P450 BM3: the very model of a modern flavocytochrome

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Flavocytochrome P450 BM3 is a bacterial P450 system in which a fatty acid hydroxylase P450 is fused to a mammalian-like diflavin NADPH–P450 reductase in a single polypeptide. The enzyme is soluble (unlike mammalian P450 redox systems) and its fusion arrangement affords it the highest catalytic activity of any P450 mono-oxygenase. This article discusses the fundamental properties of P450 BM3 and how progress with this model P450 has affected our comprehension of P450 systems in general.

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Cytochromes P450 (or P450s) are members of a superfamily of mono-oxygenases. They reductively cleave molecular oxygen, producing an oxygenated organic product and a molecule of water. They are essential for the production of steroid hormones, eicosanoids and prostaglandins in mammals. However, their role in the breakdown of drugs and other xenobiotics has brought them the most fame. The human liver has an array of membranous (microsomal) P450s, providing a front line of defense against xenobiotics. Oxygenation, often hydroxylation or epoxidation, polarizes the substrate and might provide a functional group recognized by enzymes that catalyze conjugation reactions (e.g. sulfate transferases, UDP-glucuronyl transferases or glutathione-*S*-transferases), targeting the compound for excretion. When the modified product is finally excreted or detoxified, the P450 has fulfilled its chemoprotective role [1].

The P450 superfamily is enormous, with >1000 sequenced P450 (*CYP*) genes. Genome sequencing projects continue to provide many new P450 members. Particularly exciting have been recent findings of many P450s in the genomes of *Arabidopsis thaliana* (>200 *CYP* genes) and the pathogen *Mycobacterium tuberculosis* (~20 *CYP* genes) [2,3]. The large P450 component in the mycobacterial genome compared with other bacteria was unexpected (*Escherichia coli* has no P450s), suggesting that they have important metabolic roles and raising the possibility of new drug targets.

Most known P450s are 40–60 kDa in size and the few P450s for which atomic structures are available show great similarity in overall topology (resembling triagonal prisms), and in the position and orientation of secondary structural features [4] (Fig. 1). Few amino acids are totally conserved, and these few play key roles in structural maintenance and catalysis. The cysteine that provides a thiolate ligand to the heme iron and that is essential for P450 catalytic properties [4] is completely retained. The P450s most closely related in amino acid sequence generally have very similar substrate selectivity. However, many have <15% amino acid sequence identity. Nature has succeeded in maintaining the overall P450 fold and heme-binding site while evolving large changes in primary structure to tailor diverse P450s for different substrate selectivity and redox partner interactions.

All but two of the P450 structures that have been solved are bacterial, principally because bacterial forms are soluble whereas eukaryotic P450s are generally membranous, attached to microsomal or mitochondrial membranes by an N-terminal peptide anchor, with other regions embedded in the bilayer. In addition, all P450s require the successive delivery of two electrons to catalyze the scission of molecular oxygen and to generate oxygenated product and water. However, there are important differences in redox partner identity (Fig. 2). Microsomal (class II) P450s receive electrons directly from a membranous flavin adenine dinucleotide (FAD)- and flavin mononucleotide (FMN)-containing reductase [NADPH–cytochrome-P450 reductase (CPR)]. Mitochondrial (class I) forms (which are involved in steroid synthesis) take electrons from a ferredoxin (an iron–sulfur protein) that shuttles electrons from a membranous NAD(P)H-dependent ferredoxin reductase [5]. Conversely, soluble bacterial P450s interact (as far as we know) exclusively with cytosolic redox partners, generally a ferredoxin and a ferredoxin reductase (rather than the single CPR), and virtually all bacterial P450s are class-I systems. Bacterial P450s have proved to be easier to overexpress, purify and crystallize, and structures have been determined for at least eight bacterial P450s [6]. A breakthrough was made recently with the first mammalian P450 atomic structure [7] using extensive protein engineering, including deletion of the membrane anchor, to create a chimera of two mammalian P450s (the steroid hydroxylase P450s CYP2C3 and CYP2C5) that is soluble and monomeric after dissociation from the membrane by salt. This approach clearly points the way forward for future structural studies of mammalian P450.

Model P450 system

In searching for what really makes a P450 'tick', we therefore have a quandary. Mammalian P450s are the

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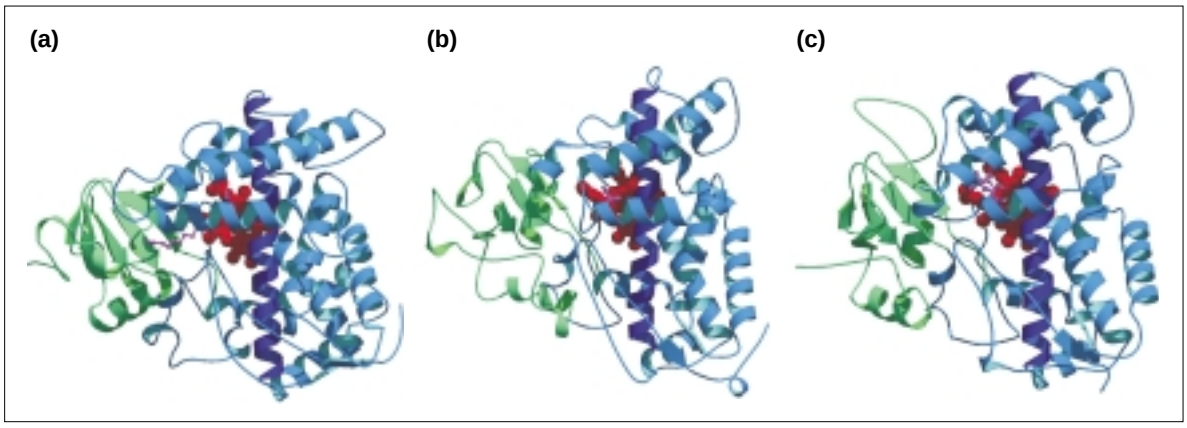

Fig. 1. Similarity of three known atomic structures of cytochromes P450: (a) BM3 (PDB code 1FAG); (b) cam (2CPP); and (c) eryF (1OXA). At least ten different P450 structures have now been solved, of which eight are prokaryotic. In each case the structures have two major structural blocks – a larger α-helix-rich domain (blue) and a smaller β-sheet-rich domain (green). The heme cofactor is indicated in red, with the substrates in each case (palmitoleic acid, camphor and deoxyerythronolide B, respectively) in purple. The long I helix is shown in dark blue in each structure. This structural element runs across the face of the heme and contains several amino acids with important roles in catalysis, including Thr268 in P450 BM3 [18].

ones for which we desire detailed enzymatic and structural detail, owing to their physiological importance. However, soluble bacterial P450 systems are most suited to the kinds of spectroscopic examination that allow a fundamental understanding of P450 properties. The *Pseudomonas putida* camphor hydroxylase P450cam (CYP101) has provided crucial information about the properties of the P450 heme system (e.g. oxidation states, thermodynamics and control of electron transfer). However, bacterial P450s generally operate a different redox system to microsomal P450s and have low sequence identity with their eukaryotic counterparts [5].

The ideal 'compromise' model was identified in 1981 by Fulco's group at UCLA. The *Bacillus megaterium* flavocytochrome P450 BM3 fatty acid hydroxylase has strong similarity to eukaryotic members of the CYP4A (fatty acid hydroxylase) family. It is soluble but uses a mammal-type (class II) redox system: a FAD- and FMN-containing NADPH-cytochrome P450 reductase [8,9]. The P450 BM3 reductase is also soluble and is fused to the C-terminal of the P450 in a single continuous 119-kDa polypeptide. Thus, P450 BM3 is an entire class II P450 system in a single polypeptide. The strategic importance of P450 BM3 was quickly recognized, and the model system has subsequently been extensively characterized.

P450 BM3 structure, function and mechanism

P450 BM3 (called CYP102A1 according to Nelson's classification system [2]) has proved to be an excellent system with which to analyze structural factors that govern substrate selectivity and electron transfer in P450s. Early studies of P450 BM3 confirmed that it hydroxylated a range of fatty acids near the ω terminus, most frequently at the ω-2 position [8]. The precise physiological function of P450 BM3 remains elusive, although a potential role in detoxification of polyunsaturated fatty acids cannot be discounted. The catalytic activity is the highest determined for a P450 mono-oxygenase (~17 000 min^{-1} with arachidonate) [10]. Underlying the high activity of P450 BM3 is the efficiency of electron transfer from the NADPH cofactor through the reductase and onto the P450 heme [11].

The first rational mutagenesis studies of P450 BM3 took place in advance of protein structural information and showed that Trp96 (a phylogenetically conserved amino acid) was important in heme binding [12]. The atomic structures of the P450 BM3 heme domain [13,14] subsequently revealed that W96 was involved in hydrogen bonds with heme propionate. The structures also showed that fatty acid was bound within a long, narrow channel that leads to the heme, and that considerable structural reorientation occurs on substrate binding, with the ω terminus of the fatty acid remaining 7 Å from the heme iron. NMR confirmed the large distance between substrate and heme, and that heme reduction provided the trigger for a second conformational change that drives the fatty acid into position for oxidative attack closer to the heme iron [15]. A major target in research on the P450 BM3 system is the elucidation of the structure of intact 119-kDa flavocytochrome. Attempts to crystallize the full-length enzyme have not yet been successful, although the structure of a construct containing the heme- and FMN-binding domains has been determined.

These atomic structures provided the basis for rational investigation of the roles of amino acids in P450 BM3. In particular, they suggested that Phe87, positioned between the ω terminus of the fatty acid and the heme iron, might have a key role. F87 'flips' from horizontal to perpendicular to the heme as substrate binds (Fig. 3). An important role in regiospecificity was confirmed when the F87A mutant was shown to oxygenate fatty acid (laurate) at the ω carbon, showing that the F87 phenyl ring protects the ω carbon from oxidative attack in

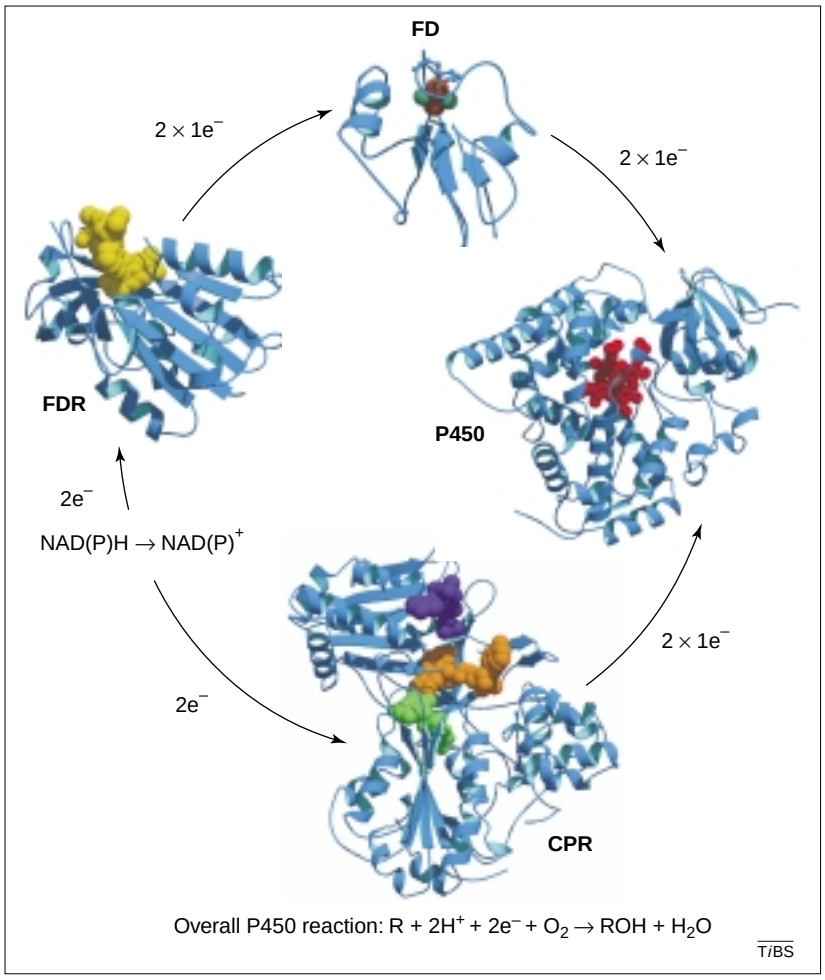

Fig. 2. Cytochrome P450 redox systems. P450s receive electrons by one of two major routes for oxygenation of substrate (R). Most P450s (including P450 BM3) generate hydroxylated products (ROH). The two electrons (e^-) are ultimately delivered from NAD(P)H. In class I P450 systems (typified by bacterial systems such as P450cam and by P450 systems from mammalian adrenal mitochondria), electrons are shuttled through a flavin adenine dinucleotide (FAD)-containing ferredoxin reductase (FDR) and a ferredoxin (FD) to the P450. In class II P450 systems (typified by mammalian hepatic drug-metabolizing isoforms and by P450 BM3), electrons are delivered by a FAD- and flavin mononucleotide (FMN)-containing P450 reductase [NADPH–cytochrome-P450 reductase (CPR)] to the P450. In eukaryotic systems, the CPR, FDR and P450 components are usually membrane-bound and, in bacterial systems, they are usually soluble. The structures of the redox protein components are represented by *Escherichia coli* FDR (FAD cofactor in yellow), *Pseudomonas putida* putidaredoxin (Fe_2S_2 cofactor in green and orange), rat CPR ($NADP^+$ in dark-blue, FAD in orange and FMN in green) and P450 BM3 heme domain (heme in red).

wild-type P450 BM3 [16]. At the opposite end of the active site are Arg47 and Tyr51, postulated to interact with the substrate carboxylate [14]. Kinetic and spectroscopic studies of mutants indicate that R47 plays the dominant role in binding fatty acid. F42 forms a hydrophobic 'lid' over the active site and is also important catalytically, with large increases in the K_d and K_M values for substrate observed for mutant F42A [10]. Other mutagenesis work has resolved important roles for reductase domain residues Gly570 and Trp574 (which are involved in FMN cofactor binding) [17], Thr268 (which controls oxygen binding and activation, and the coupling of electron transport to substrate oxygenation) [18], and Phe393 (which controls the electronic properties of the heme) [19].

Can we redesign P450 substrate selectivity?

The structure of the palmitoleate-bound form of P450 BM3 provided clear pictures of the binding mode for long-chain fatty acids and of the key amino acids that define the substrate-binding pocket [14] (Fig. 3). The site-directed mutant F87G catalyzed the accelerated oxidation of polycyclic aromatics (including pyrene and benzo-*a*-pyrene), as well as affecting the regioselectivity of fatty acid oxidation [16]. In addition, mutant F87V specifically catalyzed the production of 14*S*,15*R*-epoxyeicosatrienoic acid from arachidonic acid, as opposed to the mixture of this compound with 18*R*-hydroxyeicosateraenoic acid formed by wild-type P450 BM3 [20]. Arachidonic acid is a polyunsaturated fatty acid prone to oxidative attack at each of its four carbon–carbon double bonds. Generation of specifically hydroxylated and epoxygenated derivatives by conventional chemistry is therefore problematical. However, certain metabolites (e.g. the 20-hydroxyeicosatetraenoic acid and 14,15-epoxyeicosatetraenoic acid derivatives) have specific biological functions (e.g. in renal function and vascular tone), and there might be a biotechnological niche to be exploited through the P450-driven synthesis of arachidonate metabolites. Removal of the carboxylate-binding motif of P450 BM3 in the double mutant R47L Y51F increased the capacity of the enzyme to oxidize pyrene and other polycyclic aromatic hydrocarbons, and additional mutations to the phenylalanine (F87A) and an active site alanine (A264G) further improved the turnover and coupling of substrate oxidation to NADPH oxidation, respectively [21]. Mutant R47E also catalyzed efficient hydroxylation of fatty acid alkyl trimethylammonium derivatives, further showing the potential of the engineered P450 in organic synthesis [22].

Rational mutagenesis was also used to alter the fatty acid substrate-binding profile of P450 BM3. Wild-type P450 BM3 has negligible activity towards saturated fatty acids of ten carbons and fewer. Engineering alternative carboxylate-binding residues closer to the heme in the hydrophobic active site core resulted in improved binding and turnover of short chain alkanoic acids. Mutant L181K and double mutant L75T L181K had the largest effects, with catalytic efficiency improving >13-fold and 15-fold with butyrate and hexanoate, respectively [23]. Interestingly, removal of the R47–Y51 motif hindered the recognition and catalysis of carboxylic acid substrates of all chain lengths, emphasizing its importance for binding and turnover.

Random mutagenesis and functional selection are now also established as important tools in modern enzymology, and have been applied to the P450s. One notable success in this area has been the selection of random P450cam mutants that use hydrogen peroxide [bypassing the need for expensive NAD(P)H cofactor and redox partners] more efficiently to drive

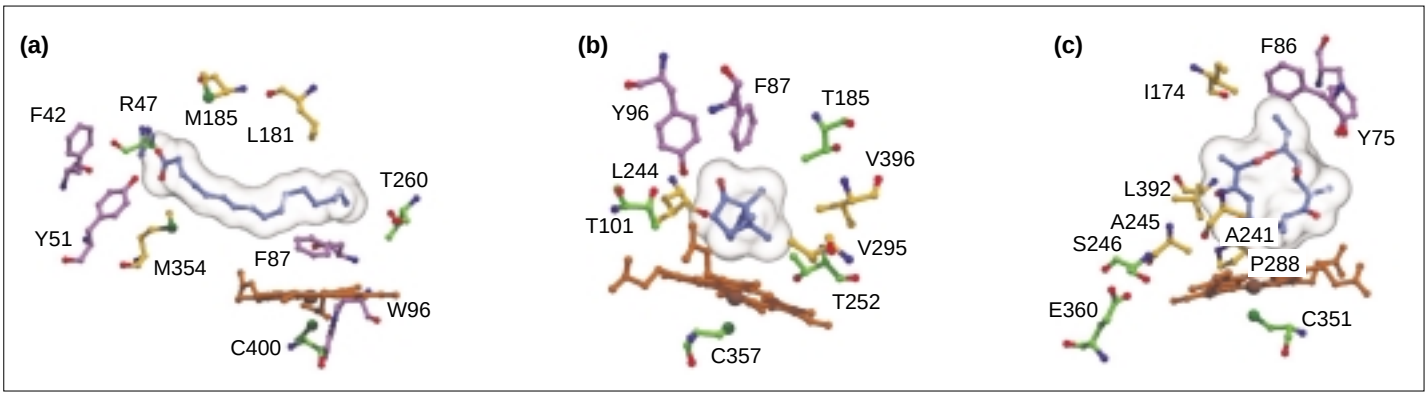

Fig. 3. Active-site structure in the cytochromes P450. The similarity between the overall topology of the P450s disguises the structural variation in the active-site cavities. It is this variation that explains the widely different substrate profiles of the P450s. The figure shows the active sites of the substrate-bound forms of (a) P450 BM3 (palmitoleic acid substrate), (b) P450cam (camphor substrate) and (c) P450 eryF (6-deoxyerythronolide-B substrate). Key amino acids include: (a) R47 (substrate carboxylate binding), F87 (regioselectivity of substrate oxidation), L181 (substrate chain length selectivity) and W96 (heme binding and spin-state maintenance); (b) T252 (oxygen coordination and activation), Y96 and F87 (substrate interaction and positioning), and C357 (heme ligation); and (c) Y75 (substrate specificity and orientation), A241 (hydrophobic interaction with substrate and control of cooperative ligand binding), F86 (creation of hydrophobic substrate pocket) and S246 and E360 (putative proton relay pathway).

catalysis by the alternative 'peroxide shunt' pathway to hydroxylate naphthalene. By using horseradish peroxidase to link hydroxylated naphthalene derivatives, fluorescent conjugates were formed and mutants with the desired activity could be selected efficiently by mass screening [24]. Cell cultures of *E. coli* producing P450 BM3 have already been used to produce gram quantities of specific hydroxylated fatty acids, reinforcing the potential of the BM3 system as a 'factory' for the production of a range of chirally pure compounds from cultures of wild-type and mutant enzymes [25].

How are electron transfer and catalysis regulated?

At the enzyme level, there are several regulatory features to consider for P450s. The most important is their ability to control electron transfer to the heme iron and to couple this to the reduction of molecular oxygen and substrate oxidation so that a productive catalytic cycle ensues. The consequences of a lack of control are wastage of energy from NAD(P)H and the production of reactive oxygen species (ROS), primarily superoxide and peroxide. Uncoupled turnovers will thus lead to cellular damage caused by ROS as well as enzyme inactivation, with the heme group itself at particular risk of oxidative destruction. For a highly active enzyme such as P450 BM3, the risks are great and regulatory control is essential. P450 BM3 imposes a thermodynamic 'block' on electron transfer to its heme iron in the substrate-free form to avoid such damage. In substrate-free P450, the heme iron is in a low-spin electronic configuration, with the iron octahedrally coordinated by four pyrolle nitrogens from the heme macrocycle (equatorial), and by cysteinate and water ligands (axial). Cysteinate heme ligation (Cys400 in BM3) is crucial for oxygen activation and is also found in the nitric oxide synthases [26]. The weakly bound water molecule is displaced by fatty acid substrate. This perturbs the spin-state equilibrium in favor of the high-spin form and results in a more positive (by ~130 mV) heme iron reduction potential [18,27]. A similar phenomenon is observed for P450cam [28]. Elevation of the heme iron potential triggers electron transfer from the reductase and this elegant redox 'switch' ensures that only substrate-bound heme is reduced. Oxygen then binds to the ferrous iron and the delivery of a second electron initiates the essentially irreversible sequence of events that complete the catalytic cycle.

A second level of control over electron transfer is exerted within the reductase domain of P450 BM3. If the enzyme is pre-incubated with NADPH, fatty acid is added at intervals and the activity is recorded, then fatty acid hydroxylation rate diminishes consistently (up to ~1 hr from pre-reduction) to ~10% of the original level [8]. The phenomenon appears to relate to accumulation of a three-electron reduced form of the reductase and the apparent inefficiency with which this form can reduce the P450 heme. Owing to rapid reduction of the reductase by NADPH, this form would be expected to accumulate rapidly, and predominates over the four-electron reduced form because of the thermodynamic properties of the flavins [27]. Thus, the reason underlying the rather slow inactivation process observed is unresolved. A change in conformational equilibrium within the three-electron reduced reductase seems the most likely. Regardless of the mechanism of inactivation, this is probably an adaptive response to an excess of NADPH over fatty acid substrate and again prevents wasteful oxidation of the former and damaging production of ROS.

Electron transfer engineering

The structures of redox proteins generally reveal that their redox cofactors come close to one another, providing rapid, highly directional electron transfer and removing the need for extensive 'through-bond' pathways using specific secondary and tertiary protein motifs [29]. For instance, in the four-heme flavocytochrome fumarate reductase (fcc_3) from *Shewanella frigidimarina*, the longest edge-to-edge distance between adjacent cofactors is <9 Å, which

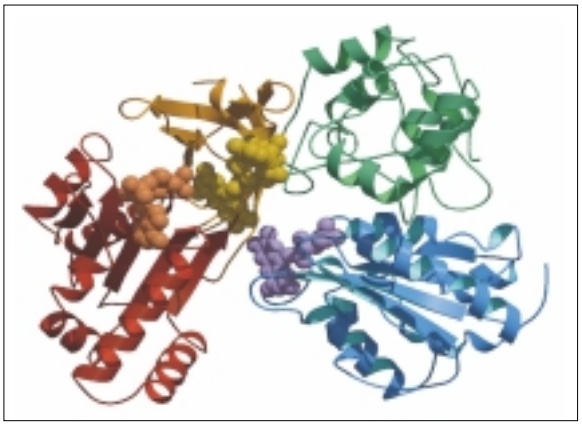

Fig. 4. Atomic structure of rat cytochrome P450 reductase. The structure of a soluble form of the rat cytochrome-P450 reductase (CPR; PDB code 1AMO) reveals discrete domain organization and arrangement of cofactors compatible with rapid and direct electron transfer between NADPH (NADP$^+$ is bound in the structure determined), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). There is a large 'hinge' domain (green) between the NADP$^+$- and FAD-binding ferredoxin-reductase-like domain (NADP$^+$-binding subdomain and cofactor in dark brown, FAD-binding subdomain and flavin in light brown) and the FMN-containing flavodoxin-like domain (domain and flavin in blue) of the enzyme. This hinge is thought to enable the movement of the reduced FMN domain to interact with cytochrome P450.

guarantees overall electron tunneling rates through the redox chain well in excess of the maximal catalytic rate (k_{cat}) [30].

The facts that P450 BM3 has a mammal-like CPR as the redox partner and that it is fused to the P450 make it an attractive model system for understanding electron transfer, both within the CPR and between the CPR flavins and the P450 heme. Although no structure is yet available for the reductase of BM3, the recent structural determination of rat CPR provided important proof that this enzyme exhibits close spatial organization of cofactors [31]. In the solved conformation, the FAD and FMN cofactors are positioned such that the distance between their respective isoalloxazine ring systems is <4 Å (Fig. 4). Similarly, the NADPH-binding site is close to the FAD and poised to effect hydride transfer.

The FAD and FMN reside on distinct domains linked by a large 'hinge' domain, which is proposed to move between a 'closed' conformation in which the two flavin domains are juxtaposed for inter-flavin (FAD–FMN) electron transfer, and an 'open' conformation, after the FMN domain has moved ~10 Å to interact with (and reduce) P450. A model for the re-positioning of the homologous FMN domain of P450 BM3 is shown in Fig. 5. A similar hinged domain motion with comparable displacement has been characterized in the cytochrome bc_1 complex [32,33]. Here, the Rieske iron–sulfur subunit moves between the cytochrome c_1 heme and the hydroquinone oxidizing site, and has been implicated as a crucial timing element that governs the transfer of two electrons from hydroquinone along two directionally and functionally distinct single-electron transfer pathways. Assuming that BM3 reductase has similar structural properties to rat CPR, the questions now focus on the nature of the FMN interaction with its redox partners and on the electron transfer from the FMN to the P450 BM3 heme, some distance away.

Because P450 enzymes interact with distinct electron transfer partners (CPR, flavodoxin, ferredoxin and cytochrome b_5), these proteins probably share a common binding region on the P450 surface. Anionic P450 residues are recognized as important in binding cationic residues on the redox partner (for an example, see Ref. [34]). This site surrounds the heme iron cysteinate ligand, and Cys400 is exposed on the surface of P450 BM3. Thus, it is likely that the redox partners dock such that their exposed cofactor (flavin, iron–sulfur or heme) is close to the cysteine, and that a short, direct electron transfer occurs rapidly from the edge of the cofactor to the P450 heme. In a model of the interaction of P450cam with its redox partner (putidaredoxin) [35], the only non-cofactor-ligated amino acids between the iron–sulfur and heme centers are Asp38 in putidaredoxin and Arg112 in P450cam. Kinetic data for relevant site-directed mutants of putidaredoxin and P450cam give strong support to this model, indicating diminished binding and/or electron transfer [35]. Similar efficient electron transfer should be evident between the FMN and the heme in P450 BM3.

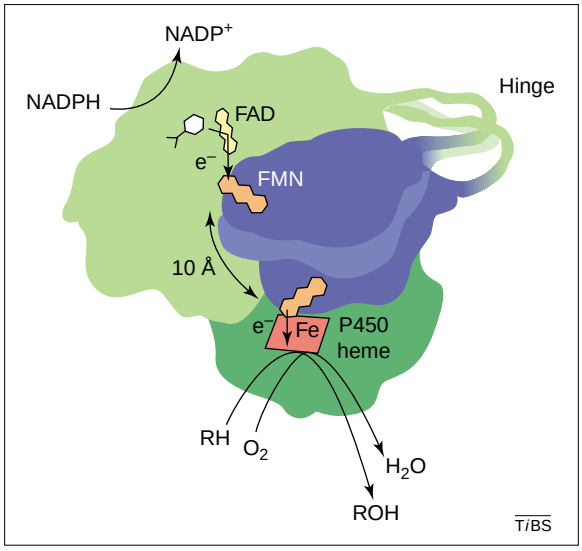

Fig. 5. Flavin mononucleotide (FMN) domain motion in flavocytochrome P450 BM3. The cartoon illustrates the movement of the FMN domain of P450 BM3 (blue), which is required to transport the FMN flavin cofactor (shown in orange isoalloxazine ring form) from near the flavin adenine dinucleotide (FAD) domain (light green, with FAD flavin cofactor in yellow) to interact with the P450 domain (dark green, with heme shown as a red box). The motion is facilitated by a linker region between the FAD and FMN domains, and facilitates the transfer of electrons (e^-) produced by NADPH oxidation from the FAD domain to the heme. At the heme, molecular oxygen is reductively cleaved to convert the fatty acid substrate (RH) to a hydroxylated product (ROH), with the formation of a molecule of water from the second oxygen atom.

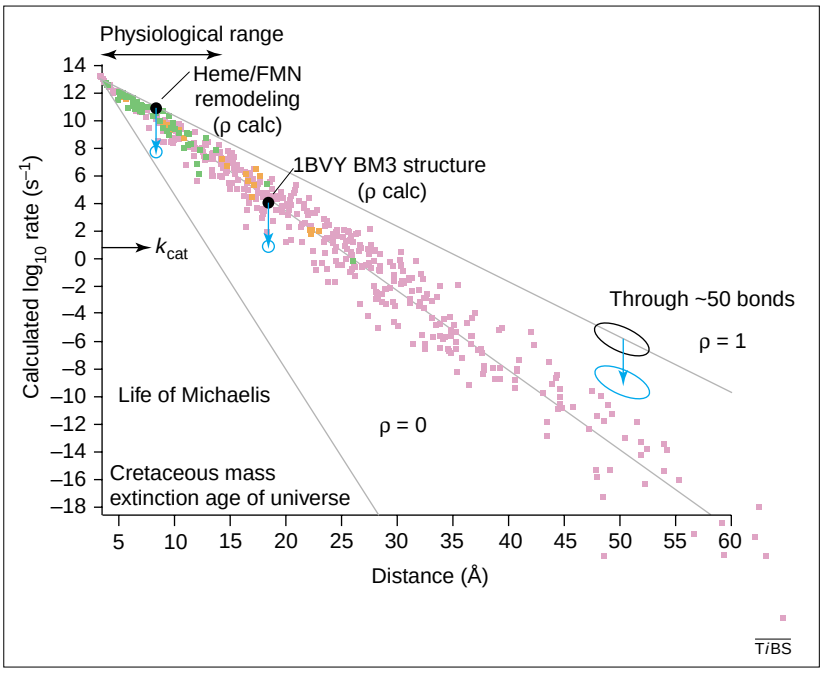

Fig. 6. Electron transfer through the protein. Extensive analysis of electron transfer rates between redox centers in a broad range of redox proteins shows the maximum tunneling rates for both physiologically productive (green squares) and physiologically unproductive (red squares) electron transfer reactions [29]. The productive reactions occur over a distance of up to 14–15 Å, whereas unproductive ones extend up to 25 Å. These are compared with calculated tunneling rates of imaginary electron transfer between randomly selected aromatic side chains in nitrogenase, hydrogenase and cytochrome oxidase that extend the calculated rates up to the 60 Å dimension of these large proteins (purple squares). The scatter of the rates at any distance represents the structural inhomogeneities (packing density) within a protein interior. The line through the points is that for an average packing density of 0.75, whereas the upper and lower lines represent electron tunneling through fully packed protein (packing density of 1) and through a vacuum (packing density of 0), respectively [29]. For P450 BM3, analysis of the tunneling through 50 σ bonds connecting the flavin mononucleotide (FMN) to the heme (~50 Å) estimates the optimal rate to be in the range of 10^{-6}–10^{-4} s^{-1} (open black oval). Assuming an unremarkable reorganization energy and considering the driving force for electron transfer provided by differences in reduction potential between centers [37], the rate is modulated to ~1000 times less (blue oval). By contrast, the shortest distance between the conjugated edges of the FMN and the heme in the FMN–heme domain structure (1BVY) is only 18 Å, giving an optimal rate of 10^{4}–10^{5} s^{-1}, or a modulated rate of 12 s^{-1}. Remodeling the interaction between FMN and heme domains ('heme/FMN remodeling') brings the inter-cofactor distance to as little as 8 Å, yielding an optimal rate of 10^{11} s^{-1}, and a modulated physiological rate of 10^{8} s^{-1}. Supplementary information is available that explains the calculations involved in producing these rate constants (http://www.uphs.upenn.edu/biocbiop/local_pages/dutton_lab.html).

Recently, the atomic structure of a polypeptide was reported that comprised the first 664 amino acids of flavocytochrome P450 BM3, encompassing the heme and FMN domains [36]. During crystal formation, the domains were proteolytically separated. The structure determined was that of a non-stoichiometric complex of two heme domains for each FMN domain. Even though the protein–protein contacts were dominated by those between the heme domains, it remains clear that the FMN domain is located near the distal face of the P450 heme, which is considered to be the binding surface, and that the FMN cofactor is orientated towards the cysteinate ligand to the heme.

An initial examination of how electrons might be transferred between flavin and heme in this structure [36] identified an extended ~50-σ-bond pathway (i.e. through amino acid residues and their peptide bonds) between the cofactors. However, a simple theoretical through-bond calculation reveals a very slow electron-transfer rate, equivalent under physiological conditions to a rate constant of 0.02 yr^{-1}, or about one electron transfer every 50 years (Fig. 6). This is thus ruled out as a functionally relevant electron tunneling route. The shortest distance between the conjugated edges of the FMN and heme cofactors in this structure is ~18 Å, somewhat outside the 14–15 Å limit found in surveys of physiological electron transfers in many redox proteins [29]. Calculations using the standard tunneling expression [33,37] yield a maximal electron transfer rate constant of ~10^{4} s^{-1}, modulated under physiological conditions to ~12 s^{-1} for the 18 Å electron tunneling between FMN and heme. Although this approaches the measured 200 s^{-1} for the reaction in the flavocytochrome [38] (Fig. 6), it remains on the slow side. To address this apparent discrepancy, we remodeled the interaction between the structurally defined FMN and heme domains. This indicates that the FMN domain can (presumably via motion around the 'hinge' between the FMN and FAD domains in the intact flavocytochrome) approach closer to the heme domain. Such motion can reduce the FMN–heme cofactor distance to as little as 8 Å, well within the physiological range for biological electron transfer. The maximum rate predicted for this flavin-to-heme electron transfer model is of the order of 10^{11} s^{-1}, which projects a rate of ~10^{8} s^{-1} under physiological conditions (Fig. 6).

Although the hinged FMN domain in P450 BM3 might simply offer restricted diffusion to move electrons in a highly controlled fashion over long distances between redox cofactors, it might function (similar to the hinge in cytochrome bc_1) as a crucial component of a timing mechanism. Thus, the motion might act as an escapement for the input into the P450 heme of the two single electrons from the NADPH–FAD–FMN chain to activate oxygen [35]. FMN motion has recently been proposed for the related *E. coli* methionine synthase, in which the flavodoxin component is predicted to move sequentially between the FAD-containing NADPH–ferredoxin reductase and cobalamin-dependent methionine synthase, facilitating electron transfer between NADPH and the synthase [39]. The possibility of interdomain motion coordinated by the hinge domain was also recognized for the domains of rat CPR [31].

Future prospects

Although P450 BM3 was discovered ~20 years ago, only the past decade has done justice to its 'model P450' tag. With a large amount of structural and biochemical information already collected, the question might be raised of what else can we learn about P450 BM3. However, when we remember just what an experimentally tractable enzyme P450 BM3 is, this question really becomes 'what do we still need

to learn about the mechanism and properties of P450s and related oxidoreductases that engage in complex oxygen chemistry?' Here, P450 BM3 has much left to offer. For instance, the high-valency metal-oxo complex responsible for substrate oxygenation is transient in P450 catalysis, has evaded spectroscopic detection and remains a 'holy grail' [4]. Cryogenic spectroscopy of P450 BM3 might provide the answer to this fundamental question in catalysis. Indeed, X-ray cryocrystallography has already provided useful information about the structural properties of oxygen-bound P450cam intermediates [40]. There are still crucial questions about the mechanism and route of proton transfer, and the activation of molecular oxygen. Flavocytochrome P450 BM3 and P450cam have again provided the lead, with important roles for a conserved threonine (T268 in BM3, T252 in P450cam) in oxygen binding and activation proved by spectroscopic and enzymatic study of site-directed mutants [18,41], and a series of acidic and basic residues predicted to shuttle protons from the protein surface to the active site in P450cam [4].

Opportunities for mechanistic investigation could lie in better describing the nature of the FMN domain movement and in determining whether it simply relies on restricted Brownian motion, or displays choreography governed by the redox states of the FMN and its interacting partners (FAD and heme). Such escapement might be the source of inactivation in the three-electron reduced form and might even modulate substrate selectivity. Its manipulation might offer new experimental routes into the cryptic mechanism of substrate hydroxylation. Rational mutagenesis of P450 BM3 continues to provide answers about the roles of phylogenetically conserved amino acids in structure and catalysis [4,6,10,16–23]. Also, both rational and random mutagenesis of P450 BM3 have the potential to generate oxygenase variants with novel substrate selectivity [21]. Many attempts have been made to mimic P450 BM3 by engineering catalytically functional fusions of eukaryotic P450s and CPRs [42], but none has had activity even ten times lower than P450 BM3. This indicates the importance of domain motion and redox-partner interactions for efficient electron transport and catalysis.

The title of this article is a play on one of the showstoppers from Gilbert and Sullivan's operetta *The Pirates of Penzance*, in which General Stanley boasts that, 'in short, in matters vegetable, animal and mineral, I am the very model of a modern major general'. Clearly, flavocytochrome P450 BM3 has already served well as such a 'model' for the P450 superfamily. As technology marches on, there is every reason to expect that further breakthroughs in understanding the intricate mechanism of cytochrome P450 will be made through study of this important flavocytochrome.

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Snap-shots of clathrin-mediated endocytosis

Matthew K. Higgins and Harvey T. McMahon

Clathrin-mediated endocytosis is one of the major entry routes into a eukaryotic cell. It is driven by protein components that aid the selection of cargo and provide the mechanical force needed to both deform the plasma membrane and detach a vesicle. Clathrin-coated vesicles were first observed by electron microscopy in the early 1960s. In subsequent years, many of the characteristic intermediates generated during vesicle formation have been trapped and observed. A variety of electron microscopy techniques, from the analysis of sections through cells to the study of endocytic intermediates formed *in vitro*, have led to the proposition of a sequence of events and of roles for different proteins during vesicle formation. In this article, these techniques and the insights gained are reviewed, and their role in providing snap-shots of the stages of endocytosis in atomic detail is discussed.

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Endocytosis is the process of vesicle formation from the plasma membrane. It has many functions, including bringing nutrients into the cell, regulating the number of signalling receptors on the cell surface and recycling synaptic vesicles at nerve terminals. The endocytic machinery can also be hijacked by pathogens for entry into the cell. Snap-shots of the engulfment of a variety of substrates, including yolk proteins [1], low density lipoproteins [2] and influenza virus [3], have been taken using the electron microscope.

The clathrin-mediated pathway (Box 1) is one of the most commonly used, and the most intensively studied, mechanisms of endocytosis. Clathrin-coated pits and vesicles were first observed by electron microscopy. The characteristic 'bristle-like' appearance of the coat in cross sections of forming vesicles, or the presence of lattices of hexagons and pentagons on the surface of the plasma membrane, makes them easily discernible (Fig. 1). Therefore, although dynamic changes occurring during endocytosis can be studied using fluorescent labelling and confocal microscopy, detailed visualization of endocytic processes is greatly aided by use of the electron microscope. In this review, we will examine how electron microscopy has given insight into the different stages of clathrin-mediated endocytosis and the proteins involved in these stages. We will review the techniques available and look forward to the future when we hope that a combination of three-dimensional reconstruction of electron microscope images, and the docking of atomic structures into these models, will reveal 'snap-shots' of the different stages of endocytosis in atomic detail.

Looking into the cell

Several invasive approaches have been used to study the formation of coated vesicles within a cell. By attaching fibroblasts to coverslips, breaking open the cells, freeze-drying and generating a carbon-platinum replica of the exposed cytoplasmic surface (reviewed in Ref. [4]), highly ordered polygonal arrays of clathrin can be seen by electron microscopy [5] (Fig. 1a). These arrays can be removed from the membrane surface with alkali, and reassembled from the components of purified clathrin-coated vesicles [6].

An alternative approach is to fix a cell with formaldehyde, embed it in resin and take sections through the resin block. In negatively stained thin-sections through a nerve terminal, different intermediates of endocytosis can be seen.

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