Unusual Cytochrome P450 Enzymes and Reactions

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Cytochrome P450 enzymes primarily catalyze mixed-function oxidation reactions, plus some reductions and rearrangements of oxygenated species, e.g. prostaglandins. Most of these reactions can be rationalized in a paradigm involving Compound I, a high-valent iron-oxygen complex (FeO³⁺), to explain seemingly unusual reactions, including ring couplings, ring expansion and contraction, and fusion of substrates. Most P450s interact with flavoenzymes or iron-sulfur proteins to receive electrons from NAD(P)H. In some cases, P450s are fused to protein partners. Other P450s catalyze non-redox isomerization reactions. A number of permutations on the P450 theme reveal the diversity of cytochrome P450 form and function.

General Aspects of Catalytic Mechanisms

Details of the cytochrome P450 catalytic mechanism are described in the accompanying minireview by Green and co-workers (1). Most of the P450 reactions can be rationalized in the context of the intermediate FeO³⁺, which corresponds to what was first described as "Compound I" in peroxidase chemistry (Fig. 1A, i). A variant mechanism has been proposed for some P450 reactions (Fig. 1A, ii), particularly those regarding aldehydes (4). Another mode of P450 reactions is the rearrangement of some oxygenated substrates (e.g. prostaglandins) (Fig. 1A, iii), as exemplified by the P450s CYP5A1 and CYP8A1, the respective prostacyclin and thromboxane synthases (see below). Another mechanistic aspect is the distinction of reactions regarding low- and high-spin FeO³⁺, proposed by Shaik et al. (7) to explain multiple reactions catalyzed by a P450. Although intriguing, this hypothesis has been examined only at the theoretical level. The experimental approaches described by Green and co-workers (1, 8) can be applied to the questions posed by the spin hypothesis.

Oxidations

Most of the unusual reactions of P450s can be understood in the context of rearrangements, either of the reaction products (due to instability) or within an enzymatic reaction intermediate. Examples of both will be considered. For a more extensive collection of unusual P450 reactions, see previous reviews (4, 9–11). The literature of these unusual reaction products is dominated by contributions from the fields of drug metabolism and plant biochemistry (which are not unrelated, in that many drugs are plant secondary metabolites). Most drugs are complex molecules, and regulatory agencies require elucidation of chemical structures of metabolites prior to registration (12). Plants have large numbers of P450 genes (hundreds per species) and utilize these in the synthesis of complex secondary metabolites, e.g. alkaloids, modified terpenes, flavonoids, etc.

Rearrangements of Oxidized Products—Many examples are known of α-hydroxy heteroatom products that break down, e.g. carbinolamines, hemiacetals, and gem-haloxydrins (Fig. 1B). However, some stable carbinolamines (5, 6) and hemiaminals (13) are known. In many cases, the initial products have not been observed directly, but indirect approaches have been used to demonstrate their existence, e.g. oxygenated halogen atoms (halo compounds) (supplemental Fig. S1) (14, 15).

Sometimes, alcohol products dehydrate to yield olefins. However, olefins can also be formed directly by an oxidative mechanism resembling those of recognized desaturases (9, 16). Arene oxides (epoxides) rearrange to phenols (17). In addition, vinyl compounds with good leaving groups can form epoxides that rearrange, e.g. to α-halocarbonyls (18, 19).

In more complex (cellular) settings, leaving groups may be attached to the alcohols generated by P450s. Accordingly, the elimination or nucleophilic addition products may be recovered and identified. The latter type of reaction with a macro-molecule (i.e. DNA and protein) is a major issue in chemical carcinogenesis and toxicity (20).

A dramatic example of rearrangement in a product involves the drug candidate BMS-690514 (supplemental Fig. S2). The reaction is rationalized by an electrophilic attack of a P450 (CYP3A4?) on a pyrrole ring to form a hydroxyppyrrole, which rearranges to open the pyrrole ring and eventually involves reaction of a neighboring aniline ring to form a stable carbinallamine product (21).

Rearrangements Involving Enzyme Intermediates—In general, rearrangements of enzyme intermediates are more complex, more interesting, and often more difficult to rationalize. A brief discussion of kinetics and mechanism is in order before consideration of examples.

Strained cycloalkyl entities have been utilized as "radical clocks" (22, 23). Abstraction of a hydrogen atom is an energetically unfavorable reaction, but, once formed, the "oxygen rebound" step (Fig. 1A, i) is very rapid, as recently demonstrated in direct experiments with P450 119A1 Compound I by Green and co-workers (1, 8). The rates of rearrangement of some cycloalkyl radical systems are known (in solution) and can be used to estimate the rates of rearrangement and oxygen rebound (radical recombination, i.e. reaction of FeOH³⁺ + •OH) in enzymes. Although this field has not been without controversy (24), rates of recombination of ~10³ s⁻¹ are considered to occur in several P450s (25). Such studies have caveats regarding rates of rearrangement, in that atoms of these molecules are
undoubtedly restrained in active sites through various bonding forces, and this can change the rates compared with those measured in simple chemical systems (26).

With this background, we can consider some of the rearrangements. Another aspect to consider is one already mentioned, that of desaturation resulting from the abstraction of a second hydrogen atom to achieve a net 2-electron loss. One recent example of an unusual rearrangement in a P450 complex involves the P450 enzyme CYP125 (from Mycobacterium tuberculosis and rhodococci) and the oxidation of cholesterol (Fig. 2). An unusual product is the formate ester (M2 in Fig. 2A). The reaction is shown as releasing formate in the proposed mechanism in Fig. 2A, presumably without extrusion into the medium (equilibration with any formate in the medium was not examined.) An alternative concerted Baeyer-Villiger reaction is shown in Fig. 2B.

Many other P450 reactions are known in which rearrangements undoubtedly occur with a P450 enzyme-intermediate complex. Notable examples are the conversion of vinyl halides to α-haloacetaldehydes (18, 28). A recent similar example involves the “direct” conversion of 7,8-dehydrocholesterol to 7-ketocholesterol by the human P450 CYP7A1 (29). Strained cycloalkyl rings undergo rearrangements (2, 30). Other examples in which cleavage of a substrate occurs in such a mechanism involve the ipso cleavage of bisphenol A (supplemental Fig. S3) (31) and the conversion of nabumetone to 6-methoxy-2-naphthylacetic acid by the human P450 CYP1A2 (supplemental Fig. S4) (32, 33).

**Coupling Reactions**—P450-catalyzed C–C and C–O bond couplings are common in plant biosynthetic pathways (e.g. alkaloid biosynthesis) and in bacteria (e.g. antibiotic synthesis in Actinomycetes). These reactions are often critical in the biosynthesis of plant secondary metabolites. An interesting example from Taxol biosynthesis (plus a possible mechanism) is shown in Fig. 3A (34).

Some other interesting examples include C–O bond coupling in the biosynthesis of grayanic acid (supplemental Fig. S5) (37), coupling in the synthesis of isoquinoline alkaloids (supplemental Fig. S6) (38), and oxidative coupling in early steps of morphine biosynthesis (supplemental Fig. S7) (39, 40). These coupling reactions, generally believed to involve radical chemistry, are not restricted to plants and bacteria. Human P450s have been shown to also catalyze steps in morphine synthesis (supplemental Fig. S8) (41). Most of these reactions are internal couplings, in which rings are in juxtaposition to fuse if a radical pathway is initiated. The coupling of two flavilin molecules together has been observed in a Streptomyces coelicolor CYP158A2 reaction, in which the two molecules are both bound in the active site (42). A dimer of capsaicin is formed during its oxidation by human liver microsomes (although which P450 is involved remains unknown) (43). The general reaction supports a view of an aromatic radical pathway but is rare among oxidations of unlinked substrates. Recently, the environmental contaminant BDE-47, a polybrominated bisphenyl ether flame retardant, was reported to be converted to a...
(brominated) p-dioxin molecule by the introduction of a second ether oxygen (supplemental Fig. S9) (44).

Nitration—A highly unusual reaction is involved in the synthesis of a phytotoxin, thaxtomin, in Streptomyces turgidiscabies (Fig. 3B) (35). The FeO$_2^{2+}$ form of the enzyme is proposed to react with nitric oxide to form an iron peroxynitrite-like species that can produce a nitrating species capable of nitrating tryptophan.
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Cyclopropanation—This is not a physiological reaction but nevertheless demonstrates the versatility of P450s (this is not an oxidation per se but is included here in the context of mechanistic similarity). The ferrous form of a mutant of bacterial CYP102A1 (P450_{mut}) reacted with ethyl diazoacetate to convert styrene to a cyclopropyl derivative (Fig. 3C) (36). Multiple turnovers were observed, and the stereochemistry was distinct from that seen in reactions with only free heme.

Methane Hydroxylation—Although P450s are well known catalysts of alkane hydroxylation, the oxidation of methane presents a special challenge due to the strong C–H bond strength (104 kcal mol⁻¹). Two groups have employed a strategy of “activating” CYP102A1 by using short perfluorinated fatty acids (with no C–H bonds available for oxidation) to “activate” the enzyme and then also adding short alkanes, which can be oxidized as substrates (45, 46). The activation process includes both a spin-state change in the iron and a constriction in the size of the active site. Watanabe and co-workers (46) were not able to oxidize methane or ethane in their system, but Reetz and co-workers (45) were able to achieve multiple turnovers of methane, an unusual feat.

Reductions

Although the vast majority of P450 reactions are oxidations, reductions are also known. Most are observed more readily under anaerobic or hypobaric conditions. For rather unclear reasons, the substrates for such reactions accept electrons from ferrous P450 at a rate that is competitive with the rates of binding and reduction of oxygen. These reactions can occur in cells with low oxygen tension, e.g. those most distant from capillary arteries in animals. The electrons must be transferred one at a time.

The nature of the 1-electron reduced intermediates and, in some ways, the fate of an oxygen in a substrate remain unclear. One unusual case is the conversion of benzo[a]pyrene epoxides to the polycyclic hydrocarbon itself, i.e. benzo[a]pyrene (47). For reasons that are not clear, the only reactions of the human “orphan” P450 CYP2S1 that have been reproducibly observed to date are all reductions (48–50).

Non-redox Reactions

At least three non-redox P450 reactions have been reported, including a phospholipase D-type hydrolysis by several mammalian P450s (51), pyrophosphatase (hydrolytic) activity of S. coelicolor CYP170A1 (52), and the rearrangement of a bicyclic pentaenone to an oxetane by CYP154A1 from the same bacterium (53). Those reactions are likely to involve elements of acid-base chemistry, but the details are unknown. In the case of CYP170A1, an alternative active site for the hydrolytic reaction has been identified (the P450 also catalyzes oxidative reactions).

Unusual P450 Enzymes

The “classical” eukaryotic P450 enzymes are membrane-associated P450s that interact either with the NADPH-dependent diflavin enzyme cytochrome P450 reductase (CPR); for Class II microsomal P450s) or with the flavoprotein adrenodoxin reductase (ADR) and the iron-sulfur protein adrenodoxin (ADX; for Class I mitochondrial P450s) (Fig. 4). Typical prokaryotic Class I systems have similar flavoprotein reductase and ferredoxin (FDx) partners (60). Interestingly, some of the mammalian microsomal P450s are also imported in the mitochondria and use ADx (an FDx) and its ADR (64). Recent years have seen the identification of several distinct types of P450s that do not fall into the general Class I/II types, many of which have been identified through characterization of P450 enzymes identified from genome sequencing projects.
P450-Redox Partner Fusion Proteins

P450<sub>BM-3</sub>—The discovery of a high-activity fatty acid hydroxylase in <i>Bacillus megaterium</i> led Narhi and Fulco (65) to identify the first P450 linked to its redox partner. P450<sub>BM-3</sub> (CYP102A1) has a eukaryote-like CPR fused to the C terminus of the P450. Both domains lack the membrane anchor regions typical of their eukaryotic relatives, and P450<sub>BM-3</sub> was the first example of a prokaryotic CPR (54). The P450-CPR fusion arrangement of P450<sub>BM-3</sub> facilitates rapid electron transfer from NADPH to the P450 heme, and the CPR portion (of P450<sub>BM-3</sub>) is also reduced by NADPH much faster than are eukaryotic CPRs (66), leading to P450<sub>BM-3</sub> having the fastest substrate oxidation rate yet reported for a P450 enzyme, ~250 s<sup>-1</sup> (67). The catalytic proficiency of P450<sub>BM-3</sub> has aroused great interest in its capacity for oxychemical synthesis, and notable successes in re-engineering P450<sub>BM-3</sub> include transforming the enzyme from a fatty acid (ω-1, ω-2, and ω-3) hydroxylase into variants capable of oxidizing short chain alkanes and alkenes as well as steroids (68, 69). P450<sub>BM-3</sub> is a dimeric enzyme, and intermonomeric electron transfer occurs in this system, as also seen for the structurally related eukaryotic nitric-oxide synthases (70–72). Several other P450<sub>BM-3</sub>-type P450-CPR enzymes are now known (e.g. see Ref. 73). The physiological function of P450<sub>BM-3</sub> still remains unclear, although a role in bacterial quorum sensing mediated by oxidative inactivation of acylhomoserine lactones has been proposed (74). Similar types of P450-CPR fusion enzymes have been found in lower eukaryotes, most notably the <i>Fusarium oxysporum</i> membrane-associated fatty acid hydroxylase P450<sub>oxys</sub> (CYP505) (75).

<i>The CYP116B Family</i>—Several years elapsed between the discovery of P450<sub>BM-3</sub> and the characterization of the next type of P450-redox partner fusion enzyme. Genome sequence data led to the identification of a small number of bacterial P450s fused to partners resembling phthalate dioxygenase reductases (76). Heterologous expression of two CYP116B family members (CYP116B1 and CYP116B2) enabled their purification and the demonstration that the reductase component binds FMN and 2Fe-2S iron-sulfur prosthetic groups and that P450 turnover is driven by NAD(P)H (55, 77). In the case of CYP116B1, the enzyme was shown to be catalytically active in the oxidation of thiocarbamate herbicides, consistent with the sequence similarity between its P450 domain and the herbicide-degrading Class I P450 CYP116A1 from <i>Rhodococcus</i> sp. strain DSM 17069 (78). The CYP116B-type module has recently been mimicked in artificial P450 fusion enzymes in efforts to enhance eukaryotic P450 activities and produce metabolites of interest (e.g. see Ref. 79). Although electron transfer kinetics in the CYP116B-type reductase may be ~30-fold slower than in the P450<sub>BM-3</sub> reductase, fusing exogenous P450s to this CYP116B type of reductase may circumvent the problem of dimerization of the P450<sub>BM-3</sub> reductase and provide a more robust fusion system for P450 engineering.

<i>Other P450-Redox Partner Fusion Protein Systems</i>—The P450<sub>BM-3</sub> (CYP102A)- and CYP116B-type P450-partner fusions are catalytically self-sufficient in terms of having all protein partners required for function covalently linked together, thus requiring only an electron donor (NAD(P)H) and a P450 substrate for oxidative catalysis. A different type of self-sufficient system was described in <i>Rhodococcus ruber</i> strain DSM 44319, composed of Fdx, FMN-containing flavoprotein reductase, and P450 modules and able to catalyze NADPH-dependent oxidation of polycyclic aromatic hydrocarbons (80). However, other systems have been characterized in which “partial” fusions decrease the number of redox partners required to reconstitute P450 function. In <i>Methylococcus capsulatus</i>, a CYP51-FDx fusion was shown to be composed of a P450 linked to a 3Fe-4S FDx at its C terminus. The enzyme (CYP51FX) was demonstrated to catalyze NADPH-dependent 14α-demethylation of lanosterol when spinach NADPH-FDx reductase was added to reconstitute a complete Class I-like system (56). A soluble biotechnologically relevant P450 enzyme from <i>Rhodococcus rhodochrous</i> strain 11Y (XplA) has an FDX module fused to the P450 at its N terminus. XplA receives electrons from an NADP-dependent flavoprotein reductase (XplB) and catalyzes the reductive degradation of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine, producing nitrite as a final product (57).

A flavodoxin-like module in CPR is ultimately responsible for electron delivery to mammalian microsomal P450s, and flavodoxins were also shown to support electron transfer to the bacterial P450s involved in biotin synthesis and cineole catalysis, replacing ferredoxins in Class I-like systems (62, 81). XplA has unusual properties, including unusually weak binding of the FMN cofactor and the absence of a phylogenetically conserved Ser/Thr residue in the P450 I helix. The latter structural deviation is consistent with a primarily reductive role for XplA because the Ser/Thr residue is implicated in hydrogen bonding and/or proton transfer to iron-bound oxygen in the normal P450 catalytic cycle (82).

P450s Acting Independently of Redox Partner Proteins

Although the classical P450 catalytic cycle requires the timed delivery of 2 consecutive electrons to the heme iron (1) to allow (i) formation of ferrous iron, enabling O<sub>2</sub> binding and formation of the ferrous-oxy (FeO<sub>2</sub>') state, and (ii) reduction of the FeO<sub>2</sub>' form (followed by further oxygen activation), a number of P450 enzymes bypass this mechanism. In doing so, these P450s either use an alternative route to achieve substrate oxidation or exploit the P450 scaffold for unconventional catalytic functions.

<i>P450 Peroxygenases</i>—The “peroxide shunt” is a long-used chemical procedure for driving P450 oxidase reactions in the absence of NAD(P)H-dependent redox partners. The reaction of H<sub>2</sub>O<sub>2</sub> or organic peroxides with a ferric P450 can result in its direct conversion to the ferric-hydroperoxo (Compound 0) intermediate (FeO<sub>2</sub>-) (Fig. 1A, ii), protonation of which leads to loss of a water molecule and formation of the reactive Compound I species (ferryl-oxo with a porphyrin π cation radical, FeO<sup>3+</sup>; see above) that oxidizes the substrate (4, 83). The peroxide is rarely efficient and is often highly destructive in oxidizing the protein and the heme prosthetic group (non-physiological high-valent iodine compounds, e.g. iodosylbenzene (84, 85), can give high rates but are also destructive). However, this class of P450s uses this mechanism and avoids excessive oxidative activity.
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Non-redox Partner-P450 Fusion and Partner Proteins

Genome sequencing projects have led to the identification of a number of novel P450 enzymes covalently attached to protein modules that are not obvious NAD(P)H-dependent redox partners or domains thereof. In several cases, these modules are homologs of structurally/biochemically characterized enzymes, although the functions of these fused enzymes are unknown in most cases. Many of these P450 fusions likely catalyze consecutive reactions or otherwise interact productively with the P450s to perform important physiological functions.

The PpoA Dioxygenase/Peroxidase-P450 Fusion—The filamentous fungus Aspergillus nidulans encodes a P450 fused at the C terminus of a heme-binding dioxygenase/peroxidase domain. Characterization of this PpoA enzyme demonstrated that the peroxidase domain catalyzed oxidation of linoleic acid to form (8R)-hydroperoxyoctadecadienoic acid, with this product then being isomerized by the P450 domain to form 5,8-dihydroxyoctadecadienoic acid. Isomerization is predicted to occur via a ferryl heme iron intermediate, as in the case of CYP5A1 and CYP58A1 (97). The Ppo enzymes, which have now been detected in a number of fungi (97), are responsible for production of Psi (precocious sexual inducer) factors, which are important for controlling the balance between sexual and asexual life cycles.

Fungal Mycophenolic Acid Synthesis—The fungal secondary metabolite mycophenolic acid has immunosuppressant activity and may also have other useful properties (e.g. antiviral and antibacterial). Initial steps in its synthesis in Penicillium brevicompactum are catalyzed by the MpaDE fusion protein, composed of a P450 (CYP631B5) with a hydrolase fused to its C terminus (59). The substrate 5-methylsellinic acid undergoes MpaD P450-dependent hydroxylation on a methyl group to generate 4,6-dihydroxy-2-(hydroxymethyl)-3-methylbenzoic acid. A lactonization reaction is then likely catalyzed by the MpaE domain of MpaDE, forming 5,7-dihydroxy-4-methylphthalic acid, which is then acted on by later pathway enzymes. Other fungal genomes have separate mpaD/E genes, and thus, the gene fusion in P. brevicompactum may provide kinetic advantages in product formation.

Other P450 Fusions of Unspecified Function—Among several uncharacterized P450 fusions identifiable from database searches are enzymes fused to modules related to acyl-CoA dehydrogenase (CYP221A1), cinnamyl alcohol dehydrogenase, and F-box motif-containing proteins. The first viral P450 (CYP5253A1) was discovered in the Mimivirus genome and is fused at its C terminus to a protein of unknown function but containing several post-translational modification sites (phosphorylation, myristoylation, and glycosylation) (98, 99). Recent studies showed that the Saccharopolyspora erythraea glycosyltransferase enzyme EryCIII, which produces erythromycin D
(from the substrates thiamine diphosphate-δ-desosamine and 3-α-mycarosylerythronolide B), is activated by the addition of the protein EryCII, with which it forms a heterotetrameric (2:2) complex (63). EryCII is homologous to P450s but lacks the cytochrome P450, and the future promises to provide more interesting vignettes into this important superfamily.

Conclusions and Future Considerations

Characterization of P450 enzymes has revealed considerable diversity in the types of protein arrangements that can be used for electron transfer and catalysis. In one sense, the chemical mechanisms have a great deal of commonality (Fig. 1), but these are manifested in a wide variety of reactions because of the natures of the substrates. Today, we have insight into the physiological roles of a small minority of the >18,000 P450 genes whose sequences have been obtained, and the future promises to provide more interesting vignettes into this important superfamily.

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