these layers is however an interesting question. Pure radiolabelled DPPC layers deposited on platinum electrodes readily desorbed onto the surface of the electrolyte when the electrode was placed into the electrochemical cell. If all of the DPPC component is also lost from a cytochrome-DPPC coating then the resulting exposed area of metal, which on the basis of a 1 : 33 ratio would be as much as 60% of the total, would make a substantial 'bare metal' contribution to the voltammogram. This is clearly not the case in fig.1b, where the voltammogram peaks have been reduced to less than 5% of the bare metal electrode values. It appears therefore that the integrity of the proteolipid layers is maintained and the likely explanation is that the lipid and protein are bound strongly to each other and to the metal.

As table 1 shows, the cytochrome b_5-DPPC layer confers specificity on the platinum electrode. Whereas the bare metal will reduce all of the dyes tested, only some of these reactions are catalysed when the cytochrome b_5-DPPC layer is present. In the cases where there is a significant enhancement of the reactivity (see for example the anodic peak for triphenyl tetrazolium, table 1) it is important to realise that the reaction is proceeding through multilayer films. As stated above these effects can be observed even with as many as six layers deposited on the metal electrode.

It is likely that the specificity arises from the functional groups which characterise the protein surface. We propose that its origin is to be found in the electrostatic interactions between the charged dye molecules and the groups on the surface of the cytochrome since dyes which carry negative charge show reduced electron transfer activity at the coated electrode, while positively charged dyes show enhanced activity. It is highly significant that a number of negatively charged groups are known to occupy the surface of the cytochrome b_5 molecule in the haem binding domain [13]. The local electrostatic forces arising there would attract the positively charged dye molecules thus facilitating electron transfer. The converse would occur with negatively charged dyes. The shifts in the potentials of the peak positions (table 1) do not follow the same trends but these will depend on the details of the association which some of the dye molecules make with the proteolipid layer which would alter their electronic states.

If the above model is valid, then curve b in fig.1 provides good evidence that our films are free from pinhole defects, at least down to atomic dimensions. This conclusion is based on the fact that the Debye screening length about a point charge in 0.1 M KCl solution is ~1 nm. Since the redox peaks of potassium ferricyanide are almost totally eliminated by the coating then if pinholes are present in the film then these must be less than 2 nm in diameter, otherwise the negatively charged dye will reach the metal electrode and contribute a bare metal peak to the voltammogram.

Our results do not allow us to speculate on the conformation or enzymic activity of the protein when bound to the electrode in the phospholipid matrix. However, preliminary experiments with a sensitive reflectance spectrometer suggest that at least at the air-water interface the protein has its normal absorbance spectrum. Furthermore, since washing the protein off the electrode surface yields a protein solution which is spectroscopically and functionally identical to native cytochrome b_5, we assume that if structural changes due to binding into the phospholipid film and onto the metal surface do occur, then these are not gross changes and are readily reversible.

Having shown that electrode selectivity may be achieved with a cytochrome b_5-DPPC layer on platinum, we believe that our work opens up the possibility of utilising other proteins in a similar way, with various surface features such as charge configurations, hydrogen bonding, hydrophobic and hydrophilic groups, allowing a range of selective electrochemical reactions to be achieved.

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