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Voltammetric studies on the electrochemical behaviour of membrane-entrapped hemes

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Summary. The electrochemical behaviour of Fe(III)-protoporphyrin IX entrapped into a cellulose triacetate membrane has been investigated by cyclic voltammetry. The physical entrapment into a solid matrix does not modify the redox properties of the entrapped hemes, which also act as efficient promoters in the electrochemistry of cytochrome *c*. Such a system represents a promising example of a simple 'solid-state' promoter, and stimulates further investigations in order to obtain more complex systems that may be of significance for basic and applied bioelectrochemistry.

Key words: Fe(III)-protoporphyrin IX - Electrochemical promoter - Cyclic voltammetry - Metalloproteins

Introduction

The electrochemical behaviour of biological systems has been investigated from a variety of viewpoints, in order to shed light on the complex mechanism(s) governing the heterogeneous electron transfer between a macromolecule and the electrode. In particular, electrochemical studies on metalloproteins appear of great interest in order to clarify the experimental conditions to achieve rapid, reversible and specific electron transfer between a soluble macromolecule and an electrode surface (Armstrong et al. 1986; Frew and Hill 1988). Generally, rapid electron transfer is unlikely because the active site of a metalloprotein often lies buried in a crevice, surrounded and shielded by the polypeptide chain (as it occurs, for example, for the heme in hemoproteins). Organic molecules adsorbed onto an electrode surface (Eddows and Hill 1979; Eddows and Hill 1981; Frew and Hill 1988) may enhance considerably the rate of heterogeneous electron transfer, acting as promoters and thereby allowing electrochemical investigations of soluble proteins also by relatively quick techniques, such as direct-current cyclic voltammetry. The latter is a powerful experimental tool because, though relatively simple, it yet provides quantitative information on the thermodynamic and kinetic aspects of an electrochemical process.

In this paper we report the results of an electrochemical investigation of Fe(III)-protoporphyrin IX (the active site of a wide class of metalloproteins), either in solution or entrapped into a solid matrix. The results provide new information relevant to: (a) the effect of a solid matrix (in the form of a membrane) in affecting the redox properties of entrapped electroactive systems, and (b) the role of an immobilized system in promoting the electrochemical activity of soluble metalloproteins, such as cytochrome *c*. Engineering a 'solid-state' promoter is of great potential value since, coupled to efficiency, this approach excludes complexities which may arise with soluble promoters, such as aspecific binding to the protein or undesired side-reactions.

Materials and methods

Fe(III)-protoporphyrin IX (hemin) and cytochrome *c* were obtained from Sigma Chem. Co (USA) and used without further purification. Cellulose triacetate was purchased from Fluka (Switzerland).

All the experiments were performed at neutral pH, in 20 mM phosphate buffer containing 0.1 M $NaClO_4$ as supporting electrolyte. The gelled cellulose triacetate membranes, obtained as previously described (Campanella et al. 1986), were kept in aqueous solution containing 0.4 mM hemin, for 24 h and then dried (for about 30 h) at 5° C. The membranes containing the entrapped hemin were then carefully washed and used.

Cyclic voltammetry was performed at 25° C in a glass microcell equipped with a calomel electrode (Ingold) as reference, and a Pt wire as counter electrode. A gold (Amel, 2 mm diameter) or a glassy carbon (Amel, 3 mm diameter) electrode were used as working electrodes.

Cyclic voltammograms of the entrapped hemin were run after fixing the membrane onto the working electrode by an O-ring and then dipping the electrode into the buffer solution. Oxygen was

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removed under mild stirring by a gentle flow of pure nitrogen kept just above the solution surface. Voltammograms were recorded after 60–90 min, in order to achieve full rehydration of the membrane and, thus, stable diffusion conditions ensuring reproducible voltammograms.

An Amel 473 multipolarograph equipped with an Amel 863 digital recorder was used for the voltammetric measurements.

Results and discussion

The *direct current* (dc) cyclic voltammograms at a glassy carbon electrode of protoporphyrin IX are illustrated in Fig. 1, either in solution (A) or entrapped (by physical immobilization) into a cellulose triacetate membrane (B). Soluble hemes display well shaped, quasi-reversible voltammograms over the whole scan rate range explored (20 to 200 mV/s, see Fig. 1A). At higher scan rates the reverse waves are less intense, indicating that only a fraction of the molecules undergoing the redox process is able to be reoxidized quickly; dimerization or some higher level of aggregation probably occurs in the system upon reduction. Well-shaped dc cyclic voltammograms are also obtained when Fe(III)-protoporphyrin IX is entrapped in a cellulose triacetate membrane (see Fig. 1B), indicating that immobilization into the solid matrix does not affect the electrochemical behaviour of the entrapped hemes. Similarly to observations on microperoxidase (Santucci et al. 1988; Brunori et al. 1989), dc voltammograms of entrapped Fe(III)-protoporphyrin IX display waves of similar shape and magnitude at both the forward and the reverse sweep, indicating that all the hemes reduced undergo re-oxidation. The voltammeric peak currents vary linearly with scan rate, showing that the process is diffusion-controlled (Nicholson and Shain 1964), in line with a high rehydration of the membrane. The peak-to-peak separation, ΔE_p, increases with the scan rate, the lowest value being 70 ± 7 mV at 20 mV/s, close to the theoretical value expected for a fully reversible one-electron reaction (Nicholson and Shain 1964; see also Bard and Faulkner 1980). The redox potential, $E_{1/2} = -107 \pm 7$ mV *vs* NHE, is consistent with the potentiometric value reported in the literature (Clark 1972), confirming that the membrane does not alter the redox properties of entrapped Fe(III)-protoporphyrin IX. Well defined dc cyclic voltammograms are also obtained at a gold electrode (not shown), although the electron transfer between protohemes (soluble or entrapped into the membrane) and the electrode surface is slow, as compared with the glassy carbon electrode.

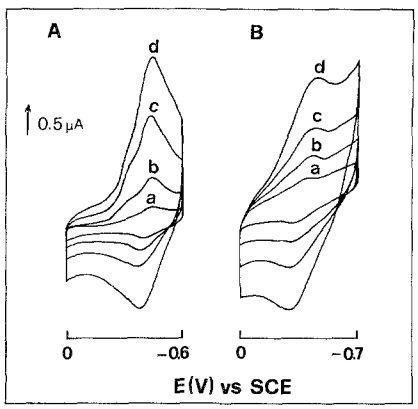

Fig. 1. Dc cyclic voltammetry of Fe(III)-protoporphyrin IX at a glassy carbon electrode, either in solution (**A**) or entrapped into a cellulose triacetate membrane (**B**). The buffer was 20 mM phosphate pH 7.0, containing 100mM $NaClO_4$ as supporting electrolyte. Scan rate: 20 mV/s (a); 50 mV/s (b); 100 mV/s (c); 200 mV/s (d)

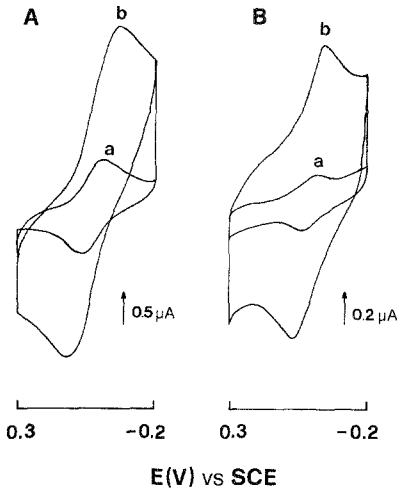

Fig. 2. Dc cyclic voltammograms of soluble cytochrome *c* at a glassy carbon (**A**) or gold (**B**) electrode, both in the presence of membrane-entrapped Fe(III)-protoporphyrin IX. The buffer was 20 mM phosphate pH 7.0, containing 100 mM $NaClO_4$ as supporting electrolyte. Scan rate: 20 mV/s (a); 200 mV/s (b)

We have also investigated the efficiency of entrapped Fe(III)-protoporphyrin IX in promoting the electrochemical activity of cytochrome *c* in solution. It is known that this protein displays very low or no electrochemical activity at naked glassy carbon or gold electrodes (Eddows and Hill 1979; Armstrong et al. 1986). The dc cyclic voltammograms of cytochrome *c* are shown in Fig. 2 at the glassy carbon (A) or gold (B) electrodes in the presence of membrane-entrapped hemin. The well defined, quasi-reversible voltammograms obtained at both electrodes, also at high scan rates (i.e. 200 mV/s), indicate that entrapped hemes promote very efficiently the electrochemical activity of cytochrome *c*. In accordance with previous data on other systems (Armstrong et al. 1982; Armstrong et al. 1985; Santucci et al. 1988; Brunori et al. 1989), we found that the peak-to-peak separation, ΔE_p, increases with scan rate. The smallest values (≈ 70 mV at both electrodes, sweep rate 20 mV/s) are close to the theoretical value for a fully reversible one-electron reaction [i.e. 57 mV, independent of the scan rate, at 25° C (Nicholson and Shain 1964)]. The calculated redox potential, $E_{1/2} \approx 260$ mV vs NHE, is in good agreement with previous data reported in the literature (Henderson and Rawlinson 1956; Hawkridge and Kuwana 1973; Eddows and Hill 1979). At present, a molecular mechanism to account quantitatively for this observation is not available. It may be suggested that the entrapped

hemes favour, through appropriate electrostatic interactions involving the lysines of cytochrome *c* and the propionates of hemin, the rapid adsorption and desorption of the macromolecules diffusing to the electrode. However, since we are not dealing with a single monolayer, the problem is more complex and demands additional investigations.

Concluding remarks and perspectives

The electrochemical behaviour of biological systems (such as metalloproteins) at several electrodes has been widely investigated in the recent years with interesting results, which have in part clarified the mechanism(s) controlling the rapid and reversible electron transfer between the protein and the electrode surface, and have pointed out the essential role of promoters.

However, in spite of the stimulating results, the problem is far from being fully understood and a deeper knowledge of the redox events occurring between proteins and the electrode surface demands further investigations. Engineering the surface of an electrode is an important task of great potential value, as illustrated in this paper. An electrode suitable for detection and analysis of redox proteins should satisfy, in fact, some fundamental requirements such as miniaturisation, long-term stability (when chemically modified), and high degree of selectivity. In this perspective, the use of membrane-entrapped compounds as promoters is pertinent and novel. As reported above, these systems are non-invasive, and thus avoid possible complexities related to aspecific binding or undesired side-reactions; moreover, the solid matrices employed are usually inert, allow entrapment also of molecules possessing high molecular mass, and the physical immobilization involves relatively simple methodologies. The results obtained using entrapped semi-biological compounds (such as Fe(III)-protoporphyrin IX described in this paper, or microperoxidase, the heme-peptide obtained from cytochrome *c*; see Brunori et al. 1989) indicate that these systems represent a promising example of simple 'solid-state' promoters successfully employed in voltammetric analysis of redox proteins in solution. Though preliminary, these results stimulate further investigations in order to obtain more complex membrane-entrapped systems which, coupling efficiency to good biological selectivity, may be of great significance (as, for example, long-life electrochemical detectors or voltammetric biosensors) for basic and applied bioelectrochemistry.

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