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Mechanistic aspects of CYP74 allene oxide synthases and related cytochrome P450 enzymes

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Abstract

The existence of CYP5, CYP8A, and the CYP74 enzymes specialized for reaction with fatty acid peroxide substrates presents opportunities for a “different look” at the catalytic cycle of the cytochrome P450s. This review considers how the properties of the peroxide-metabolizing enzymes are distinctive, and how they tie in with those of the conventional monooxygenase enzymes. Some unusual reactions of each class have parallels in the other. As new enzyme reactions and new P450 structures emerge there will be possibilities for finding their special properties and edging this knowledge into the big picture.

Keywords

allene oxide; allene oxide synthase; hydroperoxide lyase; divinyl ether synthase; CYP74; cytochrome P450; fatty acid hydroperoxide; thromboxane synthase; prostacyclin synthase; mechanism; Compound I; Compound II; catalytic cycle

1. Introduction

The synthesis and reactions of allene epoxides (allene oxides) was an academic topic in synthetic chemistry before the biosynthetic connection appeared on the scene just over twenty years ago (Hamberg, 1987, 1989; Corey et al., 1987; Brash et al., 1987; Crombie and Morgan, 1991b). Chemically produced allene epoxides were considered of interest for their potential utility in total synthesis, and allene oxides with the sensitive epoxy moiety shielded by bulky non-polar groups were the first ones isolated (Chan and Ong, 1980; Smadja, 1983). The chemical route involved epoxidation of an allene, hence the common name of these epoxides, while the biochemical route involves dehydration of an unsaturated fatty acid hydroperoxide, Fig 1 (top). Nowadays, work on the biosynthetic hydroperoxide-derived allene oxides has set a new standard for the practical handling and study of these unstable molecules and has led the way in the exploration of allene oxide chemistry (e.g. references Brash et al., 1988, 1990; Hamberg, 1988; Grechkin et al., 1991; Grechkin and Hamberg, 2000). Knowledge of the enzymes has blossomed after identification of the prototypical flaxseed allene oxide synthase (AOS) (Zimmerman and Vick, 1970) as a cytochrome P450 (Song and Brash, 1991; Song et al., 1993b). Current understanding of the CYP74 family and its products (Fig. 1) encompasses their occurrence in plants, bacteria and lower animals (Lee et al., 2008; Andreou et al., 2009;

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Hughes et al., 2009). Study of the enzymes involved is also producing new insights into the structure-function of the hemoprotein classes of cytochrome P450s (Grechkin, 2002; Tijet and Brash, 2002; Hughes et al., 2009) and catalase-related peroxidases, each of which have members acting as an allene oxide synthase (Tijet and Brash, 2002; Lee et al., 2008; Andreou et al., 2009).

As I assume that many readers of this review will be familiar with the CYP74 enzymes and their functioning in biology, and as they crop up many times in this Special Issue, I decided to approach the issue of their mechanism by a slightly roundabout route with an emphasis on comparisons to other P450 enzymes to which they are structurally and mechanistically related.

2. P450 enzymes using fatty acid peroxides as their natural substrate

When viewed in retrospect, the discovery that a P450 was responsible for the conversion of fatty acid peroxides (to thromboxane A₂ (TxA₂) and prostacyclin (PGI₂) in higher animals or allene oxide in plants) perhaps might look like a simple case of putting two and two together. But in practice the biochemical reactions were identified years before the class of enzyme involved was established. Indeed, each of the prototypical examples from mammalian and plant biology came as a distinct surprise. The first cytochrome P450s shown to use peroxides as their natural substrate were TxA synthase, now classified as CYP5, and PGI₂ synthase, CYP8A (Fig. 2). It took considerable insight to come up with the idea of P450 involvement in thromboxane and prostacyclin biosynthesis, as it represented quite a departure from the typical biochemical transformation of the cytochrome P450s (Ullrich et al., 1981; Haurand and Ullrich, 1985). TxA and PGI₂ synthases rearrange the oxygens in the endoperoxide group of prostaglandin H₂, the product of the heme-dioxygenase enzyme, cyclooxygenase (Hecker and Ullrich, 1989; Ullrich and Brugger, 1994; Tanabe and Ullrich, 1995). Cyclooxygenase, in turn, is a relative of the plant α -dioxygenase (PIOX) (Sanz et al., 1998) and fungal linoleate 8- and 10-dioxygenases (Hornsten et al., 1999; Garscha and Oliw, 2009). The CYP5 and CYP8A products, TxA₂ and PGI₂, are of critical importance in mammalian biology in the control of blood platelet reactivity and cardiovascular hemostasis and they are central players in the heart protective effects of the cyclooxygenase inhibitor, aspirin (e.g. Egan and FitzGerald, 2006).

Although precedent was established by the identification of TxA synthase and PGI₂ synthase as cytochrome P450s with fatty acid peroxide substrates, prior to the purification and spectral characterization of flaxseed AOS (Song and Brash, 1991; Song et al., 1993b) there was no inkling of its cytochrome P450 structure. As events later proved, the plant AOS need not have been a P450 – indeed the AOS of marine invertebrates turned out to be a distant relative of catalase, with no P450 homology whatsoever (Koljak et al., 1997; Oldham et al., 2005).

3. P450 enzymes and the conventional mono-oxygenation mechanism

3.1. Eliminating H₂O and forming Compound I

The vast majority of cytochrome P450 enzymes use one of the oxygen atoms from a molecule of O₂ to hydroxylate or epoxidize their substrate (e.g. for a recent review see (Guengerich, 2007)). In the typical reaction cycle, illustrated as the full circular pathway in Fig. 3, the enzyme has to reduce and dispose of the first oxygen atom from O₂ before using the second to oxygenate the substrate. Hence the need for the two reducing equivalents from a molecule of NADPH to allow the elimination of H₂O prior to the oxygenation reaction; the two electrons are transferred to the P450 one at a time (Fig. 3) via the electron transfer protein known as cytochrome P450 reductase or the alternative flavoprotein-adrenodoxin system (McLean et al., 2005). Accordingly, in the first two thirds of the reaction cycle the enzyme has bound substrate (RH), but does nothing to metabolically transform it – it is still sitting in the P450 active site as RH, waiting for the last steps in the cycle. It is only when the first oxygen atom is released as H₂O

that the enzyme is fully activated as the ferryl (iron-oxo) species called Compound I (“Compound one”) and poised for chemical interaction with the substrate. In simple terms, Compound I is oxidized by two equivalents over the original ferric enzyme (Fe^{III}) and might be written as $\text{Fe}^{\text{V}}=\text{O}$. More correctly, the extra two equivalents in Compound I are distributed one on the iron (which is therefore iron(IV)oxo) and one as a radical cation on the porphyrin ring, and hence Compound I is best represented as $\text{Por}^+\bullet\text{Fe}^{\text{IV}}=\text{O}$ (e.g. Ortiz de Montellano and De Voss, 2002; Denisov et al., 2005).

3.2. First reactions with substrate, last steps in the cycle

In cytochrome P450s, Compound I is a very strong oxidant that will attack any molecule close to it, which in our case is the substrate RH. One of the hallmarks in the protein structure of cytochrome P450s is the thiolate ligand to the heme, and this is of critical importance in the chemistry of the P450 Compound I (e.g. Denisov et al., 2005; Green, 2009). The thiolate, an ionized thiol coming from a cysteine directly under the heme iron (the proximal side) is the so-called fifth ligand to the heme iron, and it facilitates reactions with substrate occurring on the opposite or distal face of the heme. Compound I abstracts a hydrogen atom from RH, producing the substrate radical $\text{R}\bullet$ and itself being reduced by one equivalent to Compound II (“Compound two”), represented as $\text{Fe}^{\text{IV}}-\text{OH}$. The lifetime of $\text{R}\bullet$ is so short (estimated as around 10^{-9} to 10^{-10} of a second) because Compound II is poised for immediate “oxygen rebound”, the nicely descriptive technical term for the last stage of the reaction that forms the hydroxylated product. After that, product release returns the enzyme to the resting ferric state (Fig. 3).

There are a great many nuances to this prototypical reaction cycle, and much energetic debate on the best ways to represent the different forms of the $\text{Fe}[\text{O}_2]$ and $\text{Fe}[\text{O}]$ iron-oxygen species involved (Bach and Dmitrenko, 2006; Behan et al., 2006; Makris et al., 2006). It is also debated whether species in addition to Compound I are responsible for the synthesis of certain P450 products (e.g. (Vaz et al., 1998; Ortiz de Montellano and De Voss, 2002; Chandrasena et al., 2004; Jin et al., 2004; Nam, 2007)). Some argue that two different spin states of Compound I can account for the different chemistries sometimes observed (Shaik et al., 2004, 2005). Much effort has gone into the detection of the different forms of the heme throughout the reaction cycle, culminating in the recent spectroscopic detection of Compound II within an activated cytochrome P450 (Newcomb et al., 2008). For the purposes of this review, it will suffice to consider the resting ferric enzyme as Fe^{III} , Compound I as $\text{Fe}^{\text{V}}=\text{O}$ or $\text{Por}^+\bullet\text{Fe}^{\text{IV}}=\text{O}$, and Compound II as $\text{Fe}^{\text{IV}}-\text{OH}$.

4. Conventional P450 enzymes and interaction with peroxides

From the earliest days of studying P450 reaction mechanism, it was correctly deduced that the activated enzyme, Compound I, could be formed purely by chemical means. This understanding was riding on much earlier studies of catalase and peroxidases in which Compounds I and II were spectroscopically characterized before P450s were discovered (Chance, 1949). Using a hydrophobic oxygen donor, iodosobenzene, the ferric P450 can be transformed directly to Compound I:

Similarly, treating the P450 enzyme with an organic peroxide such as cumene hydroperoxide will also produce Compound I:

Here, the peroxide is reduced by two equivalents to the hydroxy derivative (cumyl alcohol) while the P450 is oxidized by two equivalents to Compound I. If the regular substrate (RH) is present in these incubations, the last steps of the conventional reaction cycle can take place normally and give the mono-oxygenated reaction product, ROH. This short-circuited P450 reaction cycle is illustrated in Fig. 3 as the Peroxide Shunt Pathway. As can be seen, there is no need for molecular oxygen or NADPH in this short route to product.

5. CYP74 AOS catalytic cycle: peroxide and substrate are one and the same

The CYP74 enzymes use their fatty acid hydroperoxide substrate to activate the enzyme and substrate in one and the same step. The ferric enzyme induces cleavage of the substrate hydroperoxide through a homolytic scission of the O-O bond. This means that the electron pair forming the O:O hydroperoxide bond is split evenly with one electron going with each oxygen. Thus, the substrate is converted to RO• (an alkoxyl radical) and the heme becomes Fe^{IV}-OH (Compound II). Fig. 3 illustrates how this CYP74 pathway (orange route) short-circuits the P450 catalytic cycle even further than in the peroxide shunt pathway. Compound I is never formed.

5.1. CYP74 and reactions of Compound II

Compared to the conventional P450 cycle, the initiating step in CYP74 catalysis brings catalysis to the point at which “oxygen rebound” is about to happen. But oxygen rebound does not occur in AOS catalysis. Instead, the reacting fatty acid loses a hydrogen atom (via transfer of an electron to the heme iron, then loss of a proton, see later), resulting in net loss of H₂O from the original hydroperoxide, overall a dehydrase-type reaction. Although this does not match the typical P450 monooxygenation, the same outcome is occasionally seen in metabolism by conventional P450 enzymes. There are a few well-documented examples in which a P450 substrate is either hydroxylated or desaturated (Rettie et al., 1987, 1995; Korzekwa et al., 1990). This parallel is illustrated in Fig. 4, comparing the CYP74 catalysis with valproic acid metabolism by the major human drug metabolizing P450, CYP3A. This highlights the fact that the same dual activity, desaturation and hydroxylation, has been found with CYP74 AOS itself. Using unnatural hydroperoxy-hydroxy derivatives of arachidonic and eicosapentaenoic acids, we observed both the dehydrase and hydroxylation activity of purified flaxseed AOS (CYP74A), giving allene oxide and epoxyalcohol products, respectively, and with full retention of the hydroperoxide oxygens in the epoxyalcohols (Song et al., 1993a). There are well-characterized epoxyalcohols identified as fatty acid hydroperoxide products in plant tissues (Hamberg, 1999), and it is likely that the “EAS” (epoxyalcohol synthase) is a new member of the CYP74 family. Although the EAS enzyme(s) have yet to be cloned in plants, a bacterial CYP74 EAS was recently identified (Lee et al., 2008). In a related development, with a catalase-related AOS from the cyanobacterium *Acaryochloris marina* we have also seen epoxyalcohol production in association with AOS activity, in this case using 9*R*-hydroperoxy-linoleic acid as substrate (Gao et al., 2009). These dual activities are atypical findings, but they serve to spotlight the mechanistic similarities of conventional P450-type hydroxylases and the CYP74-type reactions.

5.2. CYP74 as an oxygenase?

A last point here returns to the fact that the AOS catalytic cycle does not form Compound I. This means that a conventional alkane substrate (not a hydroperoxide) cannot be hydroxylated. Of course, we understand that the CYP74 enzymes are not designed for interaction with a reductase/NADPH system and that completing a full P450 catalytic cycle is a practical impossibility. However, from an understanding of the principles involved, one could see that a CYP74 enzyme could potentially be converted to Compound I by using the peroxide shunt pathway. For example, treatment with iodosobenzene or cumene hydroperoxide could

potentially form Compound I in a CYP74, just as they do in conventional P450s. Indeed, this experiment has been carried out with TxA synthase (CYP5), in which a prostaglandin endoperoxide analogue was hydroxylated through this route (Hecker et al., 1987). Such an experiment has not been reported using CYP74 or the catalase-related AOS. It would be interesting to see the outcome. One might add that several of the CYP74 enzymes react sluggishly with carbon monoxide (e.g. Lau et al., 1993; Shibata et al., 1995; Tijet et al., 2001), from which it is implied that there is also limited access to O₂ on the distal side of the heme. However, by circumventing the need for O₂ in the peroxide shunt pathway, and knowing that Compound II forms naturally in the AOS catalytic cycle, there should be room for formation of Compound I, assuming that the surrogate oxygen donor can gain access to the heme iron.

6. Mechanism of AOS: radical and ionic steps

There is a general consensus on the chemistry underlying allene oxide biosynthesis based on an understanding of fatty acid hydroperoxide chemistry and from hemoprotein chemistry. As noted above, the fatty acid hydroperoxide is cleaved homolytically producing Compound II and an alkoxyl radical, Fig. 5. Reaction of the alkoxyl radical with the adjacent double bond is highly favored, resulting in formation of an epoxy allylic carbon radical intermediate (Gardner, 1989; Wilcox and Marnett, 1993). As illustrated in Fig. 5, at this point there is the potential for further reaction via radical (blue) or ionic (red) pathways. AOS catalysis requires the net loss of a hydrogen atom from the epoxy allylic radical and there is general agreement that this will occur by the ionic route (Fig. 5, red, lower middle pathway). First Compound II accepts an electron from the intermediate, producing the ferric heme (Fe^{III}) and an epoxyallylic carbocation (Ullrich and Brugger, 1994; Gerwick, 1996; Ullrich, 2003). One of the prototypical reactions of carbocations, elimination of a β -proton, then gives the allene oxide product.

The divinyl ether synthase (DES, CYP74D (Itoh and Howe, 2001)) is similarly favored to react through the homolytic-to-ionic route with the outcome from the epoxy allylic carbocation controlled to yield the divinyl ether product (Crombie and Morgan, 1991a; Grechkin, 2002) (Fig. 5, red, lower right hand side).

7. Mechanism of HPL: a purely radical pathway?

“On paper” one could draw the AOS reaction with purely radical intermediates, but the required H-atom loss is problematic. There is, however, less objection to the hydroperoxide lyase (HPL) reaction being entirely radical in character (Fig. 5, blue, left-hand side). As shown recently, the true catalytic activity of CYP74-HPL is a hydroperoxide isomerase, giving a fatty acid hemiacetal as the final enzymatic product (Fig. 1, Fig. 5) (Grechkin and Hamberg, 2004; Grechkin et al., 2006). As the authors noted, this isomerization can be accommodated using radical intermediates. At least in small measure, a reason for promoting this as a radical “homolytic pathway” is to provide a direct contrast to the long-time ago proposals of the participation of water in the reaction mechanism, and its purported quenching of a carbocation intermediate in production of the aldehydic products (Hatanaka et al., 1987; Crombie and Morgan, 1991a). Experiment has discounted the involvement of water and proven that both of the original hydroperoxide oxygens are retained in the hemiacetal product (Grechkin and Hamberg, 2004; Grechkin et al., 2006). This evidence notwithstanding, there remains the possibility that ionic intermediates could be involved. A pathway via a carbocation intermediate can be “drawn” and still explain the retention of both hydroperoxide oxygens (Fig. 5, red, right-hand side).

The question of radical or ionic pathways harks back to a long-time issue in conventional P450 catalysis in which it is questioned whether the radical reaction, “oxygen rebound” from Compound II, can completely account for substrate oxygenation (Groves et al., 1978) or are

elements of carbocation chemistry involved (e.g. Newcomb and Chestney, 1994; Auclair et al., 2002; Newcomb et al., 2003). In the conventional P450 reaction and in the HPL-CYP74 reaction, it is possible to envisage elements of both radical and ionic mechanisms in the pathway to products. The original oxygen rebound proponent J. T. Groves concedes that one explanation for “the cationic rearranged products that sometimes appear during P450 turnover derive..... from an electron-transfer oxidation of the incipient carbon radical that competes in some cases with oxygen rebound” (Groves, 2006). Ullrich would go further in proposing that “As a common step in all P450 enzymes, an extremely rapid electron uptake by Compound II allows that the primary substrate radicals are oxidized to cations which immediately combine with a neighbouring nucleophile. Thus “electron transfer” may substitute for “oxygen rebound” as the final step leading to product formation” (Ullrich, 2003).

Ultimately it comes down to a question of whether the electron resides on the reacting substrate and radical recombination ensues:

or the electron transfers to the iron-oxo immediately prior to oxygenation:

This is a difficult issue to resolve using the natural substrates that give the well characterized products and the debate on this topic with conventional P450s makes extensive use of substrates that should exhibit different products in ionic and radical reactions (Newcomb and Chestney, 1994; Auclair et al., 2002; Newcomb et al., 2003; Jiang et al., 2006). The outcomes tend to implicate predominantly (in some cases exclusively) the radical rebound route in conventional P450 catalysis (Auclair et al., 2002; Jiang et al., 2006) and quite possibly this is the correct mechanism for HPL-CYP74.

8. CYP74 AOS protein structure-function

8.1. *Arabidopsis* and guayule CYP74A enzymes

At the time of writing there are X-ray crystal structures of two CYP74s, *A. thaliana* CYP74A1 and *Parthenium argentatum* (guayule) CYP74A2, each an AOS classified as CYP74A (Lee et al., 2008; Li et al., 2008). CYP74A1 from *Arabidopsis* exemplifies some of the CYP74 members that have been isolated partly in the low spin state and that exhibit weak activity prior to activation (*in vitro*) in detergent micelles (Noordermeer et al., 2001; Hughes et al., 2006a). In describing the enzyme activation of CYP74A1 it is reported to have “very low enzyme activity that can be massively activated with detergent”, and detergent also specifically activates a selective substrate preference for 13S-hydroperoxy-linolenic acid (Hughes et al., 2006b).

CYP74A2 from guayule (*P. argentatum*) was the second AOS to be purified, cloned, and identified as a cytochrome P450 (Pan et al., 1995). Guayule is a desert shrub once cultivated and harvested as a source of natural rubber. In the homogenized tissue the rubber particles (~1 µM in diameter) float to the surface and are readily washed and purified. Intriguingly, when the rubber particle proteins are visualized on SDS-PAGE, by far the most abundant is CYP74A2, accounting for about 50% of the protein content (Backhaus et al., 1991; Pan et al., 1995). Originally this 53 kD protein was implicated as a potential rubber transferase, the enzyme that polymerizes thousands of isoprenes into molecules of rubber (Backhaus et al., 1991). To this day the biological role of this prominent protein is unclear, and whether (how?)

it relates to the biochemical pathways of rubber biosynthesis remains to be determined. Guayule AOS is highly active with 13*S*-hydroperoxy-C18:2 ω 6 (3,700 turnovers/s) and as some of the other CYP74A enzymes with an established role in the jasmonate pathway are less active with this linoleic hydroperoxide (Hughes et al., 2006a), the guayule enzyme may well have a role in something other than jasmonate biosynthesis. That is certainly true for the specific allene oxides formed from the 9*S*-hydroperoxides of linoleic and linolenic acids by maize CYP74A AOS (Gardner, 1979; Hamberg, 2000) and the potato/tomato CYP74C AOS (Hamberg, 2000; Grechkin et al., 2008).

8.2. The heme-binding loop in CYP74

Clearly there must be something different about the specialized peroxide-metabolizing P450s and a view of sequence alignments and X-ray structures offers some clues to their distinctive catalytic activities (Lee et al., 2008; Li et al., 2008). Not surprisingly, the main functional and structural differences can be seen on both sides of the heme. On the proximal side ("underneath" the heme and opposite to the distal side where substrates bind and react) the CYP74 enzymes harbor the characteristic cysteine as the fifth ligand to the heme. The heme-binding loop on the proximal side shows distinctive changes from typical P450 monooxygenases. As noted by Li et al. in their paper on the X-ray structure of guayule AOS (CYP74A2) (Li et al., 2008), in typical P450s the loop usually forms a compact β -turn and incorporates the most typical P450 consensus sequence, FXXG, followed by three residues (XRX) followed by CXG encompassing the cysteine. In CYP74 there is a distinctive nine amino acid insert (Fig. 6). This feature turns out to be a hallmark of CYP74-like proteins. The presence of such an insert was used by Lee et al. to identify novel CYP74-like sequences from database searches, thus uncovering structural and functional relatives in plants, bacteria and lower animals (Lee et al., 2008). The expanded length allows the heme-binding loop access to the surface of the protein in a region where it could disrupt the typical P450 interaction with its reductase partner (Lee et al., 2008). Of course, CYP74 enzymes need no reduction of the heme as binding and splitting of molecular oxygen are not required.

8.3. An unusual substitution near the cysteine ligand

Another characteristic of CYP74 that has not been commented on before is the occurrence of a highly unusual amino acid four residues after the CAG motif on the proximal side of the heme (Fig. 6). This position is strongly conserved as Ala or Gly in conventional P450s, yet it is Val or Ile in CYP74. This Val resides about 4Å directly under pyrrole ring B of the heme, ~5Å to the side of the cysteine, and it may function to slightly tilt the heme in CYP74. (It was noted in the guayule X-ray structure that the CYP74 heme is more strongly held in place than usual via ionic interactions with its propionate groups (Li et al., 2008)). One of the few other P450s to express Val in this position is the *Saccharomyces cerevisiae* CYP61, a C22 sterol desaturase (cf. Fig. 5). Interestingly, there is also a larger than usual amino acid in this position (a cysteine) in the C-terminal P450 domain of PpoA, a fusion protein in *Aspergillus nidulans* comprised of an N-terminal heme dioxygenase and a C-terminal cytochrome P450 (Brodhun et al., 2009). PpoA is one of the fungal psi-factors [precocious sexual inducer]. The P450 domain isomerizes the fatty acid 8-hydroperoxide produced in the N-terminal dioxygenase/peroxidase-related domain to a 5,8-diol (Brodhun et al., 2009), a CYP74-type of transformation with oxygen rebound, akin to the hydroxylation event in epoxyalcohol synthesis.

8.4. The distal side of the heme in CYP74

P450s have hydrophobic substrates, of which the fatty acid hydroperoxides are typical. These are accommodated in the P450 active site, which, suitably, is very predominantly non-polar. Accordingly there are very few residues available for hydrogen bonding interactions during catalysis. In conventional P450s there is a threonine residue poised directly above the distal

face of the heme. It extends down from the “I-helix” that passes across the middle of the enzyme and over and across the heme. This conserved Thr is crucial in facilitating splitting of the O-O bond, a required step in creating the catalytically active ferryl heme (Compound I) (Imai et al., 1989; Denisov et al., 2005). In CYP74 the Thr is replaced by Ile or another hydrophobic alkane residue that cannot participate in hydrogen bonding. But seven residues back in the N-terminal direction along the I-helix a new Asn residue hangs over the heme (Fig. 7) (Lee et al., 2008; Li et al., 2008). By mutagenesis this Asn321 is shown to be essential for catalysis (Lee et al., 2008).

8.5. Interconversion of CYP74 activities

The two X-ray crystallography studies include structures with the substrate analogue 13S-hydroxy-linoleic acid bound in the active site (Lee et al., 2008; Li et al., 2008). The fatty acid is positioned closer to the potential action in the *Arabidopsis* structure, in which it is suitably poised for interaction with the iron, the Asn321 on the I-helix, and with a Phe137 that aligns with the conjugated diene of the hydro(pero)xy fatty acid (Fig. 8). Lee et al. rationalized a role for this Phe137 in catalysis and found that its mutation to Leu switches the product outcome from AOS to HPL activity (Lee et al., 2008). The discussion in the Supplement (Lee et al., 2008) includes a scholarly argument with mechanistic rationalization of the effect of the aromatic residue on the electron transfer to the iron, carbocation formation on the reacting fatty acid, and the catalytic drive towards the allene oxide as product. Switching to 137Leu is proposed to promote the radical route to product, strongly favoring HPL activity and formation of the hemiacetal and subsequently the aldehydes (cf. Fig. 4, blue, left hand side).

There is more than one way to mutate a CYP74, and taking a conserved differences approach with the aid of sequence alignments, Grechkin and colleagues identified two other active site residues that could switch AOS to HPL catalytic activity (Toporkova et al., 2008). Using this bioinformatics-guided method, the authors identified two I-helix residues with conserved differences in AOS and HPL. The two amino acids are positioned immediately before and after the distal Asn (Asn296 in the tomato CYP74C AOS enzyme). The mutations Phe295I and Ser297A both switched the catalytic activity using the natural 9S-hydroperoxy-linoleic acid substrate, eliminating or almost eliminating allene oxide production and achieving a lyase reaction instead (Toporkova et al., 2008). Certainly the plant CYP74s are a well conserved and closely knit family and there will be further opportunities prompted by protein comparisons to identify the structural basis for AOS, HPL, DES, and EAS catalysis.

9. Concluding remarks

Despite the differences that provide everyone with their own favorite P450, there are fundamental similarities in mechanism across the spectrum. I have tried to highlight these parallels, sometimes using the exceptional case that illustrates the point. The catalytic cycle (Fig. 3) forms a conceptual framework for this discussion. To the best of my knowledge it is the first P450 cycle in the literature that clarifies the distinction between the “peroxide shunt” of the conventional monooxygenases and the shorter-short-circuit in CYP74s and their relatives, CYP5 and CYP8A.

It has been argued by others that these peroxide metabolizing P450s were the evolutionary earlier form and that the P450 monooxygenases evolved only after atmospheric oxygen became available (Lee et al., 2008). Extending that line of thinking, one could deduce that the fundamental enzymatic mechanisms were in place in the ancestors of the present-day CYP74s, which also retain these capabilities. Ullrich, who established the P450 character of the peroxide-metabolizing TxA and PGI₂ synthases, has argued over the years that there are lessons to be drawn from the mechanistic study of the unusual P450s with impact on P450 monooxygenase catalysis (Ullrich and Brugger, 1994; Ullrich, 2003; Daiber et al., 2005). Not

that this is echoed in the current reviews of P450 mechanism. Something might have been said about this by the late American comedian Rodney Dangerfield, a specialist in "I don't get no respect" jokes, the first of which, according to Wikipedia, was "I get no respect. I played hide-and-seek, and they wouldn't even look for me!".

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Abbreviations

AOS	allene oxide synthase
HPL	hydroperoxide lyase
DES	divinyl ether synthase
EAS	epoxyalcohol synthase
TxA ₂	thromboxane A ₂
PGI ₂	prostaglandin I ₂ (prostacyclin)

Biography

Alan Brash, a native of Scotland, received his Bachelor's degree in Medical Sciences from Cambridge University (1970) and his Ph.D., focused on LC and GC-MS methods for the analysis of prostaglandins, from the University of Edinburgh in 1976. After completing a Research Fellowship at the Department of Clinical Pharmacology, Royal Postgraduate Medical School in London, he moved to Vanderbilt University in Nashville, Tennessee, where he is now Professor of Pharmacology. In the course of his 30-year career at Vanderbilt his research interests evolved towards analysis of the mechanisms of formation and transformation of lipoxygenase products with an interest in their physiological role. A co-author on nearly 200 research articles, his work has helped found research on stereochemical aspects of lipoxygenase catalysis and on the role of epithelial lipoxygenases. His findings also initiated work on the biochemistry of the CYP74 family of cytochrome P450s, and on the catalase-related hemoproteins which also metabolize fatty acid hydroperoxides.

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Fig. 1. Allene oxides and their origin in chemistry and biology (top), and other CYP74 products (below).

Fig. 2. Biosynthesis of prostacyclin (PGI₂) and thromboxane A₂ (TxA₂) in higher animals
 Two cytochrome P450s, PGI synthase (CYP8A) and TxA synthase (CYP5), metabolize the prostaglandin endoperoxide PGH₂ in reactions closely analogous to the mechanisms of CYP74s.

Fig. 3. The catalytic cycle of cytochrome P450s

The typical hydroxylation or epoxidation pathway follows the complete circular route (black). In these typical hydroxylase and epoxidase P450s, the cycle can be short-circuited via the peroxide shunt pathway when an oxygen donor oxidizes the ferric enzyme by two equivalents directly to Compound I (magenta route). If H₂O₂ is used as oxygen donor it short-circuits the cycle to a Fe[O₂] intermediate prior to forming Compound I (peroxide shunt, dotted line). In CYP74 and related enzymes such as PGI synthase and TxA synthase, peroxide cleavage by the ferric enzyme leads directly to Compound II (orange route). For clarity, the steps in the CYP74 pathways leading either to dehydration (AOS), or isomerization (HPL and DES) are not illustrated on the figure. In the case of CYP74-AOS, the reacting substrate ROOH would give Compound II and the fatty acid alkoxyl radical (Fe^{IV}-OH and RO•); upon completion of reaction the original substrate is dehydrated and the allene oxide is released as product.

Fig. 4. Parallels in hydroxylation and desaturation reactions in CYP74 and conventional P450s
Conceptually, the main difference between CYP74 (left, using fatty acid hydroperoxide) and the conventional P450 CYP3A (right, using the antiepileptic drug valproic acid) is the activation of substrate and enzyme. In CYP74 this occurs via reaction of hydroperoxide with the ferric enzyme giving a substrate radical and Compound II ($Fe^{IV}-OH$). In CYP3A reaction proceeds via the conventional route involving molecular oxygen (O_2), utilization of NADPH to reduce $[O]$ to H_2O , formation of Compound I ($Fe^V=O$, not shown), and hydrogen abstraction from the alkane giving the substrate radical and Compound II. From that point forward, Compound II catalyzes either “oxygen rebound” (the hydroxylation pathway) or a second hydrogen abstraction resulting in net desaturation of the original substrate. Allene oxide synthesis is the usual outcome for CYP74, hydroxylation is the usual outcome for the conventional P450 CYP3A, but occasionally with either enzyme, a substrate will take both pathways to products.

Fig. 5. Potential intermediates in radical and ionic pathways to CYP74 products

Homolytic cleavage of the fatty acid hydroperoxide and cyclization of the alkoxyl radical produces an epoxy allylic radical, readily converted to an epoxy allylic carbocation (middle box). A purely radical pathway can account for lyase activity via hemiacetal synthesis and its spontaneous cleavage to aldehydes (in blue). Alternatively, ionic intermediates (in red) can similarly account for lyase activity and are the preferred mechanistic route to allene oxide and divinyl ether. Although not illustrated, there is also the potential for epoxyalcohol synthesis through either radical or ionic pathways.

This partial alignment begins with the readily recognizable “PERF” sequence, usually situated around position 380–430 in the primary structure. The characteristic cysteine, found in the sequence context CXG, is part of a heme-binding loop that contains the signature sequence FXXG. In CYP74 enzymes there is a 9-amino acid insert (Lee et al., 2008), (or 8 amino acids, using a different alignment (Li et al., 2008)). Note that PGI₂ synthase (CYP8A) has an amino acid insert before the FXXG, as does its close relative, the sterol monooxygenase CYP8B. CYP74 enzymes have a highly unusual amino acid four residues after the CAG motif: this is strongly conserved as Ala or Gly in conventional P450s, yet it is Val or Ile in CYP74. One of the few other P450s to express Val in this position is the *S. cerevisiae* CYP61, a C22 sterol desaturase (cf. Fig. 4).

Fig. 7. CYP74A heme, heme binding loop, I-Helix, and distal Asn. This figure was kindly provided by Dr. C. S. Raman and is adapted from the Supplement in Lee et al., 2008.

Fig. 8.

Binding of a substrate analogue in the CYP74A active site. Overlay of the active sites of wild-type *Arabidopsis* AOS (magenta) and its Phe137Leu mutant that exhibits hydroperoxide lyase activity (green). Each enzyme was crystallized in complex with 13*S*-hydroxy-linoleic acid, a close analogue of the 13*S*-hydroperoxide substrate. Note that the 13-hydroxyl points towards the center of the heme where the ferryl oxygen would be formed by homolytic cleavage of the 13*S*-hydroperoxide. It is also poised for interaction with Asn-321 on the I-helix, while Phe137 (or Leu137) lie close to the substrate 9*Z*,11*E* conjugated diene (arrow). Reproduced with permission from reference (Lee et al., 2008).