Abstract

P450s (cytochrome P450 mono-oxygenases) are a superfamily of haem-containing mono-oxygenase enzymes that participate in a wide range of biochemical pathways in different organisms from all of the domains of life. To facilitate their activity, P450s require sequential delivery of two electrons passed from one or more redox partner enzymes. Although the P450 enzymes themselves show remarkable similarity in overall structure, it is increasingly apparent that there is enormous diversity in the redox partner systems that drive the P450 enzymes. This paper examines some of the recent advances in our understanding of the biodiversity of the P450 redox apparatus, with a particular emphasis on the redox systems in the pathogen Mycobacterium tuberculosis.

Introduction

P450s (cytochrome P450 mono-oxygenases) are an ever-growing family of enzymes that show extraordinary diversity in their reaction chemistry [1]. P450s are typically thought of as mono-oxygenases, i.e. catalysing a reductive scission of the dioxygen bound to the haem iron at the core of the P450, leading to the introduction of a single atom from oxygen into an organic substrate and the production of a molecule of water, according to the following equation:

\[ \text{RH} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} \] (1)

In this equation, RH is the substrate, and protons are delivered to the catalytic centre of the P450 through active-site amino acid side chains. The proton relay mechanism is critical for the productive cleavage of the O–O bond, and structural and mechanistic studies have implicated a number of amino acids with a proton relay role in distinct P450s (see e.g. [2–4]). Electrons are usually supplied from the reduced pyridine nucleotide coenzymes NAD(P)H. The first of the two consecutively delivered electrons is used to reduce ferric P450 haem into its ferrous form (which can then bind dioxygen). In the well-studied bacterial P450 systems P450 cam (a camphor hydroxylase) and P450 BM3 (a fatty acid hydroxylase), a control mechanism is enforced whereby substrate binding is a prerequisite for efficient electron transfer to the haem iron [5,6]. The binding of camphor (P450 cam) and e.g. arachidonic acid (P450 BM3) results in a shift in the haem iron spin-state equilibrium towards the high-spin form, with a concomitant elevation of the haem iron reduction potential by approx. 130–140 mV. This thermodynamically favourable switch triggers the first electron transfer to the haem iron from the redox partner [7]. This, in turn, probably prevents wastage of reducing equivalents in the production of superoxide/peroxide and ensures that electron transfer proceeds only when a substrate is available for oxygenation. The second electron transfer to the haem iron occurs subsequent to binding of O_2 to the ferrous iron, and produces a ferric-peroxy species. This intermediate is protonated, leading to O–O bond cleavage and resulting in the formation of a molecule of water and production of the reactive, oxyferryl radical cation species that is considered to be the major oxidant in P450 substrate oxygenation reactions [8,9]. Attack of the oxyferryl species on the bound substrate leads to its oxygenation and formation of products. In the ‘classical’ P450 catalytic cycle, hydroxylation of the substrate is portrayed. However, P450s lend themselves to more diverse chemical transformations such as reductive dehalogenation, sulphoxidation, epoxidation, acyl bond cleavage and N-oxidation [10–16].

To achieve the diverse range of chemical reactions discussed above, the vast majority of P450s interact with one or more redox partners to source their reducing equivalents. Recent studies on the P450 systems, particularly those from microbial species, have revealed an unexpected diversity in the systems used to deliver electrons to the P450s. This diversity is explored in the remainder of this paper, with an emphasis on both experimental studies and recent revelations from analyses of microbial genome sequences, including that of the pathogen Mycobacterium tuberculosis.

Classes I and II: the first two P450 redox systems

In early studies of P450 enzymes, two distinct classes of redox systems emerged. P450 enzymes from the mammalian liver were found to be integral membrane proteins (bound to the endoplasmic reticulum by an N-terminal membrane anchor) and to interact with a similarly membrane-anchored NADPH-dependent reductase containing both FAD and FMN cofactors (NADPH-cytochrome P450 reductase or...
Figure 1 | Biodiversity of cytochrome P450 redox systems

Cytochrome P450 mono-oxygenase enzymes receive electrons from a range of different redox partner enzymes (examples are shown). Helices and sheets are displayed as ribbons and strands in each image. Cofactors are shown in spacefill representation. Shown in the centre is the CYP121 P450 from *M. tuberculosis* [4]. A haem cofactor is at the heart of the molecule. Top left is rat CPR [23]. FAD (left) and FMN cofactors are closely spaced for efficient electron transfer. Top right is phthalate dioxygenase reductase from *Pseudomonas cepacia* [61]. FMN (upper) and 2Fe-2S cofactors are bound. Bottom left are bovine ADR (left) and adrenodoxin [62]. ADR binds FAD and AD binds a 2Fe-2S iron-sulphur cluster. Bottom right is *E. coli* flavodoxin, with FMN bound peripherally.

In contrast, the P450 cam enzyme (CYP101) from *Pseudomonas putida* (the first microbial P450 to be enzymically and structurally characterized) was shown to be a freely soluble protein that catalysed 5-exo hydroxylation of D-camphor in the first step of the pathway for degradation of the molecule, enabling its use as a sole source of carbon and energy for growth [18]. Its redox partners were identified as the NADH-specific FAD-containing reductase, putidaredoxin reductase, and the 2Fe-2S cluster-containing ferredoxin, putidaredoxin. Both are soluble proteins. Thus it was initially envisaged that only two types of P450 redox systems might occur in nature: (i) a prokaryotic (class I) system consisting of three soluble components: an NADH-specific flavoprotein reductase that shuttled electrons to a soluble P450 via a ferredoxin, and (ii) a eukaryotic (class II) system comprising membranous P450 and CPR enzymes. In the class II apparatus, electrons from NADPH are passed to the CPR FAD cofactor and on to the P450 via the CPR FMN cofactor.

Studies of other P450 enzymes during the 1970s and 1980s gave further credibility to the concept of the prokaryotic/eukaryotic class I/class II schism. However, studies of the P450 enzymes in the soil bacterium *Bacillus megaterium* in the 1980s provided the first strong evidence for more biodiversity in P450 redox systems (see below and [19]). In recent years, structural analysis of each of the components of the P450 cam system and the CPR from rat has been undertaken [20–23]. Putidaredoxin acts as a single-electron shuttle, carrying electrons 1 and 2 between putidaredoxin reductase and P450 cam. The reaction with the P450 may be primarily electrostatic and, under physiological conditions, the second electron transfer to the P450 appears to be the rate-limiting step in catalysis [24,25]. In the eukaryotic CPR: P450 system, communication between just two partners is required. Amino acid alignments had predicted that CPR proteins were encoded by genes that had resulted from the fusion of ancestral genes encoding FDR (ferredoxin/flavodoxin reductase)-like and flavodoxin-like proteins [26]. Flavodoxins are small, FMN-binding proteins with diverse physiological roles in numerous prokaryotes [27]. Atomic structural analysis of rat CPR (Figure 1) confirmed that the protein was a fusion of an FDR domain and a flavodoxin domain, and demonstrated that the respective flavin isoalloxazine rings were juxtaposed with edges separated by only 4 Å (1 Å = 10−10 m), enabling efficient and direct electron transfer [7,23]. The recent structural elucidation of the
CPR-like reductase domain of neuronal nitric oxide synthase reductase points to a similar organization [28]. Large-scale structural changes may occur in CPR following inter-flavin electron transfer in order that the FMN-binding domain can reorientate to interact with its cognate P450 redox partners. Studies performed on the human CPR indicate that internal electron transfer is regulated by the binding of pyridine nucleotide coenzyme, and this may also be a means of regulation of conformational equilibria in the enzyme [29].

**P450 BM3: an efficient electron transferase system**

The humble soil bacterium *B. megaterium* provided the first step forward from the class I and II redox systems, when research in A.J. Fulco’s laboratory at the University of California at Los Angeles identified an unusual P450 enzyme involved in subterminal hydroxylation of long-chain fatty acids [30,31]. P450 BM3 (CYP102A1, the third P450 identified in the bacterium) was discovered to be a fusion between a P450 and a CPR. The P450 domain of the enzyme showed significant similarity to eukaryotic CYP4 family P450s, which are involved in fatty acid hydroxylations, but neither of the P450 or CPR domains of P450 BM3 had membrane anchors and the flavocytochrome enzyme was fully soluble [7,32]. Kinetic analysis of the enzyme showed that the fusion arrangement had produced an enzyme with efficient inter-cofactor electron transfer and the highest catalytic centre activity yet reported for any P450 mono-oxygenase enzyme [33]. The similarity of the P450 BM3 system to the two-component drug-metabolizing P450 redox systems found in the mammalian liver, coupled with its relative ease of expression/production and structural/biophysical analysis when compared with its membranous eukaryotic counterparts, has fuelled intensive studies on the structure and mechanism of P450 BM3 and its component domains. Just as P450 cam has become a model for the prokaryotic class I systems, so P450 BM3 has become a paradigm for researchers interested in the class II redox system (see e.g. [34,35]). Extensive protein engineering studies have been performed on P450 BM3, enabling the understanding of the roles of residues conserved across P450 and CPR enzymes (see e.g. [34–36]). In addition, the biotechnological value of this efficient mono-oxygenase enzyme has been recognized, and variants capable of oxygenation of, e.g., alkanes, alkenes and short-chain alkanoic acids have been generated by rational engineering and forced evolution methods [37,38]. Homologues of P450 BM3 (CYP102A1) have been recognized in other bacteria, including two characterized systems in *Bacillus subtilis*: CYP102A2 and CYP102A3 [39].

**Ferredoxin or flavodoxin?**

Bacterial flavodoxins and ferredoxins are small redox proteins that participate in several key metabolic pathways. Ferredoxins are obligate one-electron carriers, and typically bind iron–sulphur cofactors with 2Fe-2S, 3Fe-4S or 4Fe-4S clusters. Iron atoms are generally co-ordinated in their single-electron reduced semiquinone form (Figure 1). They also act as single-electron donors/acceptors, and the quinol/semiquinone transition is considered to be the physiologically relevant redox couple for many of their reactions. Flavodoxins are known to surrogate for ferredoxins under conditions of cellular iron limitation, so it would perhaps not be altogether surprising if they were also found to support bacterial P450 function. Given that the FMN-binding domain of CPR is evolutionarily related to the flavodoxins, the case for their exploitation as microbial P450 redox partners is further strengthened. Evidence for the involvement of flavodoxins in P450 catalysis has now been collated. The *Escherichia coli* flavodoxin reductase/flavodoxin system was shown to support the function of bovine P450c17 [44]. The cineole-metabolizing P450 cin (CYP176A1) from *Citrobacter braakii* was shown to interact with the host flavodoxin cindoxin, the gene of which was located adjacent to the P450 on the chromosome [45]. The biotin synthesis pathway cytochrome P450 BioI from *B. subtilis* (CYP107H1) was also shown to oxygenate fatty acids supported by the host flavodoxins YkuN and YkuP [27]. Recently, a P450:flavodoxin fusion protein was identified in the soil bacterium *Rhodococcus rhodochrous*, and implicated in the degradation of the...
recalcitrant explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) [46]. Further examples of flavodoxin involvement in microbial P450 pathways will undoubtedly be uncovered as biotechnologically relevant P450 systems are characterized further.

The P450 cam system uses a 2Fe-2S cluster ferredoxin (putidaredoxin) to reduce the P450, but studies performed during the last decade have highlighted microbial P450s that also exploit 3Fe-4S ferredoxins and probably 4Fe-4S ferredoxins. For example, a 3Fe-4S ferredoxin is used by the xenobiotic-transforming P450 soy from Streptomyces griseus [47]. A 4Fe-4S ferredoxin (the only ferredoxin in the genome) was also shown to support catalytic activity in the P450 Biol system [48]. Fusion of a 3Fe-4S ferredoxin to a P450 has also been observed in the CYP51FX sterol-demethylating enzyme from Methylococcus capsulatus [49]. As with the P450 BM3 system, redox partner fusions of this type may enable more efficient electron transfer to the P450 and possibly result in less wastage of reducing equivalents.

Why use a redox partner at all?

The explosion in identification of new protein redox partners for P450s was perhaps not entirely unexpected. The P450 mono-oxygenases require consecutive delivery of two electrons, and several types of redox enzymes in nature might be applied to this role (assuming evolution of appropriate cofactor redox potentials, P450 docking interfaces etc.). A phenomenon that was not expected, however, was the discovery of P450s that had done away altogether with redox partners. The plant P450 allene oxide synthase (CYP74A) uses hydroperoxides of linoleic acid and linolenic acids as substrates, performing a molecular rearrangement and dehydration of the substrate to form allene oxide. The product is then converted into jasmonic acid, a plant growth hormone with roles in plant defence signalling [50]. A similar molecular rearrangement reaction on a peroxide substrate is catalysed by mammalian thromboxane synthase (TXAS) – human TXAS is CYP5A1. The product here (thromboxane A2) is a mediator of platelet aggregation [51]. These P450s are not ‘true’ mono-oxygenase P450s, since they do not perform reductive activation of oxygen. However, they offer an elegant illustration of the exploitation of the P450 fold to undertake a diverse type of reaction.

It has long been known that P450 mono-oxygenases can be driven (albeit inefficiently) by H2O2 or organic peroxides (the ‘peroxide shunt’ mechanism), bypassing the requirement for NAD(P)H, molecular oxygen and redox partner(s). However, locating P450s that performed this reaction naturally was still unexpected. The B. subtilis P450oxo, (and its homologue from Sphingomonas paucimobilis) catalyses α- and β-hydroxylation of fatty acids, using H2O2 as an oxidant. The structure of the B. subtilis enzyme (CYP152A1) has been resolved, providing important clues to the reaction mechanism and defining the key residues for positioning the fatty acid substrate and for catalysis [52]. Another unexpected P450 was the P450 nor (CYP55A1) enzyme from the denitrifying fungus Fusarium oxysporum, which converts two molecules of nitric oxide into nitrous oxide. The requirement for redox partner(s) is again obviated – this time through the direct reduction of the P450 by NADH [53]. A cluster of positively charged residues close to the haem-binding site is essential for productive interaction with NADH [54]. It is envisaged that hydride transfer occurs directly from NADH to a ferric–NO complex [Fe(II)NO+] requiring NADH binding in the proximity of the NO group. Support for this hypothesis comes from the recent structure of the NADH analogue (nicotinic acid adenine dinucleotide)-bound form of CYP55A1, providing evidence for stereo-selective hydride transfer from NADH and indicating that a channel for proton delivery is also formed on nucleotide binding [55].

Revelations from a genome: P450 redox partners in M. tuberculosis

With complete bacterial genome sequences coming thick and fast, excellent opportunities exist to scan genomes to identify, clone and characterize novel P450 enzymes and their redox partners. Our laboratory has a particular interest in the enzmyology of the P450 systems from the pathogen M. tuberculosis (Mtbc). The genome sequence of Mtbc revealed that the pathogen encoded an extraordinarily large number of P450s (20 in all) in comparison with other genomes of similar size. For example, E. coli has no P450s. All the P450s are stand-alone class I-type, and the structures of two of the enzymes (CYP51 and CYP121) have been solved. CYP51 is the first sterol demethylase structure solved, and the 1.06 Å structure of CYP121 is the highest resolution P450 structure and provides novel insights into haem geometry and proton relay pathways supporting catalytic function (Figure 1) [4,56]. The Mtbc P450s are of interest as drug targets, in view of data indicating their tight binding to azole drugs and the potential of these agents in anti-TB therapy [57]. Genome analysis provides some interesting insights into the potential redox partners for the Mtbc P450s, as discussed below.

Potential flavodoxin partners are not obvious in the Mtbc genome, but a number of ferredoxins and ferredoxin-like proteins are distributed in the genome. The most obvious candidates for P450 redox partners are the ferredoxin products of the rol1786 gene (adjacent to the P450 CYP143) and the rol0764c gene (adjacent to CYP51). The latter 3Fe-4S ferredoxin (Fdx) has been purified and shown to support the catalytic function of CYP51 [58]. Elsewhere on the genome, fdxA is up-regulated by low pH (conditions that may mimic those experienced by phagocytosed Mtbc) and is another potential P450 partner. ADR (adrenodoxin reductase) is the NADPH-dependent flavoenzyme that drives (through the iron–sulphur protein adrenodoxin) the steroid oxygenations catalysed by eukaryotic mitochondrial P450s (Figure 1). The first example of a prokaryotic ADR is the structurally characterized Mtbc FprA (Figure 2). FprA shows unusual redox behaviour with respect to the differing levels of flavin reduction induced on reaction with NADPH (FAD reduced to semiquinone) and NADH (to quinol). Since both
Figure 2 | Structure of the NADP⁺- and FAD-binding sites in M. tuberculosis FprA

Mtb FprA is an ADR homologue from Mtb, and is probably the electron donor to ferredoxin proteins involved in cytochrome P450 reduction [59,60]. The structure of Mtb FprA reveals key amino acid residues involved in the binding of the NADP(H) coenzyme. Arg199 and Arg200 are predicted to interact with the NADP⁺ 2’-phosphate group. Arg110 may also interact with NADP⁺ phosphate, while Trp159 shields the FprA FAD cofactor.

coenzymes have near-identical reduction potentials, the difference may be explained by variations in binding modes [59,60]. Mtb also encodes two further proteins that may act as single-component P450 reductases – both fprB and fdcB appear to encode fusion proteins between FDR and ferredoxin modules. In the case of FprB, the flavoprotein domain resembles FprA in amino acid sequence. The Mtb P450s are likely to have central roles in the complex lipid metabolism of Mtb, and many of the isoforms may be vital for viability of the pathogenic form. Thus analysis of the structural, kinetic and molecular interaction properties of the P450s and their redox partners will be vital to establish their physiological roles and to define their status as drug targets.

Summary

Recent years have seen an acceleration in the number of new types of P450 redox systems identified. Perhaps the most exciting finding has been the characterization of P450s that have dispensed with protein partners altogether – relying instead on direct interactions with NADH (P450 nor) and peroxide (P450bss) for the provision of reducing equivalents. Microbial genome sequencing projects continue to highlight new types of P450 enzymes and diversity in their redox apparatus. In some cases, the novel systems recognized may be of interest for biotechnological or biomedical exploitation.

In all cases, however, the diverse redox partners identified expand our knowledge of the versatility and adaptability of the cytochromes P450, and highlight the flexibility of these enzymes in sourcing their reducing power to fuel their critical cellular functions.

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References
