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Electrochemistry of $P450_{cin}$: new insights into P450 electron transfer

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Electrochemistry of bacterial cytochrome $P450_{cin}$ (CYP176A) reveals that, unusually, substrate binding does not affect the heme redox potential, although a marked pH dependence is consistent with a coupled single electron/ single proton transfer reaction in the range 6 < pH < 10.

The cytochromes P450 (P450s) comprise a superfamily of heme-thiolate proteins that are ubiquitous in Nature. They play essential roles in a variety of biosynthetic and biodegradative pathways and catalyse an impressive array of oxidative transformations.[1] These reactions utilise O_2 as the ultimate electron acceptor, and include the energetically demanding insertion of an O-atom into an unactivated C–H bond.

P450 mechanism continues to be a major research focus.[2] Protein crystallography has revealed a ferric heme *b* active site in the resting state of the enzyme bearing an axially coordinated cysteinate residue.[3] The 'distal' sixth coordination site may be occupied by a variety of ligands during catalysis (H_2O, OH^-, O_2 or O^{2-}) or it may be vacant. The mechanistic steps in P450 catalysis have been known for some time, based on the most thoroughly investigated example, the bacterial $P450_{cam}$ from *Pseudomonas putida*.[1]Most mechanistic investigations have focused on the generation and the nature of the reactive, high-valent, oxo-iron species and its interaction with substrate.[2] Much less attention has been given to the initial stages of the reaction cycle *e.g.* substrate binding and heme reduction, although it has been proposed that they are important in controlling catalysis.[1] Reduction to ferrous P450 in the absence of substrate, but in presence of O_2, has the potential to consume electrons (and energy) and release toxic reactive oxygen species such as $O_2^{-\cdot}$ and H_2O_2. The origin of selective substrate oxidation in preference to simple O_2 reduction in P450 catalysis remains poorly understood.

Some time ago, it was reported[4] that ferric $P450_{cam}$ undergoes a low-to-high spin transformation in its electronic ground state upon substrate binding, and subsequently this has been found to be a characteristic feature of many other P450s. Substrate binding in $P450_{cam}$ was accompanied by an anodic shift from −300 to −173 mV ($E_{m,7.0}$, *vs* NHE) in the $Fe^{III/II}$ redox couple as determined by potentiometry[5] and later by cyclic voltammetry (CV).[6–10] Similar substrate-dependent anodic shifts were found for the bacterial $P450_{BM3}$[11] and a limited number of mammalian P450s.[12]This gave support to the hypothesis[5] that a substrate-induced thermodynamic switch was operative in raising the heme potential to a value above that of its natural electron donor. This model offered an attractive explanation of the tightly coupled substrate hydroxylation activity of these enzymes; ferrous P450 cannot be generated in the absence of substrate thus futile reduction of O_2 to superoxide or peroxide is avoided.

Herein we report an electrochemical study of the recently isolated bacterial $P450_{cin}$ (CYP176A)[13] that raises important questions regarding P450 electron transfer and catalysis. $P450_{cin}$ hydroxylates the hydrophobic monoterpene 1,8-cineole, and substrate saturation induces a characteristic ferric P450 low-to-high spin conversion as reflected in a hypsochromic shift in the Soret maximum. In this sense, $P450_{cin}$ resembles other bacterial P450s yet, as we will demonstrate, its electrochemical properties are unique.

Potentiometry was performed within the pH range 6 to 9 employing dithionite as reductant and 2-hydroxynaphthoquinone and benzyl viologen as mediators using standard procedures.[14] The titrations were chemically reversible in phosphate buffer by reoxidation with ferricyanide. CV experiments were performed across the pH range 5 to 10, with the enzyme immobilized within a didodecyldimethylammonium bromide (DDAB) surfactant film cast onto an edge-plane pyrolytic graphite (EPG) working electrode as described.[7,15] The potentiometrically determined $P450_{cin}$ $Fe^{III/II}$ redox potentials (E_m, Fig. 1) are essentially unaffected by substrate binding, and exhibit a pH dependence of −59 mV/pH unit both in the presence and absence of cineole. Totally reversible single electron responses were also observed with CV (Fig. 2), and the

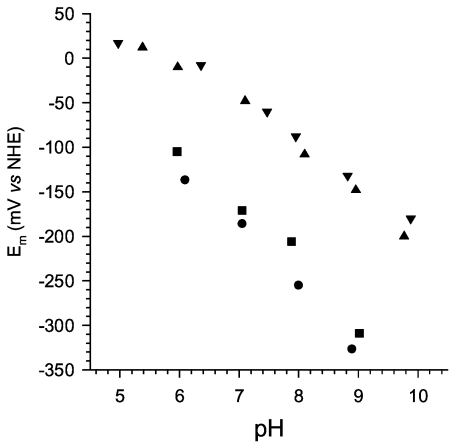

Fig. 1 Midpoint potentials of $P450_{cin}$ determined by CV (▲ substrate free and ▼ substrate bound) and potentiometry (● substrate free and ■substrate bound) as a function of pH.

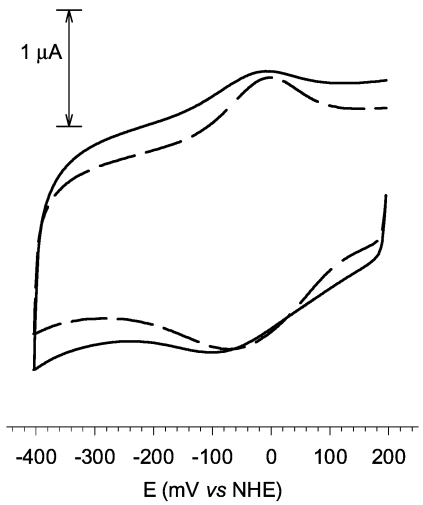

Fig. 2 Cyclic voltammograms for $P450_{cin}$ in the presence (broken curve) and absence of cineole. (Sweep rate 50 mV s^{-1}; working electrode EPG modified with a DDAB surfactant film, pH 7.4).

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same −59 mV/pH unit dependence of the redox potentials was seen.† This pH dependence is unambiguous evidence for a coupled single electron/single proton transfer reaction independent of substrate. There is no suggestion of a break in the E_m/pH profile at the high-pH end of this range, which indicates that reduction to ferrous $P450_{cin}$ liberates a strong base (pK_a > 10) at the heme active site.

Despite a complete low-to-high spin conversion of ferric $P450_{cin}$ upon addition of cineole, the heme redox potential is unchanged. A correlation between the position of the high spin/low spin equilibrium and the heme redox potential was identified in $P450_{cam}$ complexed with a range of substrates.[16] However, redox potentials are dependent upon the energies of both the oxidised and reduced forms of the couple, whereas the position of the spin equilibrium considers only the ferric form of the enzyme. Therefore, this correlation does not have a firm theoretical basis. In contrast, shifts in redox potential (ΔE) upon *complex formation* are well understood, and reflect differential binding of the substrate (*S*) between the oxidised and reduced forms of the enzyme (eqn. 1)[17]

$$\Delta E = \frac{RT}{F}\ln\left[\frac{1+K_{II}[S]}{1+K_{III}[S]}\right] \quad (1)$$

where K_{II} and K_{III} are the enzyme : substrate formation constants in the ferrous and ferric forms, respectively. In $P450_{cin}$, the absence of a shift merely indicates that ferric and ferrous $P450_{cin}$ have an equal affinity for cineole, whereas the anodic shifts seen in other P450s reflect tighter substrate binding by the reduced form of the enzyme.

The paradigm of a substrate induced gating of the heme redox potential has not been universally embraced. It has been proposed[18] that a redox based thermodynamic gate in P450s is practically flawed as, *in vivo*, the P450 redox potential is coupled to O_2 binding by the ferrous heme, thus raising its apparent redox potential (eqn 1, with O_2 as the substrate and $K_{II} \gg K_{III}$) and over-riding any substrate-induced effects. Kinetic studies have also shown that P450 reduction may occur equally rapidly in the absence or presence of substrate.[19] Our data reveal no shift in the $P450_{cin}$ redox potentials accompanying substrate binding, so some other mechanism must clearly be operative to prevent futile cycling of electrons in the absence of substrate in the tightly coupled $P450_{cin}$, and presumably other P450s.

The $P450_{cin}$ redox potential pH dependence (Fig. 1) suggests a hydroxo ligand in the distal coordination site of the ferric heme, which undergoes protonation and then dissociation upon reduction.‡ We presume that the ensuing five-coordinate ferrous $P450_{cin}$ has a structure similar to that identified crystallographically in ferrous $P450_{cam}$.[20] An amino acid residue is unlikely to be responsible for the observed pH dependence as the more than five orders of magnitude change in K_a accompanying reduction is difficult to reconcile unless the base is coordinated to the metal. Although the redox potential pH dependence of $P450_{cam}$[21] is similar to that seen here, the site of protonation in $P450_{cam}$ upon reduction remains to be explained.

The presence of the *same* pH profile in substrate free and substrate bound ferric $P450_{cin}$ indicates that *both* forms are six-coordinate hydroxo-Fe^{III} complexes. Although binding of substrate influences the position of the high-spin/low-spin equilibrium in P450s, it does not follow that the distal sixth ligand is lost in forming high spin ferric heme. In $P450_{cam}$, crystallography has indicated that the low-to-high spin transformation (upon binding camphor) is accompanied by dissociation of the axially bound aqua ligand.[20] However, there are also several crystallographically characterized examples involving $P450_{cam}$ where a high-spin (substrate bound), six-coordinate heme has been identified.[22] That is, coordination number and spin state are not necessarily coupled. In the absence of variations in coordination number, the change in electronic ground state in P450s can be explained by subtler outer-sphere interactions related to changes in local dielectric constant and the electric field of the protein upon substrate binding.[23]

Finally, the substrate free redox potential for $P450_{cin}$ ($E_{m,7.4}$ −182 mV) is atypically high for a P450. Kassner proposed that a major contributing factor to heme cofactor redox potentials was the dielectric constant of the active site;[24] hydrophobicity inducing a more positive redox potential. This model has been supported by subsequent experimental and theoretical studies.[25] Modeling studies of $P450_{cin}$ based on its known amino acid sequence predict that the distal coordination environment is more hydrophobic than seen in other bacterial P450s[3] and the relatively high potential redox couples with and without substrate are consistent with this concept.

In conclusion, we have shown that substrate induced redox potential shifts may be completely absent in P450 chemistry, the ferric heme electronic ground state and coordination number are not coupled and heme protonation in $P450_{cin}$ accompanies reduction. The questions raised by these results concerning the mechanism by which P450s oxidise substrates in preference to O_2 reduction will be addressed in future work.

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Notes and references

† The apparent *ca.* 120 mV anodic shift in the CV determined potentials relative to those derived from potentiometry has been seen elsewhere[7] and attributed to a combination of protein–lipid interactions and lipid-dependent double layer effects. Broadening of the CV responses was also seen in $P450_{cam}$ under the same conditions as employed here, and attributed to a dispersion of apparent redox potentials that result from various protein orientations at the electrode surface.[7]

‡ In contrast, ENDOR and ESEEM spectroscopic studies suggest that an aqua ligand is present in ferric, substrate-free $P450_{cam}$ at pH 7.5.[26]

1 P. R. Ortiz de Montellano, *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd edn., 1995.
2 P. R. Ortiz de Montellano and J. J. De Voss, *Nat. Prod. Rep.*, 2002, **19**, 477.
3 T. L. Poulos, *Curr. Opin. Struct. Biol.*, 1995, **5**, 767.
4 M. Sharrock, E. Muenck, P. G. Debrunner, V. Marshall, J. D. Lipscomb and I. C. Gunsalus, *Biochemistry*, 1973, **12**, 258.
5 S. G. Sligar and I. C. Gunsalus, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 1078.
6 J. Kazlauskaite, A. C. G. Westlake, L.-L. Wong and H. A. O. Hill, *Chem. Commun.*, 1996, 2189.
7 Z. Zhang, A.-E. F. Nassar, Z. Lu, J. B. Schenkman and J. F. Rusling, *J. Chem. Soc., Faraday Trans.*, 1997, **93**, 1769.
8 Y. M. Lvov, Z. Lu, J. B. Schenkman, X. Zu and J. F. Rusling, *J. Am. Chem. Soc.*, 1998, **120**, 4073.
9 J. F. Rusling, L. Zhou, B. Munge, J. Yang, C. Estavillo and J. B. Schenkman, *Faraday Discuss.*, 2000, **116**, 77.
10 K. K.-W. Lo, L.-L. Wong and H. A. O. Hill, *FEBS Lett.*, 1999, **451**, 342.
11 S. N. Daff, S. K. Chapman, K. L. Turner, R. A. Holt, S. Govindaraj, T. L. Poulos and A. W. Munro, *Biochemistry*, 1997, **36**, 13816.
12 Y.-Y. Huang, T. Hara, S. G. Sligar, M. J. Coon and T. Kimura, *Biochemistry*, 1986, **25**, 1390.
13 D. B. Hawkes, G. Adams, A. Burlingame, P. R. Ortiz de Montellano and J. J. De Voss, *J. Biol. Chem.*, 2002, **277**, 27725.
14 P. L. Dutton, *Methods Enzymol.*, 1978, **54**, 411.
15 K.-F. Aguey-Zinsou, P. V. Bernhardt, A. G. McEwan and J. P. Ridge, *J. Biol. Inorg. Chem.*, 2002, **7**, 879.
16 M. T. Fisher and S. G. Sligar, *J. Am. Chem. Soc.*, 1985, **107**, 5018.
17 W. M. Clark, *The Oxidation-Reduction Potentials of Organic Systems*, The Williams and Wilkins Company, 1960.
18 M. J. Honeychurch, H. A. O. Hill and L.-L. Wong, *FEBS Lett.*, 1999, **451**, 351.
19 F. P. Guengerich and W. W. Johnson, *Biochemistry*, 1997, **36**, 14741.
20 I. Schlichting, J. Berendzen, K. Chu, A. M. Stock, S. A. Maves, D. E. Benson, R. M. Sweet, D. Ringe, G. A. Petsko and S. G. Sligar, *Science*, 2000, **287**, 1615.
21 S. G. Sligar and I. C. Gunsalus, *Biochemistry*, 1979, **18**, 2290.
22 R. Raag and T. L. Poulos, *Biochemistry*, 1991, **30**, 2674.
23 D. Harris and G. Loew, *J. Am. Chem. Soc.*, 1993, **115**, 8775.
24 R. J. Kassner, *J. Am. Chem. Soc.*, 1973, **95**, 2674.
25 A. K. Churg and A. Warshel, *Biochemistry*, 1986, **25**, 1675.
26 D. Goldfarb, M. Bernardo, H. Thomann, P. M. H. Kroneck and V. Ullrich, *J. Am. Chem. Soc.*, 1996, **118**, 2686.