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Structural control of cytochrome P450-catalyzed ω -hydroxylation

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Abstract

The regiospecific or preferential ω -hydroxylation of hydrocarbon chains is thermodynamically disfavored because the ease of C-H bond hydroxylation depends on the bond strength, and the primary C-H bond of a terminal methyl group is stronger than the secondary or tertiary C-H bond adjacent to it. The hydroxylation reaction will therefore occur primarily at the adjacent secondary or tertiary C-H bond unless the protein structure specifically enforces primary C-H bond oxidation. Here we review the classes of enzymes that catalyze ω -hydroxylation and our current understanding of the structural features that promote the ω -hydroxylation of unbranched and methyl-branched hydrocarbon chains. The evidence indicates that steric constraints are used to favor reaction at the ω -site rather than at the more reactive (ω -1)-site.

Keywords

cytochrome P450; ω -hydroxylation; CYP4 family; carbon hydroxylation; fatty acids; branched-chain hydrocarbon acids; cholesterol degradation

1. Hydrocarbon ω -hydroxylation

In chemical terms, the regio- and stereoselective oxidation of unactivated hydrocarbon C-H bonds to the corresponding hydroxy (C-OH) products is the most difficult reaction catalyzed by cytochrome P450 enzymes. This substrate hydroxylation reaction is mediated by the “Compound I”-like ferryl species formed during the catalytic turnover of P450 enzymes. The Fe(IV) heme iron atom in this ferryl species is paired with a radical cation delocalized over the heme porphyrin ring, so the enzyme is two oxidation equivalents higher than the resting enzyme. The formation of this oxidizing intermediate via the catalytic cycle in Fig. 1 has been extensively reviewed and is therefore not discussed here [1].

The hydroxylation of a hydrocarbon chain is initiated by abstraction of the hydrogen atom of the C-H bond by the ferryl oxygen atom, yielding a transient carbon radical coupled to an Fe(IV)-OH catalytic intermediate. Rebound collapse of a hydroxyl radical equivalent from the iron with the substrate radical produces the alcohol and returns the enzyme to the resting

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ferric state (Fig. 2). Computational and experimental results indicate that the ease of oxidation of any given C-H bond is related to its bond strength; i.e., to the energy required to homolytically break the C-H into a carbon radical and a hydrogen radical ($\text{C-H} \rightarrow \text{C}\cdot + \text{H}\cdot$) [3,4]. If the ferryl species in the enzyme-substrate complex can react with more than one C-H bond, the reaction will preferentially occur with the weaker C-H bond unless steric constraints or other factors prevent it. This is common in cytochrome P450 enzymes, as their active sites often bind a diversity of substrates relatively loosely and, not infrequently, in multiple orientations. The combination of ligand mobility and dynamic protein malleability can allow the ferryl species access to more than one C-H bond in the substrate.

Oxidation of the terminal methyl of a hydrocarbon chain is referred to as ω -hydroxylation because it involves the last atom of the chain. It then follows that oxidation of the carbon next to the terminal methyl can be termed an (ω -1)-hydroxylation, and oxidation of a carbon n -atoms removed from the end of the chain an (ω - n)-hydroxylation. Based on their relative bond strengths, a tertiary C-H bond should be oxidized in preference to a secondary one, and a secondary in preference to a primary (Table 1). Thus, the terminal methyl C-H bonds are inherently more difficult to oxidize than those of secondary or tertiary C-H bonds in the hydrocarbon chain. The reactivity of C-H bonds of the terminal methyl can be increased by placing it adjacent to a double bond, an aromatic ring, or an oxygen or nitrogen atom, as these functionalities lower the energy of the resulting carbon radical by delocalizing the unpaired electron. However, here we only consider the situation in which the ferryl species differentiates between two or more hydrocarbon C-H bonds that have no adjacent activating functions. Thus, the hydroxylation of a benzylic methyl and O-dealkylation of an ether (Fig. 3) are not encompassed by the definition of ω -hydroxylation as explored in this review. The oxidation of *tert*-butyl groups in which the (ω -1)-carbon does not have an oxidizable C-H bond, as in the oxidation of terfenadone by CYP2J2 (Fig. 3), is also excluded [5]. In effect, this review explores the mechanisms utilized to promote ω - over (ω -1)-hydroxylation of hydrocarbon chains.

Given the relative reactivities of hydrocarbon C-H bonds, P450 enzymes should preferentially catalyze (ω -1)- rather than ω -hydroxylation reactions. This is indeed observed for P450 enzymes not specifically engineered to promote ω -hydroxylation. For example, CYP2B1 (ω : ω -1 ratio 3:22) [6] and CYP2E1 (ω : ω -1 ratio 1:11) [7,8] oxidize lauric acid predominantly at the ω -1 position, CYP2A6 primarily oxidizes arachidonic acid at the ω -1 position [9], and CYP102 oxidizes fatty acids at internal chain carbons (ω -1: ω -2: ω -3 36:30:34) with no ω -hydroxylation [10]. However, some cytochrome P450 enzymes preferentially catalyze ω -hydroxylation reactions. These ω -hydroxylases must enforce their regiospecificity through protein-substrate interactions that either hinder access of the ferryl species to C-H bonds other than those of the terminal methyl group or alter the intrinsic ferryl reactivity. As expected from the tight control of the bound ligand that ω -regiospecificity implies, ω -hydroxylation is generally observed as a major process with substrate-specific enzymes involved in defined biological pathways. This review focuses on these latter enzymes and on our understanding of how ω -hydroxylation is promoted.

2. Enzymes that predominantly perform ω -hydroxylation

2.1 Hydrocarbons

Bacterial P450 enzymes are known to oxidize linear hydrocarbons of C₅-C₁₅ chain lengths [11]. CYP153A1, identified in 2001 in *Acinetobacter* sp. EB104, was the first bacterial P450 specifically associated with this activity [12], but since then other members of the CYP153 family in diverse bacteria have been shown to catalyze the ω -hydroxylation of medium-length linear hydrocarbons [13,14]. The ω -hydroxylation of hydrocarbons in bacteria enables them to grow on these compounds as their sole carbon source.

The CYP52 P450 family of fungi, particularly of *Candida maltosa*, *Candida tropicalis*, and *Candida apicola* [15], also oxidize the terminal methyl groups of linear hydrocarbons. A typical example is CYP52A3 from *Candida maltosa*, which oxidizes hexadecane to 1-hexadecanol as the major product but also produces hexadecanal, hexadecanoic acid, 1,16-hexadecanediol, 16-hydroxyhexadecanoic acid, and 1,16-hexadecanedioic acid, all the products being formed with the expected extent of incorporation of oxygen atoms from molecular oxygen [16]. This enzyme is thus able to oxidize both hexadecane and the corresponding fatty acid hexadecanoic acid.

Rabbit CYP4B1 oxidizes fatty acids and an unusual range of substrates, including hydrocarbons, that it oxidizes with an $\omega/(\omega-1)$ -hydroxylation ratio that ranges from 23 for heptane to 1.6 for decane [17].

2.2 Fatty acids

The mammalian CYP4 family of P450 enzymes catalyzes the preferential ω -hydroxylation of fatty acids. Of the relevant human enzymes [18,19], CYP4A11, CYP4B1, CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12 have been reasonably well characterized, whereas the properties and functions of the other five members of the family, CYP4A22, CYP4F22, CYP4V2, CYP4X1, and CYP4Z1 remain relatively obscure. The enzymes differ in their substrate specificities in terms of fatty acid chain length and degree of unsaturation, and in some instances exhibit preferential affinities for prostaglandins and leukotrienes, but almost invariably catalyze ω - over ($\omega-1$)-hydroxylation of their substrates.

Enzymes that catalyze the ω -hydroxylation of fatty acids are widespread in other species. The catalytic specificities of the rat enzymes CYP4A1, CYP4A2, CYP4A3, and CYP4A8 have been investigated [20], as have the fatty acid ω -hydroxylase activities of CYP4 enzymes from mice [21], rabbits [22], pigs [23], and other mammals. In plants CYP76B9 [24], CYP78A1 [25], CYP86A1 [26], CYP86A8 [27], CYP86A22 [28], CYP94A1 [29], CYP94A2 [30], CYP94A5 [31], CYP94C1 [32], CYP96A1 [33], CYP96A2 [33], and CYP97B3 [33] have been shown to ω -hydroxylate fatty acids, although the substrate for the last enzyme is the thioester CoA derivative rather than the free acid.

In fungi, the members of the CYP52 family, including CYP52A3 [34], CYP52A4 [34], CYP52A5 [34], and CYP52A9 from *Candida maltosa* [34], CYP52A13 from *Candida tropicalis* [35], and both CYP52A17 and CYP52A21 from *Candida albicans* [36] are ω -selective or ω -specific fatty acid hydroxylases.

2.3 Branched fatty acids and saturated isoprenoid chains

Branched fatty acids are also substrates but have been less extensively investigated. Although linear terpene acids are formally branched, unsaturated fatty acids, they generally do not fall into the narrow class of substrates being considered here because the terminal groups, as in geraniol and farnesol (Fig. 3), are allylic to a double bond, are activated towards hydroxylation, and do not have a hydrogen on the adjacent carbon. One compound that is consistent with the present definition is phytanic acid. Phytanic acid (Fig. 4) is ω -hydroxylated in humans by most CYP4F and CYP4A human enzymes [37], with the reaction being catalyzed in order of decreasing efficiency by CYP4F3A, CYP4F3B, CYP4F2 and CYP4A11 [37,38]. CYP124 of *Mycobacterium tuberculosis* also catalyzes the ω -hydroxylation of phytanic acid [39]. In contrast, CYP102, an enzyme that is not designed to promote ω -hydroxylation, oxidizes the $\omega-1$ tertiary carbon C-H bond (40). Another terpene derivative that is oxidized by CYP4 enzymes, a reaction so far only demonstrated for human CYP4F2, is the ω -hydroxylation of γ -tocopherol and related isomers (Fig. 4) [41]. Non-isoprenoid branched hydrocarbons are also preferentially ω -hydroxylated, as

exemplified by the oxidation of 11-methyl-dodecanoic acid by CYP4A1 [42]. An impressive demonstration of the ability to hydroxylate a terminal methyl group despite the presence at the adjacent carbon of a more reactive C-H bond is the CYP4B1-catalyzed oxidation of isopropyl benzene to 2-phenyl-1-propanol [43]. In this reaction the terminal methyl C-H bond is oxidized in preference to the adjacent tertiary, benzylic C-H bond.

2.4 Sterol sidechains

Hydroxylation of the terminal carbons of the sidechain of cholesterol and other bile-acid related sterols (Fig. 5) is consistent with classification as an ω -hydroxylation. In humans, CYP27A1 oxidizes the C27 methyl group without touching the C25 secondary C-H bond [44]. In *Caenorhabditis elegans*, a similar C27 hydroxylation of cholesterol oxidation by CYP122A1 (Daf-9) leads to the production of a critical hormonal factor [45].

In *Mycobacterium tuberculosis* and related bacterial species, the oxidation of cholesterol is a critical step in the utilization of cholesterol as a carbon source and possibly in the production of factors that contribute to pathogenesis. Recent work has shown that CYP125A1 from both *M. tuberculosis* [46–48] and *Rhodococcus jostii* RHA1 ω -hydroxylates the sterol sidechain of cholesterol and its 3-keto-4-ene derivative [49]. CYP124A1 from *M. tuberculosis* also oxidizes both cholesterol and the corresponding 3-keto-4-ene structure [39,50].

3. Control of ω -hydroxylation of unbranched hydrocarbon chains

3.1 CYP4 chain length tolerance

The relationship between fatty acid chain-length and the hydroxylation regiospecificity of CYP4 enzymes has been extensively examined. The results of a typical study of rat CYP4A1, CYP4A2, CYP4A3, and CYP4A8, as well as human CYP4A11, shows that ω -regioselectivity tends to decrease for substrates longer than lauric acid (Table 2) [51]. A similar finding is obtained with rabbit CYP4A7, for which the fatty acid hydroxylation specificity gradually decreases from an $\omega/(\omega-1)$ -ratio of 15.1 for lauric acid to 1.1 for nonadecanoic acid [52]. Rabbit CYP4B1 oxidizes hydrocarbons with an $\omega/(\omega-1)$ -hydroxylation ratio that decreases from 23 for heptane to 1.6 for decane [17]. The generality of this finding is confirmed by studies of CYP52A21 from *Candida albicans* [36]. Furthermore, in the case of CYP94A2, ω -hydroxylation predominates in the oxidation of lauric acid, but shifts towards the $\omega-1$ position in longer fatty acids [53]. The bottom line is that the ω - to $(\omega-1)$ -hydroxylation ratio depends on the enzyme as well as the length and degree of unsaturation of the hydrocarbon or fatty acid that is oxidized. Although ω -hydroxylation predominates with these enzymes, increasing chain length tends to decrease the ω -regiospecificity. Thus, the mechanism(s) that govern ω -hydroxylation must allow for oxidation by a single enzyme of the terminal methyl group of hydrocarbons or fatty acids of variable length. Furthermore, they must often allow for a minor amount of $(\omega-1)$ -hydroxylation. Nevertheless, the ability to ω -hydroxylate fatty acid chains of different lengths, and the ω -hydroxylation of linear hydrocarbons by CYP4B1, preclude a simple “ruler” mechanism in which the protein anchors the fatty acid carboxyl group and hydroxylation occurs at a given distance from the carboxyl group.

3.2 Isotope effects on ω - versus $(\omega-1)$ -hydroxylation specificity

Substitution of deuterium at the terminal methyl group causes a considerable shift from ω - to $(\omega-1)$ -hydroxylation. Early deuterium substitution experiments carried out with microsomal preparations are quantitatively unreliable, as $(\omega-1)$ -hydroxylation is catalyzed by microsomal enzymes (e.g., CYP2E1) other than the CYP4 ω -hydroxylases. Nevertheless, early experiments showed that [10,10,10- d_3]-decanoic acid was oxidized to the ω -

hydroxylated alcohol product without a significant isotope effect on the overall rate of the reaction [54]. The absence of an isotope effect on the overall rate of the reaction is consistent with the fact that C-H (or C-D) bond breaking is not the rate-limiting step in P450 catalysis. However, the hydroxylation of lauric acid by recombinant rabbit CYP4B1 was shown to shift from (ω -1)- to (ω)-hydroxylation with an isotope effect of 6.2 when C11 was dideuterated, and conversely to switch from ω - to (ω -1)-hydroxylation with an isotope effect of 5.7 when the terminal methyl was trideuterated [55]. Furthermore, comparison of the oxidation of lauric acid and 12,12,12- d_3 -dodecanoic acid by recombinant, purified fungal CYP52A1 shows that the [ω /(ω -1)]-hydroxylated product ratio shifts from 11.1 ± 2.9 to 0.87 ± 0.10 upon deuteration [36]. In this same study, dideuteration of C12 in 12-chlorododecanoic acid resulted in a shift from (ω -1)-hydroxylation to chloride atom oxidation. These results indicate that hydroxylation of the ω - and (ω -1)-carbons occurs as an internally competitive process. On deuteration of the terminal methyl, which increases the bond strength, oxidation of the adjacent (ω -1) position becomes more favorable despite the structural constraints in place to promote ω -hydroxylation.

CYP4B1 has an unusually broad substrate specificity [56] and hydroxylates methyl groups on aromatic rings and other compounds in addition to fatty acids. An analysis of the intramolecular isotope effects on hydroxylation of *ortho*-xylene, *para*-xylene, 2,6-dimethylnaphthalene, and 4,4'-dimethylbiphenyl in which one of the two methyls in each compound is trideuterated shows that the isotope effect is highly masked when the methyl groups are not adjacent to each other [43]. In contrast, the isotope effect is fully expressed in similar hydroxylations by CYP2B1, an enzyme that is not an ω -hydroxylase. These results suggest that the ability of the substrate to rotate within the active site is more highly restricted in CYP4B1 than in CYP2B1, as might be expected if the substrates are tightly restrained in the CYP4B1 active site.

3.3 Fatty acid substrate analogues

CYP4 enzymes are sensitive to steric bulk at the hydrocarbon chain terminus, as revealed by studies of the oxidation of fatty acids with terminal modifications. Thus, placing a methyl group on the (ω -1)-carbon of the chain to give an isopropyl-like terminus is tolerated, but replacing the resulting isopropyl terminus with a cyclopropyl ring is not [42]. Likewise, although phenyl groups could replace some of the internal carbon atoms of the hydrocarbon chain, no activity was observed when the phenyl group was located at the hydrocarbon chain terminus even though it binds as a Type I ligand [57]. Low activity was detected if the terminal methyl group was attached to a phenyl ring at the end of a hydrocarbon chain (Fig. 6). 12-Dodecenoic acid with a less sterically bulky double bond at the chain terminus is oxidized to the epoxide [6,42,58], and fatty acids with an even less sterically hindered terminal acetylenic group are oxidized to ketene metabolites that are either hydrolyzed to the diacids or bind covalently to the P450 protein, causing its inactivation [6]. In contrast, replacement of the (ω -1)-carbon by a sulfur atom results in oxidation of the sulfur to the sulfoxide by both CYP4A1 and CYP94A1 (Fig. 6) [29,59]. The free thiol expected from ω -hydroxylation (S-dealkylation) was not observed.

The oxidation of halogen substituents to halonium oxides by cytochrome P450 enzymes is difficult due to the high electronegativity of the halogen atoms. However, in view of the ω -oxidation regioselectivity of CYP4 enzymes, the oxidation of 12-halododecanoic acids by CYP4A1 was investigated [60]. Oxidation of 12-bromo- and 12-chlorododecanoic acids yielded both 12-hydroxydodecanoic acid and 12-oxododecanoic acid (Fig. 7). The 12-oxo metabolite is the product expected from hydroxylation on the carbon bearing the halogen, essentially an (ω -1)-hydroxylation. The finding that the oxygen in the 12-hydroxydodecanoic product derived primarily from the medium and not molecular oxygen, however, is best interpreted as resulting from oxidation of the halogen to the halooxonium

intermediate, followed by displacement of this function by water. Furthermore, if the hydrogens of the terminal carbon of 12-chlorododecanoic acid were replaced by deuteriums, there was a 2–3 fold increase in halogen oxidation at the expense of (ω -1)-hydroxylation. Interestingly, 12-iodododecanoic acid, the substrate with the most oxidizable halogen atom, gave very little product from halogen oxidation. This suggests that access to the ferryl species is tightly restricted and allows the chloride and bromide, but not iodide, atoms to approach the ferryl. This is consistent with an effective diameter greater than 3.90 Å but smaller than 4.30 Å (Fig. 7).

3.4 Mechanism-based inhibitors

The most specific mechanism-based inhibitors of CYP4 enzymes incorporate a triple bond between the ω - and (ω -1)-carbons of the fatty acid hydrocarbon chain [61]. The most widely used inhibitors of this type are 11-dodecynoic acid [62], 10-undecynoic acid [62], and 17-octadecynoic acid [63]. Extensive studies have shown that transfer of the ferryl oxygen to the internal carbon of the triple bond results in alkylation of the heme group of the enzyme by the inhibitor, whereas oxygen transfer to the terminal carbon results in the formation of ketene metabolites that can bind to nucleophilic protein residues [63,64]. The relevant information in terms of the control of ω -hydroxylation is that oxidation of terminal acetylenic fatty acids of various lengths by CYP4 enzymes results primarily in protein rather than heme alkylation, indicating that the oxidation occurs largely or exclusively at the ω -carbon despite the small steric profile of the acetylenic group.

3.5 Heme covalent binding

A unique feature of the CYP4 family of P450 enzymes is that in most, but not all, members of this family the heme is covalently bound to the protein via an ester link between the heme 5-methyl group and the carboxyl group of an active site glutamic acid residue [65]. In CYP4A3, the residue is Glu318 [66]. The presence of these links has been unambiguously established for CYP4A1 [66], CYP4A2 [66], CYP4A8 [66], CYP4A11 [66], CYP4A5 [67], CYP4A7 [67], CYP4F1 [67], CYP4F4 [67], CYP4F3 [67], and CYP4B1 [67]. In contrast, the absence of a covalent heme in a CYP4 family enzyme has been demonstrated for CYP4F5 and CYP4F6 [68]. The heme is also not covalently bound in the fatty acid ω -hydroxylase from *Candida albicans* [36]. The role of covalent heme binding in CYP4 enzymes has not been convincingly defined. Covalent heme binding may improve ω -hydroxylation regiospecificity by rigidifying the active site (Fig. 8), decreasing its deformability, and thereby more tightly constraining the presentation of the ω -carbon of the chain for oxidation. Support for such a role is provided by the finding that the ω /(ω -1)-hydroxylation ratio for lauric acid by CYP4A1 is 20:1, but is only 5:1 for the CYP4A1 E320A mutant that does not bind the heme covalently [65]. Likewise, the ratio is 17:1 for CYP4A11, but only 2.8:1 for the CYP4A11 E312A mutant that again does not bind the heme covalently. However, covalent heme binding is not a requirement, as shown by the fact that the two mutants still strongly favor ω -hydroxylation and the observation that some members of the CYP4 family of ω -hydroxylases do not have a covalently bound prosthetic heme group.

3.6 Mutagenesis

Based on sequence alignments and homology structure models, site-specific mutagenesis has been utilized to explore the relationship of the protein sequence to the ω /(ω -1)-hydroxylation ratio. In one such study, the difference in the ω /(ω -1)-hydroxylation ratios of CYP4A2 (6:1) and CYP4A3 (3:1), which differ by only 18 amino acids, was shown to be determined by the first 119 amino acids, as a chimera in which CYP4A2 contributed these residues was characterized by an ω /(ω -1)-hydroxylation ratio (7:1) similar to that of CYP4A2 itself [20]. A more detailed analysis of the roles of the individual residues by site-

specific mutagenesis revealed that the $\omega/(\omega-1)$ -hydroxylation ratio was controlled by the presence or absence of three residues (in CYP4A3, Ser114, Gly115, and Ile116) and by a residue at position 119 (Fig. 9) [69].

CYP94A2 from plants exhibits a lauric acid $\omega/(\omega-1)$ -hydroxylation ratio of 19:1, but loses this ω -regioselectivity with longer fatty acids. Based on sequence alignments and a structure model, residue Phe494 was proposed to be important for controlling this specificity. Mutation of this residue into a leucine, valine, or alanine caused a major shift from ω -hydroxylation to $(\omega-1)$ -hydroxylation [53].

The mutagenesis results indicate that mutations in the protein usually result in loss of ω -hydroxylation specificity, in accord with the view that tight substrate control is required to enforce ω -hydroxylation. However, in the absence of true crystal structures, the role of specific residues in controlling regiospecificity remains ambiguous.

4. ω -Hydroxylation of methyl-branched hydrocarbon chains

4.1 Crystal structure of CYP124A1

The position in the *M. tuberculosis* genome of the gene coding for CYP124A1 adjacent to the gene for an enzyme that sulfates a terminal hydroxyl on a saturated isoprenoid chain led to the discovery that it catalyzes the ω -hydroxylation of branched hydrocarbon acids [39]. It has much lower activity for the oxidation of linear fatty acids and no detectable activity for the oxidation of branched or unbranched hydrocarbons (Table 3) [39]. Furthermore, the low activity for unbranched fatty acid oxidation is associated with a mixture of ω -, $(\omega-1)$ -, and $(\omega-2)$ -hydroxylation, whereas only ω -hydroxylation is observed with terminally branched hydrocarbon acids. It was subsequently found that CYP124A1 also oxidizes cholesterol and cholest-4-en-3-one to the 27-hydroxy derivatives in which the methyl-branched terminus of the sterol side-chain is ω -hydroxylated (Fig. 5) [50].

The basis for these substrate specificities has been clarified by determination of the crystal structure of CYP124A1 complexed with phytanic acid [39]. As shown in Fig. 10, the carboxylic acid is oriented towards the surface with the hydrocarbon chain extending down into the active site. The key interaction with respect to the high ω -hydroxylation specificity for a methyl-branched hydrocarbon tail involves specific binding of the branching methyl group in a small lipophilic cavity. This interaction positions the terminal methyl for hydroxylation and prevents the chain from slipping, thus preventing $(\omega-1)$ -hydroxylation. This is shown schematically in Fig. 10 next to the model of the active site interaction built from the electron density maps.

The low catalytic activity and loss of ω -regiospecificity in the oxidation of linear fatty acids is readily understood from this model. If the terminal methyl of a linear hydrocarbon chain binds in the lipophilic cavity otherwise occupied by the branching methyl, it would be sequestered and its hydroxylation would be impaired. To the extent that the terminal methyl escapes from this confinement, hydroxylation can occur, but no longer as a regiospecific process because the unrestrained chain can move and allow internal chain carbons to compete for hydroxylation.

4.2 Crystal structure of CYP125A1

The mechanism employed by CYP124A1 to direct the ω -hydroxylation regiochemistry of methyl-branched hydrocarbon chains focuses almost entirely on the last four carbons of the methyl-branched chain. The enzyme can therefore oxidize a diversity of substrates, so long as they have the methyl-branched terminus. CYP125A1 differs from CYP124A1 in that it is a highly substrate-specific enzyme that hydroxylates the terminal methyl of the cholesterol

and cholest-4-en-3-one sidechain [46,47,48]. Unlike CYP124A1, it does not oxidize fatty acids or branched chain hydrocarbon acids [50]. Its regiospecificity can therefore be more easily controlled by tight binding interactions of the entire substrate with the enzyme. The structures of *Mycobacterium tuberculosis* CYP125A1 without a bound ligand [47] and with cholest-4-en-3-one bound in the active site [48] show that ligand binding causes only minor perturbation of the protein structure. The structure of the enzyme complexed with cholest-4-en-3-one reveals that the sterol substrate is oriented almost perpendicular to the heme plane, with the branched side-chain terminus close to the heme iron and the A-ring of the sterol near the surface of the protein. The sterol is very tightly encased by amino acid residues, with two residues, Val115 and Met200 in contact with the sterol 19- and 20-methyl groups and located between these methyl groups and the heme group (Fig. 11). These residues are so placed that they serve as “door stops” that prevent the sterol from slipping closer to the heme group. As a result, only the two terminal methyl groups are positioned where they can be hydroxylated. This specificity is strengthened by the fact that the C25 C-H bond is oriented almost parallel to the heme plane and thus is in an orientation that is not conducive to hydroxylation by the iron-bound ferryl species.

The mechanism employed by human CYP27A1 to achieve regiospecific hydroxylation of the terminal methyl of the cholesterol side-chain is not known. However, as this enzyme is also highly substrate selective, it can be speculated that a mechanism similar to that of CYP125A1 may apply to CYP27A1. CYP27A1-catalyzed ω -hydroxylation of the 5 β -cholestane-3 α ,7 α ,12 α -triol side chain specifically gives the 27-hydroxy and 27-carboxy metabolites. However, the F215K mutant of this enzyme also produces small amounts of the 24-hydroxy and 25-hydroxy metabolites [70]. This regiospecificity relaxation is consistent with the hypothesis that 27-hydroxylation is imposed by close steric constraints on the substrate and that relaxation of these constraints by the mutation allows some access to the more reactive C24 and C25 C-H bonds.

5. Conclusions

To achieve preferential ω -hydroxylation of hydrocarbon chains, cytochrome P450 enzymes must physically constrain their substrates so that the more facile (ω -1)-hydroxylations become disfavored. Analysis of all the ω -hydroxylation data indicates that this is achieved by steric interactions between the substrate and the protein residues rather than by an alteration of the reactivity of the ferryl species. The two crystal structures now available of *M. tuberculosis* enzymes that catalyze the ω -hydroxylation of methyl-branched hydrocarbon chains reveal two approaches to controlling the regiochemistry of branched chain oxidation. In one, the branching methyl located adjacent to the terminal methyl is specifically bound in a lipophilic cavity, locking the chain terminus in place and positioning the other terminal methyl for oxidation. In the second, a substrate-specific enzyme grips its sterol substrate tightly and places two residues that block further displacement of the sterol into the active site. These interactions position the terminal methyl group and suppress access to the (ω -1)-C-H bond.

As no crystal structures are yet available for a P450 enzyme that catalyzes the ω -hydroxylation of a linear hydrocarbon chain, indirect evidence must be used to draw inferences about the nature of regiochemical control in these proteins. The almost universal observation of (ω -1)-hydroxylation as a minor reaction, the dependence of the ω /(ω -1)-ratio on the hydrocarbon and fatty acid chain length, and the shift to (ω -1)-hydroxylation when the terminal methyl is trideuterated, indicate that the ω -regiospecificity is not absolute. However, the fact that the halide atom in 12-chloro- and 12-bromododecanoic acid is oxidized to the halonium ion emphasizes the extent to which the ferryl species is constrained to oxidize the terminal atom of the chain. The evidence that the intramolecular isotope effect

in the oxidation of probes such as 4,4'-dideuterobiphenyl by CYP4B1 are highly suppressed, the general failure to oxidize the terminus when it is a cyclopropyl or bulkier group, and the fact that 12-iodododecanoic acid is not oxidized even though the bromo- and chloro-analogues are, suggests that the ω -regiospecificity is imposed, at least in part, by steric barriers that make access of the ferryl species to any part of the hydrocarbon chain other than the terminal methyl difficult, but not impossible. It is likely that the control of the regiospecificity in CYP4 enzymes is largely centered, as in CYP12A1, near the hydroxylation site because the variable length of the fatty acid chain does not allow tight control of the bound substrate. However, a clear delineation of how this is achieved must await determination of the substrate-bound crystal structure of a relevant ω -hydroxylase.

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Fig. 1.

The cytochrome P450 catalytic cycle with the Compound I-like ferryl species highlighted by a blue square. The heme is represented by the iron between two bars, which stand for the porphyrin framework. RH is a hydrocarbon substrate, and ROH its alcohol product. Reproduced with permission from reference [2].

Fig. 2.

Schematic outline of the hydroxylation of a terminal methyl group. The heme is abbreviated as two solid bars, the iron oxidation state is shown, as is a radical cation on the porphyrin in the first structure.

Fig. 3.

Benzylic hydroxylation of tolbutamide by CYP2C9 and O-demethylation of 4-methoxyacetanilide by CYP1A2 do not conform to the definition here of ω -hydroxylation. Furthermore, the oxidations of terfenadine by CYP2J2 and farnesol by a variety of enzymes occur on what is formally the ω -position of a hydrocarbon chain. However, there is no possible competition between the ω - and an (ω -1)-sites in these reactions, in addition to the fact that the farnesol terminal C-H bond is activated by the double bond. These reactions are also not considered as ω -hydroxylations for the present purpose.

Fig. 4. The oxidation of phytanic acid and 11-methyldodecanoic acid by CYP124, and γ -tocopherol by CYP4F2, are examples of the ω -hydroxylation of terminally branched hydrocarbons.

Fig. 5.
The ω -hydroxylation of the side-chains of cholesterol and cholest-4-en-3-one by enzymes such as CYP125. The numbering of the carbons at the chain terminus is indicated.

Fig. 6. Lauric acid analogues with modifications of the hydrocarbon terminus, some of which are substrates of CYP4A1 and some of which are not.

Fig. 7.

The oxidation of 12-halododecanoic acids by enzymes that normally ω -hydroxylate fatty acids. In this instance, the terminal atom is a halogen. Although halogens are not usually oxidized by P450 enzymes due to their high electron affinities, they are oxidized in the 12-halododecanoic acids because the competition for other oxidizable sites is limited. Oxidation of the Cl and Br, but not I, analogues suggests that the channel that gives access to the ferryl is sterically limited and allows Cl and Br, but not I, ready access.

Fig. 8.

The structure of the covalently bound heme in CYP4 P450 enzymes (left) and a schematic model of how tethering of the heme to the protein might rigidify the active site to promote ω -hydroxylation (right). In the figure on the right, the heme is shown schematically as a square of nitrogens with an iron center, and the link between a heme methyl group and an Asp or Glu residue of the protein as a black line. The curved arrow negated by the x indicates that motion of the heme is restricted by the covalent heme-protein bond. A schematic of a hypothetical narrow channel is shown to represent a sterically restricted access channel through which the terminal methyl has access to the ferryl species.

Fig. 9.

Computer model of the CYP4A3_2 chimera based on a homology model of CYP4A11. Residues 1-119 from CYP4A2 are in green, and the residues from CYP4A3 are in blue. The side chains in red indicate the residues that differ in CYP4A2 and CYP4A3. The three yellow residues are the Ser-Gly-Ile present in CYP4A3 but not CYP4A2. The heme group and Arg96, a possible binding site for the fatty acid carboxylic acid, are in black. On the bottom, a computer model of CYP4A11 shows the proposed location of the three residues (His117, Gly118, and Ser119) shown by site-specific mutagenesis to control the hydroxylation regioselectivity. The top figure is reproduced, with permission, from reference [20] and that on the bottom from reference [69].

Fig. 10.

The crystal structure of phytanic acid bound in the active site of *Mycobacterium tuberculosis* CYP124A1. Phytanic acid is in green, the heme iron in red, and the heme group in orange. The volumes of open cavities available for additional substrate binding are indicated. On the right is a schematic representation of the binding of one methyl in a cavity that positions the other methyl for oxidation. The panel on the left is reproduced from reference [39].

Fig. 11.

The crystal structure of cholest-4-en-3-one bound in the active site of *Mycobacterium tuberculosis* CYP125A1, based on reference [48]. The superimposed structures on the left present two orthogonal slices through the protein that show the tight packing of the ligand in the active site. The panel on the right, reproduced with permission, shows how the ligand is bound so that the two terminal methyls of the sterol side chain are placed where they can be preferentially hydroxylated. It also shows how Val115 and Met200 interact with the 18- and 19-methyl groups of the sterol, preventing it from sliding closer to the heme group.

Table 1

C-H bond strengths [1]

Bond	kcal/mol	Bond	kcal/mol
CH ₃ -H	104	HC≡CCH ₂ -H	88
Me ₂ CH-H	95	CH ₃ OCH ₂ -H	94
Me ₃ C-H	92	H ₂ NCH ₂ -H	89
CH ₂ =CH ₂ CH ₂ -H	89	CH ₂ =CH-H	108
C ₆ H ₅ CH ₂ -H	85		

Table 2

Comparison of the ω : ω -1 regioselectivity of some rat and human CYP4A enzymes for several fatty acids [51].

Fatty Acid	CYP4A1	CYP4A2	CYP4A3 ω : ω -1	CYP4A8	CYP4A11
Lauric (C12)	40 : 1	6 : 1	3 : 1	2.5 : 1	17 : 1
Myristic (C14)	3 : 1	3 : 1	1.2 : 1	1.6 : 1	4 : 1
Palmitic (C16)	1 : 1	-	-	1.6 : 1	2.2 : 1
Arachidonic (C20)	6 : 1	2.4 : 1	2 : 1	not done	not done

Table 3

Hydroxylation of methyl branched hydrocarbons, hydrocarbon acids, and unbranched fatty acids by *Mycobacterium tuberculosis* CYP124 [39]

	Chemical structure	Dissociation constant K_D (μM)	Specific activity ($\text{nmol min}^{-1} \text{nmol}^{-1}$)
Lauric acid		> 100	not detected
Palmitic acid		> 100	0.07 ± 0.03^d
Arachidic acid		not detected	not detected
15-Methyl palmitic acid		1.01 ± 0.07	7.6 ± 1.5
Phytanic acid		0.22 ± 0.006	9.9 ± 2.7
Phytane		205 ± 14	not detected
Pristane		178 ± 18	not detected