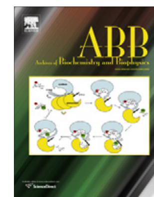
Archives of Biochemistry and Biophysics 507 (2011) 86–94

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi



Review

Structural control of cytochrome P450-catalyzed ω-hydroxylation

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A R T I C L E   I N F O

Article history:
Available online 19 August 2010

Keywords:
Cytochrome P450
ω-Hydroxylation
CYP4 family
Carbon hydroxylation
Fatty acids
Branched-chain hydrocarbon acids
Cholesterol degradation

A B S T R A C T

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.

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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 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 ($C–H \rightarrow C^{\cdot} + H^{\cdot}$) [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

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doi:10.1016/j.abb.2010.08.011

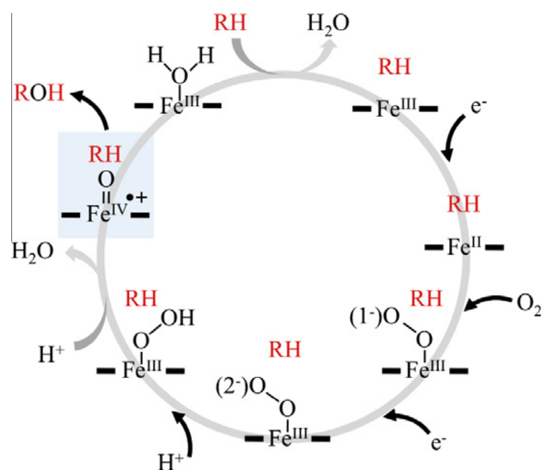

Fig. 1. The cytochrome P450 catalytic cycle with the Compound I-like ferryl species highlighted by a blue square. The heme is represented by the iron between two bars, which stand for the porphyrin framework. RH is a hydrocarbon substrate, and ROH its alcohol product. Reproduced wtih permission from Ref. [2].

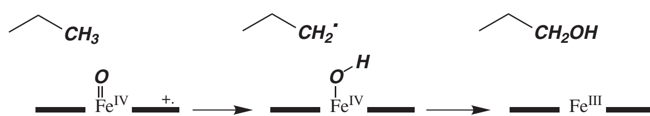

Fig. 2. Schematic outline of the hydroxylation of a terminal methyl group. The heme is abbreviated as two solid bars, the iron oxidation state is shown, as is a radical cation on the porphyrin in the first structure.

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.

Table 1
C–H bond strengths [1].

Bond	kcal/mol
CH_3–H	104
Me_2CH–H	95
Me_3C–H	92
CH_2=CH_2CH_2–H	89
$C_6H_5CH_2$–H	85
HC≡CCH_2–H	88
CH^3OCH_2–H	94
H_2NCH_2–H	89
CH_2=CH–H	108

Enzymes that predominantly perform ω-hydroxylation

Hydrocarbons

Bacterial P450 enzymes are known to oxidize linear hydrocarbons of C_5–C_{15} 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 oxidizes the terminal methyl groups of linear hydrocarbons. A typical example is CYP52A3 from *C. 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 ω/(ω−1)-hydroxylation ratio that ranges from 23 for heptane to 1.6 for decane [17].

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 (ω−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 *C. maltosa* [34], CYP52A13 from *C. tropicalis* [35], and both CYP52A17 and CYP52A21 from *Candida albicans* [36] are ω-selective or ω-specific fatty acid hydroxylases.

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

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