Direct electrochemistry of cytochrome P450cam

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The direct, unpromoted electrochemistry of cytochrome P450cam on an edge-plane graphite electrode and the conditions for the first electron transfer are reported.

The cytochrome P450cam monooxygenase system, which is native to the bacterium *Pseudomonas putida* grown on camphor, is an ideal system to study the regulation of electron transfer between two redox centres in separate protein molecules. Cytochrome P450cam catalyses the monooxygenation of D-(+)-camphor to give exclusively 5-*exo*-hydroxycamphor, for which an external source of two reducing equivalents is required. These electrons are transferred from NADH to the haem iron of P450cam through the combined action of the FAD–flavoprotein putidaredoxin reductase and the iron–sulfur protein putidaredoxin.[1,2] The mechanism of the first electron transfer step (ferri- to ferro-haem reduction) has been the subject of intense interest.[3–5] Dramatic changes observed in the haem optical spectrum upon camphor binding[6] have been shown by Mössbauer[7] and EPR[8] studies to be due to a shift in the spin-state of the ferrihaem iron, from low-spin in the absence of substrate to high-spin in the substrate-bound protein. In addition, it has been noted that this spin-state change upon camphor binding is accompanied by a redox potential change from −540 to −414 mV (*vs.* SCE).[4] This shift in the haem reduction potential is a key feature of the P450cam catalytic cycle, allowing the first electron transfer from putidaredoxin to occur and thus initiating catalytic turnover. Similar shifts upon substrate binding have also been observed with mammalian P450 enzymes.[9]

All previous redox studies on the P450cam system have been carried out using spectroscopic methods.[6,7,9] Herein we report for the first time the direct, unpromoted electrochemistry of recombinant P450cam on an edge-plane graphite (EPG) electrode. In the past decade there has been increasing interest in the direct electrochemistry of enzymes.[10,11] Without a doubt, cytochrome P450cam represents one of the most challenging enzymes yet studied. The achievement of its electrochemistry should allow the turnover of substrate to product.

The P450cam used in this study was expressed in *E. coli* and purified according to literature methods.[2,12] The direct electrochemistry of P450cam was investigated at 6 °C using cyclic voltammetry, under strictly anaerobic conditions (< 1.5 ppm oxygen) in order to prevent formation of the Fe^{II}–dioxygen complex and the possibility of a second electron transfer. We found that only freshly purified protein gave reliable and reproducible electrochemistry. The cyclic voltammogram of camphor-free P450cam on a bare EPG electrode (Fig. 1) shows two waves with a mid-point potential of −526(±11) mV *vs.* SCE. The camphor-bound form of the enzyme shows a similarly shaped response (Fig. 1) at −390(±10) mV. These potentials, and the shift in potential on camphor binding of 136 mV to a more positive value, are in reasonable agreement with earlier potentiometrically determined values of −540 and −414 mV respectively.[4]

The cathodic peak current exhibits a linear dependence on the square root of the scan rate ($v^{1/2}$) for camphor-bound P450cam, indicating that the overall process is diffusion controlled (Fig. 2). However, deviation from linearity was observed for the camphor-free enzyme. This may be caused either by adsorption to the electrode surface or by the fact that the heterogeneous electron transfer process is no longer diffusion controlled. The first of these is unlikely because the global structures of camphor-bound and camphor-free P450cam are very similar,[13] and thus both forms are expected to have similar affinities for the electrode surface. This therefore implies that the rate of heterogeneous electron transfer to the camphor-free form is slower than that to the camphor-bound form. This could be due to the relatively high reorganisation required to produce the five-coordinate, high-spin ferrous form from the six-coordinate, low-spin camphor-free enzyme, which entails the loss of the haem-bound water ligand. This requirement is similar to that for the reduction of myoglobin, which has been shown to have a high reorganisation energy compared to the reduction of cytochrome *c*, which has six-coordinate haem irons in both the

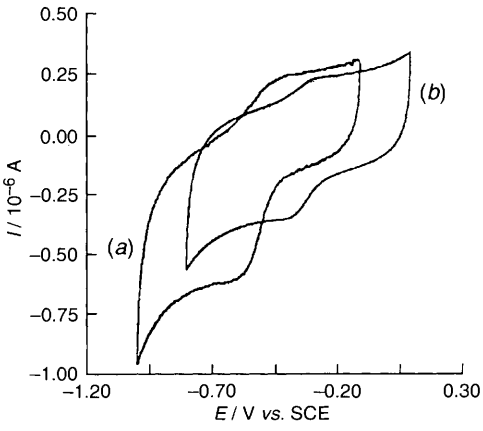

Fig. 1 Cyclic voltammograms on an edge-plane graphite electrode of (*a*) 15 μM cytochrome P450cam in 40 mM potassium phosphate buffer, pH 7.4, and (*b*) 18 μM cytochrome P450cam in 40 mM potassium phosphate buffer, pH 7.4, containing 1 mM D-(+)-camphor. Potential scan rate 50mV s^{-1}, temperature 6 °C and area of the working electrode 0.25 cm^2.

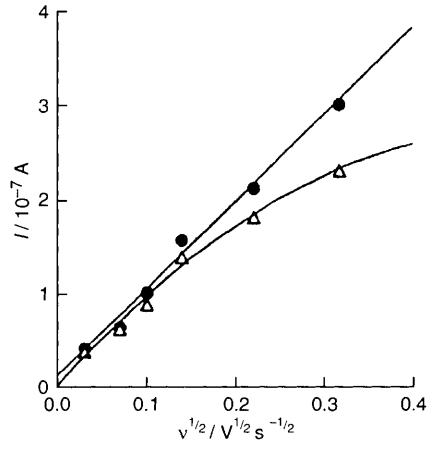

Fig. 2 The dependence of cathodic peak current on the potential scan rate for camphor-bound (●) and camphor-free (△) cytochrome P450cam. Conditions as in Fig. 1, except the temperature was 18 °C and working electrode area was 0.18 cm^2.

ferric and ferrous forms.[14] With the camphor-bound P450cam, both the ferric and ferrous forms of the enzyme have a five-coordinate, high-spin haem iron, and the reorganisation barrier to both homogeneous and heterogeneous electron transfer should be low.

The electrochemical response of P450cam at a bare, negatively charged EPG electrode strongly suggests that, despite the overall negative charge of the protein at pH 7.4 (p*I* = 4.55),[2] a specific pattern of positively charged surface amino acid residues favours an interaction between the enzyme and the electrode surface in such a way that heterogeneous electron transfer can take place. Computer modelling[15] and mutageneis studies[16,17] indicate that the basic residues Arg-72, Arg-112, Arg-364 and Lys-344 on the surface of P450cam interact with acidic residues on the surface of putidaredoxin in the complex between these two proteins. From the high resolution crystal structure of P450cam,[18] these surface residues are on the promixal side of the haem and in the region of the enzyme where the haem is closest (*ca.* 10 Å) to the surface.

Since the electron transfer rate constant generally decreases with donor–acceptor separation, it is reasonable to assume that this region on the surface of P450cam will form both the binding site for putidaredoxin and the region of interaction with the electrode. Thus we propose that these positively charged residues are also involved in the interaction between P450cam and the bare EPG electrode.

Having achieved the electrochemistry of cytochrome P450cam, it was obvious to attempt the electrochemically-driven conversion of substrate to product. We have observed the slow but catalytic oxidation of camphor to 5-*exo*-hydroxy-camphor in an electrochemical cell. The conditions are being optimised and full details will be published at a later date.

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References

1 *Cytochrome P450: Structure, Mechanism, and Biochemistry*, ed. P. R. Ortiz de Montellano, Plenum, New York, 1986.
2 I. C. Gunsalus and G. C. Wagner, *Methods Enzymol.*, 1978, **52**, 166.
3 S. G. Sligar, *Biochemistry*, 1976, **15**, 5399.
4 S. G. Sligar and I. C. Gunsalus, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 1078.
5 M. J. Hintz and J. A. Peterson, *J. Biol. Chem.*, 1981, **256**, 6721.
6 J. A. Peterson, *Arch. Biochem. Biophys.*, 1971, **144**, 678.
7 M. Sharrock, E. Munck, P. G. Debrunner, V. Marshall, J. D. Lipscomb and I. C. Gunsalus, *Biochemistry*, 1973, **12**, 258.
8 R. L. Tsai, C.-A. Yu, I., C. Gunsalus, J. Peisach, W. E. Blumberg, W. H. Orme-Johnson and H. Beinert, *Proc. Natl. Acad. Sci. USA*, 1970, **6**, 1157.
9 S. G. Sligar, D. L. Cinti, G. G. Gibson and J. B. Schenkman, *Biochem. Soc. Trans.*, 1979, **90**, 925.
10 F. A. Armstrong and A. M. Lannon, *J. Am. Chem. Soc.*, 1987, **109**, 7211.
11 L. H. Guo, H. A. O. Hill, G. A. Lawrence and G. S. Sanghera, *J. Electroanal. Chem.*, 1989, **266**, 279.
12 W. M. Atkins and S. G. Sligar, *J. Biol. Chem.*, 1988, **263**, 18842.
13 T. L. Poulos, B. C. Finzel and A. J. Howard, *Biochemistry*, 1986, **25**, 5314.
14 J. R. Winkler and H. B. Gray, *Chem. Rev.*, 1992, **982**, 369.
15 P. S. Stayton, T. L. Poulos and S. G. Sligar, *Biochemistry*, 1989, **28**, 8201.
16 P. S. Stayton and S. G. Sligar, *Biochemistry*, 1990, **29**, 7381.
17 K. Nakamura, T. Horiuchi, T. Yasukochi, K. Sekimizu, T. Hara, and Y. Sagara, *Biochim. Biophys. Acta* 1994, **207** 40.
18 T. L. Poulos, B. C. Finzel and A. J. Howard, *J. Mol. Biol.*, 1987, **195**, 687.

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