Flavocytochrome P-450 BM3: a paradigm for the analysis of electron transfer and its control in the P-450s


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Introduction

The cytochromes P-450 (P-450s) are a 'superfamily' of haem b-containing oxidase proteins [1,2], which catalyse an array of oxidative reactions with a plethora of organic substrates. P-450 enzymes are found throughout Nature, from organisms as simple as bacteria (and archaeons [3]) to higher eukaryotes. The P-450s have enormous biotechnological potential because they catalyse the controlled activation of O₂, with the potential for stereospecific and regiospecific insertion of oxygen atoms into organic molecules. This potential has already been realized with the use of the P-450s in the commercial manufacture of steroids [4], and the process is continually being developed and improved [5].

Mammals have numerous forms of membrane-bound P-450 that catalyse various forms of reaction (e.g. hydroxylation, epoxidation, N-oxidation and reductive dehalogenation) [6] and are vital to a number of physiological processes, including steroid syntheses and interconversions, the manufacture of eicosanoid derivatives for cellular signalling, and xenobiotic detoxification. The importance of mammalian hepatic P-450s in the metabolism of drugs is a subject of great importance to the pharmaceutical industry. The P-450s, the so-called 'phase I' enzymes, represent a 'first line of defence' when the body is exposed to xenobiotics, and the multiple hepatic forms of P-450 catalyse oxidative reactions on thousands of drugs and other foreign chemicals to which the body is exposed. Examples are the oxidations of ethanol, aspirin, chloroform and polycyclic aromatic hydrocarbons. These oxidations are often designed to increase the water-solubility of xenobiotics to facilitate their excretion directly, or to provide functional groups for recognition by 'phase II' drug-metabolizing enzymes, such as glutathione S-transferases or UDP-glucuronyl transferases. However, factors such as industrial pollution and the massive development of the pharmaceutical industry in the past century has meant that we have been exposed to a vast number of new chemicals that can act as substrates for the P-450s (many forms of mammalian P-450 are rather non-specific). It is now well recognized that the P-450s are capable of converting a number of compounds into more dangerous, even genotoxic, derivatives. Examples are the epoxidations of benzo[a]pyrene (P-450 1A1) and aflatoxin B1 (P-450 3A4) [7]. It is ironic that an enzyme system that evolved to defend the body against harmful organic compounds can also be tricked into acting against it, due to the rate of human evolution of new chemicals outstripping the genetic evolution of the P-450s.

Although there is intense medical and pharmaceutical interest in the mammalian P-450s, the expression and study of the structural properties of these enzymes is hampered by the fact that they are integral membrane proteins (as are their redox partners). However, the bacterial P-450s are soluble; this has simplified the overexpression and purification of these forms [8]. In many respects, the entire P-450 field has been led forward by the advances made through kinetic, spectroscopic and structural analysis of a small number of bacterial forms. There are currently six atomic structures available for bacterial P-450s [9-14]. For the two most important of these enzymes (P-450cam and P-450 BM3), the structures of both substrate-bound and free forms have been determined [9,10,15,16]. The camphor hydroxylase P-450cam from Pseudomonas putida has been one of the most intensely studied of all enzymes over the last quarter of a century [17]; the analysis of this enzyme has provided us with most of our knowledge on the structure and mechanism of P-450. However, in the past 5-10 years there has been enormous interest in the characterization of the fatty acid hydroxylase P-450 BM3 from Bacillus megaterium [18]. This shift in emphasis results from the realization that P-450 BM3 uses a simi-

Abbreviations used: hq, hydroquinone; ox, oxidized; sq, semiquinone.

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lar reductase system to that of most eukaryotic P-450s, and to its homology with the nitric oxide synthases [8,19].

**Flavocytochrome P-450 BM3: an important model enzyme**

The P-450s can be divided conveniently into two major classes, based on the type of redox systems used. In class I (or B-class), the P-450 is the terminal component of a three-protein electron transport chain, receiving electrons one at a time from either a small FMN-containing flavodoxin or an iron-sulphur ferredoxin. Typical of this class are eukaryotic mitochondrial forms and the vast majority of bacterial P-450s (e.g. P-450cam).

In class II (or E-class) P-450s, the P-450 is reduced by a single NADPH-dependent FAD- and FMN-containing P-450 reductase. The hepatic drug-metabolizing P-450s are good representatives of the class II system ([8]; see also Kirill Degtyarenko’s web site, www.icgeb.trieste.it/p450/).

P-450 BM3 (CYP 102A1) is a special example of the class II system (see accompanying article in this colloquium by Chapman et al.) and has many intriguing properties. P-450 BM3 has the highest mono-oxygenase activity reported for any P-450 (more than 4500 min⁻¹ with palmi-
toate and pentadecanoate) and catalyses the NADPH-dependent hydroxylation (usually at the ω-1 to ω-3 positions) of a wide range of satu-
rated and unsaturated fatty acids, alcohols and amides (from approx. C₁₂ to C₃₀) [8]. Expression of the gene (cyp102A1) is inducible by barbitu-
rates and certain fatty acids and is controlled at the transcriptional level by both positive and negative regulatory DNA-binding proteins [21].

P-450 is soluble and uses a similar redox partner to that of the mammalian drug-metabolizing forms (a diflavin P-450 reductase). This reduct-
ase (C-terminal) is also fused to the P-450 (N-terminal) via a short peptide linker; a similar arrangement as seen in the nitric oxide synthases [19]. The gene has been cloned and over-
expressed in *Escherichia coli*, and the flavocytochrome has been purified to homogeneity [22].

More recently, the PCR-generated subgenes encoding the isolated P-450 (haem) and reduct-
ase (diflavin) domains were also overexpressed and the proteins purified, as were the ferredoxin reductase-like and flavodoxin-like subdomains of the diflavin domain [18,23]. The atomic structures of the substrate-free and substrate (palmi-
toate)-bound forms of the haem domain of P-450 BM3 have been determined [10,16]. These structures have revealed targets for rational site-
directed mutagenesis aimed at the determination of the roles of active-site amino acids. Such studies have identified determinants of haem binding and haem iron spin-state control (Trp-
96 [24]), of regiospecificity of substrate oxidation (Phe-87 [25]) and of fatty acid ligation at the active site (Arg-47 [26]) (Figure 1). Our recent studies have shown that there is conformational communication between the haem and diflavin domains of P-450 BM3. Binding of NADP⁺ to its site on the diflavin domain induces a structural change in the haem domain that results in a marked increase in affinity for substrate-like inhibitor compounds [27].

**Redox chemistry of the P-450s**

In P-450 BM3 and other P-450s, mono-oxygenation of substrate is achieved by two successive one-electron transfers to the haem from the redox partner (Scheme 1). The first transfer (reducing Fe³⁺ resting haem to the Fe²⁺ state) occurs after substrate binds at the P-450 active site. After reduction, O₂ binds to the Fe²⁺ haem to form 'oxy-P-450'. Further reduction by a second electron from the redox partner allows the completion of the P-450 catalytic cycle, although intermediates after the second electron transfer to oxy-P-450 remain to be identified. These intermediates are so short-lived that they have been impossible to isolate spectroscopically. However, a likely model involves the second reduction leading to the formation of a ferric peroxo species. Two protons, probably from solvent water, are then delivered to the active site and one atom of oxygen is lost to form water. This leaves a high-valency iron-oxy species, possibly Fe³⁺=O, which can be stabilized by a number of resonance structures involving the haem ring and the oxygen. This compound then abstracts a hydrogen atom from bound substrate to form a substrate radical and this, in turn, collapses via an oxygen rebound mechanism to generate ferric P-450 and oxidized product [17]. The product then dissociates to regenerate the resting P-450 form (Scheme 1).

The catalytic cycle of various P-450s can be driven artificially and by non-physiological protein redox partners. Oxygen-donor molecules such as H₂O₂ and alkyl hydroperoxides can provide both the oxygen and electrons required for P-450 function [28]. It has also proved possible
to drive \( P-450 \) BM3-catalysed and mammalian \( P-450 \)-catalysed oxidations with reasonably good efficiency via an electrode [29]. In the past few years, breakthroughs have been made in the expression of mammalian \( P-450 \) s in \( E. \) coli. This has led to the discovery that the \( E. \) coli NADPH-flavodoxin reductase and flavodoxin electron transport system can support the activity of heterologously expressed \( P-450 \) s [30]. This occurs through a Ping Pong mechanism in which the flavodoxin acts as a single-electron shuttle between the flavodoxin reductase and the \( P-450 \) [31].

The ability to overexpress and purify the component domains and subdomains as well as the intact flavocytochrome has simplified the study of the redox properties of \( P-450 \) BM3. We have analysed these properties by using techniques such as stopped-flow absorbance spectrophotometry, EPR and redox potentiometry to understand the electron transfer processes and their mechanisms of regulation.

**Electron transfer and its control in flavocytochrome \( P-450 \) BM3**

How does the electron transfer pathway through \( P-450 \) BM3 operate and how is electron transfer to the haem iron regulated to avoid oxygen reduction (and hence toxic radical production) in the absence of substrate? We have examined this problem by using stopped-flow kinetics to examine individual steps in the catalytic process, and potentiometric titrations to determine the reduction potentials of the flavin and haem cofactors (Figure 2). The availability of the isolated domains (particularly the flavin-containing domains) proved vital to deconvolute the complex spectral changes observed during anaerobic reductive and oxidative titrations of intact flavocytochrome \( P-450 \) BM3, changes that (at certain

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**Figure 1**

*Organization of key amino acid residues at the active site of substrate (palmitoleate)-bound \( P-450 \) BM3 [16]*

The active site is a long channel extending from the protein surface to the haem, lined with hydrophobic residues (e.g. Leu-437, Leu-181 and Met-354). Cys-400 is the proximal haem iron ligand [10]. The side chain of Trp-96 is in hydrogen-bonding distance to a haem propionate and is important in maintaining haem iron in the low spin state [24]. Just above the haem plane is the side-chain of Phe-87, which interacts with the \( \alpha \)-terminus of fatty acid substrates and prevents \( \alpha \)-oxidation [25]. At the mouth of the binding channel, the side-chains of Arg-47 and Tyr-51 stabilize the carboxy group of the fatty acid by electrostatic and hydrogen-bonding interactions respectively (26), and M. A. Noble, C. S. Miles, S. K. Chapman, G. A. Reid, R. P. Hanzlik and A. W. Munro, unpublished work). The phenyl side chain of Phe-42 'caps' the channel and is critical for efficient fatty acid oxidation (M. A. Noble, C. S. Miles, S. K. Chapman, G. A. Reid, R. P. Hanzlik and A. W. Munro, unpublished work).
Schematic representation of the catalytic cycle of flavocytochrome P-450 BM3

Electrons are represented as filled circles. Two electrons are derived ultimately from NAD(P)H and are transferred (as a hydride) to the FAD (dehydrogenation). Electrons are passed to the FMN, which acts as a shuttle, passing electrons one at a time to the haem iron (electron transfer). Electrons cannot pass from FMN to haem until fatty acid substrate (SH) is bound, owing to SH-induced elevation of the reduction potential of the P-450 BM3 haem iron. Thereafter, one electron is transferred to form ferrous haem, which binds O₂. A second electron is passed to this higher potential oxy-P-450 species; hydroxylation of substrate follows (mono-oxygenation). Dissociation of product (oxygenated substrate [SO₂H]) regenerates the resting state, ferric P-450. The cycle is regulated positively by a redox switch (induced by binding of SH to the P-450), and also negatively by the accumulation of FMN hydroquinone, which occurs when [NADPH] ≫ [SH].

Dehydrogenation Electron transfer Monoxygenation

The reductant NADPH transfers two electrons (as a hydride ion) to the FAD of the P-450 BM3 reductase. The flavin reduction rate is biphasic, with a ‘fast’ phase of 758 s⁻¹ and a ‘slow’ phase of 118 s⁻¹ at 25°C [32]. The midpoint potential for this two-electron transfer [FAD oxidized/hydroquinone (ox/hq) = −332 mV] is close to that of NADP⁺/H itself (−320 mV) but the reduction potentials for both the first [FMN oxidized/semiquinone (ox/sq) = −213 mV] and second [FMN semiquinone/hydroquinone (sq/hq) = −193 mV] electron transfers to the FMN are much more positive, and one or both electrons are transferred to this flavin [33]. The rate of electron transfer between flavins (measured by the formation of a blue flavin semiquinone at 600 nm) is very fast: 452 s⁻¹ at 25°C compared with the rate of fatty acid hydroxylation (only approx. 77 s⁻¹ at 30°C for pentadecanoate [34]). Thus electron transfers through the flavins of P-450 BM3 seem unlikely to limit fatty acid oxidation to any significant extent. Indeed, it has been reported that the reduction of ferricytochrome c (via the FMN of P-450 BM3) can occur simultaneously with the hydroxylation of myristic acid, without affecting the rate of either process [35]. In the absence of fatty acid, NADPH converts P-450 BM3 to a three-electron reduced state. The FAD sq/hq couple is approx. 50 mV more negative than the midpoint potential of NADP⁺/H and the FAD remains as a neutral blue semiquinone [33]. This is confirmed by EPR measurements of NADPH-reduced P-450 BM3 [32]. However, during the oxidation of tetradecanol (a ‘slow’ substrate; kₗ ≈ 730 min⁻¹) two flavin semiquinones are detected (one neutral, one anionic) by EPR. This suggests that, during steady-state fatty acid oxidation, the diflavin domain of P-450 BM3 (which can hold up to four electrons) cycles between reduction states 0, 2, 1 and 0 and that the binding of NADP⁺ results in the stabilization of the two-semiquinone state [36]. This might be achieved through the modulation (i.e. increase) of the ox/sq and sq/hq reduction potentials of the FAD of P-450 BM3 by the binding of oxidized nucleotide. We have recently observed such a phenomenon induced by the binding of adenosine 2’-monophosphate to the E. coli FLDR enzyme [31]. In the absence of fatty acid substrate, the three-electron-reduced state of the flavins accumulates and this leads to a time-dependent irreversible inactivation of the activity of P-450 BM3, probably through a slow
conformational change [34,37]. It is tempting to speculate that this is a regulatory mechanism by which the enzyme can 'switch off' and avoid wasteful NADPH oxidation if fatty acid substrate is in short supply [38].

Reduction of the P-450 BM3 haem iron cannot occur until fatty acid is bound at the active site. Binding of fatty acid induces a low-spin to high-spin shift in the P-450 haem iron and results in a change in the visible absorbance maximum (the Soret band) from 419 to 390 nm. Binding and spin-shift are extremely fast (more than 800 s⁻¹ at 25°C). The best substrates [e.g. arachidonate or palmitate (Figure 2)] convert most (more than 80%) of the resting low-spin P-450 haem iron (S = 1/2) to the high-spin form (S = 5/2). The spin-state conversion is achieved by substrate-dependent dehydration of the active site, specifically the displacement of the sixth ligand to the haem iron (a water molecule). Accompanying the spin-state shift is an elevation of the reduction potential of the haem iron of up to 130 mV (Figure 2) [33]. An increase of similar magnitude has been observed for the binding of camphor to P-450cam [39]. It seems that the spin-state of P-450 BM3 is poised deliberately close to the equilibrium between the low-spin and high-spin forms, such that removal of the sixth water ligand by substrate shifts the equilibrium strongly in favour of the high-spin form and acts as a redox 'switch' that opens the gate for electron transfer to the haem iron when substrate is bound [17,33]. This elegant mechanism avoids the production of harmful superoxide/H₂O₂ in the absence of fatty acids.

As shown in Figure 2, the reduction potential of P-450 BM3 haem iron increases to such an extent that the equilibration of electrons from FMN becomes feasible. The rate of the first electron transfer to the fatty acid-bound haem is dependent on the nature of the substrate. For instance, rates of 223 s⁻¹ (with myristate) and 130 s⁻¹ (with laurate) were measured at 25°C by stopped-flow kinetics, after the formation of the CO-adduct of ferrous P-450 BM3 at 450 nm [32]. During catalysis, the ferrous iron binds O₂ rapidly (Scheme 1) and this species is further reduced by a second electron transfer from FMN. It is likely that the reduction potential of the ferrous 'oxy-P-450' is considerably more positive than that of the ferric substrate-bound form, and that the second electron transfer occurs almost simultaneously [32,33]. Subsequent steps leading to product formation and the regeneration of ferric P-450 BM3 occur too fast to be measured by stopped-flow techniques. P-450 BM3 is a very well evolved enzyme and it is difficult to pinpoint a single rate-limiting step for the wild-type protein. However, the fact that the first FMN-to-haem electron transfer becomes rate-limiting in a number of active-site mutants suggests that this is the key step (M. A. Noble, C. S. Miles, S. K. Chapman, G. A. Reid, R. P. Hanzlik and A. W. Munro, unpublished work).

Figure 2

Comparison of the individual reduction potentials for the FMN, FAD (in the diflavin domain) and haem iron (in the P-450 domain) of flavocytochrome P-450 BM3

Values are relative to the normal hydrogen electrode. The midpoint potential for NADPH (hatched bar: - 320 mV) is close to that for the two-electron reduction of the FAD at - 312 mV (FAD₄ (ox/sq) = - 292 mV, FAD₄ (sq/hq) = - 372 mV). However, after the transfer of two electrons (as a hydride ion) to the FAD, electrons can be readily transferred to the FMN. Both the redox couples for the FMN are much more positive than those for FAD [FMN₋₋₁ (ox/sq) = - 213 mV, FMN₋₋₁ (sq/hq) = - 193 mV]. In the absence of fatty acid substrates, the enzyme can be reduced only to a three-electron form, because the FAD sq/hq and substrate-free haem Fe⁺/Fe²⁺ couples are much more negative than that of NADPH. Binding of fatty acid substrates palmitate (palm) or arachidonate (arac) to the P-450 elevates the reduction potential of the haem iron by approx. 130 mV (dependent on the extent of low-spin to high-spin haem iron shift that is induced), permitting the equilibration of electrons from the reduced FMN and initiating the P-450 catalytic cycle [33].

Future prospects in the study of flavocytochrome P-450 BM3

The study of P-450 BM3 over the past decade has provided many significant insights into the
structure and mechanism of its mammalian homologues. As with all important enzymes, many of these findings have led to new topics for investigation. Among the most pertinent current problems are the understanding of the conformational properties of the enzyme that underlie such phenomena as the time-dependent inactivation by NADPH [34, 37] and the haem reduction-dependent movement of substrate at the active site [40]. However, perhaps the most important question regards the route of electron transfer from the FMN to the haem iron of P-450 BM3. We have investigated this problem by using site-directed mutagenesis [24], and examination of the crystal structure of the haem domain indicates that a convenient site for electron transfer from the reductase might exist at the region where the cysteinyl ligand to the iron (Cys-400) is exposed to solvent. Further insight might be obtained from the forthcoming atomic structure of the FMN–haem domain of P-450 BM3 [23]. However, the real answer might come only with the solution of the structure of the intact (119 kDa) flavocytochrome, a task that has until now defeated some of the world’s top crystallographers. We wait and hope.

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Electron transfer in trimethylamine dehydrogenase and electron-transferring flavoprotein


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Introduction

Trimethylamine dehydrogenase (TMADH) is an iron–sulphur flavoprotein from the bacterium Methylophilus methylotrophus. TMADH catalyses the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde [1]:

\[(\text{CH}_3)_3\text{N} + \text{H}_2\text{O} \rightarrow (\text{CH}_3)_2\text{NH} + \text{CH}_2\text{O} + 2\text{H}^+ + 2e^-\]

The crystal structure [2] reveals that the enzyme is a homodimer, and each subunit contains an unusual covalently linked 6-S-cysteinyl FMN cofactor [3–5], a bacterial ferredoxin-type 4Fe–4S centre [6] and ADP [7]. The physiological electron acceptor of TMADH is an electron-transferring flavoprotein (ETF) [8], a heterodimer containing one equivalent of FAD [8] and AMP [9], that cycles between the quinone and anionic semiquinone states. The reaction catalysed by TMADH can be divided into reductive and oxidative half-reactions (Figure 1) [10–14]. The reductive half-reaction is resolved into three sequential kinetic phases [15] ([15]; M.-H. Jang, J. Basran, N. S. Scrutton and R. Hille, unpublished work): an initial fast phase that represents the reduction of the 6-S-cysteinyl FMN by substrate, an intermediate phase that represents the electron transfer from the reduced 6-S-cysteinyl FMN to generate flavin semiquinone and a reduced iron–sulphur centre, and a slow phase that involves the formation of an unusual spin-interacting state of the enzyme caused by the strong ferromagnetic coupling of the unpaired magnetic moments of the reduced electron-transferring flavoprotein.

Figure 1

Reaction sequence of TMADH

Abbreviations: F, flavin; FeS, 4Fe–4S centre; ox, oxidized; red, reduced. Reaction 1, two-electron reduction of flavin by amine substrate; reaction 2, one-electron transfer to 4Fe–4S centre; reaction 3, eT complex formation and one-electron transfer to ETF; reaction 4, internal one-electron transfer to 4Fe–4S centre reaction; 5, eT complex formation and one-electron transfer to ETF.