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Use of 'solid-state' promoters in the electrochemistry of cytochrome *c* at a gold electrode

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The direct electrochemistry of cytochrome *c* at a gold electrode was investigated by cyclic voltammetry using, as promoters, microperoxidase (the haem-undecapeptide obtained by hydrolysis of cytochrome *c*), Fe(III)-protoporphyrin IX or protoporphyrin-IX, all entrapped in a cellulose triacetate membrane. The results indicate that these immobilized systems strongly enhance the rate of electron transfer between the protein in solution and the electrode surface, and thus behave as 'solid-state' promoters, though with differing efficiencies. These results are of interest because they raise the possibility of engineering an efficient and versatile promoter active also at inert electrode surfaces.

INTRODUCTION

Heterogeneous electron-transfer reactions of redox proteins at a variety of electrodes have been widely investigated [1,2] because of their significance in understanding the mechanism of biological electron transfer and also because of their potential applications in the area of biosensors. The electrochemistry of cytochrome *c* has been extensively investigated at gold electrodes modified by adsorption, for example, of 4,4′-bipyridine, 1,2-(4-pyridinyl)-ethylene or cysteine, on the electrode surface [3,4]. The studies of the quasi-reversible electron-transfer reaction of the protein at the modified gold electrodes have underlined the importance of modifiers in providing a suitable interface for interaction of the macromolecule with the electrode and in preventing its irreversible and degradative adsorption at the electrode surface.

We have shown [5] that microperoxidase, the haem-containing undecapeptide obtained by hydrolysis of cytochrome *c* (see Fig. 1), is electrochemically active at a glassy carbon electrode, even in the absence of promoters or mediators. Moreover, we have made [6] the novel observation that a cellulose triacetate membrane containing entrapped microperoxidase and fixed on a glassy-carbon-electrode surface successfully promotes the electrochemistry of soluble metalloproteins such as cytochrome *c* or azurin.

Here we report the results of an investigation into the voltammetric behaviour of cytochrome *c* at an inert gold electrode coated with a cellulose triacetate membrane containing microperoxidase, Fe(III)-protoporphyrin IX or metal-free protoporphyrin IX. The new results provide further information on the potentialities of entrapped porphyrins or porphyrin complexes in promoting the electrochemistry of metalloproteins at electrodes with different features (such as, for example, different degrees of polarization); moreover, they shed light on the mechanism by which microperoxidase enhances the electron-transfer rate between a metalloprotein in solution and the electrode surface. It is well known that no electrochemical activity of cytochrome *c* is detected at a naked gold electrode [1,3]; by contrast, we show here that the protein displays well-shaped cyclic voltammograms at the same electrode once coated with a cellulose triacetate membrane containing entrapped microperoxidase or Fe(III)-protoporphyrin IX. These immobilized systems are therefore also efficient 'solid-state' promoters at a gold electrode. It may be recalled (see Eddowes & Hill [7]) that promoters act by a mechanism different from conventional mediators insofar as they are not electroactive in the potential range of the investigated redox process and their role is to provide a suitable interface favouring orientation and rapid electron transfer between a macromolecule and the electrode surface. Engineering a 'solid-state' electrochemical promoter is a matter of great interest, because some of the problems related to the use of soluble promoters (such as a specific binding to the protein or undesired side reactions) would be minimized. A hypothesis on the mechanism governing the heterogeneous electron-transfer reaction at the electrode is also discussed.

MATERIALS AND METHODS

Microperoxidase was obtained from Sigma or prepared by the method of Harbury & Loach [8]. Cytochrome *c*, Fe(III)-protoporphyrin IX (haemin) and metal-free protoporphyrin IX were purchased from Sigma and used without further purification. Cellulose triacetate was obtained from Fluka.

Physical entrapment was obtained by dipping the cellulose triacetate membrane (prepared as described in [9,10]), in gelled form, into an aqueous solution of microperoxidase or haemin or

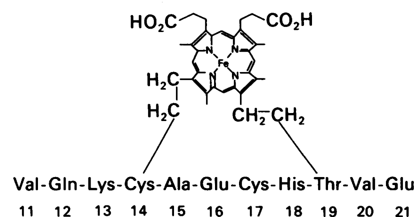

Fig. 1. Structure of microperoxidase, the haem-peptide obtained from the hydrolysis of horse heart cytochrome *c*

The numbers refer to the amino acid sequence of the native protein.

Abbreviation used: ΔE_p, peak potentials separation.

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protoporphyrin IX (2 mg/ml) for 24 h at 5 °C. Before use, the membranes were allowed to dry for 24–36 h and then carefully washed in water until no more colour (as tested spectrophotometrically) was released. Electrochemical experiments were carried out at neutral pH, in 20 mM-phosphate buffer containing 100 mM-sodium perchlorate as supporting electrolyte. A 0.5 mm-thick membrane, entrapping one of the investigated systems, was fixed with an O-ring on to a gold microelectrode. The electrode was then dipped in the electrochemical cell containing dissolved cytochrome c (5 mg/ml) for cyclic voltammetry. A gold electrode (Amel, Milan, Italy), 2 mm in diameter, was used for the voltammetric measurements; a saturated calomel microelectrode (Ingold, Urdorf, Switzerland) was the reference and a platinum gauze acted as the counterelectrode. Cyclic voltammograms were run in an anaerobic environment obtained by removing O_2 from the cell by a gentle flow of pure, CO_2-free N_2 maintained just above the solution surface and previously passed through a 0.2 M-NaOH solution. Experiments were performed 60–90 min after the introduction of the working electrode into the cell, in order to achieve full rehydration of the membrane and thus stable voltammograms. Voltammetric measurements were performed by using an Amel 473 multipolarograph equipped with an Amel 863 recorder.

RESULTS

D.c. cyclic voltammograms of soluble cytochrome *c* at a gold electrode, coated with a membrane containing entrapped microperoxidase, are shown in Fig. 2. A well-defined cyclic voltammetry is observed; the cathodic and the anodic peaks are similar in shape and the I_{pa}/I_{pc} (anodic and cathodic peak current ratio) ratio is about unity. The value of $\Delta E_p = 60 \pm 4$ mV, obtained at a scan rate of 10 mV/s, is very close to that expected for a reversible one electron-transfer reaction, i.e. $\Delta E_p = 57$ mV [11]. However, it increased with the scan rate (up to 100 mV/s), as already reported for other proteins [12,13]. The values of voltammetric peak currents were found to be proportional to the square root of the scan rate, consistent with a diffusion-controlled redox process [11]. Following Nicholson & Shain [14], the rate constant for the heterogeneous electron transfer (K_s) was estimated to be $(6 \pm 2) \times 10^{-3}$ cm·s^{-1}, on the basis of *n* (electrons per molecule oxidized or reduced) being 1, α (charge-transfer coefficient) being 0.5 and the temperature being 25 °C. The calculated redox potential ($\simeq +260$ mV versus normal hydrogen electrode) is in good agreement with the values reported in the literature [3,14,15]. By contrast, at naked gold electrodes cytochrome *c* shows no electrochemical activity, indicating that the electron-transfer rate between the protein and the electrode surface is too low [1]. In addition, a plain cellulose triacetate membrane fixed on the electrode surface has no effect, contrary to what has been reported for a glassy carbon electrode [16]. Thus the electrochemical activity of cytochrome *c* observed at a gold electrode coated with membrane-entrapped microperoxidase is to be attributed to the haem peptide, which promotes electron transfer.

The behaviour of membrane-entrapped Fe(III)-protoporphyrin IX at the same electrode was also investigated. In contrast with microperoxidase (which displays no detectable electrochemical activity at a gold electrode), membrane-entrapped Fe(III)-protoporphyrin IX shows quasi-reversible d.c. cyclic voltammograms (potential range: 0 to −0.6 V versus standard calomel electrode), as illustrated in Fig. 3, although the low current values are the relatively broad peaks observed indicate that electron transfer between entrapped haems and the gold surface is slow. Thus, under the experimental conditions investigated, the polypeptide of microperoxidase lowers the electron-transfer rate between the haem iron and the gold electrode surface; some degree of aggregation masking the electroactive group may occur.

Similarly to microperoxidase, membrane-entrapped Fe(III)-protoporphyrin IX promotes the electrochemistry of cytochrome *c*. In Fig. 4 the d.c. cyclic voltammograms of cytochrome *c* at a gold electrode coated with a cellulose triacetate membrane containing entrapped Fe(III)-protoporphyrin IX are shown. Well-shaped, quasi-reversible, d.c. cyclic voltammograms of the protein are obtained and, apart from small differences to be attributed to capacitive currents, the voltammograms shown in this Figure appear very similar, in shape and magnitude, to those illustrated in Fig. 2, thus suggesting that entrapped haem and microperoxidase both promote electron transfer, possibly via the

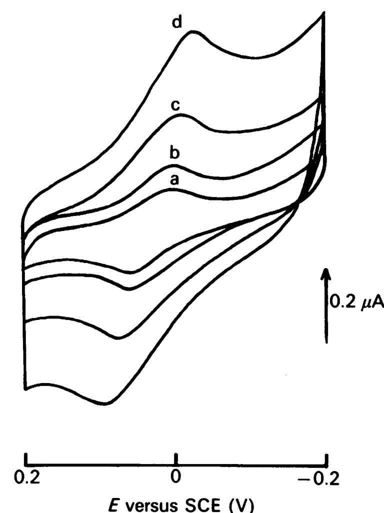

Fig. 2. D.c. cyclic voltammetry of cytochrome *c* (5 mg/ml) at a gold electrode coated with a cellulose triacetate membrane containing entrapped microperoxidase

Experimental conditions: 20 mM-phosphate, pH 7.0, containing 100 mM-sodium perchlorate; sweep rate: 10 mV/s (a); 20 mV/s (b); 50 mV/s (c); 100 mV/s (d); temperature, 25 °C. Abbreviations: *E*, working electrode potential; SCE, standard calomel electrode.

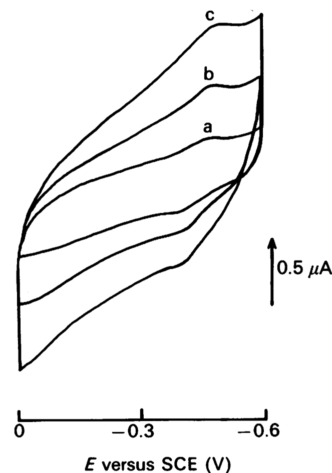

Fig. 3. D.c. cyclic voltammograms of membrane-entrapped Fe(III)-protoporphyrin IX at a gold electrode

Sweep rate: 20 mV/s (a); 50 mV/s (b); 100 mV/s (c). Other experimental conditions and abbreviations were as for Fig. 2.

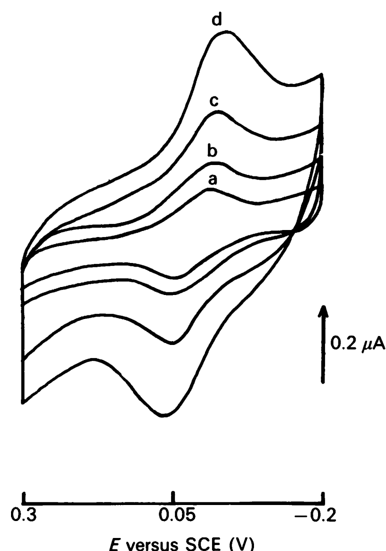

Fig. 4. D.c. cyclic voltammetry of cytochrome c (5 mg/ml) at a gold electrode coated with a cellulose triacetate membrane containing entrapped Fe(III)-protoporphyrin IX

Other experimental conditions and abbreviations were as for Fig. 2.

same mechanism. In order to explore whether the metal may enhance the rate by acting as an electron carrier, parallel experiments were carried out using entrapped protoporphyrin IX (i.e. metal-free haemin). Also, in this case, quasi-reversible d.c. cyclic voltammograms were obtained up to scan rates of 20 mV/s (results not shown); at higher scan rates, however, a progressive loss of reversibility in the process was observed. This result indicates that the presence of the metal in the porphyrin ring is not essential in order to obtain promotion of electron transfer between cytochrome c and the electrode surface; however, when present it exerts a measurable effect on the rate of the process.

DISCUSSION

It has been reported [2,3] that direct electrochemistry of soluble metalloproteins at gold electrodes can be achieved with chemisorbed compounds that promote rapid reversible binding of the macromolecules at the electrode modified surface and prevent degradation of the protein, owing to irreversible adsorption at the gold surface. These compounds generally posses functional groups that interact with the charged amino acid residues of the protein surface (such as Lys-13, Lys-72 and Lys-86 in cytochrome c, distributed around the exposed edge of the haem [17]) and provide a favourable orientation of the macromolecule. It is known [17] that interaction between cytochrome c and its oxidase is sharply affected by modification of a few lysine residues, presumably involved in the formation of an active complex. Therefore it may be expected that, when membrane-entrapped microperoxidase (which is negatively charged at neutral pH; pI 4.7 [18]) is employed as a 'solid-state' promoter, the groups which may interact with the positively charged lysine on the cytochrome c may be: (a) the two propionates of the haem, highly exposed to the solvent, or (b) the two carboxylates of Glu-21, the C-terminal residue of microperoxidase (see Fig. 1). From the results reported above, it appears that membrane-entrapped microperoxidase and Fe(III)-protoporphyrin IX both promote rapid electron transfer between cytochrome c and the electrode surface; thus they should operate via a similar mechanism, possibly involving the two propionates of the haem, which interact electrostatically with the positively charged lysine residues on the surface of cytochrome c. This interaction, by promoting a favourable orientation of the solvated macromolecule, is supposed to enhance electron transfer between cytochrome c and the modified electrode surface. However, since the active membrane is very thick, the mechanism by which electron transfer occurs across such a large distance is not clear to us, although the role of the entrapped aromatic macrocycles is experimentally proven and therefore crucial.

Finally, it should be noted that, in the case of cytochrome c, the polypeptide of microperoxidase has no significant effect on the promotion of electron transfer to the gold surface; on the other hand, the same peptide is crucial in promoting electron transfer of azurin, a copper-containing metalloprotein which displays well-defined cyclic voltammograms at a glassy carbon electrode once coated with a membrane containing microperoxidase [6], while showing no detectable signal with the same membrane containing Fe(III)-protoporphyrin IX. The different overall charge of azurin (pI 5.1 [19]) and the lack of positively charged groups in the putative electron-transfer hydrophobic patch of azurin, may provide a molecular explanation of this different behaviour [20]. These observations provide direct support for the role of electrostatic interactions in the protein–electrode electron-transfer mechanism and focus on the importance of entrapped polypeptides in promoting direct electrochemistry of metalloproteins. Suitable polypeptides may provide charged or hydrophobic amino acids, forming a patch enhancing favourable interactions and thereby promoting electron transfer between the protein in solution and the electrode surface.

In conclusion, the results reported above indicate that membrane-entrapped haems, either free [as in Fe(III)-protoporphyrin IX] or bound to a short amino acid chain (as in microperoxidase), act as efficient 'solid-state' promoters in the electrochemistry of cytochrome c at a normally inert gold electrode surface. These results stimulate further studies designed to engineer a system which couples, with efficiency and versatility, also a certain degree of molecular recognition. Obviously, 'solid-state' promoters of this type would be of great significance in applied biochemistry, biotechnology and sensors science.

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