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CATALYTIC REACTIVITIES AND STRUCTURE/FUNCTION RELATIONSHIPS OF CYTOCHROME P450 ENZYMES

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Key Word Index—Plants; cytochrome P450; catalytic reactivities; structure/function relationship.

Abstract—The catalytic reactivities of the enzymes in the P450 supergene family are reviewed. Emphasis is given to the recent novel reactivities catalysed by plant P450s. Common principles in the underlying mechanisms involving the interaction of oxygen and various redox states of the iron atom are discussed. Structure/function relationships of P450 enzymes based on the available three-dimensional structures are reviewed. Recent advances in the determination of residues important for substrate specificity are highlighted. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Cytochromes P450 (P450s) derive their name from the characteristic peak at 450 nm in the carbon monoxide difference spectrum of reduced cytochrome P450. The carbon monoxide binding pigment first found in liver microsomes [1, 2] was shown to be a heme-containing protein [3]. Today, P450s constitute a supergene family of numerous enzymes found in mammalian tissues, insects, plants, yeasts and bacteria [4]. The prokaryotic P450s constitute a large class of soluble proteins, in contrast to the eukaryotic enzymes that, with few exceptions, are membrane-bound to either the mitochondrion or the endoplasmic reticulum.

Cytochromes P450 are powerful oxidizing catalysts, which activate molecular oxygen and insert typically one oxygen atom (as a hydroxyl group) into lipophilic substrates. P450s catalyse the oxidation of many exogenous and endogenous compounds [5,6]. In P450-dependent monooxygenases, reducing equivalents from NADPH are transferred via a flavin-containing reductase in the microsomal system, or a ferredoxin reductase and a non-heme iron protein in the mitochondrial and bacterial systems, to the terminal cytochrome P450, which is the specific component that binds the substrate and activates molecular oxygen. Generally, the cytochromes P450 involved in detoxification (e.g. in the liver) have broad substrate-specificities, whereas the ones involved in biosynthesis of endogenous compounds (e.g. of steroids and secondary plant products) have narrow substrate-specificities.

Our knowledge of chemical reactions that are catalysed by cytochromes P450 is continuously expanding. Especially amongst the rapidly increasing number of cytochromes P450 that are being identified in plants and microorganisms, we see some very unusual catalytic reactivities [7]. The aim of this review is to summarize the current knowledge on the catalytic reactivities and the structure/function relationships of cytochrome P450 enzymes.

THE CATALYTIC CYCLE

P450s are b-type heme proteins containing iron protoporphyrin IX imbedded in a hydrophobic environment in the active site. The ferric, prosthetic heme group of P450s are characterized by having a cysteinyl thiolate as a fifth ligand, and a water molecule placed as the sixth ligand. The catalytic mechanism for P450s involves reductive activation of molecular oxygen in a process in which several steps are one-electron transfers, i.e. movement of either an electron or a hydrogen atom [8]. The generation of radicals within the active site of P450s is responsible for their unusual catalytic properties (Figure 1). Upon binding of substrate, the water molecule is displaced and the iron spin state changes from low spin state to high spin state as the iron shifts from a hexa-coordinated to a penta-coordinated state [9, 10]. Some P450s already exist in the high spin state without the addition of substrate [11]. Binding of substrate facilitates reduction of the ferric prosthetic group to the ferrous state [9]. In P450cam the binding of substrate is correlated with an increase in reduction potential from about −270 mV to −170 mV [12]. The corresponding change in reduction potential

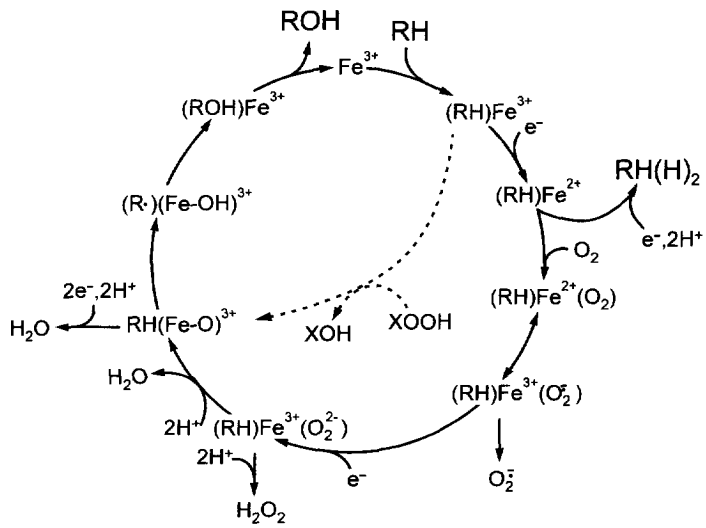

Fig. 1. The catalytic cycle for cytochrome P450. RH is substrate, $RH(H)_2$ a reduction product, ROH a monooxygenation product, and XOOH a peroxy compound that can serve as an alternative oxygen donor. (From reference [6], with permission.)

upon binding of substrate is less obvious in microsomal P450s [13, 14]. The substrate-bound, high-spin, ferrous cytochrome P450 readily binds molecular oxygen [9]. Limited structural information is available for the intermediates beyond the ferrous dioxygen complex due to a very transient existence of the involved radicals. The information available is primarily derived from model systems. The ferrous dioxygen complex is reduced by a second electron transferred from NADPH-cytochrome P450-reductase or ferrous cytochrome b_5 [15]. Depending on the substrate or the species of P450 involved, the role of b_5 in supplying the second electron can vary. Only in one case has an obligatory requirement for b_5 been demonstrated [16]. Under certain conditions, ferrous dioxygen P450 complex can reduce oxidized b_5, resulting in the release of ferric P450 and molecular oxygen [17]. This may explain why some P450-associated activities are stimulated by cytochrome b_5, while others are inhibited [18]. If the second electron is not delivered soon enough, the ferrous dioxygen complex can autoxidize to ferric P450 and superoxide anion, which dismutates to form hydrogen peroxide and molecular oxygen [19]. Alternatively, the hydrogen peroxide that is released might derive from the two-electron-reduced oxygen-bound complex [20]. When the substrate has structural complementarity to the active site of the enzyme, the peroxide shunt (as the uncoupling to hydrogen peroxide is called) is suppressed [21].

The part of the catalytic cycle in which the dioxygen is split is not very well understood. The cysteinyl thiolate ligand assists by 'pushing' electrons into a heterolytic cleavage of the oxygen–oxygen bond resulting in the formation of the activated oxygen species, $[FeO]^{3+}$, and the release of water [9, 22]. The formation of the activated oxygen species is the only non-radical process in the catalytic cycle [8]. The activated species of the iron-oxo complex is a radical that may be represented by several resonance forms, e.g. $Fe^3O \leftrightarrow Fe^5 = O \leftrightarrow Fe^4 - O^{\cdot} \leftrightarrow [Fe^4 = O]^{+\cdot}$ [8, 23]. When assigning the charge to the iron atom in these structures, it is assumed that the pair of electrons forming the coordination bond resides entirely on the ligand, and is not shared with the metal [23]. The activated species in the general catalytic cycle of P450s is believed to be the oxoiron(IV) porphyrin radical cation, $[Fe^4 = O]^{+\cdot}$, similar to compound I in horseradish peroxidase [10]. For convenience, the activated oxygen species will be presented as $[FeO]^{3+}$ in the present review. In the successful P450 cycle, the $[FeO]^{3+}$ abstracts a hydrogen atom from the substrate, yielding a carbon radical and iron-bound hydroxyl radical, followed immediately by radical recombination (oxygen rebound), to form an alcohol. The hydroxylated molecule is believed to be bound with less affinity to the active site than the substrate, favouring release of the product and subsequent binding of a new substrate molecule [9]. Under certain conditions, the P450 catalytic cycle follows an oxidase path in which a four-electron reduction of the dioxygen results in the formation of water [20]. There is strong indications that the branch point for this uncoupling reaction is at the level of activated, single oxygen-bound iron, the $[FeO]^{3+}$ species [24].

Loida and Sligar [20] have demonstrated that the uncoupling of the cytochrome P450 reaction through the peroxide shunt or the oxidase pathway can be changed in a predictable way in P450cam. The key determinants which control uncoupling at the two-electron dioxygen-bound state and result in the release of peroxide are the hydration level in the active site and the access of solvent to the iron [20]. Accordingly, addition of steric bulk to the periphery of the substrate binding pocket by site-directed mutagenesis reduces the liberation of hydrogen peroxide by 'pushing' the substrates towards the active site [20]. The ratio of oxidase of hydroxylation activity can be increased by over 400-fold upon engineering steric bulk around the

perimeter of the active site, thereby 'pushing' the substrate away from the active site and allowing the input of additional electrons [20]. This indicates that tight coupling of the substrate to the active site is a key factor for favouring the hydroxylation reaction over the release of water. Control of the specificity at the peroxide shunt and oxidase branch point in the P450 catalytic cycle is essential for the successful design of new substrate specificities for P450s.

Peroxides and peroxy acids can substitute for molecular oxygen in certain cytochrome P450-catalysed reactions. In these reactions, the P450 enzymes follow either a peroxygenative or a peroxidative pathway, neither of which involves transfer of reducing equivalents from NADPH *via* the reductase. The peroxygenative pathway differs from the peroxidative pathway in that an oxygen from the peroxide is incorporated into the substrate. The peroxygenative pathway is generally believed to follow a heterolytic cleavage of the O–O peroxide bond, which generates an oxygen-activated species similar to the one generated by the classical $NADPH/O_2$ mechanism (Figure 1). The peroxidative pathway generally follows a homolytic cleavage of the O–O peroxide bond [10]. In the peroxidative reactions, the P450 enzymes couple reduction of peroxides (e.g. cumene hydroperoxide, linoleic peroxide) to oxidation of the substrates [25, 26]. The operation of the peroxidative and peroxygenative pathways explains most of the differences in product profiles obtained with hydroperoxides versus $NADPH/O_2$ [10]. An example is the conversion of benzo[*a*]pyrene to phenols with $NADPH/O_2$, and to quinones with cumene hydroperoxide. The latter can be explained by secondary peroxidative oxidations of the phenol initially produced by a peroxygenative reaction [10].

The P450 catalytic cycle is involved in a vast number of chemical reactions. A unified mechanism for P450 reactions can be viewed in the light that the formal $[FeO]^{3+}$ species abstracts either electrons or hydrogen atoms from the substrate, followed by a radical recombination event (oxygen rebound). Novel P450-dependent reactions are continuously being discovered, expanding our view of P450 catalysis. The currently known catalytic reactivities of P450s will be presented in the following sections.

REACTIVITIES INVOLVING C ATOMS

Hydroxylation and oxidation of C *atoms*

Insertion of hydroxyl groups into lipophilic substrates is the earliest known reactivity of P450 enzymes (Figure 2). The hydroxylation reaction is the classical P450 reactivity and is found in all kingdoms. The carbon hydroxylation reactions are thought to follow a non-concerted mechanism, which requires the initial removal of a hydrogen atom by the activated oxygen species, rather than a concerted interplay between a carbon, a hydrogen and the activated oxygen atom. Evidences for a non-concerted mechanism include the observation of large intrinsic isotope effects [27], a

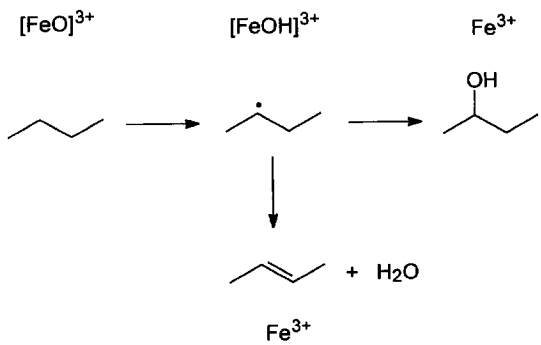

Fig. 2. Carbon hydroxylation and dehydrogenation (desaturation) reactions catalysed by cytochrome P450 enzymes. (Reprinted with permission from *Crit. Rev. Biochem. Mol. Biol.* **25**, 97. Copyright CRC Press, Boca Raton, Florida.)

correlation between the strength of the C–H bond and its susceptibility to hydroxylation [28], and scrambling of stereochemistry in the hydroxylation of camphor and norbornane [28, 29].

The high regio- and stereoselectivity typical of P450-catalysed hydroxylation reactions is thought to be imposed by structural constraints within the active site rather than by the intrinsic nature of the hydroxylation mechanism [10]. The selectivity contributes to the overwhelming diversity in the biosynthesis of secondary plant products, e.g. phenylpropanoids, terpenoids and alkaloids (for a review see Bolwell *et al.* [7]). P450-catalysed hydroxylations of aromatic rings, which are important for detoxification purposes, typically follow a NIH shift mechanism [30]. In the NIH shift mechanism, an initial epoxidation of the aromatic ring is followed by a chemical opening of the epoxide ring, migration of a hydride to the vicinal carbon, and tautomerization of the keto-enol formed (see Figure 3). The intermediacy of an arene oxide results in several hydroxylation products, as exemplified by the formation of 1,2-naphthadiol and 1- and 2-naphthol in mammalian liver [30]. Direct insertion of the oxygen atom into the C-H bond (like the aliphatic hydroxylations) has been reported [31]. In plants, the colour of flowers indicates the level of hydroxylation of the aromatic B-ring in the anthocyanin molecules. The presence of product-specific flavonoid 3′- and 3′,5′-hydroxylases [32], suggests direct insertion of the oxygen into the C–H bond. Similarly, the product-specificity of the novel benzoic acid 2-hydroxylase, which catalyses production of the plant signalling molecule salicylic acid, suggests a similar direct insertion mechanism for this enzyme. Benzoic acid 2-hydroxylase is a unique P450 by being the first soluble, high molecular weight P450 isolated from eukaryotes [33].

The transfer of oxygen to olefins, e.g. unsaturated fatty acids, typically results in epoxidation rather than hydroxylations reactions [10]. Several examples from plants show that the same P450 enzyme catalyses the epoxidation of a terminally unsaturated fatty acid and the ω-hydroxylation of the saturated analogue (for a

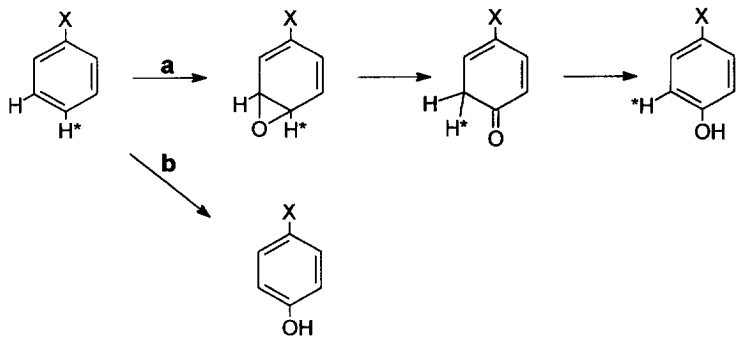

Fig. 3. Different mechanisms for hydroxylation of aromatic rings catalysed by cytochrome P450 enzymes. The asterisk denotes a labelled hydrogen. *a* designates the 'NIH' pathway and *b* designates the pathway for 'direct' hydroxylation. X indicates an unspecified substituent. (Modified from reference [10], with permission.)

review see Bolwell *et al.* [7]). The P450-catalysed epoxidation of terminal olefines is often paralleled by *N*-alkylation of the prosthetic heme group resulting in inactivation of the enzyme (Figure 4) [10]. Mechanism-based inactivation by self-catalysed heme-alkylations are relatively common with terminal olefins provided they are accepted as substrates in the active site and do not have substituents that delocalize charge or electron density from the double bond [34]. Similarly, oxidation of terminal acetylenes is accompanied by heme-alkylations, a function that has been useful for design of isozyme-selective or isozyme-specific irreversible inhibitors [10].

P450 enzymes catalyse dehydrogenation (desaturation) reactions, where for example, alkanes are oxidized to olefines without the intermediacy of an alcohol (Figure 2). In plants, no example of this reactivity has (yet) been identified, but in animals, dehydrogenation of the antiepileptic drug valproic acid by a liver P450 results in the formation of the toxic Δ^4-valproic acid [35]. In the dehydrogenation reactions, the P450 enzyme functions as an oxidant in which the iron-bound hydroxy radical $[FeOH]^{3+}$ abstracts a second hydrogen atom from the substrate, rather than recombining with the substrate radical (Figure 2).

A parallel to the partitioning between hydroxylation and dehydrogenation processes in the $NADPH/O_2$-dependent P450 reactions is seen in the peroxide-dependent P450 reactions catalysed by allene oxide synthase (AOS). AOS is found in higher plants, as well as in algae, corals and starfish [36]. In plants, AOS is involved in the biosynthesis of the signalling molecule jasmonic acid. AOS catalyses the conversion of unsaturated hydroperoxy fatty acid substrates into a single product, the corresponding allene oxide. Mechanistically, AOS follows the peroxidative pathway of P450s (see above). It is generally accepted that the process is initiated by homolytic cleavage of the hydroperoxide giving an iron-oxo complex and an alkoxyl radical, often stabilized by conjugated double bonds (Figure 5). The alkoxyl radical cyclizes to form a more stable epoxy carbon radical. One-electron oxidation of the epoxyallylic radical by the iron-oxy complex results in the formation of an epoxyallylic cation, which upon loss of a proton gives the allene oxide [37]. By using model hydroperoxy fatty acid substrates, Song *et al.* [37] have demonstrated the production of epoxyalcohols by AOS. The formation of epoxyalcohols results from radical recombination of the iron-bound hydroxy-radical and the epoxyallylic carbon radical [37]. In this reaction the AOS follows the peroxygenative pathway by incorporation of an oxygen from the peroxide. There is no clear understanding of what it is that determines the partitioning between hydroxylation or dehydrogenation in either $NADPH/O_2$- or hydroperoxide-dependent P450 reactions. An AOS-homologous hydroperoxide-

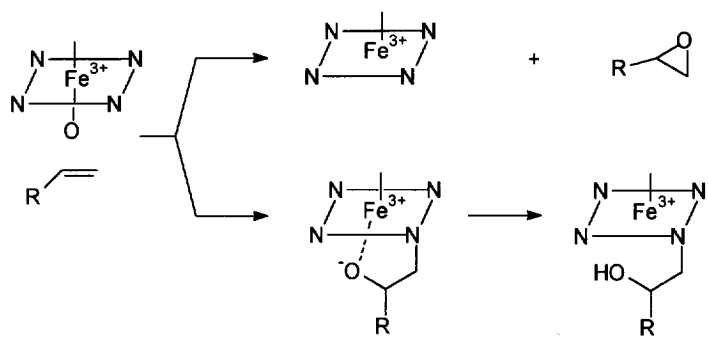

Fig. 4. P450-catalysed heme alkylation or epoxidation during the oxidation of terminal olefins. (From reference [10], with permission.)

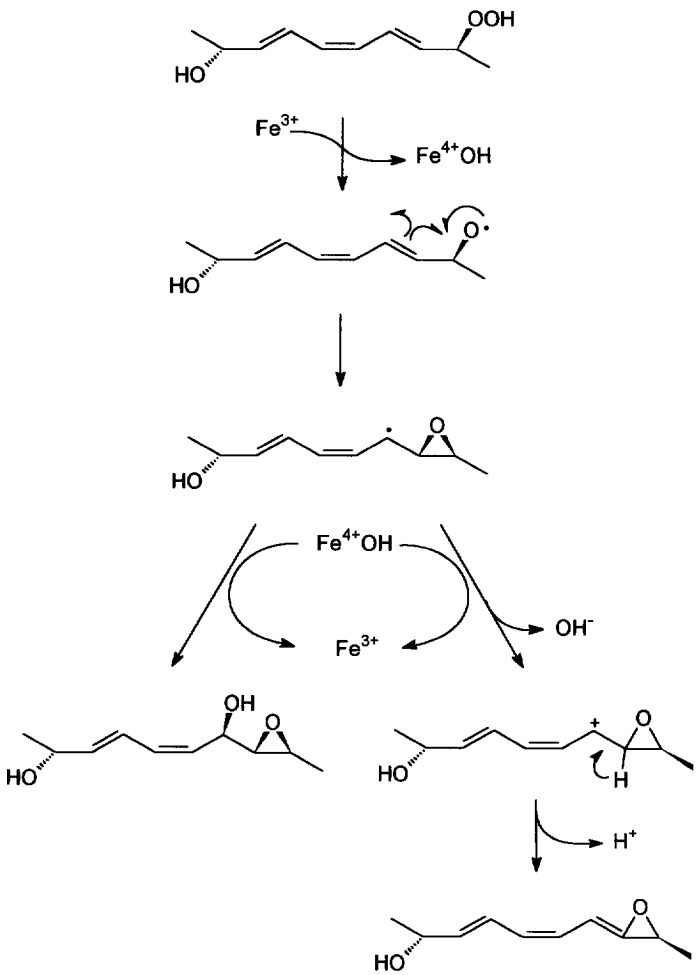

Fig. 5. Formation of epoxyalcohols and allene oxides by allene oxide synthases. (From reference [37], with permission.)

dependent P450 called rubber particle protein (RPP) has recently been isolated from rubber particles of the desert shrub guayule (*Parthenium argentum*) [38]. The function of RPP, which constitutes 50% of the protein in the rubber particles, is unknown. RPP is unique by being the first eukaryotic P450 to be identified outside endoplasmic reticulum, mitochondria, or plastids [38].

Mechanistically, allene oxide synthase is closely related to prostacyclin synthase and thromboxane synthase, which are involved in biosynthesis of the prostaglandin derivatives, prostacyclins and thromboxanes, respectively. These enzymes catalyse internal rearrangements (isomerizations) of endoperoxide precursors by a cage radical mechanism as shown for thromboxane synthase in Figure 6 [39, 40]. The reaction is initiated by homolytic cleavage of the prostaglandin endoperoxide, followed by rearrangement of the alkoxide radical to an allylic radical. Oxidation to an allylic cation is followed by cyclization to the respective products. Alternatively, the allylic radical in the active site of thromboxane synthase can fragment into malondialdehyde and a polyunsaturated fatty acid (Figure 6). At least 50% of the prostaglandin precursors are converted into these products, for which no physiological role has been found [40].

Oxidative C–O *and* C–C *coupling*

A novel P450 catalytic reactivity involving intermolecular oxidative phenol coupling in alkaloid biosynthesis has been demonstrated in plant cell cultures of *Berberis stolonifera* [41]. In this reaction, the P450 enzyme, berbemunine synthase, catalyses the highly stereo- and regiospecific dimerization of two benzyltetrahydroisoquinoline substrates without insertion of the activated oxygen atom (Figure 7) [41]. In the proposed mechanism, the active site features two substrate binding sites to accommodate each chiral benzyltetrahydroisoquinoline alkaloid involved. In the initial steps, sequential abstraction of hydrogen atoms from each substrate results in the generation of two radicals within the active site. The close proximity of the two radicals results in a biradical oxidative coupling to form the dimerization of the benzyltetrahydroisoquinolines by an ether linkage [41]. The proposed active oxygen species involved are compound I (Fe(IV)O-porphyrin

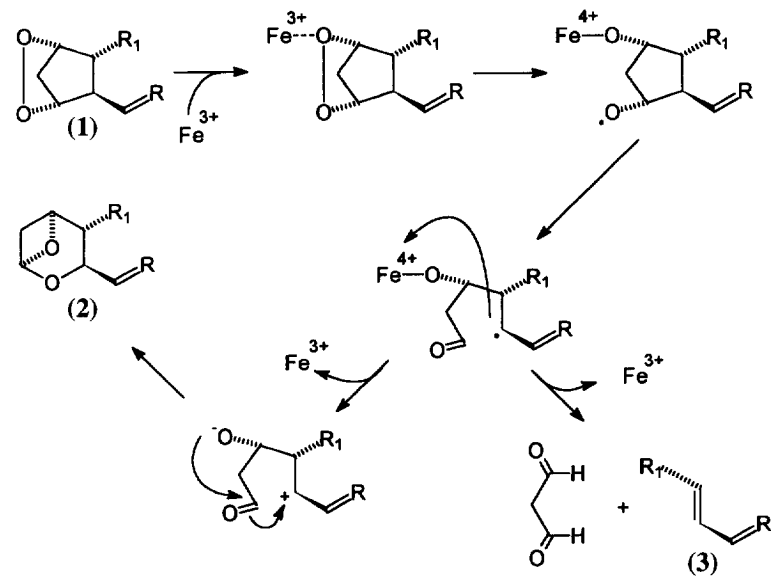

Fig. 6. The mechanism for the conversion of prostaglandin H_2 (**1**) into thromboxane A_2 (**2**) or malondialdehyde and 12(*S*)-hydroxy-5(*Z*),8(*E*),10(*E*)-heptadecatrienoic acid (**3**) by thromboxane synthase. (From reference [39], with permission.)

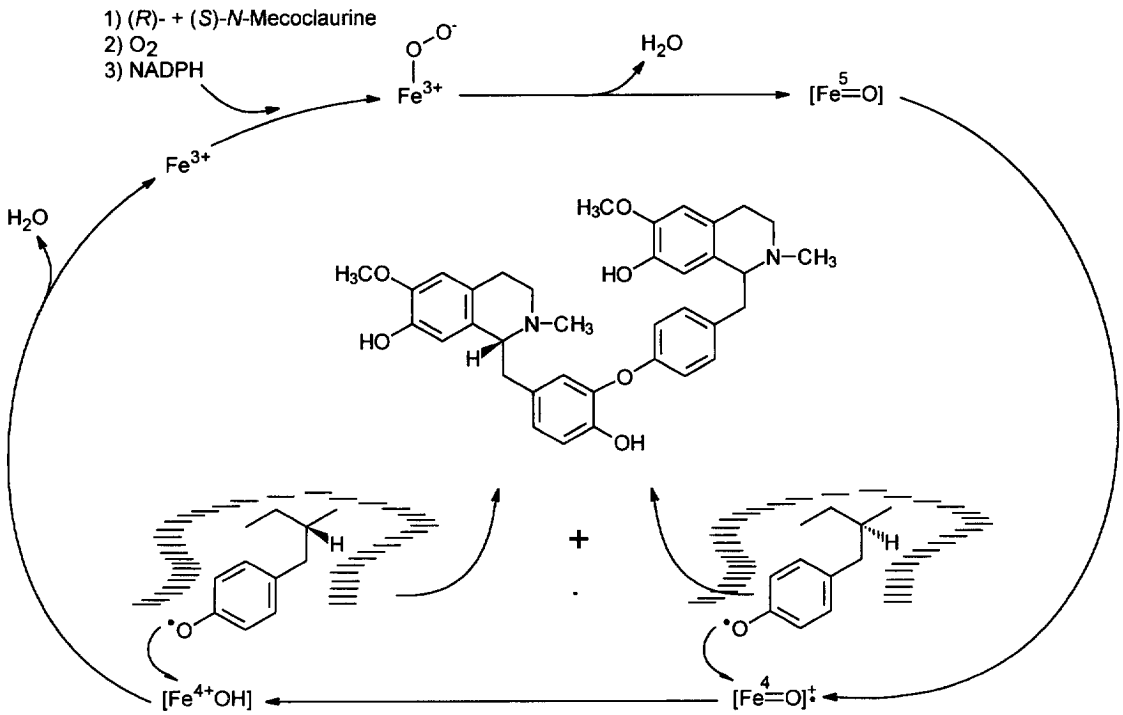

Fig. 7. The proposed mechanism for intermolecular oxidative phenol coupling of chiral benzyltetrahydroisoquinoline alkaloids involving two one-electron steps. (From reference [41], with permission.)

cation radical) and compound II (neutral oxoiron(IV) complex) of the horseradish peroxidase cycle [41]. The overall reaction resembles the dehydrogenation reactions in which the P450 functions as oxidase, and the reduced oxygen leaves the active site as water (see above). Cytochrome P450-dependent formation of methylenedioxy bridges (-OCH_2O-), derived from *o*-methoxyphenol structures, occurs in a number of natural plant products, particularly alkaloids [42, 43]. Intramolecular oxidative C–C phenol coupling of (*R*)-reticuline to salutaridine, a crucial step in biosynthesis of morphine, has been shown to be catalysed by a P450 in *Papaver somniferum* [44]. These two types of reactions are likely to proceed as biradical couplings similar to the dimerization of benzyltetrahydroisoquinolines in *B. stolonifera* cells.

In the yeast spore wall, dimerization of two *N*-formyltyrosine molecules to a *N,N'*-bisformyl dityrosine, which serves as precursor for a spore wall-specific macromolecule, is catalysed by the enzyme Dit2 (CYP56) [45, 46]. A possible mechanism could involve oxidative biradical coupling as is suggested for the C–C or C–O coupling reactions in alkaloid biosynthesis.

Aryl migration and C–C *bond cleavage*

A P450 reactivity in which C-hydroxylation is associated with 1,2-aryl migration has been identified in the conversion of the secondary plant product flavanones into isoflavones [47]. In elicitor-induced *Pueraria lobata*, the flavanone liquiritigenin [**4**] is converted in daidzein [**6**], via 2,7,4'-trihydroxyisoflavanone (**5**) (Figure 8). The abstraction of a hydrogen atom at C-3 of liquiritigenin is followed by a 1,2 shift migration of the phenyl group. The carbon radical formed at C-2 becomes hydroxylated as evidenced by $^{18}O_2$ labelling experiments [47, 48]. The hydroxylation associated with rearrangement is a new category of P450 reactivities, although the underlying reaction, hydrogen abstraction and subsequent hydroxylation, is typical for P450s. The aryl migration involves cleavage of a C–C bond. P450-dependent cleavages of C–C bonds explain many reactions in the biosynthesis of secondary plant products [48]. As an example, the biosynthesis of the furanocoumarin psoralen is initiated by abstraction of a hydrogen atom, followed by C–C cleavage which yields psoralen and an isopropyl radical [49]. The latter is further converted into acetone [49]. P450-dependent C–C bond cleavages have also been identified in the biosynthesis of steroids (see under multifunctional P450s).

REACTIVITIES INVOLVING HETEROATOMS

Oxidation of heteroatoms

P450s metabolise compounds containing the heteroatoms N, S or O resulting in a variety of hydroxylation, oxidation and C_α-hydroxylation (dealkylation) products [5, 10]. In parallel to carbon hydroxylation reactions, the key step in the heteroatom oxygenation reactions appears to be the abstraction of an electron from the heteroatom to the activated iron-oxo complex (Figure 9) [5, 10]. The resulting heteroatom radical subsequently collapses with the activated oxygen atom. Alternatively, a hydrogen atom is abstracted from the adjacent C_α-atom followed by a rapid hydroxyl radical rebound. The α-hydroxylated product readily decomposes to the observed dealkylation products (Figure 9). An observed preference is that S-containing compounds decompose to S-oxides, and N-containing compounds decompose to N-hydroxy compounds, which is explained by the relative stability

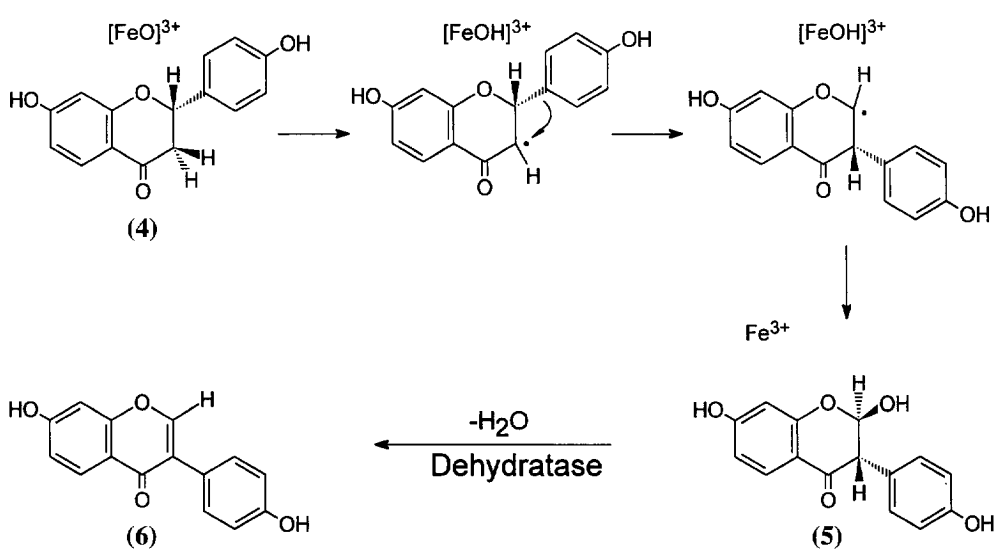

Fig. 8. The proposed mechanism for the oxidative aryl migration in the isoflavone biosynthesis. Liquiritigenin (**4**) is converted into daidzein (**6**) via 2,7,4'-trihydroxyisoflavanone (**5**). (From reference [48], with permission.)

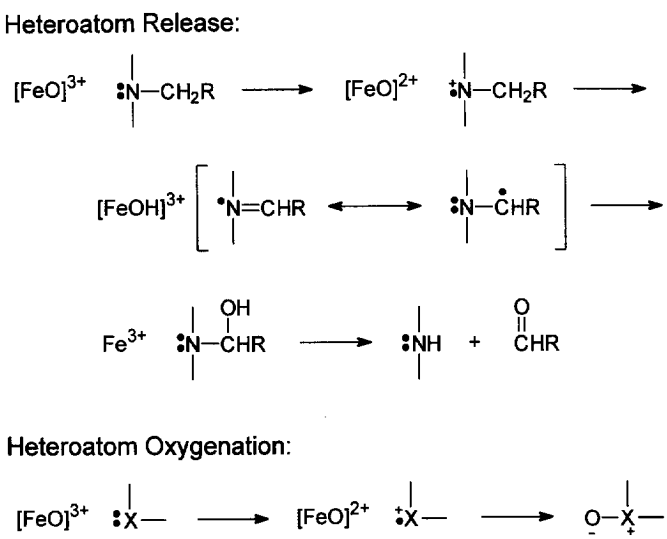

Fig. 9. The mechanism for release and oxygenation of heteroatoms. (Reprinted with permission from *Crit. Rev. Biochem. Mol. Biol.* **25**, 97. Copyright CRC Press, Bota Raton, Florida.)

of the oxides [50]. In addition, S-oxidation reactions predominate over S-dealkylation reactions due to the relative stability of the sulphur radical [50, 51]. Generally, N-hydroxylation reactions are only observed when α-hydrogens are not available. Several exceptions have been observed for N-hydroxylation reactions which occur in the presence of α-hydrogens. These include the conversion of amphetamine derivatives to hydroxylamines [52], the N-oxide formation of the tertiary amine in the pyrrolizidine alkaloid senecionine [53], and the two consecutive N-hydroxylation reactions that convert amino acids into the corresponding oximes in the biosynthetic pathway of cyanogenic glucosides (see under multifunctional P450s) [54].

Due to the high electronegativity of oxygen, O-dealkylation reactions are thought to be initiated by hydroxylation on the adjacent carbon atom rather than by electron abstraction from the oxygen atom [10]. Accordingly, O-dealkylation reactions exhibit large intrinsic isotopic effects, e.g. $k_H/k_D = 13$–14 for O-dealkylation of 7-ethoxycoumarin [55]. In contrast, the intrinsic isotope effects of N-dealkylations, which are initiated by an electron abstraction from the nitrogen atom, are in the range $k_H/k_D = 1.3$–3.0 [56–58].

P450 enzymes catalyse oxidative cleavage of esters with formation of an aldehyde (or ketone) from the alcohol moiety [59, 60]. $^{18}O_2$ studies indicate that the reaction involves hydroxylation of the α-carbon of the alcohol to yield an unstable geminal hydroxy ester, which decomposes by a nonhydrolytic rearrangement to the aldehyde (or ketone) [59]. A novel P450 catalytic reactivity involving oxidative cleavages of RR'C = N(OH) has recently been demonstrated in liver microsomes [61]. The reaction results in the formation of RR'C = O and nitrogen oxides such as NO^{2-}, NO^{3-}, and NO (61). A mechanism for this reaction has not yet been proposed, but the reaction resembles the oxidation of N^{ω}-hydroxy-L-arginine to citrulline and NO catalysed by nitric oxide synthase (see later).

DeMaster *et al.* [62] have described a P450-catalysed conversion of butyraldoxime into butyronitrile and nitrobutane in rat liver. The nitrobutane production results from N-oxidations of the oxime, whereas the dehydration of aldoxime into nitrile represents a novel P450 reactivity. The dehydration is catalysed by P450 in its ferrous state, requires stoichiometric amounts of NADPH, and is inhibited by oxygen and CO [62]. Boucher *et al.* [63] have demonstrated that the reaction is general for (Z)-aldoximes, and is catalysed by rat liver microsomes as well as by a heterologously expressed P450 3A4. The latter suggests that the P450-dependent aldoxime dehydration is a general reaction of P450 enzymes. Two mechanisms have been proposed for the reaction (Figure 10) [63]. Initially, the (Z)-aldoximes bind by their N-atom to the ferro-P450. In the proposed mechanism (via mesomeric form A_1) a charge is transferred from the C=N moiety to the ferro complex. This would make the CHR hydrogen more acidic, and would facilitate its abstraction by a basic amino acid in the active site, possibly favoring loss of the oxime hydroxyl group assisted by its protonation. In the second proposed mechanism (via mesomeric form A_2) a charge is transferred from the ferro-P450 to the C=N moiety based on the electron-richness of ferro-P450 and the thiolate anion from the fifth axial cysteine ligand. Such a charge transfer should lead to a negative charge on the aldoxime carbon with subsequent elimination of the aldoxime hydroxyl group after protonation. Loss of the activated β-hydrogen atom yields the nitrile, and regenerates the ferro-P450. The importance of P450-catalysed dehydration of aldoximes in living organisms remains unknown. In the biosynthesis of amino acid-derived secondary plant products cyanogenic glucosides, oximes are converted into ni-

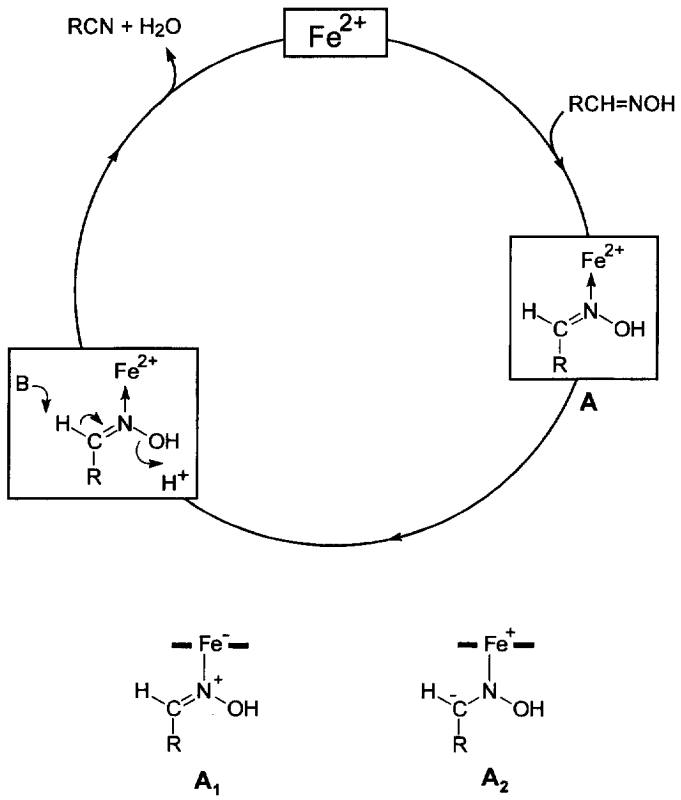

Fig. 10. Possible mechanisms for the P450-dependent dehydration of (Z)-aldoximes. A_1 and A_2 are possible mesomeric forms of complex A, suggesting two possible mechanistic pathways. B is a basic amino acid in the active site. (Reprinted with permission from *Biochemistry* **33**, 7811. Copyright 1994 American Chemical Society.)

triles (see under 3.4.2) [64]. Subsequently, the nitriles are hydroxylated into the corresponding α-hydroxynitrile [64]. The oxime-metabolizing enzyme activity and the nitrile-hydroxylating activity have been shown to be dependent on P450 enzymes [65]. Recent results indicate that a single enzyme designated cytochrome P450ox catalyses the conversion of *p*-hydroxyphenylacetaldoxime to the corresponding α-hydroxynitrile, *p*-hydroxymandelonitrile [66]. This shows that the oxime-metabolizing activity in the biosynthetic pathway of cyanogenic glucosides is different from the anaerobic P450-dependent aldoxime dehydration activity present in rat liver.

Reduction of nitric oxide

A unique cytochrome P450nor from the fungus *Fusarium oxysporum* is involved in denitrification of nitrate of nitrite to N_2O. P450nor catalyses the reduction of nitric oxide to nitrous oxide by NADH, and is unique in that it catalyses an anaerobic reaction and receives its reducing equivalents directly from NADH after binding of NO [67]. In addition, P450nor was the first soluble eukaryotic P450 to be described. On the basis of spectroscopic and kinetic evidences, a mechanism has been proposed for the NO reduction by P450nor (Fig. 11) [68]. The catalytic cycle consists of (i) binding of NO to the Fe^{3+} enzyme, (ii) reduction of the $Fe^{3+}NO$ enzyme with NADH to $(Fe^{3+}NO)^{2-}$, and (iii) the reaction of the activated NO species with another NO to form N_2O and Fe^{3+} [68]. The proposed mechanism is comparable to the usual P450 reaction where the two-electron reduced O_2 on the iron site reacts with the substrate RH as follows (the P450nor reaction is indicated in parentheses): O_2^{2-} $((NO)^{2-}) + RH$ $(NO) + 2H^+ \rightarrow ROH$ $(N_2O) + H_2O$ [68]. In spite of the unique features of P450nor, the reaction mechanism follows the common principle of the typical P450 reaction.

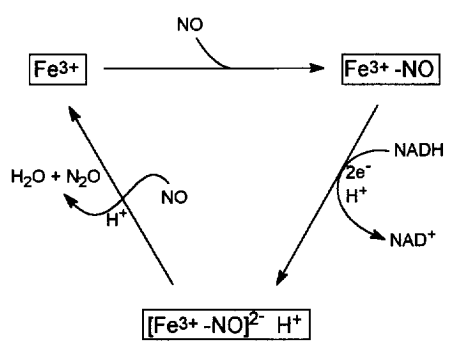

Fig. 11. The proposed mechanism for P450nor catalysing the anaerobic reduction of nitric oxide to nitrous oxide with electrons directly transferred from NADH. (From reference [68], with permission.)

Reactivities involving reductive dehalogenations

Under low oxygen tension, P450s have been shown to be capable of reductive dehalogenation of a variety of halogenated ethanes [69]. The proposed mechanism for the reductive dehydrogenolysis of the carbon halogen bond and the corresponding oxidation of heme-iron(II) proceeds in three steps: (i) ligation of halocarbon to iron, (ii) carbon-halogen cleavage, (iii) rapid scavenging of the radical by heme (Figure 12) [70, 71]. When the radical contains a halogen on the adjacent carbon, conversion to alkene can ensue. Reductive dehalogenation of polyhalomethanes has been demonstrated by the metalloporphyrin iron(II)deuteroporphyrin IX, P450cam and phenobarbital-induced rat liver microsomes [69]. This suggests that reductive dehalogenation is a general P450 reactivity. At low oxygen tension, polyhalomethanes will compete with oxygen for binding at heme iron(II) (step (i) in Figure 12). If the bond cleavage step proceeds rapidly enough, reduction of the halide can ensue [71]. The size of a substrate is limited for reactivity of P450cam as illustrated by the ready dehalogenation of insecticide DDT by ferro-porphyrins, whereas P450cam does not react with this large substrate.

Uotila and coworkers [72, 73] have isolated several strains of actinomycetes which are able to *p*-dehalogenate and *p*-hydroxylate pentachlorinated, -fluorinated, and -brominated phenols to the corresponding halogenated hydroquinones. These activities may be catalysed by membraneous P450 enzymes [72, 73]. Evidence includes the effect of P450 inhibitors on the activities, and an increase in A_{448} and A_{457}, respectively, in the CO-difference spectra of membrane preparations from two different, induced actinomycete strains. Under aerobic conditions, the *p*-hydroxylation activity requires molecular oxygen as source of oxygen. However, in the presence of sulphite, dithionite, or iodosobenzene, the oxygen atom incorporated is taken from water [74]. The mechanism by which anaerobic dehalogenation reactions catalysed by the actinomycetes takes place in the presence of sulphite and dithiotreitol may resemble that of the reductive halogenation reactions described above, whereby the initial aromatic radical formed after cleavage of the C-X bond escapes the active site and reacts with water to form the hydroquinone. The ability of iodosobenzene to make dehalogenating P450 enzymes utilize water as oxygen source, provides an example of involvement of iron-coordinated water molecules in oxygen exchange during iodosobenzene-supported P450-catalysed reactions [10].

(*i*) ligation at iron

$$P\,Fe^{2+} + RX \longrightarrow P\,Fe^{2+}(XR)$$

(*ii*) carbon—halogen bond cleavage

$$P\,Fe^{2+}(XR) \rightarrow [P\,Fe\cdots X\cdots R]^{2+} \rightarrow P\,Fe^{3+} + R^{\bullet} + X^{-}$$

(*iii*) rapid scavenging of the radical by heme

$$P\,Fe^{2+} + R^{\bullet} \xrightarrow{\text{fast}} (P\,FeR) \xrightarrow[\text{or Solv-H}]{H^{+}} P\,Fe^{3+} + RH$$

Fig. 12. The proposed mechanism for reductive dehalogenation reactions catalysed by ferro-cytochrome P450s.

MULTIFUNCTIONAL P450 ENZYMES

Biosynthesis of steroids

P450 enzymes which are involved in biosynthetic pathways generally have a high substrate-specificity compared to the P450 enzymes involved in xenobiotic metabolism. In the biosynthesis of steroids there are several multifunctional P450 enzymes in which the same active site catalyses two or three sequential oxidative reactions, each step requiring the consumption of one molecule of oxygen and one molecule of NADPH [10]. The first cycles typically introduce hydroxyl groups, whereas the final cycle results in a unique carbon–carbon bond cleavage. In a normal P450 cycle, the hydroxylated product is released from the active site due to its low binding affinity compared to the substrate. In contrast, efficient catalysis of multi-step reactions in single active sites is possible due to an increased affinity of the hydroxylated intermediates compared to the substrate. This is exemplified by P450scc where the hydroxylated intermediates bind 300 times stronger than the substrate cholesterol (see below) [75].

The mitochondrial P450ssc catalyses the side chain cleavage of cholesterol to pregnenolone by two hydroxylations and one cleavage event, the intermediates being 22-hydroxycholesterol and 20,22-dihydroxycholesterol (Figure 13) [76, 77]. Two mechanisms have been proposed for the final step in the cholesterol side chain cleavage reaction (Figure 13) [10]. In one mechanism the activated oxygen species $[FeO]^{3+}$ intercepts one of the hydroxyl groups which results in the formation of a peroxide. Proton removal from the adjacent hydroxyl group initiates the C–C cleavage. In an alternative mechanism, abstraction of a hydrogen atom from one of the hydroxyl groups by the activated oxygen species produces an alkoxyl radical. Homolytic cleavage of the C–C bond followed by a second electron abstraction to $[FeOH]^{3+}$ completes the reaction.

Aromatase is involved in the conversion of androgens to estrogens and 14α-demethylase is involved in sterol biosynthesis. Both enzymes catalyse the oxidation of a methyl group to an aldehyde, followed by an acyl-carbon cleavage to yield a double bond and the release of formic acid (Figure 14) [23]. 14α-Demethylases are the most highly conserved P450 species as evidenced by comparison of the yeast and mammalian proteins [78]. The plant homologue, obtusifoliol 14α-demethylase, has not yet been isolated, but its activity has been detected which demonstrates that 14α-demethylases are widespread in all eukaryotic organisms [79]. P45017α involved in andrenal androgens catalyses an acyl-carbon cleavage reaction which results in the conversion of the pregnenolone

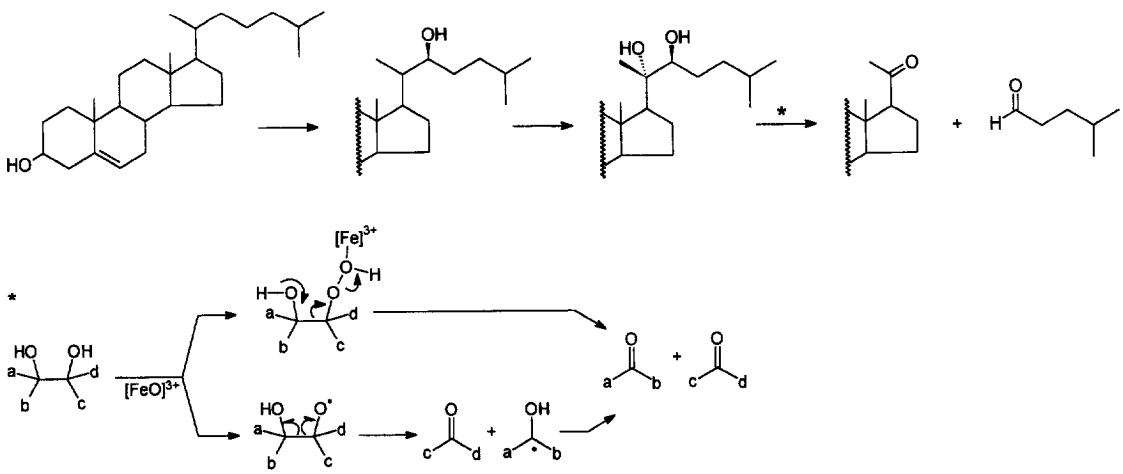

Fig. 13. The catalytic reactivity of P450scc that cleaves the side chain of cholesterol. The insert illustrates two plausible mechanisms for the final step in the side chain cleavage. (From reference [10], with permission.)

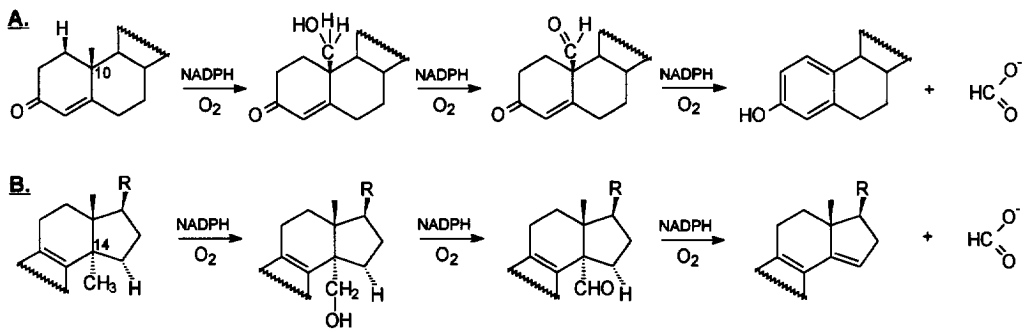

Fig. 14. The intermediates in the catalytic turnover of the multifunctional aromatase (A) and 14α-demethylase (B). (From reference [81], with permission.)

into acetic acid and 17keto-, 16-en or 17OH steroid products (Figure 15) [23]. Akhtar and colleagues have suggested that these three multifunctional enzymes carry out their catalysis at a single active site, but by two distinct activated oxygen species: a $[FeO]^{3+}$ being involved in the hydroxylation reactions as in the general P450 hydroxylation mechanism, whereas a $[FeOOH]^{3+}$ species is involved in the acyl-carbon cleavage [23, 80–82]. The novel feature of this proposal is that the $[FeOOH]^{3+}$ intermediate in the cycle

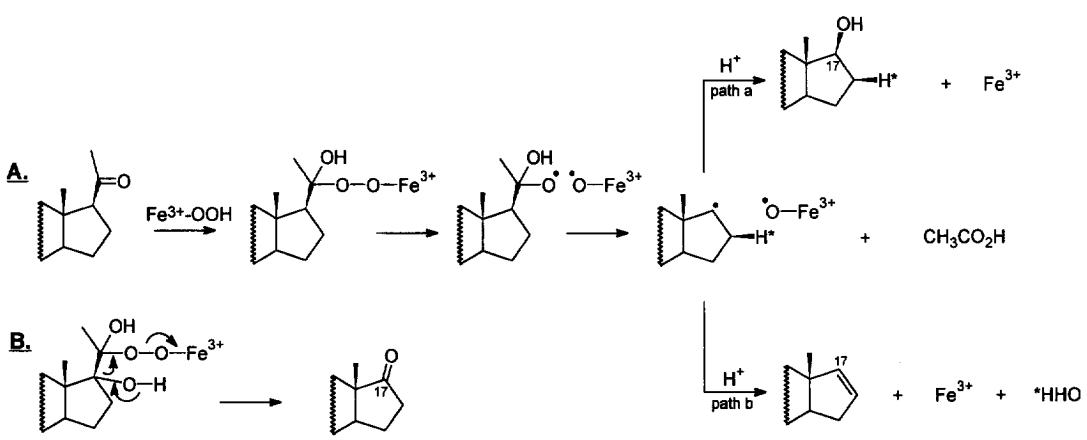

Fig. 15. The proposed mechanism for C–C bond cleavage via a peroxy adduct of the $[FeOOH]^{3+}$ species in the biosynthesis the pregnenolone steroids by P45017α. The side chain cleavage can proceed via a free radical mechanism (A) or an ionic mechanism (B). (Reprinted with permission for *Biochemistry* **33**, 4410. Copyright 1994 American Chemical Society.)

of P450 enzymes is trapped by the electrophilic property of the carbonyl group and produces a peroxy adduct that decomposes homolytically to give the products according to the fragmentation path (Figure 15A). For aromatase and 14α-demethylase, the mechanism involving the $[FeO]^{3+}$ or $[FeOOH]^{3+}$ species also involves the incorporation of an oxygen atom into the cleavage fragment. For the P45017α, however, the formation of the 17OH product excludes the involvement of the $[FeO]^{3+}$ species, and provides strong evidence for the involvement of a $[FeOOH]^{3+}$ species [82]. In the formation of 17keto-steroid from 17-hydroxypregnenolone by P45017α, the peroxy adduct may fragment following an ionic mechanism instead of the homolytic cleavage as shown in Figure 15B.

Biosynthesis of cyanogenic glucosides

The biosynthetic pathway of the tyrosine-derived cyanogenic glucoside dhurrin from *Sorghum bicolor* (L.) Moench consists of two multifunctional cytochrome P450 enzymes designated P450tyr and P450ox (Figure 16). P450tyr catalyses the conversion of tyrosine into *p*-hydroxyphenylacetaldoxime [54], which is metabolized by P450ox to *p*-hydroxymandelonitrile [66]. P450tyr is a multifunctional *N*-hydroxylase which catalyses the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime by two consecutive N-hydroxylations [54, 83]. In the proposed mechanism for P450tyr, tyrosine is initially hydroxylated to form *N*-hydroxytyrosine, which in a second reaction forms the *N,N*-dihydroxytyrosine. *N,N*-dihydroxytyrosine is unstable and spontaneously dehydrates into 2-nitroso-3-(*p*-hydroxyphenyl)propionic acid, which subsequently decarboxylates to *p*-hydroxyphenylacetaldoxime. The dehydration and decarboxylation reactions may proceed as concerted reactions. According to the proposed biosynthetic pathway for cyanogenic glucosides, P450ox catalyzes a dehydration step followed by a C-hydroxylation reaction (Figure 16). The mechanism for the conversion of oxime to the corresponding α-hydroxynitrile by P450ox has not yet been solved [66].

The biosynthesis of glucosinolates resembles the biosynthesis of cyanogenic glucosides in that amino acids are precursors and oximes are intermediates of the pathways. Recently, Du *et al.* [84] have demonstrated the involvement of P450 in the conversion of tyrosine to the *p*-hydroxyphenylacetaldoxime in the biosynthesis of *p*-hydroxybenzylglucosinolate in *Sinapis alba* L. This indicates that homologous P450s catalyse the multistep conversion of amino acid into oxime in the biosynthesis of glucosinolates and cyanogenic glucosides.

Nitric oxide synthases

The enzymes responsible for the synthesis of nitric oxide (NO), and citrulline from L-arginine in mammalian tissues, are known as NO synthases (NOSs) (for a review see Knowles and Moncada [85]). NOSs are

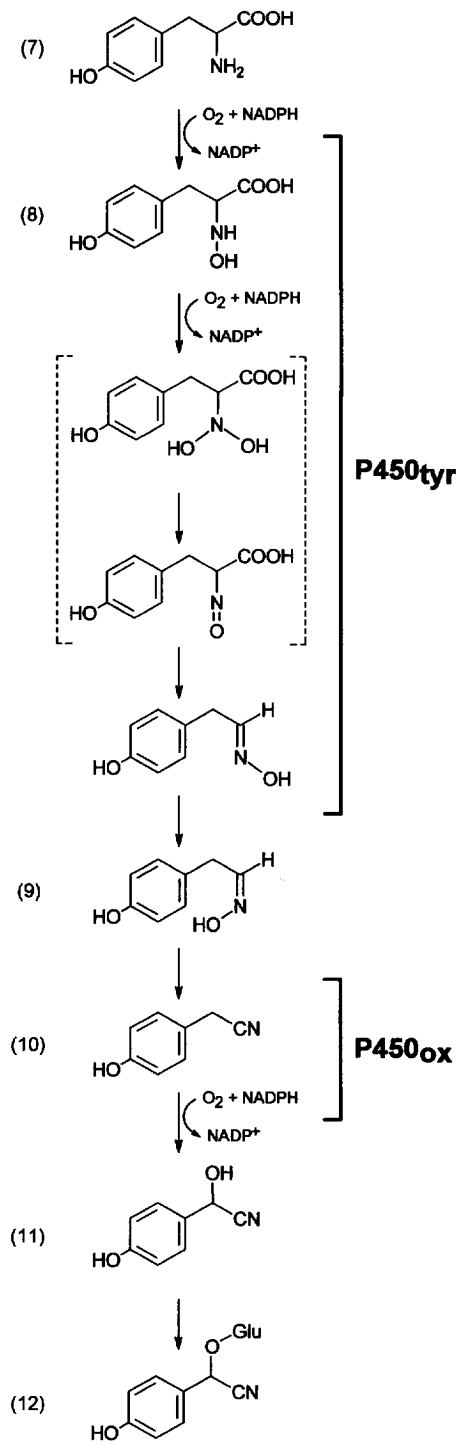

Fig. 16. The biosynthetic pathway of the tyrosine-derived cyanogenic glucoside dhurrin (12) involving two multifunctional cytochrome P450 monooxygenases, P450tyr and P450ox. P450tyr catalyses the conversion of tyrosine (7) to (*Z*)-*p*-hydroxyphenylacetaldoxime (9) via *N*-hydroxytyrosine (8), and P450ox catalyses the conversion of (*Z*)-*p*-hydroxyphenylacetaldoxime to *p*-hydroxymandelonitrile (11) via *p*-hydroxyphenylacetonitrile. The reactions in the dotted bracket may proceed by as concerted reactions. (From reference [54], with permission.)

proteins of 125–155 kDa with an N-terminal domain containing a heme group [86, 87], and a C-terminal domain containing the binding sites for NADPH, FAD and FMN [88]. The C-terminal domain has 30–40% sequence identity with NADPH-cytochrome P450-reductase [88]. In addition, NOSs contain variable amounts of the prosthetic group tetrahydrobiopterin [89]. NOSs exist in several isoforms which differ with respect to Ca^{2+}/calmodulin-dependency, cytokinin-inducibility, and whether found in the cytosol or associated with membranes [85]. NOS represents a eukaryotic equivalent to the soluble P450BM-3 from *Bacillus megaterium* (see later), which also contains an N-terminal heme domain and a C-terminal flavin-containing domain [90].

The heme group of NOSs displays certain spectroscopic properties similar to those of cytochromes P450. These include an absorbance maximum at 445 nm which is diagnostic for the formation of a reduced carbon monoxide-heme adduct [86], and 'type I' substrate difference spectra with the substrates L-arginine and *N*-hydroxyarginine [91]. Resonance Raman spectra of NOSs in the resting, reduced and CO-bound states are consistent with a thiolate axial ligand bound to the heme characteristic of the P450 class of heme proteins [92]. However, NOSs are missing the conserved heme-binding domain, require the cofactor tetrahydrobiopterin for activity, and furthermore are active only as dimers [86, 87, 89, 93].

NOSs catalyse sequentially an N-hydroxylation reaction and an oxidative cleavage of a C=N(OH) bond (see Figure 17). Formation of N^{ω}-hydroxy-L-arginine follows the general P450 cycle in which an activated $[FeO]^{3+}$ species N-hydroxylates one of the guanidino nitrogens of L-arginine. N^{ω}-hydroxy-L-arginine is not released from NOS under normal assay conditions, indicating that it has high affinity for the enzyme, as characteristic of multifunctional P450s. An odd-electron stoichiometry is required for the conversion of N^{ω}-hydroxy-L-arginine to NO, which results in the consumption of only 0.5 mol NADPH. A thermodynamic mechanism has been proposed by Korth *et al.* [94], based on a previous mechanism proposed by Marletta [95] (Figure 17). After the initial reduction of Fe^{3+} by one electron from NADPH followed by the binding of O_2, a free radical hydrogen atom is abstracted from N^{ω}-hydroxy-L-arginine. The resulting ferri-hydroperoxy species supports a nucleophilic attack on the iminoxyl radical $\rangle C{=}NO\cdot$, yielding an amino radical which results in the production of NO and citrulline. It is not yet clear how tetrahydrobiopterin affects NOS activity, but there is indirect evidence that tetrahydrobiopterin is involved in both of the reactions [96].

THE STRUCTURE–FUNCTION RELATIONSHIP OF P450 ENZYMES

Three-dimensional structures of P450 enzymes

Cytochromes P450 are divided into classes, depending on the nature of the redox partner. Class I P450s are found in the mitochondrial membranes of eukaryotes and in most bacteria and require a flavin adenine dinucleotide (FAD)-containing reductase and an iron-sulphur protein as redox partner; class II P450s are bound to the endoplasmatic reticulum and interact directly with NADPH-cytochrome P450-reductase which contains both FAD and flavin mononucleotide (FMN). Recently, a class III of P450s has been defined, which represents those proteins that do not require an electron-transfer partner [97]. Class III P450s do not catalyse monooxygenation reactions, but rather rearrange endoperoxides or hydroperoxides [97]. Today, the three-dimensional structure has been solved for four soluble, bacterial P450 enzymes. These include P450cam [98], the heme-domain of BM-3 [99], P450terp [100], and P450eryF [101]. The structure of P450cam, a soluble 5-exo-hydroxylase of camphor from *Pseudomonas putida*, was the first atomic structure of a cytochrome P450 to be solved (Figure 18) [98]. P450cam has functioned as a model structure for all P450s, although P450cam belongs to class I and

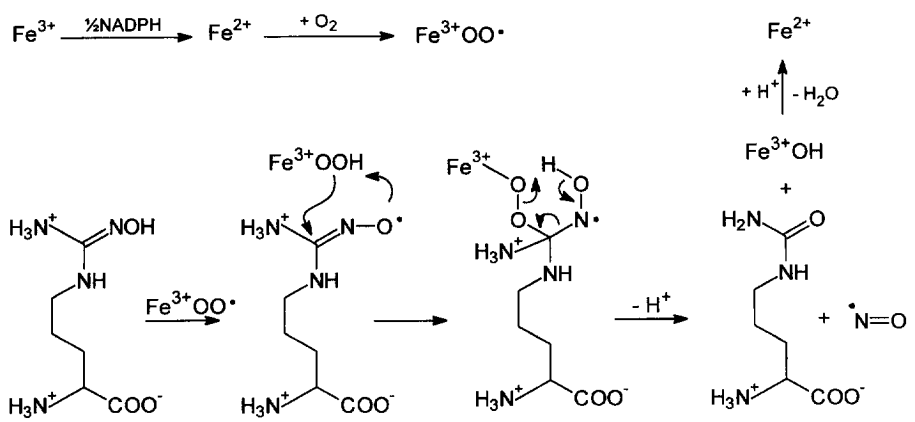

Fig. 17. A proposed mechanism for the formation of NO and citrulline from N^{ω}-hydroxy-L-arginine by NO synthase. (From reference [94], with permission.)

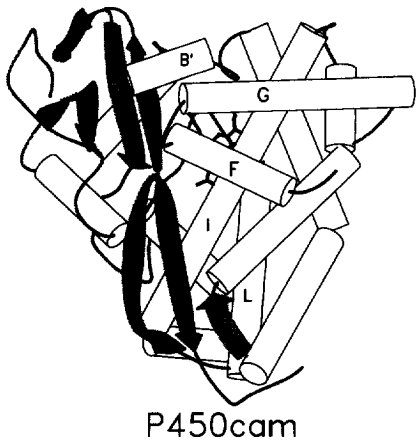

Fig. 18. Schematic representation of the P450cam structure. Helices are indicated by bars and β-sheets structure by sheets. The shaded region highlights the antiparallel β pair and β bulge that contains the axial heme ligand, Cys-357. (Courtesy of T. L. Poulos.)

shares only 10–20% sequences identity with the microsomal P450s. P450BM-3 is a soluble fatty acid monooxygenase of 119 kDa from *B. megaterium*. P450BM-3 consists of an N-terminal P450 domain and a C-terminal flavoprotein reductase [90]. P450BM-3 shows functional and structural similarity to the eukaryotic class II enzymes as evidenced by about 35% sequence identity between the reductase domain and microsomal P450-reductases [102] and about 25–30% identity between the P450 domain and the microsomal fatty acid ω-hydroxylase and the *n*-alkane-inducible microsomal P450s [103]. The structure of BM-3 is considered a prototype for microsomal P450s [99]. P450terp is a soluble class I P450 which hydroxylates the monoterpene α-terpineol as part of the metabolic assimilation of this compound as the sole carbon source for the pseudomonad [104]. P450eryF is a soluble class I P450, which participates in the biosynthesis of the antibiotic erythromycin [101].

A three-dimensional structure has not yet been obtained for any plant P450s. However, the availability of several structures representing different P450 classes allows insights to be gained regarding structure/function relationships for this important superfamily of proteins. The structures exhibit a significant degree of tertiary and secondary structural homology with an overall shape resembling that of a triangular prism [99]. Secondary structure elements are found in similar locations in all four molecules, with approximately 50% of the amino acids found in α-helices and approximately 15% found in β-sheets. Although the helices are distributed non-sequentially throughout the polypeptide chain, the tertiary structure is composed of an asymmetric arrangement of helices clustered on one side of the protein and the β-structure located predominantly on the opposite side [99, 100]. P450terp and BM-3 contain several large insertions compared with P450cam, which may represent the minimal P450 structure [100]. The heme binding core structure is highly conserved among the protein structures. The heme group is embedded between the helices designated I and L with no part of it directly exposed to the aqueous medium (see Figure 18). The COOH-terminal portion of the I helix, the L helix, and the loop containing the cysteine that coordinates the heme are structurally similar. All P450s exhibit high amino acid sequence similarity in these regions suggesting a conserved structural motif. Most differences between the enzymes are observed around the substrate binding pocket and regions suggested to be important for binding of the redox partner [105].

Based on sequence homology, a strong structural similarity is evident between membrane P450s and soluble bacterial P450s [106]. In contrast to earlier models predicting 6–10 transmembrane segments, the membrane-bound P450s are not believed to have the catalytic domain exposed to the cytoplasm and to differ from the soluble P450s by having an N-terminal transmembrane anchor. In the bacterial P450 structures, the heme has an orientation parallel to the plane of the protein [100]. EPR spectroscopy has demonstrated that in membrane-bound P450s the heme plane is nearly parallel to the membrane surface [107]. It has been proposed that membrane-bound P450s lie flat on the membrane surface, with the distal end of the active site and the substrate channel facing the membrane [108].

The presence of a hydrophobic membrane anchor is a major obstacle to crystallization of microsomal P450s. Schunk and co-workers have provided a strategy for generation of the soluble and functional cytosolic domain of the microsomal P450 52A3 [109]. High levels of expression of the membrane-bound state of the protein was followed by sequence-specific proteolysis at a factor Xa restriction site introduced between the membrane anchor and the catalytic domain. The truncated, soluble P450 protein exhibited unchanged spectral characteristics and maintained 85% of the activity of the wildtype protein. This method may facilitate structure–function studies on the catalytic domain of membrane-bound P450s provided that crystallization conditions can be found.

The I Helix

A prominent feature of P450 structures is the long I helix (50 Å in length in BM-3), which is disrupted in the immediate vicinity of the heme at the highly conserved threonine position (Thr-252 in P450cam). Amino acid substitutions of the threonine residue in several mammalian P450s result in altered substrate selectivities and binding characteristics, indicating that this threonine plays an important role in defining these properties [110–112]. As examples, mutants of P450(ω-1) and P450(16α) with His and Ile substitutions at this position, respectively, result in inactive enzymes, and mutant enzymes with Val and Asn at this

position retain limited activity toward some substrates [110, 111]. The hydroxyl group of the threonine, however, is not essential to function of P450 enzymes, which is evidenced by the existence of at least 11 native, functional P450 sequences which do not have a threonine at this position [113].

When Thr-252 in P450cam is replaced with Ala, the monooxygenase reaction is uncoupled, resulting in H_2O_2 production [114]. This points to a role for the Thr-252 hydroxyl group in cleavage of the dioxygen bond during catalysis, and as the center for an oxygen binding pocket. In the X-ray structure of the Thr252Ala mutant, the local distortion in the I helix becomes more exaggerated and is accompanied by an additional solvent molecule in the active site [114]. This suggests that solvent molecules are responsible for the observed uncoupling of camphor hydroxylation. The uncoupling may be due to both steric interference of the extra solvent molecule on dioxygen binding as well as easier access of dioxygen to solvent protons, promoting H_2O_2 and/or H_2O production rather than formation of hydroxylated substrates. Substitution of the conserved threonine with isoleucine in the native allene oxide synthase [115] and thromboxane synthase [116], two atypical P450s catalysing rearrangements of fatty acid peroxides without incorporation of oxygen, is in agreement with this threonine being important for oxygen binding.

The I helix is bent and elongated in the region around the conserved threonine, which forms a large groove [100]. P450eryF has an alanine at the position homologous to that of Thr252 in P450cam. Despite this substitution, the I helix in P450eryF also has the characteristic bend and groove [101]. Recent modelling studies have indicated that the preserved groove cannot accommodate either substrate or dioxygen. Instead, the groove is occupied by water molecules, which may serve as proton source during catalysis [100]. Most P450s have a serine or threonine as the C-terminal neighbour to the conserved threonine and an acidic residue as the N-terminal neighbour. It has been proposed that the acidic residue plays a central role in proton transfer to the threonine hydroxyl group either directly or via a water molecule located in the groove [99, 117]. The acidic residue would become reprotonated either by a charge relay mechanism as proposed for P450cam [117] or by accessible solvent molecules as proposed for P450terp and BM-3 [100]. In an alternative view, the ordered water molecules inside the groove, along with the many cooperative interactions (hydrogen bonds and salt bridges) between this section of the I helix and other regions of the structure, may function in maintaining the disruption of the normal α-helical bonds [100]. A more elongated and flexible structure may allow the active site of P450s to accommodate (1) a wider range of substrates (important for detoxification of xenobiotics); (2) the entrance of dioxygen into the binding site; and subsequently (3) the entrance of protons or exit of water.

The ERR-triad

Comparison of P450 sequences has revealed a conserved Glu-Arg-Arg triad located just N-terminal to the heme-binding domain [100]. In the crystal structures the ERR triad forms salt-bridge interactions which result in a highly conserved three-dimensional structure in all the proteins, despite the lack of any typical secondary structures in the region. Mutations of these residues have led to the production of inactive proteins. It has been proposed that the ERR-triad acts to establish a folding motif that locks the heme-binding domain in position, assuring the stable association of heme with the protein [100]. As a result, distribution of the ERR-triad would lead to the loss of heme binding and of P450 activity, as observed in the mutants.

The molecular dipole

Examination of electrostatic charge distribution of the P450 structures, led to the discovery of a molecular dipole, which qualitatively is the same in all the enzymes [100]. The molecular dipole arises from a negatively charged distal face and a positively charged proximal face, of which the latter has been shown to form a complementary charge interaction with the redox partner. It is proposed that the molecular dipole accelerates the diffusion-limited interaction of the P450 and its redox partner by steering the negatively charged region of the electron donor away from the negatively charged distal face, and towards the proximal face [100]. Furthermore, the molecular dipole may influence the electrochemical flow of the reactants involved in the activation of oxygen by assisting both the proximal-to-distal flow of electrons from the redox partner and the distal-to-proximal flow of protons from the solvent [100].

The substrate binding pocket

The substrate binding pocket is adjacent to the heme and is accessible through a long hydrophobic channel, the substrate binding pocket. Binding of substrates to cytochrome P450s appears to be largely determined by hydrophobic interactions, suggesting that the active site of cytochrome P450 is comparable to an organic solvent in its ability to accommodate hydrophobic compounds [118]. The little variation among cytochrome P450 enzymes with respect to the nature of the reactive species formed and in catalytic mechanism used, suggests that the primary catalytic role of the P450 protein is the formation of reactive oxygen species. The reaction between the activated oxygen species and the substrate is then primarily determined by the relative reactivities of the oxidizable sites on the substrate, the orientation of the substrate relative to the activated oxygen species, and the degree of mobility of the substrate within the active site. Collectively P450 enzymes display a broad range of substrate specificities,

but the individual P450s show a high degree of substrate and product selectivity. Particularly, the number of highly regio- and stereospecific P450s involved in the biosynthesis of secondary plant products seems almost unlimited.

A major interest in P450 research is the elucidation of molecular mechanisms underlying substrate specificity. In principle this would allow the design of specific inhibitors as well as P450 enzymes with specific substrate-specificities. Many approaches have been undertaken to investigate this problem, including chemical modifications with substrate analogues [119], site-directed mutagenesis [111, 112, 120–122], protein engineering with chimeric constructs [123–127], searching for similar subsequences in other protein families with similar substrate or binding specificities [128, 129], and sequence alignment with the bacterial P450s whose three-dimensional structure has been solved [130]. These studies have not given a unified view of the substrate recognition sites in P450s, which is not unexpected in view of findings that very minor amino acid substitutions result in significant changes of P450 substrate specificity. As an example, change of a single amino acid Phe209Leu in P450coh resulted in a conversion of substrate specificity from coumarin to steroid hydroxylation [121].

In a recent study, Gotoh has identified putative substrate recognition sites, SRSs, in the drug-metabolizing cytochrome P450 family 2 (CYP2) by sequence comparison to bacterial P450s (Figure 19) [131]. CYP2 is the largest and most diverse of the P450 families. An improved sequence alignment took into consideration group-to-group alignment, secondary structure prediction and hydropathy indices. The six SRSs identified were dispersed along the primary sequence and constituted about 16% of the amino acid residues. All the known point mutations and chimeric fragments that in previous studies have been shown to significantly affect the substrate specificities fell within or overlapped some of the six SRSs (Figure 19). Interestingly, analysis of nucleotide substitutions in four subfamilies, CYP2A, 2B, 2C, and 2D, consistently indicated that the SRSs had accumulated more non-synonymous (amino acid-changing) substitutions than the rest of the sequence [31]. This was interpreted as an indication that diversification of duplicate genes of drug-metabolizing P450s occurs primarily in substrate recognition regions to cope with the increasing number of foreign compounds, in parallel to the enormous diversity of antigen recognition sites of immunoglobulins and the major histocompatibility complex [132, 133]. The combined knowledge from the four P450 X-ray structures and the identified SRS regions within CYP2 has provided a structural basis for the controlled modification of substrate specificities of members of CYP2 by changing the identity of specific amino acid side chains within the access channel. It is an open question to what extent the SRS regions can be found in P450s from other families. The wide diversity in CYP2 function suggests

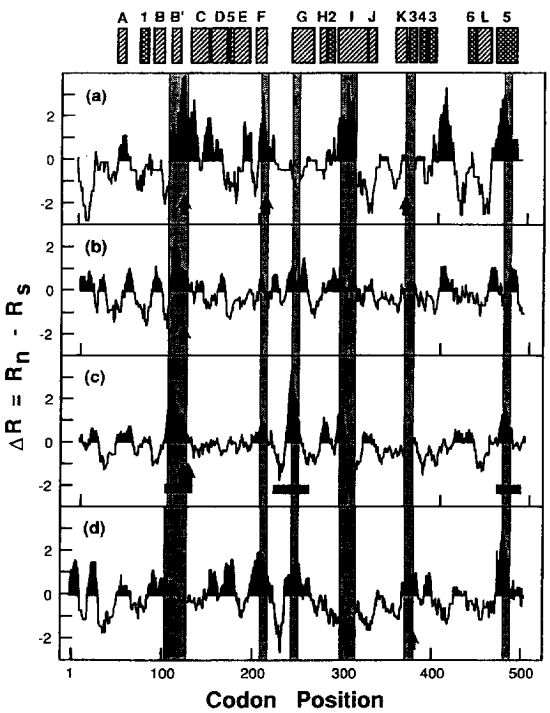

Fig. 19. Localization of substrate recognition sites in cytochrome P450 family 2 (CYP2) as inferred from comparative analyses of amino acid and coding nucleotide sequences. Positive ΔR_t means that nucleotide substitutions replacing amino acids within a window (nine codons) have occurred more frequently than expected from the global number of non-synonymous substitutions after correction for local variation in nucleotide mutation rates. Calculations were made individually for four CYP2 families: 2A (a), 2B (b), 2C (c), 2D (d). Shaded areas indicate substrate recognition sites. The locations of the residues identified experimentally as responsible for substrate recognition are indicated by arrowheads. The three filled boxes in (c) indicate the locations of the fragment that affect substrate specificities in chimeras between rabbit P450 2C2 and 2C14. (From reference [131], with permission.)

that the identified region should be present in other CYP families.

Regio- and stereoselectivity of P450-catalysed reactions depend on the constraints provided by the active site and by the flexibility of the substrate. This has been illustrated by comparison of the three-dimensional structure of P450cam solved in complex with differing substrates [134]. The looser fit of norcamphor within the active site pocket apparently results in at least three detectable hydroxylated products, whereas camphor only gives a single product. Comparison of the stereoselectivity of thioanisole sulfoxidation and styrene epoxidation shows that P450terp has a sterically constrained active site resulting in absolute stereochemistry of thioanisol and styrene oxidation [135]. In contrast, P450BM-3 and P450cam, which have relatively larger active sites, exhibit low stereospecificity of the same reactions. These results establish that the

bacterial enzymes, like the detoxifying mammalian liver P450s, can oxidise a diversity of substrates other than just the endogenous substrate for which they are commonly identified. In plants, the herbicide-metabolizing P450s may represent biosynthetic P450s with relatively large active sites, which allow exogenous compounds to be metabolized.

The importance of substrate flexibility is illustrated in a study comparing two substrates: benzphetamine consisting of two phenyl rings joined by a linker, and 7-ethoxycoumarin, a small rigid molecule consisting of two fused aromatic rings [112]. Substitutions of residues in the substrate binding pocket of P450IA2 resulted in reduced activities towards benzphetamine and higher activities towards 7-ethoxycoumarin, indicating that flexible substrates such as benzphetamine are more sensitive to the active site environment of P450s and require active site features such as hydrophilic groups or projecting side chains to facilitate their orientation in the active site prior to catalysis [112]. More rigid substrates, like 7-ethoxycoumarin, have fewer possible conformations and consequently need less direction from the enzyme to assume a correct orientation.

In P450tyr and Dit2, the only P450s known to metabolize tyrosine, the highly conserved threonine (corresponding to Thr-252 in P450cam) is replaced with asparagine, and the typical C-terminal neighbour replaced by proline [113]. This suggests that these residues are important active site features for defining the tyrosine-specificity of these enzymes.

Recently, computer-based substrate-docking analysis has been used to identify potential substrates for P450cam [136]. 20 000 compounds in the Available Chemicals Directory were searched for a good 'fit' within the P450cam active site. A mutant of P450cam with an enlarged active site was used to search for non-substrates. Eight out of eleven compounds were correctly predicted to promote catalytic turnover, and five predicted non-substrates were not substrates. This shows that a computer search method can be sufficiently discriminating to distinguish reactivities of different active sites. As three-dimensional structures of eukaryotic P450s appear, this method could be very useful in the design of active sites with new specificities.

Topology of membrane-bound P450 enzymes

Microsomal cytochromes P450 are integral membrane proteins with a large cytoplasmic catalytic domain, bound to the membrane by a hydrophobic amino-terminal region which consists of 20–25 amino acids [108]. The short amino-terminal segment functions both as an insertion signal and as a stop-transfer sequence [137]. The co-translational insertion into the ER membrane requires the signal recognition particle [138]. Fusion of secretory, soluble or mitochondrial proteins (at their N-terminal end) to the hydrophobic N-terminal region of cytochrome P450s results in retention of the fusion proteins in the ER [137, 139–141]. These observations indicate that the large cytoplasmic domain of cytochromes P450 is not required for retention in the ER and suggest that a specific sequence or structure within the N-terminal region functions as an ER retention signal. The N-terminal region of microsomal P450s contains four common structural motifs: a negatively charged amino acid at the amino terminus, a long stretch of hydrophobic amino acids, a few positively charged amino acids and a proline-rich region [142].

The orientation of the N-terminal signal-anchor segment of P450s in the ER membrane has been controversial, and therefore subject to numerous studies. Nelson and Strobel [108] have proposed a hairpin loop conformation which would result in the N-terminus being exposed to the cytoplasm, as is the case for type II and uncleaved type I membrane proteins. However, many data strongly indicate that the N-terminus of P450s faces the lumen of ER. Evidence is provided by glycosylation of introduced and native N-glycosylation sites [141, 143], antibody studies [144], FITC labelling of N-terminal methionine only after Triton X-100 treatment [145, 146], and by the combined use of a 'topological' screen based on growth of modified *his4* yeast mutant on histidinol-medium and subcellular immunoprecipitation [147]. Monier *et al.* [139] have provided evidence for the P450 N-terminus being initially inserted as a loop, and subsequently reoriented to a single membrane-spanning polypeptide chain with a head-in orientation. Replacement of negatively charged amino acids in the N-terminus by positively charged amino acids converts the signal-anchor sequence of P450IIC2 into a type II signal, in which the protein is translocated across the membrane [140]. This is in agreement with studies showing that the balance between the N-terminal charge and the length of the hydrophobic segment determines the topogenic function of the N-terminus [147, 148]. The positively charged amino acids following the hydrophobic stretch increase the efficiency of the stop-halt signal, particularly when the hydrophobic stretch is relatively short [149]. In summary, the microsomal P450s are neither type I nor type II membrane proteins, but share characteristics of both. They resemble the type I proteins by having the C-terminal in the cytoplasma and the type II proteins by having a stop-halt transfer signal.

In contrast to the above investigations, heterologously expressed recombinant P450s lacking the N-terminal hydrophobic region have been shown to be tightly bound to the membrane [150–152]. Furthermore, Ohta *et al.* [152] demonstrated that high salt treatment did not remove truncated P450IA1 from the yeast membrane, indicating that the N-terminal hydrophobic segment is not solely responsible for attachment to the membrane. This provides evidence that for some P450s additional segments are involved in membrane binding.

Retention of microsomal P450 enzymes to the ER

Little is known about the mechanism by which cytochromes P450 are retained in the ER. P450s contain neither a C-terminal double lysine motif [153] nor a KDEL sequence [154] which represent retention signals for type I transmembrane or luminal ER proteins, respectively. Several studies involving endoglucosidase H sensitivity and alkaline phosphatase resistance have demonstrated that microsomal P450s are not processed by Golgi-associated proteins [141, 155, 156]. This indicates that P450s are restricted to the ER membrane by a mechanism different from recycling through the Golgi compartment. A protein segment consisting of the 29 amino acids at the N-terminus is sufficient for retention of P4502C1 to the ER, indicating that the ER residency of P450s seem to be attained through a unique transmembrane segment-mediated mechanism [141]. Alternatively, the transmembrane region of microsomal P450s may assemble into a network of membrane proteins which cannot enter the transport vesicles exiting from the ER [156].

The proline-rich region

The highly conserved proline-rich region present in many P450s including plant P450s follows the signal-anchor segment and is not involved in retention of P450s in the ER [156]. The proline-rich region forms a hinge between the cytoplasmic catalytic domain and the hydrophobic membrane anchor. In addition, the proline-rich region has been shown to be important for the proper incorporation of heme as evidenced by the absence of a P450 · CO spectrum in proteins mutated in one or several of the prolines [157]. Furthermore, mutated proteins, in which more than one proline residue have been exchanged, are more sensitive to trypsin digestion than the wild type. From these results it has been proposed that the proline residues in the proline-rich region are crucial for the formation of the correct conformation of microsomal P450s [157].

CONCLUDING REMARKS

In the superfamily of cytochrome P450 enzymes over 500 different enzymes have been identified in bacteria, fungi, worms, plants, insects, fish and mammals (D. R. Nelson, personal communication). Particularly within the group of plant and microorganism P450s, the field is expanding rapidly with many new enzymes and new catalytic reactivities being identified each year. The application of the oxidizing power of P450 enzymes for industrial purposes has great potential. The heterologous expression systems now available allow the production of significant amounts of specific cytochrome P450s, which can be used in commercial application to chemical processes, pollution control or in the synthesis of pharmaceuticals or secondary plant metabolites. Knowledge of the structure–function relationship of a given P450 enzyme should allow the rational design of specific inhibitors (e.g. herbicides or pharmaceuticals) or of P450 enzymes with altered substrate specificities. The application of P450 enzymes in biotechnology has a promising future.

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