Variations on a (t)heme—novel mechanisms, redox partners and catalytic functions in the cytochrome P450 superfamily†

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The catalytic potential of the P450s in organic biotransformations is the subject of this review—with emphasis on the breadth of P450 redox systems now recognised and the catalytic versatility of these biotechnologically important enzymes.

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1 Introduction

The regio- and stereoselective oxygenation of organic molecules is notoriously difficult to achieve by standard organic synthesis approaches (*e.g.* using Sharpless asymmetric hydroxylation or Jacobsen's salen catalyst for asymmetric epoxidation) and these methods often do not lend themselves to large scale synthesis.[1,2] Enzyme-based approaches have the advantages of being "cleaner" and usually safer (*i.e.* involving use of less hazardous reagents, fewer reaction steps and requiring less fractionation of product mixtures) and usually produce a smaller spectrum of oxygenated products. Indeed, a single product may be feasible—as seen *e.g.* in the exclusive 5-*exo* hydroxylation of camphor by the *Pseudomonas putida* cytochrome P450cam enzyme (CYP101A1).[3] That said, there are relatively few types of enzyme catalyst known to catalyse oxygenation reactions. These include the flavin-containing mono-oxygenases (FMOs) and dioxygenases, which are involved in diverse reactions such as xenobiotic metabolism and detoxification, collagen and antibiotic biosynthesis.[4,5] The most prominent class of oxygenases is the cytochrome P450 (P450) enzyme superfamily, members of which are found widespread in nature and which perform a wide array of oxygenation and other biochemical transformations on countless endogenous biochemicals and xenobiotics.[6–9] The P450s are heme-containing biocatalysts that typically have a relatively hydrophobic active site cavity for substrate binding, as revealed by atomic structural studies.[10–14] There are several examples of the mutagenesis (both by rational design and forced evolution methods) of different P450 enzymes in order to facilitate alteration of substrate selectivity, and the position of oxygenation of the substrate *e.g.*[15–18] The catalytic potential of the P450s in organic biotransformations is the subject of this review—with emphasis on the breadth of P450 systems now recognised and the catalytic versatility of these biotechnologically important enzymes.

2 Cytochromes P450: a brief history

The P450s were first recognised as unusual pigments in mammalian liver microsome samples (*i.e.* the membranous material from the liver endoplasmic reticulum). Their ability to form a complex with carbon monoxide (CO) led to their recognition as a distinctive type of cytochrome (*i.e.* a heme-binding electron-transferring protein), with absorption maximum of the ferrous–CO complex shifted to ~450 nm (hence Pigment 450 or P450)[19,20] (Fig. 1). This characteristic shift of the main (Soret) band of the P450 heme is a consequence of the nature of the proximal axial ligand to the heme iron (*i.e.* the ligand *trans* to CO in the complex), which is a phylogenetically conserved cysteine thiolate. The electron donating character of this bond is critical for the P450's catalytic activity.[21] The ligand *trans* to the cysteinate is typically a water molecule (as opposed to another amino acid from the protein), and the water is readily displaced during the enzyme's catalytic cycle in favour of dioxygen (*vide infra*).[22]

From the earliest stages of research into the P450s, their involvement with oxidation of organic molecules was recognised. In particular, carbon monoxide (or other ligands) was found to inhibit the oxidative transformation and detoxification of various drugs and other organic compounds by the hepatic P450s (*e.g.* references 23, 24). This stimulated intensive research into the

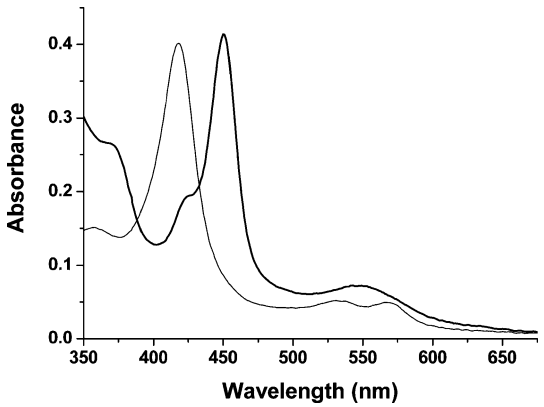

Fig. 1 Absorption spectra for cytochrome P450 and its ferrous–carbon monoxide complex. A typical absorption spectrum for a P450 enzyme (CYP51B1 from *Mycobacterium tuberculosis*, ~4 μM enzyme) is shown in its oxidised (ferric) state (thin solid line, A_{max} = 418 nm) and in its dithionite-reduced Fe(II)–CO complex (thick solid line, A_{max} = 450 nm). The major absorption (Soret) band shift to ~450 nm in the CO complex is a hallmark of the cysteinate-coordinated heme iron of P450 enzymes. A small shoulder originating from the inactive (thiol-coordinated) P420 form of CYP51B1 is seen at ~420 nm in the spectrum of the Fe(II)–CO complex.

properties of these enzymes, and a huge amount of progress has been made over the last 50 years into the understanding of the structure, mechanism, biological function and diversity of the P450s.[25] It was soon established that P450s catalysed the reductive activation and scission of molecular oxygen (dioxygen) that binds to their heme iron. This leads to insertion of an atom of oxygen into the substrate (frequently resulting in hydroxylation) and production of a molecule of water from the other oxygen atom.[26] This typical P450 reaction requires delivery of two electrons and two protons to the P450 heme iron, as shown in eqn (1).

$$RH + O_2 + 2e^- + 2H^+ \rightarrow ROH + H_2O \quad (1)$$

However, as will be discussed later in the article, reactions far more diverse than hydroxylations are catalysed by P450s, and their means of sourcing protons and electrons are also variable[27,28] (Fig. 2).

3 The P450 catalytic cycle

To facilitate P450 catalysis, the vast majority of these enzymes require the delivery of two electrons ultimately derived from pyridine nucleotide coenzymes (*i.e.* NADPH or NADH). The electrons are transported to the P450 heme *via* one or more redox partner proteins. Electrons are delivered at discrete points in the P450 catalytic cycle, as illustrated in Fig. 2. The first electron reduces the ferric heme iron to ferrous, which can then bind dioxygen. As a prelude to reduction, substrate binding usually facilitates dissociation of a water ligand bound weakly to the iron and *trans* to the cysteinate, which in turn favours an electronic reorganisation in the heme iron d-orbitals. This results in a shift in ferric heme iron spin state equilibrium from low-spin ($S = 1/2$) towards high-spin ($S = 5/2$).[29,30] In turn, this leads to an increase in the heme iron reduction potential that favours electron transfer to the heme iron from the redox partner (*e.g.* references 31, 32).

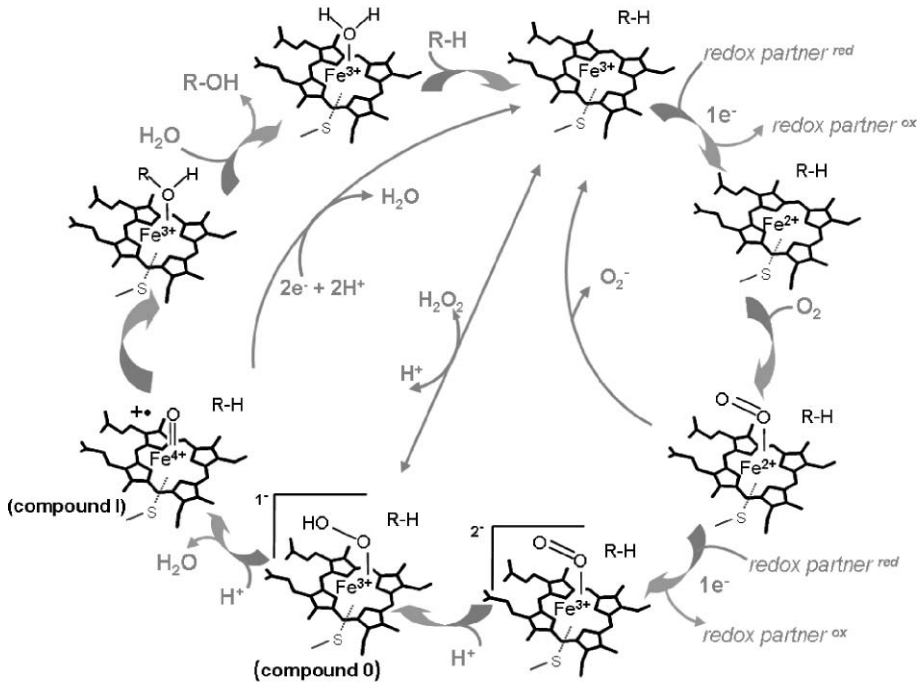

Fig. 2 The catalytic cycle of cytochrome P450. The intermediate stages of a P450 catalytic cycle leading to substrate (R–H) hydroxylation are shown. The heme macrocycle is depicted to represent heme, with the oxidation state of the heme iron indicated. The proximal heme ligand (cysteine thiolate, indicated as a S-atom linked to the iron) and distal ligand (a water molecule, changing to dioxygen as the cycle progresses) are also indicated. In the first step, substrate binding leads to displacement of the distal water ligand. This effects a shift in ferric heme iron spin-state equilibrium from low-spin ($S = 1/2$) towards high-spin ($S = 5/2$). This leads to a more positive potential of the iron and favours electron transfer from the redox partner to reduce the heme iron to the ferrous state. Ferrous heme iron binds oxygen to form the ferrous–oxy intermediate (which is isoelectronic with the ferric–superoxy form). Delivery of a second electron from the redox partner reduces heme iron to the ferric peroxy state. Protonation produces the ferric hydroperoxo form (also known as compound 0). A further protonation leads to scission of the bound dioxygen and the production of a water molecule. The remnant intermediate on the heme is a ferryl–oxo species (compound I) with a porphyrin π radical cation. This is likely to be the catalytically relevant substrate oxidant in most P450 reactions. Compound I attacks the nearby substrate and effects its hydroxylation. The departure of product (R–OH) allows water to rebind the ferric iron and complete the cycle. Non-productive pathways leading to collapse of oxy intermediates in the cycle are also indicated. The ferrous–oxy species can decay to reform ferric P450 with production of superoxide. Compound 0 collapses with production of peroxide, and the reaction can be driven (productively) in the reverse direction (albeit inefficiently in most cases) by mixing H_2O_2 (or organic peroxides) with ferric, substrate-bound P450. Compound I collapses with production of water. Collapse of these species may occur if *e.g.* electron/proton delivery is not timely or if substrate is inappropriately positioned or otherwise resistant to oxidative attack.

This may be an elegant mechanism to ensure that electron transfer to the P450 and subsequent formation of reactive oxygen species occurs only when substrate is available for oxygenation. While this phenomenon is observed for many "well-regulated" P450 enzymes (*e.g.* the *P. putida* camphor hydroxylase P450cam [CYP101A1] and the *Bacillus megaterium* fatty acid hydroxylase P450 BM3 [CYP102A1]) which also often have relatively restricted substrate selectivity, it clearly does not hold for all P450s (*e.g.* references 33, 34). The ferrous oxy species formed on binding of dioxygen is isoelectronic with a ferric superoxy state, and the equilibrium may be in favour of the latter form. The delivery of a second electron leads to the production of the ferric peroxy form, which is rapidly protonated to the ferric hydroperoxy state (compound 0). A further protonation leads to collapse of this species with the production of a ferryl–oxo form (compound I) considered to be the primary species responsible for the attack on, and oxygenation of, the substrate. A molecule of water is also produced at this stage in the cycle. Compound I oxygenates the substrate, and dissociation of the product results in the re-binding of a water molecule as the 6th ligand to the restored ferric heme iron, completing the cycle.[8,35]

The apparent simplicity of the cycle hides the fact that later P450 intermediates (particularly compounds 0 and I) are transient and difficult to characterise. In particular, compound I is extremely elusive and it has proven almost impossible to obtain convincing structural or spectroscopic evidence for its formation. However, recent studies have provided evidence for the existence of the ferryl–oxo state.[36] In addition, there is some proof that both the ferryl intermediate and its predecessor compound 0 may be viable substrate oxidants.[37,38] The source of protons in the cycle is ultimately bulk solvent, although active site amino acid residues or substrate can clearly participate in relay pathways to facilitate proton delivery to ferrous oxy intermediates,[12,39,40] as discussed in more detail later.

The nature of electron donor proteins for P450s was, until relatively recently, considered to be quite well understood—with only two major classes of protein systems thought to be responsible for transport of NAD(P)H-derived electrons to members of the P450 superfamily. However, studies in recent years have revealed a diversity of redox partner systems that almost matches the diversity of reactions performed by the P450s themselves.

4 The biological diversity of P450 redox systems

As detailed above, the stereotypical P450 requires consecutive delivery of two electrons to its heme iron at distinct points in its catalytic cycle (Fig. 2). However, the means by which this process is achieved in nature is considerably more diverse than was first thought. In early years of P450 research, two distinct types of redox partners came to prominence and for several years were considered to reflect the only major classes of P450 redox systems. These were the mammalian hepatic type which consists of two protein components (a P450 and a NADPH-dependent reductase, both membrane bound) and the bacterial-type system, which (on basis of studies with the *P. putida* CYP101A1) consists of a soluble P450 to which electron transfer is mediated by an iron–sulfur protein, which in turn is reduced by a flavin-containing reductase.[41–43] More detailed kinetic and product formation studies of the systems revealed that both were dependent on NADPH or NADH, with the mammalian system favouring NADPH, and the CYP101A1 system favouring NADH. The redox partner in mammalian liver (and subsequently shown to participate in several P450-dependent reactions in other mammalian organs and eukaryotic cells) is a FAD- and FMN-containing enzyme termed cytochrome P450 reductase (CPR).[44,45] The FAD is the entry point for electrons derived (by hydride ion transfer) from NADPH, with FMN receiving single electrons from the FAD and passing these to the P450.[46–49] In the bacterial system, detailed analyses of the CYP101A1 redox partners demonstrated that these were a FAD-containing reductase (putidaredoxin reductase) that transferred electrons from NADH to a 2Fe–2S ferredoxin (putidaredoxin). Putidaredoxin (a one electron carrier) then mediated single electron transfer to CYP101A1.[50–52]

Over the years, these types of P450 redox systems became referred to as class I (for the CYP101A1-like 3-component redox system) and class II (for the eukaryotic CPR/P450 2-component system) (Fig. 3). The class II system components were shown to be membrane anchored as a consequence of hydrophobic N-terminal peptide sections that embedded them into the microsomal membranes. Large numbers of eukaryotic P450s belong to class II. The class I components of the CYP101A1 system were all cytosolic proteins encoded on a transmissible plasmid that conferred on *Pseudomonas putida* the ability to grow on camphor as a sole carbon source.[53] However, early studies on P450s from mammalian adrenal mitochondria revealed them also to have a redox system diverse from liver-type class II systems. These mitochondrial P450s are critical for conversion of cholesterol into pregnenolone (catalysed by CYP11A1; the committed step in synthesis of steroid hormones) and for other steroid transformations (notably the transformations of deoxycorticosterone to corticosterone, and of 11-deoxycortisol to cortisol catalysed by CYP11B1, and oxygenations of corticosterone to aldosterone catalysed by CYP11B2).[54–56] It was established that these mitochondrial reactions are supported by the FAD-binding NADPH-dependent adrenodoxin reductase (ADR) and the 2Fe–2S protein adrenodoxin (AD), and detailed studies of the molecular structures of these redox partner proteins (in isolation and in complex) have been done,[57–60] as have analyses of their catalytic and spectroscopic characteristics, and molecular interactions (*e.g.* references 61–67). The mitochondrial system has parallels with the CYP101A1 system (although ADR and the mitochondrial P450s are membrane anchored proteins), perhaps pointing to the evolutionary relationships between the organelle and bacterial cells. While the mitochondrial-type P450 redox apparatus can be embraced into a "wider" class I system, the 1980's brought in a revolution in our recognition of the broader

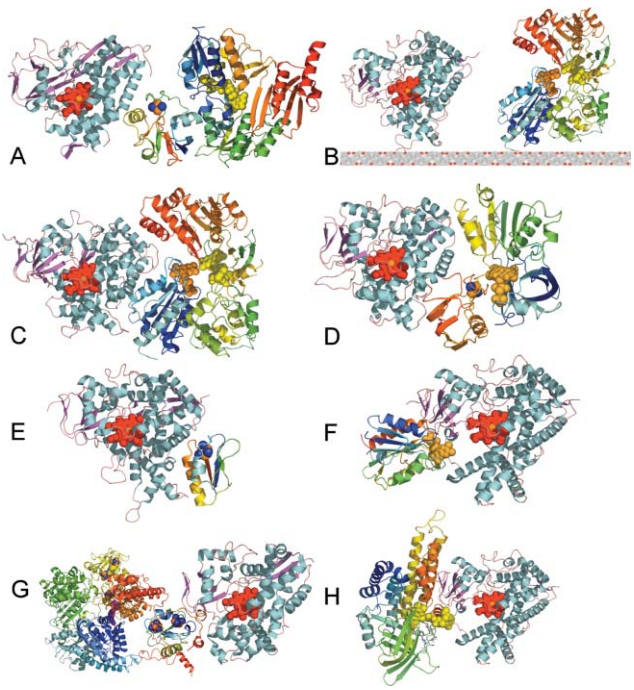

Fig. 3 Biodiversity of P450 systems and redox partners. Recent years have seen the discovery of several new types of natural redox systems for driving P450 catalysis. These are illustrated schematically, using the appropriate atomic structures to represent the individual P450s and their partner proteins as available. A, The soluble class I P450cam camphor hydroxylase system, shown (left to right) as the structure of P450cam, putidaredoxin and putidaredoxin reductase (PDB codes 2CPP, 1PDX and 1Q1R, respectively).[22,228,229] B, the membrane associated class II (liver-type) redox system illustrated as the structures of rabbit CYP2C5 and rat CPR (1DT6 and 1AMO).[114,196] C, The *B. megaterium* flavocytochrome P450 BM3 fatty acid hydroxylase represented as the substrate (palmitoleate)-bound BM3 heme domain (1FAG) and the rat CPR structure (1AMO).[114,197] D, The P450-phthalate dioxygenase reductase (PDOR)-type fusion protein as exemplified by the system from *Rhodococcus* sp. NCIMB 9784.[101] The structure is represented as that for *Saccharopolyspora erythraea* P450eryF (CYP107A1) linked to the PDOR enzyme from *Burkholderia cepacia* (1OXA and 2PIA).[98,205] E, The *Methylococcus capsulatus* CYP51-Fdx fusion protein, illustrated as the linked *M. tuberculosis* CYP51B1 and the *Pyrococcus furiosus* 3Fe–4S ferredoxin (1X8V and 1SJ1).[11,117,306] F, The *Rhodococcus rhodocrous* P450/flavodoxin fusion protein (XplA), illustrated as the structures of rabbit CYP2B4 and *E. coli* flavodoxin (1PO5 and 1AHN).[125,214,307] G, The pyruvate-dependent P450 system from *Sulfolobus solfataricus*, in which a soluble P450 is reduced by a pyruvate-ferredoxin oxidoreductase and a 3Fe–4S- and 4Fe–4S-cluster-binding ferredoxin (7-Fe ferredoxin).[126] The system is represented by *S. solfataricus* CYP119A1, a *Desulfovibrio africanus* pyruvate ferredoxin oxidoreductase and an *Azotobacter vinelandii* 7-Fe ferredoxin (1F4U, 2C3M and 1FD2).[216,308,309] H, The hypothetical *Pseudomonas fluorescens* P450-acyl CoA dehydrogenase (P450-ACAD) fusion protein, represented by the *Polyangium cellulosum* P450epoK (CYP167A1) fused to the *Sus scrofa* (pig liver) medium chain acyl coA dehydrogenase (1Q5E and 3MDE).[10,310] In each case, bound protein cofactors (heme: red, FMN: orange, FAD: yellow, iron–sulfur clusters: orange and blue) are shown in spacefill representation.

biodiversity of P450 redox systems. The expansion of types of P450 redox systems continues today, and is continually fuelled by novel data from genome sequencing projects.

The first major "outlier" in the neat class I and class II system arose through elegant studies of bacterial lipid oxidation done in the laboratory of Armand Fulco at UCLA.[68] The presence of fatty acid hydroxylation activity in the soil bacterium *B. megaterium* was established, and the positions of hydroxylation on the fatty acid chains were shown to be sub-terminal (*i.e.* at the ω-1 to ω-3 positions), rather than at the ω-terminal, as seen for many eukaryotic family 4 P450s.[69,70] When the relevant gene (classified as *CYP102A1*) was isolated, its sequence indicated that the encoded protein (P450 BM3) was a fusion enzyme consisting of a fatty acid hydroxylase P450 (at the N-terminal end) covalently attached to a CPR by a short peptide linker[71,72] (Fig. 3). There was no evidence of any membrane anchor region for either of the two major domains of P450 BM3, and subsequent expression studies demonstrated that P450 BM3 is a soluble multidomain flavocytochrome.[72–74] P450 BM3 catalyses oxygenation (epoxidation and hydroxylation) of a wide range of saturated and unsaturated fatty acids, favouring lipids of carbon chain length ~12–20, and having the highest reported rate of any P450 oxygenase (*e.g.* approx. 17000 min^{-1} with arachidonic acid as substrate).[72,75–78] The key factor underlying this efficient oxygenase activity is the rapid rate of electron transfer through the P450 BM3 system. The coenzyme favoured is NADPH, and the apparent rate of electron (hydride ion) transfer from NADPH to the FAD in the CPR domain of P450 BM3 is >700 s^{-1} (the fast phase from biphasic stopped-flow absorption transients) at 25 °C, substantially faster than respective rates in *e.g.* human CPR.[79,80] Internal electron transfer between FAD and FMN cofactors in the P450 BM3 CPR domain is also much faster than in human CPR. Cytochrome *c* reduction (which occurs *via* electron transfer from the FMN cofactor, and thus requires FAD-to-FMN electron transfer) by P450 BM3 has a k_{cat} value of ~44 s^{-1} for P450 BM3 at 25 °C, whereas a similar rate for human CPR is achieved only at 37 °C in a similar buffer system, and rat CPR is also much slower.[75,81,82] Temperature jump equilibrium perturbation studies of 2-electron reduced human CPR also indicated that FAD-to-FMN electron transfer rate is only ~11 s^{-1} in sodium dithionite reduced enzyme, with stimulation of the rate observed in NADPH-reduced CPR (55 s^{-1}).[83] The stimulation of internal electron transfer achieved with NADPH as electron donor was assigned to conformational effects in the coenzyme-bound form, perhaps particularly in the region of a "gatekeeper" tryptophan residue, the side chain of which shields the CPR FAD in the oxidised enzyme and must be displaced to enable electron transfer from the NADPH nicotinamide.[83] Stopped-flow measurements of FMN-to-heme electron transfer in P450 BM3 (determined by following rate of formation of the ferrous–carbon monoxide complex at 450 nm) indicated that the limiting rate for the first FMN-to-heme electron transfer reaction was ~223 s^{-1} at 25 °C (for myristic acid-bound enzyme), and that this is likely a major rate-limiting step in the entire substrate oxygenation catalytic cycle of the flavocytochrome.[79,84] Comparable rates of CPR FMN-to-P450 heme iron in eukaryotic P450s were reported as *e.g.* ~80 s^{-1} for the fast phase of heme reduction in benzphetamine-bound CYP2B4 and only 0.85 s^{-1} for substrate-free CYP1A1 (*e.g.* references 85, 86). Due to its relative ease of expression and purification, its soluble and single component nature, and to the fact that it was the first prokaryotic system to mimic the mammalian P450/CPR redox system—P450 BM3 has been adopted as an experimental model of the eukaryotic class II redox system. It has been extensively engineered to explore such aspects as substrate selectivity, cofactor binding and electron transfer (*e.g.* references 87–89). Atomic structural studies have also provided critical information for the general understanding of P450 mechanism, as detailed in the *P450cam and P450 BM3—paradigms in the P450 superfamily* section below.

The advent of genome sequencing in the 1990's led to the recognition that P450 BM3-like enzymes exist in several other bacteria, including various Bacilli, *Ralstonia metallidurans* and *Bradyrhizobium japonicum* (*e.g.* references 90, 91). Two BM3 homologues (CYP102A2 and CYP102A3) are present in *Bacillus subtilis* and both catalyse hydroxylation of fatty acids at the ω-1 to ω-3 positions.[92] BM3-type enzymes are also present in lower eukaryotes. In the fungus *Fusarium oxysporum* (a rice pathogen) a fatty acid hydroxylase activity (oxygenation at ω-1 to ω-3 positions, as for BM3) was discovered in the 1980's and shown to be due to a BM3-like P450-CPR fusion protein.[93,94] The enzyme (P450 foxy or CYP505A1) was strongly NADPH-specific and membrane associated (although apparently devoid of a true membrane anchor region).[95,96] Similar enzymes are evident in the genomes of other eukaryotes, including *Neurospora crassa* and *Aspergillus oryzae.* In *Fusarium verticilloides*, CYP505B1 likely functions as a hydroxylase in production of the polyketide mycotoxin fumonisin.[97]

Genome analysis also led to identification of another type of bacterial P450–redox partner fusion involving the fusion of a soluble P450 (N-terminal) to a FMN- and 2Fe–2S cluster-containing reductase resembling phthalate dioxygenase reductase (PDOR). The original enzyme originated from *Rhodococcus* sp. NCIMB 9784.[98] This type of enzyme is also observed in various species of the genera *Burkholderia* and in *Ralstonia metallidurans*[99] (Fig. 3). The isoforms from *Rhodococcus* sp. NCIMB 9784 (CYP116B2), *Ralstonia metallidurans* (CYP116B1) and *Rhodococcus ruber* strain DSM 44319 (CYP116B3) have been expressed and characterised, and constructs expressing the respective P450 and PDOR domains of the *Rhodococcus* enzyme have also been reported. All these enzymes have been shown to be NAD(P)H-dependent electron transferases.[100–103] Weak 7-ethoxycoumarin deethylase activity of the CYP116B2 protein was demonstrated, and CYP116B3 hydroxylates polycyclic aromatic hydrocarbons (including fluorene, indene and naphthalene) at low rates.[101,103] The physiologically-relevant enzymatic functions of these enzymes remain to be resolved, but it is of note that a non-fused P450 (ThcB or CYP116A1 from *Rhodococcus erythropolis*) has extensive amino acid identity (>50%) to the heme domains of these P450-PDOR enzymes, and catalyses oxidative attack and degradation of atrazine and thiocarbamate herbicides (*e.g.* EPTC: *S*-ethyl dipropylthiocarbamate).[104]

The BM3-type and P450-PDOR type fusion proteins are *bona fide* fusions of P450s with NAD(P)H-dependent reductases and are clearly catalytically self-sufficient P450s (*i.e.* requiring no other protein partner: simply coenzyme, dioxygen and substrate for oxygenation).[27] However, other types of P450 redox systems and fusion proteins have also been uncovered in recent years. Studies of the P450 BioI (CYP107H1) enzyme from *B. subtilis* confirmed its involvement in the biotin (vitamin H) synthesis pathway, and

showed that the P450 communicated productively with the two flavodoxin proteins from *B. subtilis* (YkuN and YkuP), as well as with the sole ferredoxin in the genome—the 4Fe–4S protein Fer.[105–110] In *Citrobacter braakii*, P450cin (CYP176A1) is involved in oxygenation of the monoterpene 1,8-cineole to facilitate its utilisation as a carbon source for growth, and also uses a flavodoxin redox partner (cindoxin) (Fig. 3).[111]

Given the ability of ferredoxin and flavodoxin proteins to act as single electron shuttles in various important microbial processes (*e.g.* reductive activation of pyruvate-formate lyase and biotin synthase), the ability of flavodoxins to participate in bacterial P450 electron transport chains was not entirely unexpected.[27,28,112,113] The fact that eukaryotic CPRs and the reductase domain of P450 BM3 have a flavodoxin-like domain responsible for mediating electron transport between the FAD/NADPH-binding domain and the P450 also demonstrates that the flavodoxin module is widely exploited in P450 systems.[84,114] The fact that flavodoxins can supplant ferredoxins in a class I-like system is now well established, with NAD(P)H-dependent FAD-containing reductases acting to reduce the flavodoxins. Indeed, the *E. coli* flavodoxin reductase–flavodoxin system has been shown to support activity of mammalian, as well as bacterial, P450s (*e.g.* progesterone 17α-hydroxylase activity of bovine P450c17).[115,116]

P450s have now been discovered in which flavodoxin or ferredoxin modules are fused to the hemoprotein (Fig. 3). In the bacterium *Methylococcus capsulatus*, a soluble P450 (N-terminal) fused to a ferredoxin occurs, with the two major structural domains attached *via* an alanine-rich linker.[117] The protein (named McCYP51FX) is a member of the CYP51 P450 family. The CYP51s are sterol demethylases which play essential roles in eukaryotes.[7] They are perhaps best known for their roles in yeasts and fungi, where the 3-step oxidation of lanosterol (resulting in cleavage of the 14α-methyl group) produces the membrane sterol ergosterol, essential for maintenance of membrane fluidity. This enzyme is the target for azole antifungals (*e.g.* fluconazole), and loss of membrane fluidity and integrity is a consequence of accumulation of methylated sterols.[118] The discovery of prokaryotic CYP51s was unexpected, since it was considered that sterol biosynthetic pathways did not exist in these organisms. The first prokaryotic CYP51 (CYP51B1) was characterised in the pathogen *Mycobacterium tuberculosis*, and has been structurally and biochemically analysed.[11,119–121] CYP51B1 catalyses demethylation of dihydrolanosterol and the plant sterol obtusifoliol, but there does not appear to be a true sterol biosynthetic pathway in *M. tuberculosis* (or in *M. bovis* or other mycobacterial or actinobacterial strains in which the enzyme also occurs).[122,123] However, in *Methylococcus capsulatus* there is compelling evidence for cholesterol biosynthesis and hence a physiological role for McCYP51FX. The fusion of the CYP51 to a ferredoxin (notionally a 3Fe–4S ferredoxin from amino acid sequence, although firm spectroscopic evidence for its identity has yet to be presented) likely offers advantages of coordinate expression of the P450 and its redox partner, and may (as in BM3) afford improved electron transfer kinetics as a consequence of enhanced domain interactions, optimised protein geometries and inter-cofactor (iron sulfur-to-heme) distances. However, this type of fusion enzyme must still source electrons from another redox partner, likely a NAD(P)H-dependent FAD-containing reductase.[27,124] Recently, a fusion between a soluble P450 and a flavodoxin was identified in the bacterium *Rhodococcus rhodocrous* (strain Y-11) (Fig. 3). The enzyme, XplA, has a flavodoxin domain at the N-terminal of the protein and participates in degradation of the explosive RDX (Royal Demolition eXplosive, hexahydro-1,3,5-trinitro-1,3,5-triazine). The host strain was isolated from RDX-contaminated soil, and plants expressing XplA may offer the possibility of degrading RDX in explosive contaminated soil.[125] At present, a physiological role for XplA is not clear, but it may be the case that the degradative reaction catalysed is reductive and thus that the reaction occurs efficiently only at low environmental oxygen concentrations (see below).

In studies from the Ortiz de Montellano group, a completely novel electron source for P450 catalysis was identified, necessitating operation of a further different type of redox partner chain. CYP119A1 from the archaeon *Sulfolobus solfataricus* was shown to be reduced in a non-pyridine nucleotide coenzyme-dependent manner by a 2-oxoacid-ferredoxin oxidoreductase and a ferredoxin from the related organism *Sulfolobus tokodaii*[126] (Fig. 3). The ferredoxin is a 7Fe protein (binding 3Fe–4S and 4Fe–4S clusters) and the 2-oxoacid-ferredoxin oxidoreductase catalysed pyruvate dehydrogenation and was reported to bind TPP, 4Fe–4S and CoA cofactors. The system supports lauric acid hydroxylation by CYP119A1 with a temperature optimum close to 70 °C. Subsequently, the comparable redox partners were cloned from *S. solfataricus* itself, and were also shown to support laurate hydroxylation by CYP119A1.[126,127] While the "traditional" P450 redox chain depends on NAD(P)H oxidation, the fact that the P450s do not interact directly with the dehydrogenase (but instead with an intermediate electron donor protein or domain, *e.g.* a ferredoxin) suggests that P450 reducing equivalents may be sourced from substrates much more diverse than pyridine nucleotide coenzymes in certain systems, as in the *Sulfolobus* system discussed above.

Recent developments in the characterisation of novel P450 redox partner systems and fusions clearly indicate that simplistic views of class I and II P450 redox systems are outmoded and that a wide variety of distinctive P450 redox systems likely remain to be characterised in nature. In this respect, the apparent presence of an acyl CoA dehydrogenase-P450 fusion in the genome of the bacterium *Pseudomonas fluorescens* may indicate a further novel class of P450 enzyme[128] (Fig. 3).

5 The major reactions of cytochromes P450

The P450s are oxygenases, and the classical reaction catalysed is the hydroxylation of organic substrates on carbon atoms—for example the hydroxylation of saturated fatty acids at the ω-carbon (as catalysed by eukaryotic CYP4 family P450s), or at ω-1 to ω-3 positions (by P450 BM3).[77,129] However, P450s are considerably more versatile in their reactivity and perform several other forms of chemical transformations.[130,131] Several different molecular conversions known to be catalysed by various P450 isoforms are shown in Fig. 4, along with examples of the P450s that perform these reactions. One of the most famous P450 reactions is the demethylation of sterols, with the 14α-demethylation of lanosterol catalysed by fungal CYP51 enzymes being the target reaction inhibited by the azole and triazole classes of antifungal drugs (including clotrimazole, fluconazole and ketoconazole).[132] In addition to dealkylation at carbon atoms, N-, S- and

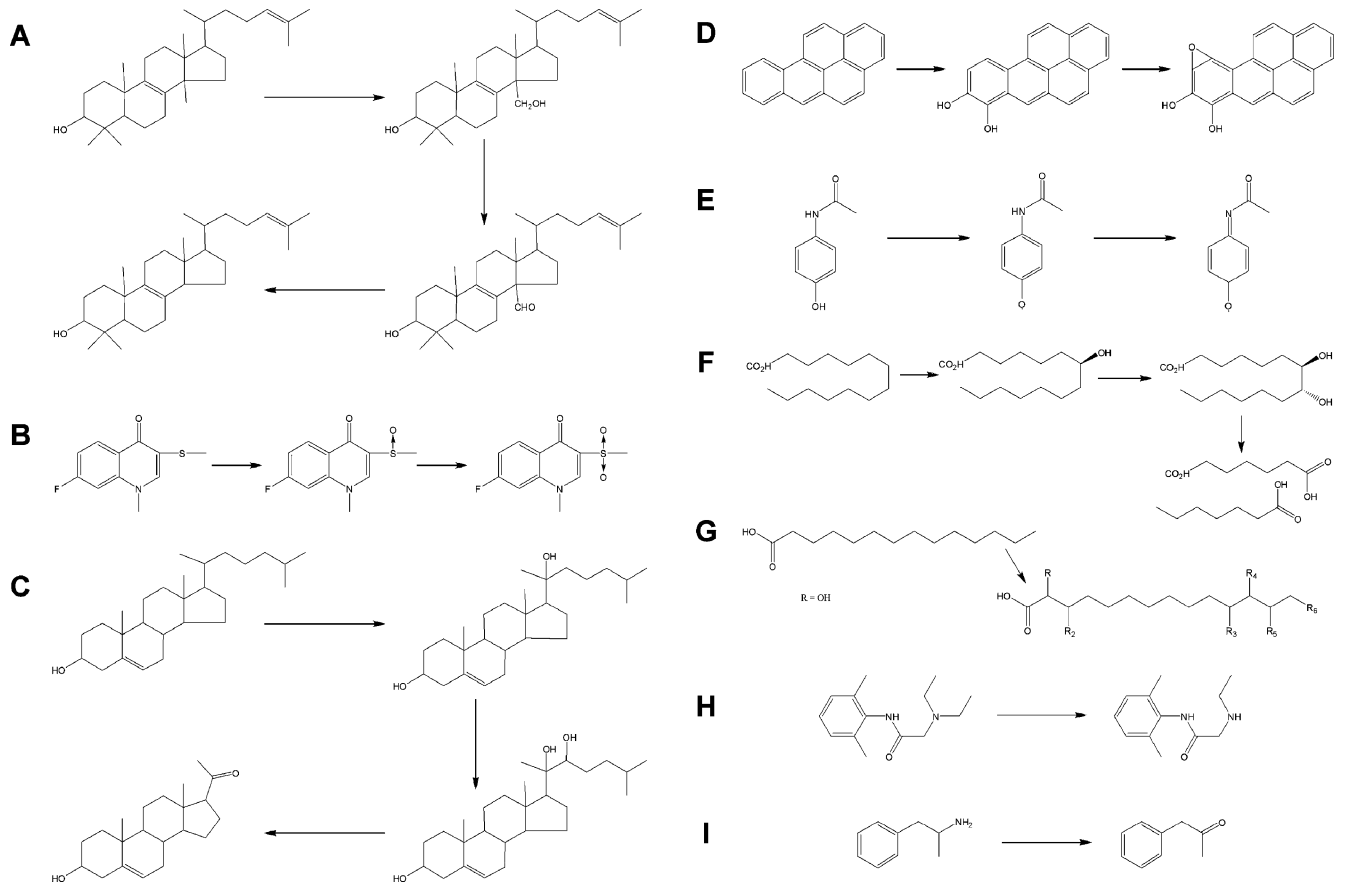

Fig. 4 Reaction classes catalysed by cytochromes P450. Typical reactions representing different major classes of P450-dependent oxidations are shown. A, Demethylation: the 3-step oxidation of the fungal sterol lanosterol catalysed by the sterol demethylase (CYP51). The product is the 14α-demethylated ergosterol.[7] B, S-oxidation: the stereoselective S-oxidation of the heart failure drug flosequinan by CYP3A2 in rat and human liver microsomes.[311] C, C–C bond cleavage: the 3-step oxidation of cholesterol resulting in its dihydroxylation, followed by side chain cleavage and the formation of pregnenolone, catalysed by P450scc (CYP11A1).[312] D, Polycyclic aromatic hydroxylation and epoxidation: the successive hydroxylation and epoxidation reactions catalysed by mammalian CYP1A1 with benzo[*a*]pyrene, leading to formation of genotoxic and carcinogenic epoxide products (the (+) and (−) benzo[*a*]pyrene-7,8-diol-9,10-epoxides).[313] E, Dehydrogenation: the P450-mediated dehydrogenation of acetaminophen to its iminoquinone, likely catalysed by CYP2E1 and other human P450s.[151,314] F, C–C bond cleavage: the successive hydroxylation and scission of tetradecanoic acid (or of tetradecanoyl coenzyme A) by *B. subtilis* P450 BioI (CYP107H1).[106] G, C–H bond hydroxylation: the hydroxylation of tetradecanoic acid at the ω-1, ω-2 or ω-3 position (by *Bacillus megaterium* P450 BM3 [CYP102A1]), at the ω position (by eukaryotic CYP4 enzymes), and at the α/β positions (by *Bacillus subtilis* P450 BS_β [CYP152A1]).[69,129,162] H, N-deethylation: the N-deethylation of lidocaine by rat liver CYP2B1 and CYP2B2.[315] I, deamination: deamination of amphetamine by rabbit CYP2C3.[316]

O-dealkylations are also catalysed by P450s; for example the N_3-demethylation of caffeine catalysed by human CYP1A2 and the O-demethylation of 5-methoxytryptamine by CYP2D6.[133,134]

The oxidative cleavage of carbon–carbon bonds is also catalysed by P450s. Perhaps the most famous conversion being the 3-step reaction catalysed by the mammalian P450scc (P450 side chain cleavage, CYP11A1), which results in removal of the cholesterol side chain and formation of pregnenolone and 4-methylpentanal as products.[135,136] This reaction occurs in mitochondria of the adrenal glands, and is the committed step in steroid hormone synthesis.[137] A similar reaction scheme is thought to be catalysed by *B. subtilis* P450 BioI (CYP107H1) in its conversion of long chain fatty acyl CoA esters (and/or fatty acids) to pimeloyl CoA (and/or pimelic acid) in the biotin synthesis pathway.[104]

Dehydrogenation reactions include the well-studied oxidation and desaturation of the anticonvulsant valproate to 2-*n*-propyl-4-pentenoic acid catalysed by various eukaryotic P450s, including CYP2B1 and CYP2C9.[138–140] Epoxidations of alkenes and of carbon–carbon double bonds in *e.g.* unsaturated fatty acids are well known P450 reactions.[141,142] A concerted mechanism of olefin epoxidation is inferred from the retention of stereochemistry observed in certain P450-mediated reactions (*e.g.* epoxidation of *cis*-stilbene and oleic acid).[138,143,144] However, a non-concerted pathway may also occur, since alkylation of heme at one of its pyrrole nitrogens can occur during P450-catalysed oxidation of terminal olefins to their epoxides.[145] Epoxides themselves do not alkylate the heme, and alkylation must occur due to formation of a reactive intermediate produced during the oxidative attack on the olefin.[138] Further evidence in favour of an alternative non-concerted pathway comes from the observation that carbonyl-containing products are formed during oxidation of some olefins, *e.g.* the formation of both oxide and aldehyde products from oxidation of trichloroethylene.[146] Again, such products do not appear to be due to rearrangement of epoxide products, but would

be consistent with the formation of a carbocation intermediate in an alternative reaction pathway to the olefin epoxidation route.[138,146,147] The presence of alternative pathways might be consistent with the two-state reactivity model of de Visser *et al.*, in which the ferryl porphyrin radical intermediate in the P450 catalytic cycle (*i.e.* compound I) exists in either doublet or quartet states. Different energy barriers to the closure of the epoxide occur according to nature of the intermediate, with the higher barrier from the quartet state transition enabling alternative reactions to compete with epoxidation (including heme alkylation).[148–150] The oxidation of acetylenes is also performed by P450s, although oxidation of terminal acetylenes is prone to result in covalent modification of the heme macrocycle if oxygen addition is to the internal carbon of the acetylenic bond. Addition to the external carbon leads to a ketene product.[138]

A particularly important P450 reaction in human physiology is that of aromatase (CYP19A1). Aromatase creates an aromatic steroid A ring in the oxidative conversion of the androgens androstanedione and testosterone to the estrogens estrone and 17β-estradiol, respectively.[151] This is another 3-step reaction which ultimately leads to removal of the substrate C19 methyl group. An interesting aspect of the reactions catalysed is the possibility of involvement of compound 0 (*i.e.* the ferric hydroperoxy species in Fig. 2) in the final step of side chain removal.[152,153] Other reactions of physiological and biotechnological relevance include the oxygenation of aromatic molecules, as exemplified by the epoxidation and hydroxylation of the cigarette smoke component benzo[*a*]pyrene to a carcinogenic diol epoxide, catalysed by human CYP1A1.[151] Human CYP1B1 also oxygenates and activates a spectrum of carcinogens, including polycyclic aromatic hydrocarbons (PAH's), heterocyclic and aromatic amines (*e.g.* benz[*a*]anthracene and 2-aminoanthracene).[151,154,155] Active site mutations also enhanced the capacity of *P. putida* CYP101A1 to oxygenate a range of PAH's, including phenanthrene and pyrene.[156,157] Oxidation at nitrogen atoms is another well known P450 reaction, and is often competitive with N-dealkylation. Examples include the N-oxygenation of *N*,*N*-dimethylalanine and *N*,*N*-dialkylarylamines by mammalian CYP2B1 and CYP2B4.[158,159] Other types of P450-mediated reactions include oxidative deamination and dehalogenation, reduction of epoxides and reductive β-scission of alkyl peroxides.[130,131] Detailed descriptions of the multiple reactions of human and other P450 enzymes, and of their mechanisms, are given in the excellent reviews by de Voss and Ortiz de Montellano, and by Guengerich.[138,151]

6 Unorthodox P450 systems and reactions

The reactions above all involve a relatively "traditional" P450 pathway whereby oxygen is reductively activated. The reactions are thus oxygen-dependent and also require interaction of the P450 with a redox partner and (ultimately) electron transfer from NAD(P)H. However, a series of other P450-dependent reactions are now recognised which do not involve the "orthodox" P450 pathway. As shown in Fig. 2, the pathway of collapse of the ferric hydroperoxy intermediate in the cycle generates hydrogen peroxide (H_2O_2) as a by-product as the ferric form of P450 is regenerated. The reaction can be forced in the productive direction by addition of peroxide to ferric substrate-bound P450, in the so-called "peroxide shunt" pathway.[160] This can be done with H_2O_2 itself or with organic peroxides (*e.g.* cumene hydroperoxide), but is often inefficient and usually results in at least partial oxidative destruction of the heme and in oxidative damage to the protein.[161] The discovery that nature had adopted this method of driving P450s came as a surprise to the field. P450 BS_β (CYP152A1) from *B. subtilis* catalyses fatty acid hydroxylation at the β-carbon, using H_2O_2 as a source of electrons/proton to drive the P450 from the ferric form directly to compound 0.[162] The enzyme is well adapted as a peroxygenase, and negligible heme destruction is observed in turnover (Fig. 5). CYP152's are not confined to *B. subtilis*. Homologues are found in other organisms, *e.g.* the bacterium *Sphingomonas paucimobilis* (CYP152B1).[163] Another novel P450 reaction that deviates from requirement for dioxygen is the reduction of two molecules of nitric oxide (nitrogen monoxide,

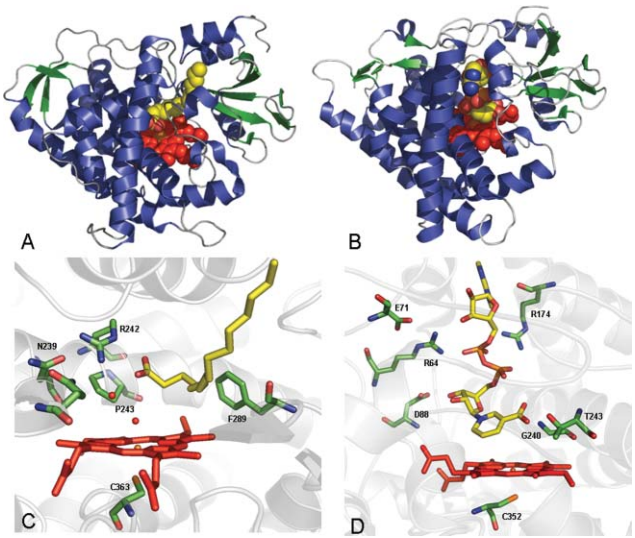

Fig. 5 Atomic structures of P450nor and P450BS_β. Atomic structures are shown for *B. subtilis* P450 BS_β (CYP152A1) and *Fusarium oxysporum* P450nor (CYP55A1). Panel A shows the topology of P450 BS_β and panel B that for P450nor, with helices and β sheets represented in blue and green, respectively. The heme cofactor is shown in red spacefill at the centre of both structures. The fatty acid substrate (palmitic acid) is shown in yellow spacefill bound to P450 BS_β, while the NADH analogue NAAD (nicotinic acid pyridine dinucleotide) is shown in atom coloured spacefill on the distal face of the heme in P450nor. Panels C and D show atomic detail from the active sites of P450 BS_β and P450nor, respectively. The alkyl chain of palmitic acid is indicated in yellow stick format in panel C, while the NAAD is shown as atom coloured sticks in panel D. The axial heme ligands for P450 BS_β (Cys 363 and the oxygen of a water molecule) and P450nor (Cys 352) are shown. Key amino acids in the active site at the distal face of the heme iron are also indicated in both cases. In P450 BS_β (panel C), the I helix is distorted by Pro 243, which is located close to the 6th coordination (water) position on the heme iron. Palmitic acid is stabilised by both hydrophobic and electrostatic interactions with Arg 242. The Phe 289 side chain also makes hydrophobic contacts with palmitic acid. The side chains of Asn 239 and Arg 242 (and that of Gln 85) create a polar environment to accommodate the fatty acid carboxylate and the H_2O_2 substrate.[162] The fatty acid carboxylate is proposed to assist in cleavage of the peroxide O–O bond. In P450nor (panel D), Arg 64 and Arg 174 bind and stabilise the coenzyme analogue, the latter forming a strong interaction with the dinucleotide pyrophosphate group. A salt bridge network involving Glu 71, Arg 64 and Asp 88 is disrupted on NAAD binding. Thr 243 and Glu 240 also make stabilising hydrogen bonds to the NAAD.[169]

NO) to dinitrogen oxide (N_2O) catalysed by the fungal P450nor (CYP55A1). The original P450nor was characterised from the rice pathogen *Fusarium oxysporum*, and there appear to be separate cytosolic and mitochondrial isoforms.[164]

CYP55A1 is nitrite/nitrate inducible and participates in an energy generating pathway for reduction of these molecules to N_2O. This pathway could be important to the host under oxygen limiting conditions, perhaps explaining CYP55A1's presence in mitochondria and how toxic effects of oxidative decay of NO and NO complexes of CYP55A1 can be minimised. CYP55A1's reductive removal of NO from mitochondria also prevents its action as an inhibitor of the fungal respiratory chain.[165] Other fungal isoforms have been analysed in *Trichosporon cutaneum* and *Cylindrocarpon tonkinese*.[166] In the latter case there are two separate mitochondrial and cytosolic isoforms (CYP55A2 and A3), and the cytosolic form has higher affinity for NADPH, suggesting a NO detoxification role in the cytoplasm.[167,168] P450nor, like P450 BS_β, bypasses requirement for an accessory redox partner, and the enzyme reacts with pyridine nucleotide coenzyme directly (NADH is preferred over NADPH for CYP55A1) to source reducing equivalents. Atomic structural data for a pyridine nucleotide (PAAD: 3-pyridine aldehyde adenine dinucleotide) complex provided insights into the mode of coenzyme binding (in the active site cavity above the heme plane) and the mechanism of hydride transfer. It was concluded from structural data that most PAAD molecules were oxidised to form NAAD (nicotinic acid pyridine dinucleotide) and that a large structural change accompanied ligand binding. Two arginines (Arg 64 and Arg 174) interacted with the ligand pyrophosphate, and stabilising protein side chain interactions with the nicotinic acid ring were also observed.[169] Stereo-selective hydride transfer from NADH to the NO-bound heme was suggested from the structure, with the pro-*R* side of the ligand C4 atom facing the heme-bound NO molecule (Fig. 5). The presence of an unusual active site salt-bridge network appears to destabilise the ligand-free structure, but its disruption on binding the coenzyme should also enhance rate of release of product ($NAD(P)^+$), thus helping to explain the enzyme's high catalytic rate (~1200 s^{-1} at 10 °C).[169,170] This is obviously a fascinating P450 class, but there is as yet no indication whether this type of P450 could be evolved (rationally or otherwise) from a reductase into a substrate-specific oxygenase. While the P450nor reaction is novel in terms of the direct interaction of P450 with coenzyme, the capacity of P450s to catalyse substrate reductions is well recognised. For instance, reductive dehalogenation of CCl_4 and halogenated anaesthetics such as halothane is catalysed by P450s under low oxygen tension.[171–173] In addition, degradation of the explosive RDX by the P450-flavodoxin enzyme XplA from *R. rhodocrous* (discussed above) also likely results from a reductive reaction catalysed in anoxic environments.[125]

Also mentioned above, CYP119A1 from the archaeon *Sulfolobus solfataricus* can be driven by a class I-like redox system that exploits pyruvate instead of NAD(P)H.[126,127] Thus, non-standard P450 reactions are now recognised which do not require dioxygen, exogenous redox partners or NAD(P)H. Other P450s that have dispensed with all three of these reaction components are those that catalyse either dehydration or molecular rearrangement reactions. Plant allene oxide synthases (CYP74A family, first characterised as the flax seed isoform) are involved in the pathway for synthesis of the plant growth regulator jasmonic acid. Flax CYP74A1 catalyses dehydration of fatty acid hydroperoxides to the respective allene oxides, which are reactive epoxides and transient intermediates in pathways to more stable end products (Fig. 6). The flax seed enzyme catalysed dehydration of 13(*S*)-hydroperoxylinolenic acid to the respective allene oxide at ~70–80000 min^{-1} at 25 °C, much faster than the rate for any oxygenase P450.[174] The product formed is the substrate of allene oxide cyclase, and is a precursor in the pathway to jasmonic acid. The plant allene oxide synthase P450 is the first enzyme of the octadecanoid pathway leading to synthesis of jasmonates.[175] The enzyme thus also has pivotal roles in *e.g.* plant development and defence.[176]

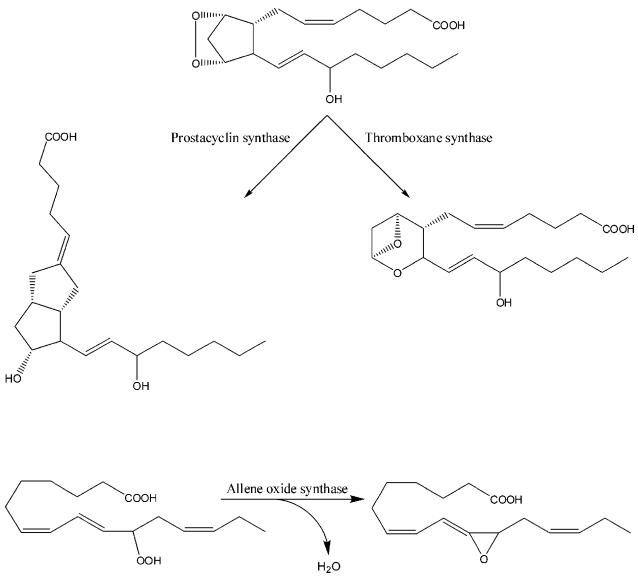

Fig. 6 Non-oxygen-dependent cytochrome P450 reactions. The reactions catalysed by the mammalian P450 enzymes thromboxane synthase (CYP5A1) and prostacyclin synthase (CYP8A1), and by the plant enzyme allene oxide synthase (CYP74A1), do not require molecular oxygen or interactions with (and electron delivery from) exogenous redox partners or cofactors. Instead, these enzymes catalyse molecular rearrangements of their respective substrates. In the upper reaction scheme the transformations of prostaglandin H_2 catalysed by thromboxane synthase (conversion to thromboxane A_2) and prostacyclin synthase (conversion to prostacyclin, also known as prostaglandin I_2) are shown. Reactions are hypothesised to progress *via* homolytic cleavage of the endoperoxide.[151,178–180] In the lower scheme, the allene oxide synthase-catalysed dehydration of 13(*S*)-hydroperoxylinolenic acid to the respective allene oxide is shown.[174]

The mammalian thromboxane synthase (CYP5A1) catalyses conversion of prostaglandin (PG) H_2 to thromboxane A_2. The product induces platelet aggregation and vasoconstriction.[177,178] The CYP5A1-dependent conversions of prostaglandins H_1, H_2, H_3 and G_2 into various products have in common the fact that all are molecular rearrangements for which neither dioxygen or electron transfer from NAD(P)H are required[151,178] (Fig. 6). The reaction is hypothesised to take place *via* homolytic cleavage of the endoperoxide in the PG substrate, with a ferryl heme iron bonded to one of the oxygens and a radical located on the other oxygen atom. This rearranges with transfer of the radical to a carbon atom, followed by electron transfer to the heme iron (which dissociates from the intermediate product and is restored to the ferric form), and then the collapse of the carbocation intermediate to yield the final stable product in the physiological reaction.[151,179]

A very similar reaction scheme is envisaged for prostacyclin synthase (CYP8A1). This has been purified from human and bovine cells and catalyses prostaglandin transformation (including PGH_2 and PGG_2) into the corresponding prostacyclins[151,179,180] (Fig. 6). Prostacyclin (PGI_2) is the CYP8A1 product derived from PGH_2 rearrangement, and has strong platelet anti-aggregation and vasodilation effects. The two products, derived from the actions of CYP5A1 and CYP8A1 on PGH2, are antagonistic in cellular function, and the balance of products is clearly important in human health.[151] Thus, as our knowledge of P450s and P450 reactions continues to expand, we become increasingly aware of their diversity, of their differing reactivity and mechanisms, and the differing requirements (or lack of these) for redox partners. There is, in fact, no longer an "orthodox" P450 system. However, hydroxylation reactions remain the most frequently observed and "stereotypical" P450 reactions, and oxygenations catalysed by P450s are frequently preliminary reactions that ultimately lead to end points such as cleavage of C–C bonds.

7 Insights from P450 redox system atomic structure

The age of structural biology has truly arrived for the P450 superfamily, with several distinct P450 isoforms now structurally resolved by X-ray crystallography, as well as substrate- and inhibitor/ligand-bound structures determined for certain P450s. Structural features general to the P450s are exemplified in the P450cam and P450 BM3 atomic structures. These P450s have the general shape of a triangular prism and two major structural domains that sandwich the heme *b*.[22,181] The so-called α and β domains are named according to their prominent secondary structural elements. The general fold (as shown in Fig. 7 for P450cam, CYP101A1) is conserved across the P450 superfamily, and there has yet to be any non-P450 enzyme shown to adopt this structural arrangement.[182] Thus, while eukaryotic and prokaryotic nitric oxide synthases perform similar reaction chemistry to P450s and have cysteinate-coordinated heme iron, their structural topology is dramatically different *e.g.*[183,184] At the time of preparation of this article, there were structures of 27 distinct P450s available (or pending release) on the PDB database (Table 1). While α helical and β sheet elements and their three dimensional arrangement are broadly conserved in the P450s (Fig. 7), there are substantial differences in the relative organisation of these elements and thus in the structural organisation of the P450 active site and other key regions of the enzymes.[182] While several amino acids are highly conserved within P450 families (families formally being P450s with ≥40% amino sequence identity and generally showing similar substrate selectivity[185]), there are few amino acids completely conserved across the P450 superfamily. The cysteine ligand to the heme iron is absolutely conserved and critical to P450 oxygenase function, and resides in a loop region (the β-bulge) preceding the L-helix (Fig. 7). Structural organisation of this segment protects the Cys ligand and enables it to accept hydrogen bonds from peptide NH groups. The structural arrangement of the Cys ligand is shared by two other heme *b*-containing monooxygenases (NOS and chloroperoxidase), which otherwise have protein folding patterns distinctive from P450s.[182,186,187] This points to the importance of the cysteine loop region in facilitating oxygenase catalysis in the P450s, and it appears clear that proximal coordination of the heme iron is by cysteinate anion in active P450s.[188] Protonation of the thiolate

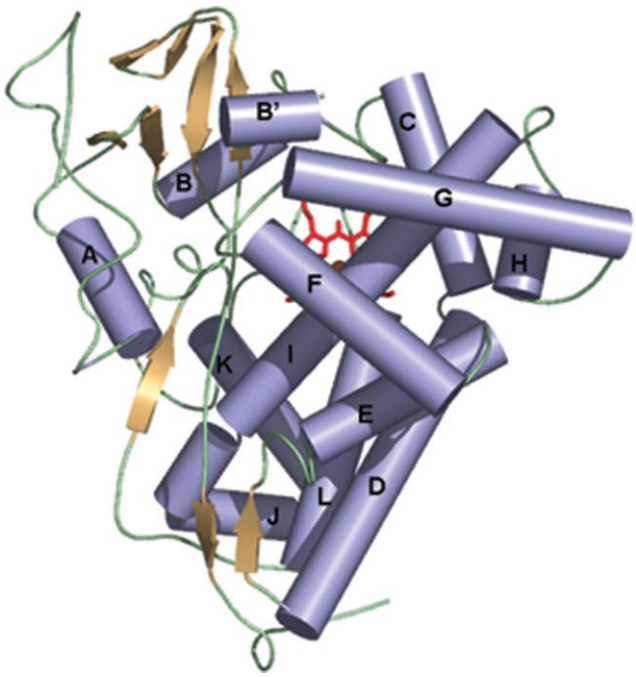

Fig. 7 General topology of cytochrome P450. The general fold for a P450 enzyme is shown, using the *P. putida* camphor hydroxylase P450cam (CYP101A1) as an example. The major secondary structural elements are shown and selected helices labelled according to standard nomenclature.[22] The α helices are represented as blue cylinders, with β sheet components as brown arrows. Interconnecting loop regions are shown in cyan string representation. The P450s adopt an overall structural fold resembling a trigonal prism, with the heme cofactor (shown in red) buried at the centre of the molecule. There are two major domains of a P450: the larger α (helix-rich) domain and the smaller β (sheet-rich) domain. The β domain is evident at the top left of the P450cam structure as represented here. Major structural elements include the long I helix, which runs across the distal face of the heme and contains several residues important for catalysis (including Thr 252 and Val 247, with important roles in coupling of electron transfer to substrate oxygenation and in substrate specificity, respectively).[201,202,250] The positions of structural elements close to the heme (including the I and L helices) are generally well conserved in the P450s, while there is considerable deviation in structural organisation of elements that control substrate specificity, including the B′ helix.[182]

to a thiol likely underlies the Soret optical transition from ~450 nm to ~420 nm for the ferrous–CO adduct of the heme iron, and this P450-to-P420 transition is associated with inactivation of P450 oxygenase function.[189] Substrate-binding stabilises the thiolate-coordinated (P450) form in certain P450s, and can even re-convert inactive P420 into active P450 enzyme.[121,190]

A heavily conserved residue located seven amino acids prior to the absolutely conserved cysteine is a phenylanine (Phe 393 in P450 BM3) which interacts with the Cys–Fe bond and plays an important role in tuning the reduction potential of the heme iron and its reactivity with dioxygen in P450 BM3.[191] Studies of BM3 F393A/H variants revealed a more positive heme iron potential and a concomitant acceleration of rate of electron transfer from the fused CPR redox partner. However, this led to a more stable ferrous–oxy intermediate and a substantial decrease in steady-state rate of fatty acid hydroxylation.[87] Thus, the conserved Phe plays a key role in poise of heme iron redox potential to facilitate both efficient electron transfer to the heme iron from the redox partner,

Table 1 Atomic structures of cytochrome P450 enzymes At the time of preparation of this article, the structures of 27 distinct P450 enzymes had been reported. All structures were solved by X-ray crystallography. In certain cases (*e.g.* for P450cam and P450 BM3) there are a number of structures available for individual P450 isoforms on the PDB (www.pdb.org) in various substrate/ligand bound forms. The table provides data for the first atomic structure solved in each case for all of the P450 enzymes. The table shows the current list in chronological order of the date of the first reported structure for each P450 isoform

P450	PDB code	Organism	Function
CYP101A1[22] (P450cam)	2CPP	*Pseudomonas putida*	Camphor 5-hydroxylase
CYP102A1[181] (P450 BM3)	2HPD	*Bacillus megaterium*	Fatty acid monooxygenase *e.g.* arachidonic acid hydroxylase
CYP108A1[292] (P450 terp)	1CPT	*Pseudomonas* sp.	α-Terpineol hydroxylase
CYP107A1[293] (P450eryF)	1OXA	*Saccharopolyspora erythraea*	6-Deoxyerythronolide B hydroxylase
CYP55A1[294] (P450nor)	1ROM	*Fusarium oxysporum*	Nitric oxide reductase
CYP2C5[196]	1DT6	*Oryctolagus cuniculus*	Unspecific oxidoreductase *e.g.* progesterone 21-hydroxylase
CYP119A1[295]	1F4T	*Sulfolobus solfactaricus*	Monooxygenase *e.g.* lauric acid hydroxylase
CYP51B1[11] (CYP51)	1EA1	*Mycobacterium tuberculosis*	Sterol demethylase *e.g.* obtusifoliol 14α-demethylase
CYP165B3[296] (OxyB)	1LFK	*Amycolatopsis orientalis*	Oxidative phenol coupling of CD-ring during vancomycin biosynthesis
CYP154C1[297]	1GWI	*Streptomyces coelicolor* A3(2)	12- and 14-Carbon macrolactone monooxygenase *e.g.* narbomycin hydroxylase
CYP121[12]	1N4O	*Mycobacterium tuberculosis*	Potentially involved in polyketide metabolism
CYP175A1[218]	1N97	*Thermus thermophilus*	β-Carotene hydroxylase
CYP152A1[162] (P450 Bsβ)	1IZO	*Bacillus subtilis*	Fatty acid monooxygenase *e.g.* myristic acid hydroxylase
CYP2C9[298]	1OG2	*Homo sapiens*	Unspecific oxidoreductase *e.g.* warfarin hydroxylase
CYP2B4[213]	1PO5	*Oryctolagus cuniculus*	Unspecific oxidoreductase *e.g.* aryl-4-monooxygenase
CYP167A1[10] (P450epoK)	1Q5D	*Polyangium cellulosum*	Epothilone C and D epoxidation
CYP165C4[299] (OxyC)	1UED	*Amycolatopsis orientalis*	Oxidative phenol coupling of AB-ring during vancomycin biosynthesis
CYP154A1[300]	1ODO	*Streptomyces coelicolor* A3(2)	Possibly involved in polyketide metabolism
CYP2C8[301]	1PQ2	*Homo sapiens*	Unspecific oxidoreductase *e.g.* cerivastatin monooxygenase
CYP176A1[111] (P450cin)	1T2B	*Citrobacter braakii*	1,8-Cineole monooxygenase
CYP119A2[302] (P450st)	1UE8	*Sulfolobus tokodaii*	Possible fatty acid monooxygenase
CYP3A4[208]	1W0E	*Homo sapiens*	Unspecific oxidoreductase *e.g.* progesterone 21-hydroxylase
CYP158A2[303]	1SE6	*Streptomyces coelicolor* A3(2)	Oxidative phenolic coupling involved in flaviolin polymerisation
CYP2A6[13]	1Z10	*Homo sapiens*	Unspecific small molecule monooxygenase *e.g.* 7-ethoxy coumarin hydroxylation
CYP2D6[304]	2F9Q	*Homo sapiens*	Unspecific oxidoreductase *e.g.* debrisoquine 4-hydroxylase
CYP107L1[291] (PikC)	2BVJ	*Streptomyces venezuelae*	12- and 14-Carbon macrolactone monooxygenase *e.g.* narbomycin hydroxylase
CYP8A1[305] (Prostacyclin synthase)	2IAG	*Homo sapiens*	Prostaglandin H_2 isomerisation

and the P450-mediated reduction of dioxygen.[87,191] The Phe is absent from a small number of P450s, including CYP10 from the pond snail *Lymnaea stagnalis* and members of the CYP74 (allene oxide synthase) and CYP8A (prostacyclin synthase) families. In the case of the CYP74/CYP8A enzymes (at least), these P450s do not perform oxygenation chemistry.[175]

Glycines in the β-bulge heme-binding region immediately around the conserved cysteine are also heavily, but not absolutely, conserved in P450s. The only other residues considered absolutely conserved in the P450 superfamily are a glutamate and an arginine in a EXXR motif in the K helix region (Glu 320 and Arg 323 in P450 BM3). These residues appear important for hydrogen bonding and maintaining a "meander" region of peptide ~10–15 residues to the N-terminal side of the heme binding loop and on the proximal side of the heme. These conserved residues are thought important for maintenance of P450 tertiary structure, and likely contribute to heme binding.[192,193] However, recent expression studies of the *Streptomyces coelicolor* CYP157C1 showed that this P450 binds heme and forms a normal (P450) ferrous–carbon monoxide complex, despite absence of the conserved arginine in this sequence. Mutagenesis to restore the arginine resulted in proteins that bound heme, but formed P420 CO complexes. Other CYP157 family members and *S. coelicolor* CYP156A1 also do not have the EXXR motif, and it may now be the case that only the cysteine iron ligand (or possibly the cysteine and the glutamate from the EXXR motif) is conserved across the P450 superfamily (which currently has >6500 members).[194]

The bacterial P450s are usually soluble enzymes, and X-ray crystal structures for the prokaryotic P450s are typically solved for proteins expressed from unmodified versions of the respective genes. However, eukaryotic P450s are membranous, and successes with structural characterisation of many of these P450s in recent years has resulted from protein engineering to remove the transmembrane domain at the N-terminus of the P450, and sometimes with secondary mutagenesis to prevent aggregation of soluble domains produced.[182,195,196] The structures solved to date confirm the typical topology of the P450s shown in Fig. 7. However, intriguing structural variations exist that give rise to the differing *e.g.* substrate selectivity and binding, redox partner interactions and conformational heterogeneity within the P450s. Examples include the dramatically different active site architecture observed in the first two P450s for which atomic structures were resolved. In P450 BM3 the active site is ~1 nm in diameter at the mouth of the entrance, with the active site extending ~2 nm down to the heme. Atomic structures of fatty acid-bound forms (both palmitoleic acid and the esterified substrate *N*-palmitoylglycine) show the long chain substrates extending down the active site channel towards the heme.[197,198] By contrast the active site in P450cam is more compact and substantial conformational rearrangements of the P450 do not occur on binding camphor (see *P450cam and P450 BM3—paradigms in the P450 superfamily* section below).[22,199] The high resolution atomic structure of *Mycobacterium tuberculosis* CYP121 shows a large active site cavity which is highly constricted in the immediate vicinity of the heme iron, suggesting a very specific type of substrate recognition.[12]

The *M. tuberculosis* sterol demethylase (CYP51B1) remains the only representative of this enzyme family to be structurally characterised, and catalyses eukaryotic CYP51-like 14α-demethylation of various sterol substrates (including the plant sterol obtusifoliol).[122] CYP51B1 exhibits a disordered I helix region in the ligand-free form, and a profound distortion of the I helix is observed in estriol- and ligand (4-phenylimidazole or fluconazole)-bound forms. This deformation, combined with the extended conformation of the BC loop region, creates a wide access channel to the active site. Comparisons of atomic structures of ligand-free and estriol-bound forms of CYP51B1 show that the C helix undergoes a helix-to-coil transition on estriol dissociation and that loss of C helix structure results in a more extensive opening to the heme pocket. Binding of azoles or estriol triggers substantial (4-phenylimidazole) or partial (fluconazole or estriol) C helix ordering. Azole binding does not affect conformation of the BC loop, but estriol binding releases the loop from its surface position and enables it to adopt a more closed conformation.[11,119,200]

In *P. putida* P450cam, a combination of atomic structural analysis and mutagenesis has been used to address the issue of proton delivery to heme iron–oxy species. Thr 252 on the distal (substrate) side of the heme plane is pivotal to a distortion of the I helix, with its side chain making a hydrogen bond to a peptide carbonyl oxygen atom, where the latter atom would normally be involved in hydrogen bonding to maintain the I helix.[182] It was hypothesised that this residue had a key role in proton relay and/or oxygen binding to P450cam. Mutagenesis of Thr 252 resulted in P450cam mutants in which NADH oxidation was substantially uncoupled from formation of product (5-*exo*-hydroxy-camphor).[201,202] An analogous mutant of *B. megaterium* P450 BM3 (T268A) also demonstrated considerable uncoupling of NADPH oxidation from oxygenation of saturated fatty acids.[203]

In the atomic structure of the substrate (6-deoxyerythronolide B or 6-DEB)-bound form of *Saccharopolyspora erythraea* P450eryF (CYP107A1, involved in the production of the antibiotic erythromycin[204]), an alanine replaces the threonine residue found in P450cam/P450 BM3 (and widely conserved as Thr or Ser in numerous P450s). However, a similar distortion of the P450eryF I helix is observed as in P450cam, with a water molecule occupying the same position as would be occupied by the Thr 252 side chain of P450cam.[205] Structural data for both the P450eryF–6DEB complex and the ferrous–oxy complex of P450cam are consistent with a model in which ordered solvent molecules at P450 active sites are likely proton donors to the heme-bound dioxygen to facilitate O–O bond cleavage and P450 catalysis.[205,206] In the P450cam oxy complex a new water molecule is positioned close to the oxygen and is poised for proton delivery. In the P450eryF complex, a substrate hydroxyl appears to anchor a water molecule in a similarly catalytically relevant position close to the iron.[182,205,206] Thus, both protein residues and substrates appear to be relevant to organising the active site solvent network to facilitate proton donation.[182] In *M. tuberculosis* CYP121, an intricate network of hydrogen bonding interactions between amino acid side chains and active site water molecules may also define a proton relay system.[12]

Important features from atomic structures of eukaryotic P450s include the fact that these are larger proteins than their prokaryotic counterparts, and there are typically insertions including additional helical elements between F and G helices (F′ and G′) and an extended peptide between J and K helices.[207] CYP3A4 is the major human P450 enzyme involved in clearance of xenobiotics, and atomic structures were solved for ligand-free enzyme and for complexes with metyrapone and progesterone.[208,209] Given

the large size of some known substrates for human CYP3A4 (including cyclosporine with a molecular weight of 1203 Da) and the atypical kinetics and ligand binding properties of the P450 (suggestive of two or more ligand–substrate binding sites), the finding that the active site of CYP3A4 was relatively small (*ca.* 950 Å^3) was unexpected. For instance CYP3A4 exhibits homotropic cooperativity in the oxidation of aflatoxin B1, and parallel studies are consistent with three distinct ligand binding sites in the enzyme.[210] Also, fluorescence resonance energy transfer (FRET) analysis was used to demonstrate that there were two binding sites for the fluorescent substrate 1-pyrenebutanol, and titration data were consistent with a sequential binding mechanism for the ligand.[211] Also unexpected was the fact that the ligand-bound structures did not demonstrate considerable changes in protein conformation, even despite the fact that the two ligands occupied different sites—either coordinating the heme iron in the active site (metyrapone) or bound peripherally in a postulated effector site (progesterone).[208] In view of the relatively small size of the ligands used in earlier structural studies of CYP3A4, Ekroos and Sjögren determined structures of CYP3A4 complexes with the larger inhibitory ligands ketoconazole and erythromycin.[212] Substantial conformational rearrangements were seen in the CYP3A4–ketoconazole complex, including the disruption of a large, ordered cluster of phenylalanine residues located above the active site and major changes in the organisation of the F and G helices and their interconnecting loops.[212] Importantly, two molecules of ketoconazole were bound in the CYP3A4 active site, with one coordinating to the heme iron and the second stacked above the first ketoconazole molecule. This is the first structural description of simultaneous binding of multiple ligands in a P450 enzyme, and is consistent with models of multiple ligand occupancy based on CYP3A4 kinetics. Conformational alterations in the CYP3A4–erythromycin complex were not as extensive as those in the ketoconazole complex, and the nature of the rearrangement in the F/G helix region was different, with residues moving in opposite directions. There are large increases in active site volume in both complexes, and possibly this increases to >2000 Å^3 in the erythromycin-bound form.[212] Thus, ligand-associated structural rearrangements in CYP3A4 are extremely difficult to predict, and the atypical (*i.e.* non-hyperbolic) kinetic and equilibrium binding properties of this enzyme may have their origins in multiple binding events and/or conformational states of the enzyme. Even more extensive conformational changes were observed in structures of CYP2B4, one of the most extensively studied mammalian P450s. CYP2B4's structure was determined in the ligand-free form and in complex with two azole drugs (4-(4-chlorophenyl)imidazole [CPI] and bifonazole). The ligand-free crystal form is a dimer which adopts a conformation in which the active site is wide open, with a cleft formed by F/G helices at one side of the molecule and the B′/C loop region and C helix on the other side.[213] Intriguingly, a histidine residue (His 226) from one of the monomers coordinates the heme iron in the other monomer. In the CPI inhibitor-bound crystals of a H226Y point mutant, a far more closed structure was observed with the B′/C loop region and the N-terminal portion of the I helix moving towards the active site and contacting the ligand, and with F/G helices also migrating towards the B′ helix to cap the active site.[214] However, when bound to the bulkier bifonazole ligand a distinctive open conformation of the P450 was observed, in which the I helix was distorted to accommodate bifonazole and unexpected ligand interactions with residues in the P450 C helix were observed.[215] Dramatic conformational changes are thus also a feature of CYP2B4 and are likely to be a hallmark of many of the mammalian drug metabolising P450s that have broad substrate selectivity. Conformational flexibility is also inferred from mapping positions of azole drug resistance-conferring mutations found in *Candida albicans* CYP51 onto the atomic structure of the *M. tuberculosis* enzyme. These mutations map mainly to flexible P450 regions (including the F/G and B/C helical sections and their intervening loops) rather than to the CYP51 active site, and suggest that resistance may be achieved by altered conformational dynamics in CYP51, as opposed to disruption of the active site.[11,27]

With respect to redox partner interactions, the importance of a positively charged region surrounding the proximal face of the P450 heme is well established, and several basic residues were identified in the structure of rabbit CYP2C5 (the first mammalian P450 crystal structure) that could form electrostatic interactions with an acidic face on the CPR partner.[114,196] In the case of P450nor (CYP55A1), however, the redox partner has been dispensed with in favour of direct interaction with NADH, which binds within the active site cavity. In P450nor there is a distinct lack of positive charge on the heme distal surface, as might be expected.[169]

Atomic structures have also been solved for two P450s from thermophilic organisms (CYP119A1 from *Sulfolobus solfataricus* and CYP175A1 from *Thermus thermophilus*). Obvious differences between these P450s and those from mesostable organisms are their shorter overall amino acid sequences and smaller surface loops with fewer labile residues at peptide turns and on the protein surface.[216,217] CYP119A1 also has a clustering of aromatic amino acids on one side of the P450 that has been validated (by characterisation of a site-directed mutant) as important for thermostability. An extensive network of salt bridges appears important in thermostability of both CYP119A1 and CYP175A1, with charged residues in the latter arranged into networks (rather than ion pairs) for added stabilisation.[218] Thermophilic adaptations clearly hold lessons important for rational engineering of mesostable P450s, to achieve greater thermal resistance.

8 P450cam and P450 BM3—paradigms in the P450 superfamily

The field of P450 enzymology is large and complex and the scientific focus on multiple isoforms has inevitably led to the discovery of vast variations in structural, catalytic and mechanistic features in the P450 superfamily. In the search for commonality that binds the P450s together, the detailed characterisation of model P450 systems has been of enormous use in understanding basic principles relating to structure and function in CYP enzymes. Due to the more experimentally amenable nature of soluble prokaryotic P450s, it is not surprising that the systems most studied, and from which most detail on P450 characteristics have been gleaned, are both bacterial. These systems are the *P. putida* camphor hydroxylase P450cam, (CYP101A1) and the *B. megaterium* fatty acid hydroxylase P450 BM3 (CYP102A1).[22,84]

P450cam was the first bacterial P450 studied in detail from the 1960s onwards, and the first P450 for which an atomic structure was resolved.[199] It is a representative of the class I redox system.

P450cam catalyses 5-*exo* hydroxylation of D-camphor, the first step in catabolism of the compound. P450 BM3 catalyses oxygenation of long chain saturated and unsaturated fatty acids ($\sim C_{12}$ to C_{20}). It hydroxylates saturated substrates near the ω terminus (at ω-1 to ω-3 positions). The physiological role of P450 BM3 remains uncertain, although it was postulated that BM3-type enzymes could have roles in detoxification of polyunsaturated fatty acids (*e.g.* linolenic acid).[219] P450 BM3 was the first prokaryotic class II redox system characterised from the early 1980s onwards, and is a natural fusion protein between soluble P450 and CPR proteins.[220] Its heme (P450) domain was the second P450 enzyme structurally resolved by X-ray crystallography.[181] Due to its eukaryotic-like redox system and its convenient fusion arrangement, together with the facts that its heme domain has strong similarity to eukaryotic fatty acid oxygenases and that is inducible by barbiturates in *B. megaterium* (as seen for many mammalian P450s), it was soon adopted as a tractable model for the mammalian class II systems.[221] Several defining features of P450s in general have been established through studies of P450cam and (latterly) P450 BM3.

For both P450cam and P450 BM3, substrate binding (camphor and fatty acids, respectively) induces a shift in heme iron spin-state equilibrium from low-spin towards high-spin (*i.e.* from $S = 1/2$ to $S = 5/2$) due to displacement of the distal axial aqua ligand and the creation of a pentacoordinate ferric heme iron species.[31] This results in an increase in the heme iron reduction potential (by ~130–140 mV).[32,222] This thermodynamic switch is an important regulatory feature in both P450s (and doubtless in several other P450 systems) and serves to control electron transfer between the P450 and the redox partner. If electron flow occurs in substrate-free P450, non-productive reduction of molecular oxygen occurs and reducing equivalents are wasted in formation of superoxide, H_2O_2 or water. Substrate-dependent regulation of heme iron potential ensures that electron transport to the heme iron is dramatically accelerated when substrate is available for oxygenation. While these data point to elegant thermodynamic control over electron transfer in the bacterial enzymes, it should be noted that many, particularly eukaryotic, P450s exist in a partial or predominant high spin-state in their natural environment, and that thermodynamic control of catalysis may be less important in these cases. However, conformational changes in P450 enzymes are now well established (see *Insights from P450 redox system atomic structure* section) and these are also clearly relevant to catalysis. For P450cam, there are no dramatic alterations in overall structure on substrate binding, according to atomic structural data for substrate-free and camphor-bound forms.[22,199] However, in P450 BM3 the structures of substrate-free and palmitoleic acid-bound forms of the heme domain are quite different, and a major structural rearrangement was considered to be a consequence of binding fatty acids.[197,198] In the palmitoleate-bound structure, fatty acid is bound at a considerable distance from the heme iron, with its carboxylate group interacting with residues Arg 47 and Tyr 51 at the active site mouth.[75,197] NMR studies, based on measurement of the relaxation effects of the unpaired electrons of the heme iron on protons of water and of the bound substrates, were consistent with this finding, suggesting a distance of ~7.6–7.8 Å from the terminal methyl of lauric acid substrate to the P450 heme iron in its ferric form.[223] Reduction of the complex suggested movement of the fatty acid closer to the heme iron (by ~6 Å), but as yet there is no crystal structure of a reduced fatty acid complex of the P450.[224]

Recent studies of mutant forms of P450 BM3 and other CYP102 family members cast some doubts on the general model of substrate-binding mode and substrate-induced conformational change, as developed by the aforementioned studies on P450 BM3. First, amino acid sequences available for several other CYP102 enzymes show that the Arg/Tyr motif is absent in all cases.[27] Studies of the CYP102A2 and CYP102A3 isoforms from *B. subtilis* indicate that catalytic properties (*i.e.* fatty acid oxygenation with similar regioselectivity) are like those of P450 BM3, and these data thus suggest that the fatty acid binding mode identified in the P450 BM3 palmitoleic acid-bound atomic structure is likely not that observed across the CYP102 family.[92] Obviously, structures for other CYP102 P450s in substrate-bound forms will be essential to resolve the issue. However, it is possible that the binding of palmitoleate identified in the ferric P450 BM3 complex structure reflects an unproductive mode stabilised fortuitously by interactions with substrate carboxylate, and one that would not be feasible in other CYP102 enzymes. In structural studies of mutant forms of P450 BM3, the "substrate-bound" conformation of the protein (*i.e.* as seen for wild-type P450 BM3) was also observed in atomic structures of both substrate-free (SF) and palmitoleate-bound (SB) forms of an A264E BM3 heme domain.[225] In the SF structure, the Glu-264 side chain adopts two positions—either as a ligand to the iron or interacting with the phenyl side chain of active site residue Phe 87.[226] In the SB structure, palmitoleate interacts with Phe 87 and the Glu 264 side chain is forced towards coordination with the heme iron. An interesting observation was that fatty acid substrates bound substantially tighter to A264E heme domain than to wild-type P450 BM3.[226] An obvious question then becomes whether binding of fatty acids genuinely induce the conformational rearrangement observed in the wild-type P450 BM3 (*i.e.* SF-to-SB), or whether different conformers exist naturally in solution and that substrate binds more tightly to, and stabilises, the SB conformer. Data for the A264E variant are consistent with the latter conclusion, as is the more recent structural identification of a third conformer (in both wild-type BM3 and an A264H mutant) in which there is more open access to the active site, likely reflecting a state primed for substrate entry.[227] Thus, structural studies of P450cam and P450 BM3 point towards different P450 types in which conformational rearrangements in substrate-bound forms are less or more important, respectively, to catalysis.

Abiding questions in P450 catalysis relate to mechanisms by which proton and electron transfer to the heme iron is achieved. As seen in the catalytic cycle shown in Fig. 2, delivery of two protons is required for formation of the ferryl–oxo compound I species. Seminal studies on P450cam provided key information on the mechanism of proton relay in P450s, and (as discussed above) conserved amino acids with hydroxyl side chains (Thr 252 in P450cam, Thr 268 in P450 BM3) are critical for organisation of a solvent network to facilitate proton delivery.[182,203] Electron transfer from redox partners has also been studied extensively in both systems. In P450cam, interactions with the 2Fe–2S protein putidaredoxin (Pd) have been analysed extensively by mutagenesis and by analysis of electron transfer reactions between the partner enzymes. While there is no atomic structure of a P450cam–Pd complex, there are atomic structures available for both Pd and its electron donor protein putidaredoxin reductase (Pdr).[228,229] Structural analysis of Pd indicates that it has a much lower asymmetric charge distribution compared to homologues from

eukaryotes, with only two positive residues in the vicinity of the 2Fe–2S cluster (Asp 38 and Arg 66). Pd docking with P450cam is thus likely to differ from the molecular recognition process seen in *e.g.* interaction of mitochondrial P450scc (CYP11A1) and its ferredoxin (adrenodoxin), where electrostatic recognition is important.[230] Pd residues important for productive interactions with P450cam include Asp 38 (a mutant of which all but eliminated P450 activity) and the C-terminal residue Trp 106.[231–233] The Pd–P450cam interactions are highly specific, and recent studies suggest that these interactions are also guided by redox states of both ferredoxin and P450 proteins.[234]

In P450 BM3, there is clearly great interest in determination of a structure for the intact flavocytochrome, in order to understand in atomic detail the mode of interaction between CPR and P450 domains. A note of caution is sounded by the existing atomic structure of the membrane binding region-deleted form of rat CPR, in which its component FAD/NADPH- and FMN-binding domains are closed together in the $NADP^+$-complex, demonstrating a direct route of electron transfer between FAD and FMN cofactors. The flavins are separated by only ~4 Å and direct electron tunnelling undoubtedly occurs.[114] Reorientation of domains is clearly required to enable the CPR FMN domain to interact with, and transfer its electrons to, cognate P450s.[235] Possibly, flavin redox status and/or binding/debinding of NADP(H) coenzyme are key factors in controlling CPR conformational equilibria. In recent structural studies of neuronal NOS (nNOS) reductase domain, similar "closure" of the FAD and FMN domains was observed, with direct FAD-to-FMN electron transfer also inferred in nNOS and flavin methyls separated by only ~4.8 Å.[236] Flexibility of the FMN-binding domain is observed in nNOS reductase structures, with distinct rotated positions seen for the two crystallographically distinct FMN domains in the nNOS homodimer structure. The nNOS reductase FMN domain positions are also distinct from the FMN domain position in the CPR structure.[114,236] FMN domain motion is clearly an important feature in the diflavin reductase enzyme class, and binding of the protein calmodulin is also known to induce structural reorganisation in NOS.[237] The atomic structure of an FMN–heme domain construct of P450 BM3 was determined (the first ~630 residues of the 1048 amino acid flavocytochrome being structurally resolved).[238] It was found that domains were proteolytically separated and present in a non-stoichiometric ratio (2 heme domains for each FMN domain).[238] Despite this, the FMN (flavodoxin-like) domain was positioned towards the proximal heme face of the heme domain, in a position near to that in which a realistic electron transfer complex with the P450 might be formed. However, a proposed FMN-to-heme electron transfer pathway involving multiple amino acids and their peptide bonds is unlikely due to the extended distance involved.[84] Earlier hydrodynamic studies on P450 BM3 indicated that it was predominantly dimeric, and more recent work has demonstrated that mutant forms devoid of FMN (G570D) or with hexacoordinated heme iron (A264H), and that are inactive as fatty acid hydroxylases in isolation, can reconstitute this activity when combined.[88,239,240] This indicates that the P450 BM3 flavocytochrome dimer is active as a fatty acid hydroxylase, with electron transfer occurring from FMN in the first monomer to heme in the other monomer.[240] A similar mode of operation has been shown for eukaryotic NOS.[241] Thus, understanding of the productive mode of interaction of the FMN and heme domains in P450 BM3 is likely complicated by this dimeric organisation, and may await structural resolution of the intact flavocytochrome.

Extensive rational mutagenesis studies have been done on both P450 BM3 and P450cam, and have been highly informative in terms of delineating roles for individual amino acids in dictating substrate discrimination, and also in defining mechanistic functions. More recently, forced evolution has also proven an important tool for selectively altering P450 properties (Fig. 8). In P450 BM3, Phe 87 was recognised from atomic structures as an important residue for control of regioselectivity of fatty acid oxygenation.[181] The terminal (ω) methyl group of fatty acids

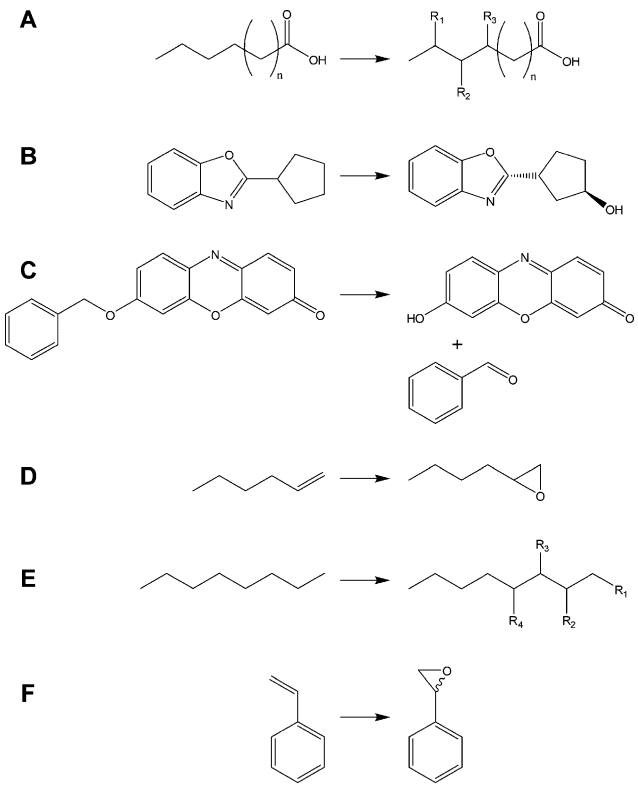

Fig. 8 Reactions catalysed by wild-type and mutants of flavocytochrome P450 BM3. Wild-type and mutant forms of P450 BM3 (created by both rational mutagenesis and forced evolution) have been shown to catalyse the oxygenation of a diverse array of organic molecules. Key examples are shown. A, The general hydroxylation of saturated, linear chain fatty acids at the ω-1, ω-2 and ω-3 positions (indicated by R_1–R_3).[69] B, The stereoselective hydroxylation of an achiral cyclopentanecarboxylic acid derivative (2-cyclopentylbenzoxazole) to the *R*,*R*-diastereomer catalysed by a P450 BM3 variant (1-12G) that was created by gene shuffling between a number of mutant BM3 P450s (themselves generated by both rational mutagenesis and forced evolution). Other mutants created favoured the production of the *S*,*S*-diastereomer.[317] C, Oxidative bond cleavage of benzoxylresorufin by wild-type BM3, leading to production of resorufin and benzaldehyde.[318] D, Enantiospecific epoxidation of terminal alkenes by engineered P450 BM3 mutants created by a combination of forced evolution and rational mutagenesis in the P450 active site. Reaction is shown as the oxidation of 1-hexene to 1,2-epoxyhexane.[141] E, Hydroxylation of octane at the ω and ω-1 to ω-3 positions, catalysed by force evolved BM3 mutants containing several active site mutations.[258,319,320] F, Stereoselective epoxidation of styrene to styrene oxide by a multiple mutant of P450 BM3, and A184 mutants thereof, which switch enantioselectivity of oxidation towards the *S*-enantiomer of styrene oxide.[321]

interacts with the Phe 87 side chain above the plane of the heme iron, and is thus protected from oxidative attack. Saturated fatty acids are instead oxygenated at ω-1 to ω-3 positions.[69] An F87A mutant was reported to catalyse fatty acid hydroxylation at the ω-carbon, using NMR to identify the product of laurate oxidation.[242] However, other studies on a F87V mutant suggested that oxygenation position of arachidonic acid moved away from the ω-end.[15] In wild-type P450 BM3 there was conversion of arachidonic acid to an almost enantiomerically pure 18(*R*)-hydroxyeicosatetraenoic acid and 14(*S*),15(*R*)-epoxyeicosatetraenoic acid mixture (*ca.* 80:20 ratio of products). In the F87V mutant the latter product accumulated as ~99% of the overall product with negligible 18(*R*)-hydroxyeicosatetraenoic acid detected.[15,78] Other P450 BM3 Phe 87 mutants were also shown to improve oxidation of certain aromatic compounds, as discussed further below. Elsewhere in the P450 BM3 heme domain, mutation of Arg 47 at the active site mouth (a likely interaction site for the carboxylate group of fatty acid substrates) resulted in weakened affinity for fatty acids (increased K_m values for lauric acid and arachidonic acid with R47A and R47G mutants) and decreased k_{cat} values.[75] A R47E mutant bound alkyltrimethylammonium derivatives of C_{12}–C_{16} saturated fatty acids much tighter than wild-type BM3 (lower K_m's and much higher k_{cat} values), again consistent with a role for Arg 47 in interaction with the fatty acid carboxylate group.[243]

In the P450 BM3 reductase domain (as with CPR and other eukaryotic diflavin reductases, including NOS and methionine synthase reductase), a "catalytic triad" of residues is conserved around the NADPH binding site and is implicated in catalysis of hydride transfer from the coenzyme to the enzyme FAD.[244] In P450 BM3 these residues are Cys 999, Asp 1044 and Ser 830. From structural and catalytic studies of wild-type and mutant forms of rat CPR, these residues are considered essential for binding and orientation of the coenzyme nicotinamide ring, and in promoting hydride transfer by the stabilisation of a transient carbocation on the nicotinamide following hydride transfer to the FAD.[244] The P450 BM3 C999A mutant catalysed ferricyanide and cytochrome *c* reduction much slower than wild-type enzyme, largely due to a considerably decreased rate of hydride transfer from the coenzyme, consistent with the postulates of Kasper and co-workers.[89,244] A stable charge transfer species was formed between reduced (hydroquinone) FAD cofactor and oxidised coenzyme ($NADP^+$) in the wild-type BM3 FAD domain, but not in the C999A variant—suggesting that the conserved triad cysteine is also important for stabilising the $FADH_2$–$NADP^+$ complex following electron transfer from the coenzyme.[89] Elsewhere in the BM3 reductase, the aromatic side chain of W1064 covers the isoalloxazine ring of the FAD, and must be displaced on binding NADPH to enable flavin reduction.[235] The comparable residue in human CPR is Trp 676, and a W676A mutant demonstrated a 1000-fold switch in specificity from NADPH towards NADH for CPR-catalysed cytochrome *c* reduction.[81] In the comparable W1064A/H mutants of P450 BM3 reductase domain, the mutations effected an ~100 mV increase in reduction potential of the FAD, and an even more substantial switch in coenzyme selectivity towards NADH was observed (~6000-fold for BM3 reductase-catalysed ferricyanide reduction).[245] Given the much lower cost of NADH, these substrate specificity conversions may be of significance for the biotechnological exploitation of P450 BM3 (see below).

In studies of P450cam, there is a long history of rational mutagenesis aimed at exploration of structure–function relationships. Among the most interesting mutants is T252A, where substantial uncoupling of NADH oxidation from camphor hydroxylation was observed.[201,202] The majority of reducing equivalents wasted were diverted into H_2O_2 production, consistent with collapse of the catalytic cycle at the compound 0 stage (Fig. 2). As discussed above, it is thought that this amino acid is essential in hydrogen bonding to a water molecule that acts as proton donor for scission of dioxygen bound to the heme iron.[182] Structural detail demonstrated that the hydroxyl side chain of active site residue Tyr 96 forms a hydrogen bond with the ketone group of D-camphor, and regioselectivity was altered in a Y96F mutant, with hydroxylation occurring at the 3 and 6 positions in addition to the 5-*exo* hydroxylation observed with wild-type P450cam.[246,247] These data were consistent with less specific substrate interactions and, possibly, greater mobility of camphor in the mutant active site. Y96A/F mutants were also shown to substantially enhance rate of production of styrene oxide from styrene, and to increase coupling of NADH oxidation to styrene oxide formation by up to ~5-fold.[248] Turnover of naphthalene to 1-naphthol and 2-naphthol was also enhanced in various Tyr 96 mutants.[249] Mutations of other active site residues proximal to bound camphor (V295I and V247A) also resulted in perturbations to product profiles with camphor analogues, again consistent with the importance of several active site contacts in maintaining the bound substrate in a position appropriate for attack at the 5-*exo* position by the P450 compound I.[250] Also apparent from mutagenic studies on redox partner (Pd) interactions was the fact that the Pd C-terminal tryptophan was important for its binding to P450cam.[233] Mutations at basic residues on the proximal face of the P450 (Arg72, Lys344 and Arg112) diminished binding to Pd, consistent with the importance of electrostatic interactions guiding productive redox partner interactions.[251,252] Combinations of mutations in the active site and heme vicinity of P450cam have also been shown to effect considerable changes in substrate selectivity (Fig. 9). For instance, variants containing 8 or 9 point mutations (including the L358P mutation that removes a hydrogen bond between the main chain amide of the leucine side chain and the heme iron cysteine thiolate) were shown to catalyse oxidation of ethane to ethanol, albeit with substantial uncoupling of NADH oxidation from product formation.[253] A rational and progressive process of decreasing active site volume (by mutations to introduce bulky amino acid side chains) was also successful in creating P450cam variants able to oxygenate the gaseous alkanes butane and propane.[254] Earlier studies used a similar approach to improve reaction efficiency and coupling with ethylbenzene as the substrate.[255,256] P450cam has also been rationally evolved to oxygenate polychlorinated benzenes (1,3-dichlorobenzene and 1,3,5-trichlorobenzene),[157] and mutations to engineer out the P450 BM3 carboxylate binding motif (Arg 47 and Tyr 51) in combination with active site mutations (including mutations to Leu 437) were also shown to enable efficient oxidation of both polycyclic aromatic hydrocarbons (phenanthrene and fluoranthrene) and short chain alkanoic acids.[17,257]

Recent years have seen the application of directed (forced) evolution in attempts to alter the properties of P450s, and the greatest successes have undoubtedly come with studies of P450 BM3. The enzyme has been evolved to produce large increases in

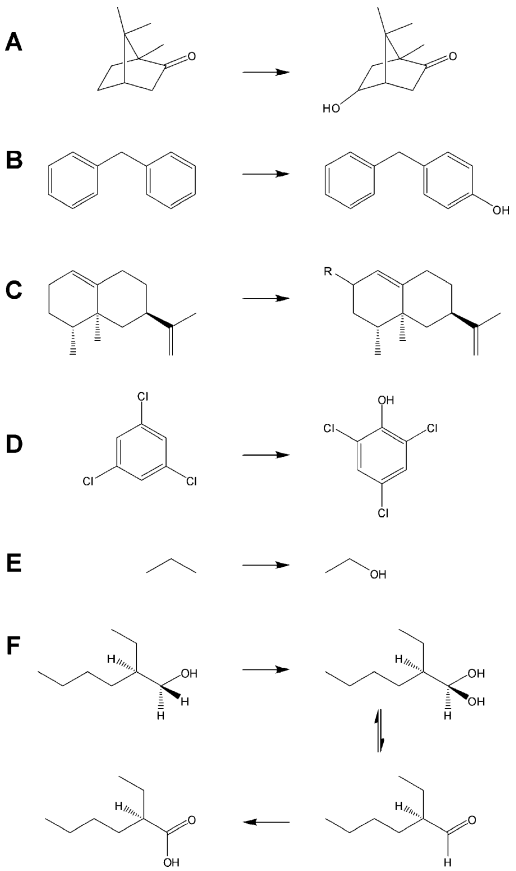

Fig. 9 Reactions catalysed by wild-type and mutants of P450cam. P450cam, the most intensively studied P450, has been shown to catalyse a range of oxidative reactions, and mutated forms further enhance the substrate specificity range of the enzyme. Key examples are shown. A, The natural reaction of wild-type P450cam: the 5-*exo* hydroxylation of D-camphor.[3] B, Conversion of diphenylmethane to 4-hydroxydiphenylmethane by variants isolated from active site mutant P450 libraries.[322] C, Oxygenation of (+)-valencene to (+)-*trans*-nootkatol (R: -OH) and the fine fragrance (+)-nootkatone (R: =O) by variants of P450cam containing the F87A/L and Y96F mutations (with (+)-nootkatone production enhanced by the L244A mutation).[268] D, Conversion of 1,3,5-trichlorobenzene to 2,4,6-trichlorophenol; catalysed poorly by wild-type P450cam and dramatically improved in a F87W, Y96F, V247L triple mutant.[157] E, Conversion of ethane to ethanol catalysed by a multiple mutant of P450cam (incorporating the L358P mutation adjacent to the Cys 357 heme ligand).[253] F, Oxidation of ethylhexanol to ethylhexanoic acid by wild-type and active site mutants of P450cam.[323]

the rate of oxidation of alkanes. In a BM3 variant named 139-3 with 11 individual mutations (only two of which are in the active site), turnover of pentane, hexane, cyclohexane and octane was increased to levels comparable with those for wild-type BM3 and some of its slower fatty acid substrates (*i.e.* from <200 min^{-1} up to 3–4000 min^{-1}).[258] Butane and propane were also oxygenated, and the 1- and 2-hydroxylation of propane was further improved in subsequent rounds of evolution of the 139-3 mutant to produce the 9-10A mutant with thirteen individual mutations.[18] It is difficult to rationalise clearly the structural reasons underlying enhanced affinity and activity for alkanes in the 139-3 variant, although disruption of a salt bridge (that might facilitate conformational change to enable alkane binding) and the fact that a number of mutations are located in the flexible F/G helix region are possible explanations.[258] Later studies used active site saturation mutagenesis to generate other derivatives of mutant 9-10A that further enhanced propane oxidation and facilitated conversion of ethane to ethanol.[259] Oxidation of methane to methanol, however, has yet to be achieved by any P450 mutant. In other directed evolution studies, BM3 variants have been created that enhance thermostability and peroxide shunt activity of the enzyme.[260,261] The peroxygenase variant was further evolved to enable production of human-like metabolites of the β-adrenergic blocker drug propranolol using H_2O_2.[262] Enzyme tolerance to solvents (dimethyl sulfoxide and tetrahydrofuran) for its lipid substrates was also improved by directed evolution.[263] The era of forced evolution will obviously usher in production of mutant P450 (and other) enzymes with novel properties and biotechnological applications. However, there are new challenges involved for structural biologists and enzymologists with respect to rationalising (for P450s containing multiple mutations) how many of the mutations identified actually contribute to producing the desired biochemical effects, and also in sorting the "wheat from the chaff" in terms of establishing which individual mutations compound the desirable effects of others, and which make negligible overall difference.

9 Technological and biotechnological advances

With their critical roles in eukaryotic and bacterial physiology, and their capacity to introduce oxygen atoms regio- and stereoselectively into organic substrates, it is no surprise that P450s have received immense attention from both academic and industrial consortia aiming to *e.g.* exploit their reaction chemistry for production of high value biomolecules, enhance their catalytic performance or alter substrate selectivity, enzyme stability or other characteristics by rational mutagenesis or forced evolution. There has been a long history of rational engineering of P450 structure–function, with a major breakthrough being conversion of substrate selectivity of murine P450coh (CYP2A5) from coumarin to testosterone hydroxylation by a single F209L mutation.[264] The Phe 209 residue is located in the F/G helix region (possibly in the interconnecting loop between helices), an important region for substrate recognition and active site entry.[182,265] A further excellent example of conversion of P450 specificity by point mutation was the switch in regioselectivity of steroid (androstanedione and testosterone) hydroxylation from the 16β to the 15α position by two point mutations (I114A/G478S) in CYP2B1.[266]

Rational approaches to P450 engineering have been aided by availability of atomic structures for several P450s (Table 1), as well as by the huge (and increasing) database of gene and translated amino acid sequences for P450s from all domains of life. This has enabled a systematic approach to investigating roles of phylogenetically conserved amino acids, and to establishing how single mutations can accomplish substantial changes to P450 properties. Examples include the demonstration that the invariant P450 cysteine is the thiolate ligand to heme iron (and is essential for oxygenase catalysis), and that the strongly conserved phenylalanine residue in the heme-binding motif is pivotal in modulating thermodynamic properties and reactivity of the heme.[87,267] In the P450cam system, structural data enabled rational active site mutagenesis that introduced catalytic activity

towards the sesquiterpene (+)-valencene, and the ability to produce the fine fragrance (+)-nootkatone.[268]

In human CYP2A6, a L160H mutation is a natural polymorphism that diminishes catalytic activity of nicotine oxidation, and leads to a switch from nicotine 7-hydroxylation to 3-hydroxylation.[151,269] It is plausible that defects in nicotine metabolism lead to reduction in an individual's smoking levels and to a lowered lung cancer risk, particularly since CYP2A6 activates tobacco carcinogens (including nornitrosonicotine [NNN] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK]).[151] However, links between CYP2A6 genotype and smoking-related cancer remain speculative. A genetic test involving CYP2A6 has been marketed (http://www.nicotest.com), but is controversial. The basis of the NicoTest™ genetic blood test is the establishment of the usefulness and extent of requirement for nicotine replacement for individuals wishing to give up smoking. The test checks for polymorphisms in the dopamine D2 receptor (DRD2) gene, which are claimed to affect relative usefulness of nicotine replacement therapy (patches) and the drug Zyban™ (bupropion, which reduces craving for nicotine) for the subject. Simultaneous assessment of the CYP2A6 gene is designed to inform on ability of the subject's P450 to metabolise nicotine, and hence on the level of nicotine replacement to be used. However, the organisation GeneWatch UK (http://www.genewatch.org) has expressed concerns relating to validity of the approach since they consider it unlikely to provide useful information for male subjects. Notwithstanding the controversy over the relevance of genetic testing for CYP2A6 polymorphisms with respect to smoking cessation, there have been other notable successes with commercialisation of expressed P450s. Systems available for analysis of P450 catalytic properties, inhibition profiles *etc.* include P450 and redox partner enzymes overexpressed in insect microsomes using the Baculovirus system (BD Biosciences) and human P450s co-expressed with CPR in *E. coli* membranes (Cypex Ltd). Assays have also been commercialised for measurement of activities of several important individual human P450 isoforms (*e.g.* from Promega), and these are of particular relevance for determining specific inhibition of key P450s involved in drug metabolism.

With respect to developing P450 enzymes with desirable features and enhanced catalytic properties, there have been great advances in recent years in scientists' ability to produce soluble, functional domains of eukaryotic P450s.[270] As discussed, these breakthroughs have also facilitated crystallisation of several important mammalian drug-metabolising P450s, leading to determination of their three dimensional structures and more detailed understanding of their mode of interaction with substrates and inhibitors.[182] The coming years will doubtless see the determination of several more P450 crystal structures, and further development of our understanding of interactions of P450s with important biomolecules. There have also been substantial efforts towards creation of P450–redox partner fusion enzymes as a means of enhancing electron transfer to P450s (and hence P450 activity) and generating catalytically self-sufficient P450s similar to P450 BM3. Most studies have involved genetic fusion of eukaryotic P450s to CPR enzymes, often resulting in moderate increases in turnover number with respect to the rates obtained by "natural" reconstitution of individual P450 and CPR enzymes.[271] However, no such fusion yet reported has an oxygenase activity at a level even 10% of that for P450 BM3, although this is at least partially explained by the much faster electron transport process within the BM3 CPR compared to those for eukaryotic CPR enzymes.[79,83] Examples of catalytically functional and stable mammalian P450/CPR fusion enzymes include CYP3A4 and CYP17A chimeras.[272,273] Another interesting P450 fusion enzyme was a "triple" fusion of P450cam to both Pdr and Pd (*i.e.* all components of this class I P450 system). While (as with P450 BM3 mimics) the camphor hydroxylase activity of this fusion was much less than that of the mixture of the individual proteins ($\sim 30\ min^{-1}$), the single component system has clear advantages in simplicity of expression and purification.[274] Although the hiatus of activity in research on P450/redox partner fusions was in the 1990's, there are obvious applications for such systems in *e.g.* diagnostics and fine oxychemical production.

Another sphere of interest in P450s, with ramifications for biotechnological applications, is the mode by which these enzymes can be supplied with electrons for catalysis. Rational engineering has provided routes to altering CPR and P450 BM3 coenzyme selectivity from NADPH towards NADH.[246,247] This has the potential to decrease operation costs substantially (possibly by up to 90%), although inclusion of a NADPH regenerating system (*e.g.* glucose-6-phosphate dehydrogenase and glucose-6-phosphate) can also enhance the cost efficiency of NADPH-dependent P450 catalytic processes. The recent studies of Ortiz de Montellano and co-workers in defining a P450 system reliant on 2-oxo-acids (pyruvate rather than NAD(P)H) as electron donor also points the way to development of novel, cheaper redox apparatus to drive P450 function.[126] Alternatives to costly coenzymes include the use of peroxides and the peroxide shunt pathway (Fig. 2). While some advances have been made in this area by generating P450s with greater stability to oxidative destruction by H_2O_2,[260] substantial work remains to be done in order to establish how oxidative destruction of the heme macrocycle and protein matrix can be minimised, and productive interactions with H_2O_2 maximised. In this case, the "natural" peroxygenase enzymes, typified by the *B. subtilis* CYP152A1 fatty acid β-hydroxylase, may be ideal model systems.[162] Electrocatalysis is another attractive method for driving P450 catalysis, since it can bypass altogether requirements for redox partners and deliver electrons directly to a P450 from an electrode. There have been successes in this area, although there are issues with respect to avoiding loss of P450 activity (aggregation, unfolding, heme dissociation *etc.*) at the electrode surface. Estabrook and co-workers reported fatty acid ω-hydroxylation by rat CYP4A1 in presence of rat CPR, or as a P450 fusion to rat CPR, using a silver electrode and cobalt sepulchrate as a mediator.[275] 12-Hydroxylauric acid was produced from lauric acid at rates comparable to those achieved by driving the reaction with NADPH. Activity was maintained for a much longer period in the presence of catalase, pointing to protein-damaging effects of oxygen radicals produced at the electrode surface, or by non-productive oxidation of the mediator. The requirement for CPR also suggests that electrons are still delivered to the P450 by this route, following CPR reduction by the mediator. This raises the important issue of requirement for temporally distinct delivery of the two electrons required for productive oxygenase catalysis (either side of the binding of dioxygen, see Fig. 2) and suggests that this could only be achieved by the CPR in the electrocatalytic system. A similar electrocatalytic approach was used by the same group to drive progesterone hydroxylation at $\sim 25\ min^{-1}$ using a

bovine CYP17A-CPR fusion protein.[276] Thus, electrocatalysis is a viable method for driving P450 oxygenations, but efficient electron transport may rely on mediators and natural redox partners to deliver electrons to a P450 whose heme may be quite well insulated at the centre of its protein matrix.

Finally, an important recent advance relevant to analysis of P450s (and several other membrane proteins) has been the development of Nanodisc™ technology by Sligar.[277,278] Nanodiscs are self-assembling, monodisperse discoidal molecules, comprised of a lipid (dipalmitoylphosphatidylcholine, DPPC) bilayer surrounded by a helical peptide "belt" of variable length, termed the membrane scaffold protein (MSP). These self-assembling nanoparticles were designed as a tool for solubilisation and isolation of membrane-bound enzymes, transporters and channels, and to provide a novel route to their biochemical and structural characterisation.[278] The technology has already been used to incorporate arabidopsis P450 (CYP73A5) and CPR into Nanodiscs, with the assemblies isolated using a hexahistidine tag attached to the MSP and by nickel affinity chromatography.[279] The Nanodisc-encapsulated P450 was shown to have native-like substrate (*trans*-cinammic acid) binding properties, and substrate hydroxylation was reconstituted when CYP73A5 and CPR enzymes were reconstituted in the same Nanodisc.[279] Other studies have focused on human CYP3A4 and showed that encapsulated P450 exhibits homotropic cooperativity in binding its substrate testosterone (using absorption spectroscopy to monitor association of substrate with P450) and that substrate hydroxylation can be driven in CYP3A4 Nanodiscs using H_2O_2.[280] More recent studies exploited Nanodisc technology to explore conformational equilibria and reduction kinetics in the CYP3A4 system.[281] Nanodiscs clearly have enormous potential for studying isolated, membranous P450s (with Nanodiscs conveniently suspended in aqueous media), and for analysis of molecular interactions between P450s and ligands/redox partners.

10 Conclusions and future prospects

The last 20 years has seen a vast increase in our understanding of structure and mechanism of P450s, and of their biological and catalytic diversity. It will undoubtedly be the case that more atomic structures of eukaryotic P450s (particularly hepatic drug-metabolising isoforms) will be solved in the near future, given the enormous pharmacological importance of these enzymes. Recent structural data for the major human P450 isoform CYP3A4 indicates that detailed atomic structures may be essential for any predictive drug metabolism to be undertaken, given the conformational flexibility of certain P450 isoforms.[212] Another key question relating to P450 structure–function is the mode of interaction of P450s with their various redox partners. This will require determination of an atomic structure of a stoichiometric complex of a P450 with the partner protein, or of one of the natural P450–redox partner fusion proteins (*e.g.* a CYP102, CYP505 or CYP116B enzyme). However, a full appreciation of modes of binding of P450s with CPR and other redox partners is also likely to require structural elucidation of crystal complexes solved in different redox states and substrate-bound forms. This is a major challenge and will require several years' effort.

Despite huge progress in recent years, numerous key questions remain relating to the definitive (spectroscopic or structural) identification of the major oxidising species (*i.e.* compound I) in the P450 cycle, and also regarding the relevance of other intermediates (primarily compound 0) in P450-mediated reactions. A substantial step towards identifying the transient compound I was made recently in studies of *Sulfolobus solfataricus* CYP119A1, in which peroxynitrite was used to oxidise the P450 heme to a moderately stable species with spectral properties consistent with that of a Fe(IV)–oxo neutral porphyrin (compound II), as reported for the heme thiolate protein choloroperoxidase.[282] Laser photoexcitation of the CYP119A1 species at 355 nm resulted in partial conversion to a new, transient spectral species with absorption characteristics similar to known compound I species in models and enzyme systems.[36] Photooxidation of compound II to compound I has been reported in other proteins (equine myoglobin and horseradish peroxidase) and thus the method used here for CYP119A1 may be generally applicable.[283] However, the relatively long half life of the compound I-like species in CYP119A1, and lack of influence of P450 substrate molecules on its decay, raise some questions as to the nature of the intermediate, and further studies are obviously needed in this area.[36] In an alternative approach to isolating reactive P450 cycle intermediates, Hoffman and co-workers used γ irradiation of a substrate-bound P450cam ferrous–oxy complex (at 77 K) to introduce the 2nd electron. EPR and ^{1}H ENDOR studies provided compelling evidence for formation of the ferric peroxy intermediate, which then abstracted a proton to form substantial amounts (up to 40% yield) of the ferric hydroperoxy form.[37] Elevating temperature ("annealing") to ~200 K led to hydroxycamphor product formation in the wild-type enzyme, but no direct evidence for compound I formation was obtained. Cutting-edge studies of this type have brought us closer to identification of compound I and appear to rule out the involvement of compound 0 in most P450 oxidations. However, there are experimental data suggesting that compound 0 is a viable oxidant for *e.g.* epoxidation of carbon–carbon double bonds and for sulfur oxidations.[284–287] These contrast with computational studies indicating that different spin states of compound I act as different oxidants, and that compound 0 may have no significant role in oxygenation chemistry.[288] The definitive identification of the ultimate oxidant species in P450 catalysis (nominally compound I) and the possibility of secondary oxidant(s) clearly remains a hotly contested and controversial area and will obviously be the subject of numerous studies in the next few years.

Other areas of interest revolve around biotechnological exploitation of P450s, and particularly their usefulness in production of chiral oxyfunctionalised compounds as fine chemicals and intermediates in organic synthetic processes. For instance, P450s are already involved naturally in the synthesis of high value lipids (*e.g.* steroids, eicosanoids) and of numerous antibiotics (see Fig. 10). There are certainly opportunities to engineer P450 variants with altered activities that could, for example, produce novel polyketide antibiotics as a result of alteration in the position of substrate oxygenation. Possibilities in this area are highlighted by the *Streptomyces venezuelae* PikC P450 (CYP107L1), which catalyses hydroxylation of two different types of macrolactone at two different positions (or both positions), creating a range of different macrolide antibiotics (methymycin, neomethymycin, novamethymycin, pikromycin, neopikromycin and novapikromycin).[289–291] Rational and forced evolution approaches have other roles to play in producing P450s that are more thermostable, pressure stable, protease and solvent resistant. This

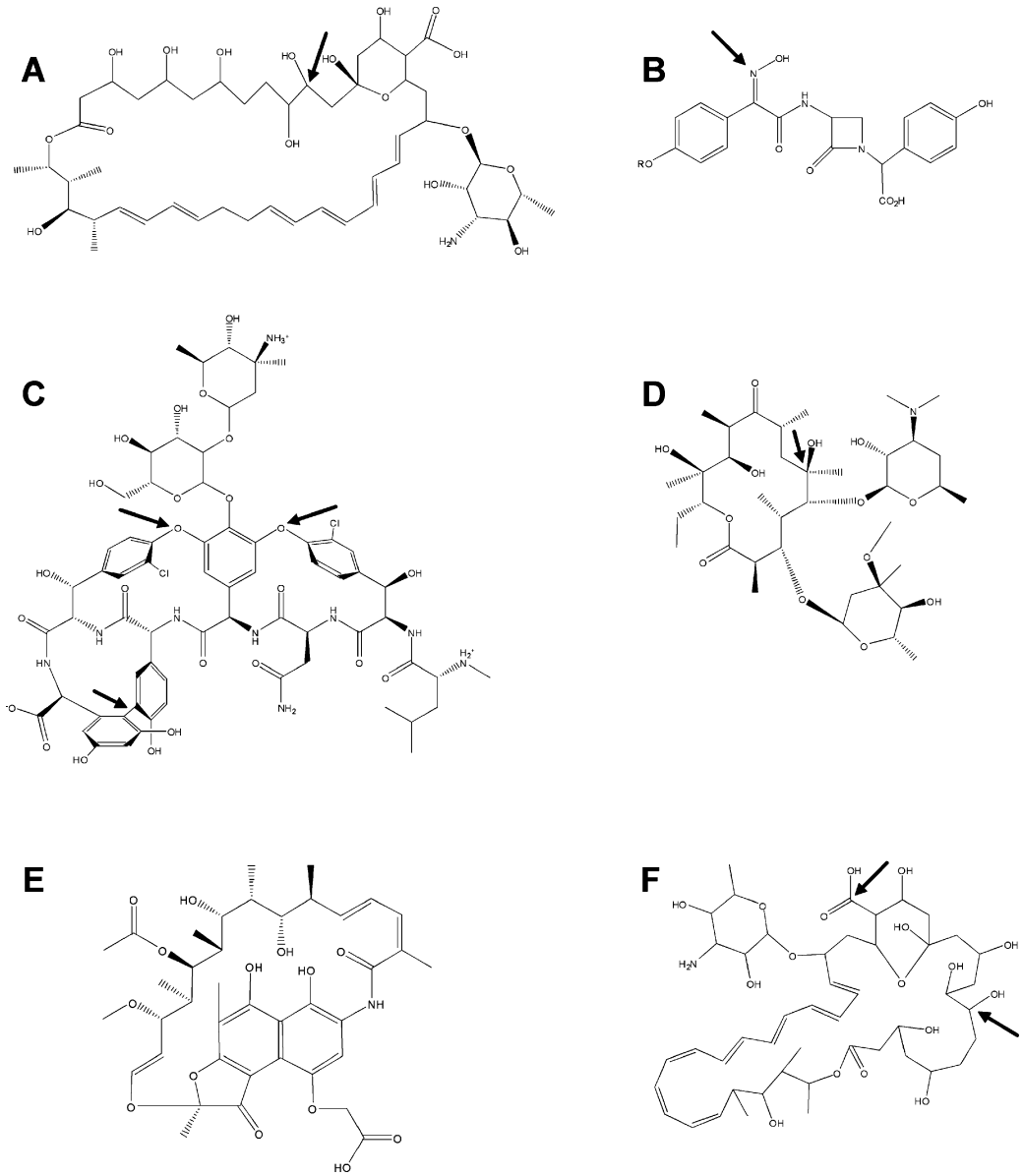

Fig. 10 Involvement of P450 enzymes in antibiotic synthesis. P450 enzymes are widely exploited for oxidative modifications of polyketide and other antibiotics. Their activity is usually essential for the biological activities of these molecules. Selected examples are shown, with the position(s) of oxidative modification performed by P450 enzymes indicated by arrows. **A**, nystatin;[324] **B**, nocardicin A;[325] **C**, vancomycin;[326] **D**, erythromycin;[204] **E**, rifamycin B;[327] **F**, amphotericin.[328] For rifamycin, an *orf0* (P450-encoding) gene deletion led to the lack of methyl hydroxylation of proansamycin X (a rifamycin B precursor) and failure of the host organism (*Amycolatopsis mediterranei*) to transform this precursor to rifamycin B *via* the intermediate rifamycin W.[327] In the case of amphotericin, gene inactivation studies indicated that two distinct P450s from *Streptomyces nodosus* (*amphL* and *amphN*) are responsible for C_8 hydroxylation and for C_{16} methyl oxidation to form the carboxylate, as indicated in the relevant panel.[329,330]

will be complemented by isolation and characterisation of natural (likely archaeal) P450 enzymes from unusual environments that already possess characteristics that make them robust and suitable for industrial applications. In the race for exploitation of P450s for biotechnological purposes, it should not be forgotten that there is a vast amount we do not understand regarding normal physiological roles of P450s in primary and secondary metabolism in numerous organisms. For instance, very little is known about the roles of most of the (>270) P450s in *Arabidopsis thaliana*, and even several of the 57 human P450s have yet to be characterised in detail. In *Mycobacterium tuberculosis*, there are 20 P450s, and azole and triazole compounds (known P450 inhibitors) have been shown to be effective antimycobacterial drugs.[123] Evidently a vast amount of research is required on these and many other P450 systems to uncover further novel activities and the place of these enzymes in the web of metabolism in their respective host organisms.

The P450 field is vibrant and its research community is thriving on novel structural and bioinformatics data. This information will provide the fuel for research in diverse areas on these oxygenases and their redox partners, in order to further develop our understanding of their biological functions and their potential as environmentally friendly biocatalysts in the chemist's tool box.

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