Biochem. J. (1989) 264, 301–304 (Printed in Great Britain)

Membrane-entrapped microperoxidase as a 'solid-state' promoter in the electrochemistry of soluble metalloproteins

Maurizio BRUNORI,*‡ Roberto SANTUCCI,* Luigi CAMPANELLA† and Gloria TRANCHIDA†
*C.N.R. Centro di Biologia Molecolare, Dipartimento di Scienze Biochimiche, and †Dipartimento di Chimica, Università di Roma 'La Sapienza', 00185 Roma, Italy

Immobilization of biological systems in solid matrices is presently of great interest, in view of the many potential advantages associated with both the higher stability of the immobilized macromolecules and the potential utilization for biotechnology. In the present paper the electrochemical behaviour of the undecapeptide from cytochrome *c* (called microperoxidase) tightly entrapped in a cellulose triacetate membrane is reported; its utilization as 'solid-state' promoter in the electrochemistry of soluble metalloproteins is presented. The results obtained indicate that: (i) membrane-entrapped microperoxidase undergoes rapid reversible electron transfer at a glassy carbon electrode; (ii) the electrochemical process is diffusion-controlled; (iii) entrapped microperoxidase acts as a 'solid-state' promoter in the electrochemistry of soluble cytochrome *c* and of azurin.

INTRODUCTION

Immobilization of biological systems on solid supports is presently of great interest, in view of several advantageous features such as the higher stability of the immobilized macromolecule or the potential use of membrane-entrapped biosystems. Recently [1] the report of the first successful example of direct electrochemistry of cytochrome *c* immobilized in a semi-rigid polymeric film has opened the way to a wider use of 'solid-state' voltammetry.

In the present paper we report on the electrochemical behavior of the undecapeptide from cytochrome *c* (called microperoxidase) entrapped in a cellulose triacetate membrane. In solution this haemopeptide undergoes direct (unmediated) electrochemical reaction at a glassy carbon electrode [2]. Information on the electrochemical behaviour of immobilized microperoxidase is of interest in view of the possible efficient utilization of this system as a 'solid-state' promoter for the voltammetry of soluble metalloproteins.

The results reported below indicate that: (*a*) membrane-entrapped microperoxidase undergoes rapid reversible electron transfer at a glassy carbon electrode in the absence of promoters; (*b*) the electrochemical process is diffusion-controlled, in agreement with a high degree of rehydration of the membrane; (*c*) entrapped microperoxidase acts as an efficient 'solid-state' promoter for the electrochemistry of soluble metalloproteins, such as cytochrome *c* and azurin.

MATERIALS AND METHODS

Experiments were carried out at neutral pH in 20 mM-sodium phosphate buffer containing 100 mM-$NaClO_4$ as supporting electrolyte. Microperoxidase was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or prepared by us from horse heart cytochrome *c* (type VI) (Sigma Chemical Co.) by the method of Harbury & Loach [3]. Azurin from *Pseudomonas aeruginosa* was kindly provided by Professor M.C. Silvestrini. An Amel 473 multipolarograph equipped with an Amel 863 recorder was used for the voltammetric measurements.

Microperoxidase was physically entrapped in a cellulose triacetate membrane (prepared as reported by Campanella *et al.* [4,5]) as follows: the membrane, in gelled form, was dipped into an aqueous solution containing 5 mg of the haemopeptide/ml at 5 °C for 24 h; then it was dried for about 30 h at 5 °C, and before use was carefully washed with distilled water until no more microperoxidase was released (as detected spectrophotometrically).

Electrochemical measurements were carried out by fixing, with an 'O'-ring, a 0.5 mm-thick membrane containing entrapped microperoxidase on to a microelectrode. The electrode was dipped into the electrochemical cell containing the appropriate buffer for voltammetry; the extent of release of microperoxidase in the buffer, always checked spectrophotometrically after each measurement, was found to be negligible. A glassy carbon electrode (3 mm diam.) or a gold electrode (2 mm diam.) was used for the voltammetric measurements.

RESULTS AND DISCUSSION

Fig. 1 shows the d.c. cyclic voltammograms of membrane-entrapped microperoxidase at a glassy carbon electrode, under the experimental conditions described above. A well-defined electrochemical behaviour is observed, indicating that a reversible process occurs at the electrode. The cathodic and the anodic peaks are symmetric and of similar magnitudes, with an i_{pa}/i_{pc} ratio about unity. Voltammetric peak currents vary linearly with the square root of the potential scan rate, indicating that the process is diffusion-controlled [6], in line with a

‡ To whom correspondence should be addressed.

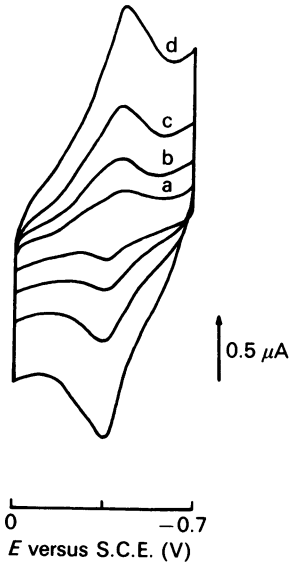

Fig. 1. D.c. cyclic voltammetry of membrane-entrapped microperoxidase (approx. 80 μM) at a glassy carbon electrode, in 20 mM-phosphate buffer, pH 7.0, containing 0.1 M-$NaClO_4$

Potential range: 0 to −0.7 V versus standard calomel electrode (S.C.E.). Sweep rate: 20 mV/s (a); 50 mV/s (b); 100 mV/s (c); 200 mV/s (d). Temperature: 25 °C.

high degree of rehydration of the cellulose triacetate membrane in aqueous media (see also Oliver *et al.* [1]). For a fully reversible one-electron transfer reaction, a peak separation of 57 mV (at 25 °C), independent of scan rate, is expected [6]. From the voltammograms obtained (see Fig. 1), a ΔE_p value of 62 ± 5 mV is determined, irrespective of the scan rate, in good agreement with theory. The calculated redox potential ($E_{\frac{1}{2}} \simeq -160$ mV versus the standard hydrogen electrode) is also in good agreement with previous results [2,7]. Comparison with the electrochemistry of microperoxidase in solution [2] shows that immobilization is without effect and indicates the higher reversibility of the entrapped haemopeptide in the voltammetric process.

It is well known that soluble metalloproteins, such as cytochrome *c* and azurin, give poorly resolved electrochemical behaviour at a naked glassy carbon electrode [8]. We have investigated the electrochemical behaviour of these two proteins after coating the microelectrode with a cellulose triacetate membrane, eventually containing entrapped microperoxidase or protoporphyrin IX.

The cyclic voltammograms shown in Figs. 2 and 3 illustrate some of the results. Essentially no response is observed at the naked glassy carbon electrode (Fig. 2*a*), in agreement with the literature [8]. When the electrode is coated with a cellulose triacetate membrane, the signal of soluble cytochrome *c* is clearly improved (Fig. 2*b*). This surprising observation may be attributed to a favourable charge effect (since the membrane and cytochrome *c* are both polarized but with opposite charge) and/or to a decrease of the diffusion coefficient and thus an increase of the signal-to-noise ratio (by reduction of the background current). When the cellulose triacetate membrane contains entrapped protoporphyrin IX (Fig. 2*c*) or microperoxidase (Fig. 3*a*), the voltammetric response of soluble cytochrome *c* is markedly enhanced, giving place to well-defined cyclic voltammograms characterized by symmetrically shaped cathodic and anodic peaks, with peak current ratios (i_{pa}/i_{pc}) near unity. The redox potential $E_{\frac{1}{2}}$ is +0.250 V versus the standard hydrogen electrode, which is in good agreement with the values from the literature [9–11]. Also, the calculated [12,13] values of the diffusion coefficient ($D_0 = 3 \times 10^{-6}$ cm^2/s) and of the heterogeneous electron-transfer rate constant ($k_s = 5 \times 10^{-3} \pm 1 \times 10^{-3}$ cm/s) are in good agreement with the data reported in the literature [14]. Therefore a membrane containing microperoxidase acts as a promoter, giving rise to well-shaped cyclic voltammograms and enhancing considerably the electron-transfer rate between the protein in solution and the

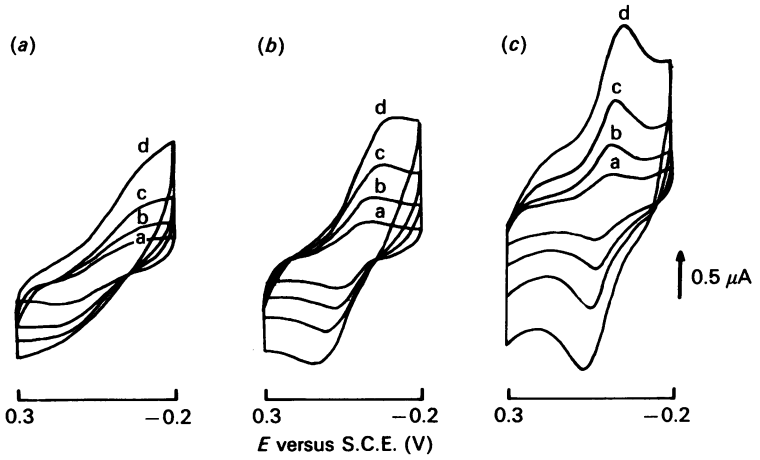

Fig. 2. D.c. cyclic voltammetry of cytochrome *c* at a glassy carbon electrode

(*a*) Naked electrode; (*b*) electrode coated with a cellulose triacetate membrane; (*c*) electrode coated with the same membrane but containing entrapped protoporphyrin IX. Sweep rates in (*a*): 5 mV/s (a); 10 mV/s (b); 20 mV/s (c); 50 mV/s (d). Sweep rates in (*b*) and in (*c*): 20 mV/s (a); 50 mV/s (b); 100 mV/s (c); 200 mV/s (d). Other experimental conditions were as for Fig. 1.

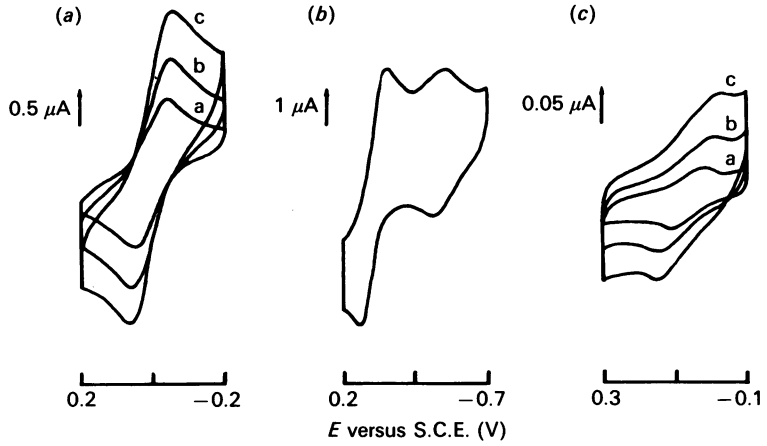

Fig. 3. D.c. cyclic voltammograms at a glassy carbon electrode of *(a)* and *(b)* cytochrome *c* (5 mg/ml) in the presence of membrane-entrapped microperoxidase and *(c)* azurin (5 mg/ml) in the presence of membrane-entrapped microperoxidase

Sweep rates in (*a*): 20 mV/s (a); 50 mV/s (b); 100 mV/s (c). Sweep rate in (*b*): 200 mV/s. Sweep rates in (*c*): 5 mV/s (a); 10 mV/s (b); 20 mV/s (c). Other experimental conditions were as for Fig. 1.

electrode surface. As shown in Fig. 3(*b*), the stable reversible d.c. cyclic voltammograms obtained over an extended potential range (+0.2 V to −0.7 V versus the standard calomel electrode) clearly indicate two distinct processes, with well-separated redox potentials characteristic of the two systems when examined independently.

The electrochemical behaviour of the copper protein azurin [15] was also investigated, with the use of a membrane containing entrapped microperoxidase. Azurin shows no activity at the naked glassy carbon electrode, in accordance with previous data [8]. However, a defined electrochemical response (see Fig. 3*c*) is obtained with the use of an electrode coated with a cellulose triacetate membrane containing entrapped microperoxidase. The electron transfer between the protein in solution and the electrode surface is fairly slow, as shown by the scan rate values necessary to obtain quasi-reversible cyclic voltammograms. The calculated heterogeneous electron-transfer rate constant is $k_s = 5 \times 10^{-5} \pm 2 \times 10^{-5}$ cm/s [12,13]. From the cyclic voltammograms shown in Fig. 3(*c*) we find that the ΔE_p value increases with the scan rate, as already observed for other proteins [16,17] (the lowest value of 80 mV being obtained at a scan rate of 5 mV/s). The calculated value for the redox potential $E_{\frac{1}{2}} = +0.300$ V versus the standard hydrogen electrode is not inconsistent with the potentiometric value reported in the literature [18] under similar experimental conditions (+0.330 V versus the standard hydrogen electrode at neutral pH).

The electrochemistry of cytochrome *c* in the absence and in the presence of entrapped microperoxidase was also investigated at a gold electrode. As expected, no measurable electrochemical activity is observed with the naked gold electrode (in the absence and in the presence of cellulose triacetate membrane); in contrast, a voltammetric response is detected in the presence of membrane-entrapped microperoxidase (results not shown). Well-shaped quasi-reversible voltammograms are in fact obtained (scan rates 5 to 20 mV/s), although the electron transfer between the protein and the electrode surface is slower as compared with the glassy carbon electrode.

The results reported above indicate that membrane-entrapped microperoxidase is characterized by a reversible and direct electrochemical behaviour, and (even more interesting) can be successfully employed in voltammetric investigations of cytochrome *c* and azurin in solution. The relatively simple methodology adopted to entrap microperoxidase, and the absence of alterations in the electrochemical behavior of the haemopeptide after immobilization, indicate that this system may be a promising example of a 'solid-state' promoter. Needless to say, the realization of an efficient and versatile 'solid-state' promoter, characterized by a certain degree of biological specificity, would be of great significance for basic and applied electrochemistry. Finally, the voltammetric experiments described above clearly underline the interesting features of cellulose triacetate membranes, which in combination with a high degree of chemical inertia show relatively low ohmic resistance and allow fast electron transfer at the electrode–membrane interface.

This work was partially supported by grants from the Ministero della Pubblica Istruzione of Italy and the University of Rome 'La Sapienza'. We express our thanks to Professor M. C. Silvestrini for the gift of azurin.

REFERENCES

1. Oliver, B. N., Egekeze, J. O. & Murray, R. W. (1988) J. Am. Chem. Soc. **110**, 2321–2322
2. Santucci, R., Reinhard, H. & Brunori, M. (1988) J. Am. Chem. Soc. **110**, 8536–8537
3. Harbury, H. A. & Loach, P. A. (1960) J. Biol. Chem. **235**, 3640–3645
4. Campanella, L., Tomassetti, M., De Angelis, G., Sammartino, M. P. & Cordatore, M. (1987) Clin. Chim. Acta **169**, 175–182
5. Campanella, L., Sammartino, M. P. & Tomassetti, M. (1986) Proc. Int. Meet. Chem. Sensors 2nd, Bordeaux, 580–583
6. Nicholson, R. S. & Shain, I. (1964) Anal. Chem. **36**, 706–723

7. Harbury, H. A. & Loach, P. A. (1959) Proc. Natl. Acad. Sci. U.S.A. **45**, 1344–1359
8. Armstrong, F. A., Hill, H. A. O. & Walton, W. J. (1986) Q. Rev. Biophys. **18**, 261–322
9. Henderson, R. W. & Rawlinson, W. A. (1956) Biochem. J. **62**, 21–29
10. Hawkridge, F. M. & Kuwana, T. (1973) Anal. Chem. **45**, 1021–1027
11. Eddows, M. J. & Hill, H. A. O. (1979) J. Am. Chem. Soc. **101**, 4461–4464
12. Nicholson, R. S. (1965) Anal. Chem. **37**, 1315–1355
13. Bard, A. J. & Faulkner, L. R. (1980) Electrochemical Methods: Fundamentals and Applications, pp. 215–231. John Wiley and Sons, New York
14. Ehrenberg, A. (1956) Acta Chem. Scand. **11**, 126–135
15. Ambler, R. P. (1963) Biochem. J. **89**, 341–349
16. Armstrong, F. A., Hill, H. A. O. & Walton, N. J. (1982) FEBS Lett. **145**, 241–244
17. Armstrong, F. A., Hill, H. A. O., Oliver, B. N. & Whitford, D. J. (1985) J. Am. Chem. Soc. **107**, 1473–1476
18. Adman, E. T. (1985) in Metalloproteins, part I (Harrison, P. M., ed.), pp. 1–42, Macmillan, London

Received 14 August 1989; accepted 20 September 1989