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FUNCTIONAL AND EVOLUTIONARY RELATIONSHIPS AMONG DIVERSE OXYGENASES

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Abstract

Oxygenases that incorporate one or two atoms of dioxygen into substrates are found in many metabolic pathways. In this article, representative oxygenases, principally those found in bacterial pathways for the degradation of hydrocarbons, are reviewed. Monooxygenases, discussed in this chapter, incorporate one hydroxyl group into substrates. In this reaction, two atoms of dioxygen are reduced to one hydroxyl group and one H_2O molecule by the concomitant oxidation of NAD(P)H. Dioxygenases catalyze the incorporation of two atoms of dioxygen into substrates. Two types of dioxygenases, aromatic-ring dioxygenases and aromatic-ring-cleavage dioxygenases, are discussed. The aromatic-ring dioxygenases incorporate two hydroxyl groups into aromatic substrates, and *cis*-diols are formed. This reaction also requires NAD(P)H as an electron donor. Aromatic-ring-cleavage dioxygenases incorporate two atoms of dioxygen into aromatic substrates, and the aromatic ring is cleaved. This reaction does not require an external reductant.

All the oxygenases possess a cofactor, a transition metal, flavin or pteridine, that interacts with dioxygen. The concerted reactions between dioxygen and carbon in organic compounds are spin forbidden. The cofactor is used to overcome this restriction.

For the oxygenases that require the NAD(P)H cofactor, the enzyme reaction is separated into two steps, the oxidation of NAD(P)H to generate two reducing equivalents, and the hydroxylation of substrates. Flavoprotein hydroxylases that catalyze the monohydroxylation of the aromatic ring carry out these two reactions on a single polypeptide chain. In other oxygenases, the NAD(P)H oxidation and a hydroxylation reaction are catalyzed by two separate polypeptides that are linked by a short electron-transport chain. Two reducing equivalents generated by the oxidation of NAD(P)H are transferred through the electron-transport chain to the cofactor on a hydroxylase component that they reduce. Dioxygen couples with the reduced cofactor and subsequently hydroxylates substrates. The electron-transport chains associ-

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ated with oxygenases contain at least two redox centers. The first redox center is usually a flavin, while the second is an iron-sulfur cluster. The electron transport is initiated by a single two-electron transfer from NAD(P)H to a flavin, followed by two single-electron transfers from the flavin to an iron-sulfur cluster.

The primary sequences of many oxygenases have been determined, and according to their sequence similarities, the oxygenases can be grouped into several protein families. Among proteins of the same family, the sequences in regions involved in cofactor binding are strongly conserved. Local sequence similarities are also observed among oxygenases from different families, primarily in regions involved in cofactor binding.

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INTRODUCTION

Dioxygen can be incorporated directly into organic compounds in reactions catalyzed by enzymes known as oxygenases or hydroxylases. These enzymes utilize transition metals or reduced flavin/pteridine to activate dioxygen, which in its ground state is unreactive. In these processes, highly reactive forms of oxygen, such as singlet oxygen and hydroxyl radicals, may be generated. The threat of reactive oxygen species has been proposed as one of the reasons why the use of dioxygen in general metabolism is limited, and why most biochemical oxidations are carried out by the transfer of electrons and/or reducing equivalents to FAD or $NAD(P)^+$, instead (17, 89).

Oxygenases play significant roles in microbial catabolic pathways. They initiate the degradation of aromatic compounds both by hydroxylating the aromatic ring in preparation for ring-cleavage and by catalyzing the ring-fission reaction (49). The initial reaction of alkane degradation is also catalyzed by an oxygenase. Microbial oxygenases in these pathways have been extensively studied in order to understand their reaction mechanisms, specificity, and regulation. These studies are also relevant to the application of microbial enzymes in environmental and industrial biotechnology.

Oxygenases in higher organisms, while less abundant, are by no means less important than those found in microorganisms. Mammalian oxygenases are involved in the hydroxylation of steroids, in the synthesis of neurotransmitters, and in the detoxication of poisonous compounds. Not all oxygenases, however, carry out essential, or even desirable, reactions. For example, plant lipoxygenases that oxidize polyunsaturated fatty acids in lipids have been studied because they produce both pleasant and unpleasant flavors in edible plant products (84).

The catabolic versatility of microbes plays an essential role in the carbon cycle and depends on the use of oxygenases in the initiation of degradative pathways. Recent studies have shed light on the structures and reaction

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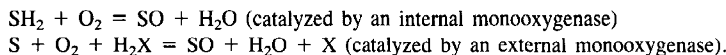
mechanisms of some of these enzymes. Biochemical and biophysical studies have been combined with molecular genetic techniques. The deduced amino acid sequences of numerous oxygenases can now be compared. Also, patterns of evolutionary relationships are emerging, and concepts of gene families have developed, from these comparisons as well as from other studies. A discussion of every known oxygenase is beyond the scope of this review. Here, we focus on microbial oxygenases involved in the utilization of organic growth substrates, although selected mammalian enzymes related to their microbial counterparts are included.

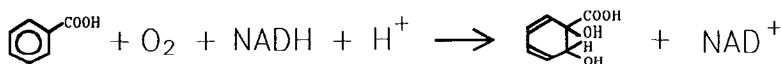
GENERAL PROPERTIES OF OXYGENASES

Oxygenases that catalyze the incorporation of only one atom of dioxygen into substrates (S) are termed monooxygenases, also referred to as *mixed-function oxygenases*. The second atom of dioxygen is reduced to H_2O either by the substrates (S) themselves or by a cosubstrate reductant (H_2X):

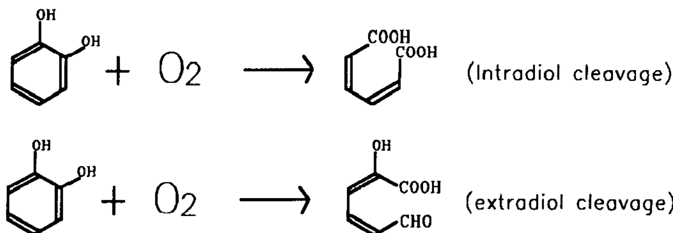


$SH_2 + O_2 = SO + H_2O$ (catalyzed by an internal monooxygenase)
$S + O_2 + H_2X = SO + H_2O + X$ (catalyzed by an external monooxygenase).

Oxygenases that catalyze the incorporation of both atoms of dioxygen in their substrates are known as dioxygenases. In this article, we discuss two types of dioxygenases, aromatic-ring dioxygenases and aromatic-ring-cleavage dioxygenases. The aromatic-ring dioxygenases incorporate two hydroxyl groups on the aromatic ring at the expense of dioxygen and NAD(P)H, e.g.:



The aromatic-ring-cleavage dioxygenases open the aromatic ring by incorporating two atoms of dioxygen into substrates, e.g.:



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Both monooxygenases and dioxygenases require cofactors capable of reacting with dioxygen because concerted reactions are spin-forbidden between paramagnetic dioxygen, with two unpaired electron spins, and carbon in organic compounds, which exists in a singlet state. Reactions of dioxygen with transition metals, which have incompletely filled *d* orbitals, are not spin-forbidden. Diamagnetic metal-oxygen complexes may be formed that can subsequently react with organic substrates (44).

Transition metals, such as iron, serve as catalysts in numerous oxygenases. Some oxygenases utilize nonheme and non-iron-sulfur Fe(II) or Fe(III), whereas others are heme enzymes. In some cases, other transition metals such as manganese, copper, and cobalt are used to bind dioxygen. Alternatively, or in addition to metal ions, flavins or pteridines may serve as cofactors in monooxygenases. These cofactors can exist in semiquinone forms in which the spin of one electron can be inverted (29). The semiquinones of flavins and pteridines can react with dioxygen, resulting in a covalently bound peroxide intermediate. In certain reactions initiated by Fe(II)-containing monooxygenases, α-ketoglutarate acts as a decarboxylating cosubstrate and ascorbic acid reduces the metal-containing catalyst (78).

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AROMATIC-RING HYDROXYLASES

In the aerobic degradation of aromatic hydrocarbons, substrates are degraded via the cleavage of the aromatic ring by dioxygenases. Substrates of the aromatic-ring-cleavage dioxygenases possess two hydroxyl groups on the aromatic ring, either *ortho* or *para* to each other. One of the first steps in the degradation of the aromatic compounds, therefore, involves the introduction of one or two hydroxyl groups on the aromatic ring (49). Introduction of single hydroxyl groups on the aromatic ring, monohydroxylation, is generally catalyzed by monooxygenases, while simultaneous introduction of two hydroxyl groups, dihydroxylation, is catalyzed by dioxygenases. The majority of monooxygenases catalyzing monohydroxylation of the aromatic ring of substituted phenols are single-component enzymes, although multicomponent monooxygenases, such as phenol and toluene-4 monooxygenase have also been found.

p-*Hydroxybenzoate Hydroxylase, a Flavoprotein Monooxygenase*

The structure and reaction mechanisms of *p*-hydroxybenzoate hydroxylase have been extensively characterized (22, 117). Its reaction cycle is initiated by binding the substrate to the enzyme. The substrate-binding provokes a conformational change that increases the rate of the hydride transfer from

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NADPH to the N5 position of the flavin isoalloxazine ring 10^5-fold. NADPH is oxidized and $FADH_2$ is formed. Dioxygen then binds to $FADH_2$ and is reduced by two single-electron transfers. The first electron transferred from the $FADH_2$ to dioxygen may produce a superoxide anion that may then be transformed to 4a-hydroperoxy-flavin by the second electron transfer. Unreactive dioxygen is thus converted to reactive 4a-hydroperoxy-flavin, which attacks the substrate aromatic ring.

In the next step, 4a-hydroperoxy-flavin is converted to a short-lived intermediate, and the substrate is hydroxylated. The chemical structure of this short-lived intermediate has not yet been identified (131, 132). If a substrate analog that does not possess an electron-donating substituent on the aromatic ring, such as benzoate or 6-hydroxynicotinate, is bound to the enzyme, the enzyme reaction is uncoupled: 4a-hydroperoxy-flavin is formed, but the substrate is not hydroxylated. The peroxide species on FAD then slowly decays to form hydrogen peroxide. This observation suggests that the hydroxylation reaction proceeds via an electrophilic attack of activated oxygen on the 3-position of the substrate. The phenolate form of the substrate is believed to be accessible to the electrophilic attack of activated oxygen (22). The products of the hydroxylation are 3,4-dihydroxybenzoate and flavin 4a-hydroxide. The latter compound is unstable, and spontaneously converts to FAD (116).

The 4-hydroxyl group of the substrate is hydrogen-bonded to the hydroxyl group of Tyr201 of the enzyme, which also hydrogen bonds with the hydroxyl group of Tyr385. Site-directed mutagenesis was used to examine the role of this hydrogen-bond network by substituting phenylalanine residues for each of these tyrosines. Both substitutions reduced the reduction rate of FAD by NADPH. In addition, substrate hydroxylation was uncoupled in the Tyr201-Phe mutant enzyme. Tyr201 therefore is assumed to stabilize the phenolate form of the substrate. The Tyr385-Phe mutant not only hydroxylates *p*-hydroxybenzoate, but also 3,4-dihydroxybenzoate. Therefore, Tyr385 appears to be a determinant of the substrate specificity (22).

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Structural Relationships of Aromatic Flavoprotein Monooxygenases

The flavoprotein monooxygenases, exemplified by *p*-hydroxybenzoate hydroxylase, are classified into several subgroups according to their subunit sizes. 4-Hydroxybenzoate, 4-hydroxyphenylacetate 3, and salicylate hydroxylases are approximately 45 kilodaltons (kDa) in size (112, 116, 150), whereas other hydroxylases weigh between 60 and 80 kDa (100, 105, 151) (Table 1). Most flavoprotein monooxygenases incorporate a new hydroxyl group *ortho* to the existing hydroxyl group. Enzymes that catalyze *para* hydroxylation are also known. 3-Hydroxybenzoate-6-hydroxylase from

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Table 1 Families of hydroxylase components

Enzyme	Organism or plasmid	Subunit size, cofactor	Reference
Aromatic-ring monooxygenases			
p-Hydroxybenzoate hydroxylase family		45 kDa, FAD	
p-Hydroxybenzoate 3-hydroxylase (EC 1.14.13.2)	*Pseudomonas fluorescens*		116
Salycilate hydroxylase (EC 1.14.13.1)	NAH7		149
Phenol 2-hydroxylase family		65 kDa	
2-4-Dichlorophenol 6-monooxygenase (EC 1.14.13.20)	pJP4		105
Phenol 2-hydroxylase (EC 1.14.13.7)	*Pseudomonas* EST1001		100
Unclassified flavoprotein monooxygenases			
o-Nitrophenol hydroxylase	*Pseudomonas putida* B2	65 kDa, FAD?	151
4-Hydroxyphenylacetate 3-Hydroxylase (EC 1.14.13.3)	*Pseudomonas putida*	44 kDa, FAD	112
2,4-Dichlorophenoxyacetate monooxygenase	pJP4	32 kDa	127
3-Hydroxybenzoate-6-hydroxylase	*Pseudmonas cepacia*	44 kDa, FAD	140
3-Hydroxybenzoate-6-hydroxylase	*Micrococcus* sp.	70 kDa, FAD	111
Multicomponent phenol 2-hydroxylase family		Similarity to methane monooxygenase 34 kDa + 10 kDa + 58 kDa + 10 kDa, Fe(II)	
Phenol 2-hydroxylase (EC 1.14.13.7)	*Pseudomonas* CF600		98
Toluene 4-monooxygenase	*Pseudomonas mendocina*		148
Aromatic-ring dioxygenases			
Benzene 1,2-dioxygenase family		(50 kDa, 20 kDa)$_n$, Fe(II) + Rieske-type [2Fe-2S]	
Benzene 1,2-dioxygenase (EC 1.14.12.3)	*Pseudomonas putida*		56

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Table 1 *Continued*

Enzyme	Organism or plasmid	Subunit size, cofactor	Reference
Toluene 2,3-dioxygenase	*Pseudomonas putida* F1		154
Benzoate 1,2-dioxygenase	*Acinetobacter calcoaceticus*		94
Toluate 1,2-dioxygenase	TOL		46
Naphthalene 1,2-dihydroxylase	NAH		66
Unclassified aromatic-ring dioxygenases			
Phthalate 4,5-dioxygenase (EC 1.14.12.7)	*Pseudomonas cepacia*	(50 kDa)$_4$, Fe(II) + Rieske-type [2Fe-2S]	8
4-Sulfonobenzoate 3,4-dioxygenase	*Comamonas testosteroni*	(50 kDa)$_2$, Fe(II) + Rieske-type [2Fe-2S]	71
4-Chlorophenylacetate 3,4-Dioxygenase	*Pseudomonas* CBS	(50 kDa)$_3$, Rieske-type [2Fe-2S]	80
Aromatic-ring-cleavage dioxygenases			
Catechol 2,3-dioxygenase I family		34 kDa, Fe(II)	
Catechol 2,3-dioxygenase I (EC 1.13.11.2)	TOL		88
2,3-Dihydroxybiphenyl dioxygenase	*Pseudomonas paucimobilis*		130
1,2-Dihydroxynaphthalene dioxygenase	NAH		45
Protocatechuate 4,5-dioxygenase family			
Protocatechuate 4,5-dioxygenase (EC 1.13.11.8)	*Pseudomonas paucimobilis*	(34 kDa, 15 kDa)$_n$, Fe(II)	91
Catechol 2,3-dioxygenase II (EC 1.13.11.2)	*Alcaligenes eutrophus*	(34 kDa)$_n$	60
Homoprotocatechuate dioxygenase	*Escherichia coli*	(30 kDa)$_n$	114
Catechol 1,2-dioxygenase family			
Catechol 1,2-dioxygenase I (EC 1.13.11.1)	*Pseudomonas arvilla*	(32 kDa, 30 kDa)$_n$, (30 kDa)$_n$, (32 kDa)$_n$	
Catechol 1,2-dioxygenase II (EC 1.13.11.1)	pP51	(28 kDa)$_n$	137

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Protocatechuate 3,4-dioxygenase (EC 1.13.1.3)	*Acinetobacter calcoaceticus*	(23 kDa, 27 kDa)$_n$	52
Gentisate 1,2-dioxygenase (EC 1.13.11.4)	*Comamonas testosteroni*	(40 kDa)$_4$, Fe(II)	50
Alkyl group hydroxylase			
Alkane hydroxylase family		41 kDa, Membrane-bound, Fe(II)	
Alkane hydroxylase (EC 1.14.15.3)	ALK		65
Xylene monooxygenase	TOL		129
Methane monooxygenase	*Methylosinus trichosporium*	Component A = (54 kDa, 43 kDa, 23 kDa)$_2$, component B (= regulatory protein 16 kDa), binuclear iron cluster on component A	27
Vanillate demethylase	*Pseudomonas* sp.	(37 kDa)$_n$	13
Unclassified			
4-Methoxybenzoate monooxygenase (putidamonooxin) (EC 1.14.99.15)	*Pseudomonas putida*	(40 kDa)$_3$, Fe(II) + Rieske-type [2Fe 2S]	142
Toluene sulfonate methyl-monooxygenase	*Comamonas testosteroni*	(43 kDa)$_3$ or (43 kDa)$_4$	72
Cytochrome P-450 family			
Camphor 5-monooxygenase (EC 1.14.15.1)	CAM		109
Mitochondrial P-450	Eukaryotes		92
Microsomal P-450	Eukaryotes		92
Cytochrome P-450$_{bm\text{-}3}$	*Bacillus megaterium*	120 kDa, bifunctional (hydroxylase + electron-transport components)	68
Aromatic amino acid hydroxylase family		Fe(II), biopterin	
Phenylalanine 4-hydroxylase (EC 1.14.16.1)	Eukaryotes	52 kDa	35
Tyrosine 3-hydroxylase (EC 1.14.16.2)	Eukaryotes	59 kDa	35
Tryptophane hydroxylase (EC 1.14.16.4)	Eukaryotes	51 kDa	35

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Pseudomonas cepacia is a monomer of 44 kDa whereas that from *Micrococcus* sp. has a molecular weight of 70,000 (111, 140).

Salicylate hydroxylase encoded on the NAH7 plasmid of *Pseudomonas putida,* which catalyzes the oxidative decarboxylation of 2-hydroxybenzoate, shares 25% amino acid sequence identity with *p*-hydroxybenzoate hydroxylase (149). The strongest sequence conservation is observed in and adjacent to the FAD-binding regions.

Phenol hydroxylase from the yeast *Trichosporon cutaneum* (118), 2,4-dichlorophenol hydroxylase encoded on the pJP4 plasmid of *Alcaligenes eutrophus* (105), and phenol hydroxylase from *Pseudomonas* species ES-T1001 (100) are 20–30 kDa heavier than *p*-hydroxybenzoate and salicylate hydroxylases. The dichlorophenol and phenol hydroxylases share 46% amino acid sequence identity, indicating that they are evolutionarily related.

Local sequence similarities between the group of dichlorophenol and phenol hydroxylases and that of *p*-hydroxybenzoate and salicylate hydroxylases are observed in two regions. In the first of these, at the amino-terminal regions of all four enzymes, a conserved amino acid sequence pattern forms an ADP-binding fingerprint associated with a $\beta\alpha\beta$ fold (144). In *p*-hydroxybenzoate hydroxylase, the conserved amino-terminal region binds the ADP portion of FAD (116). The global sequence similarity between *p*-hydroxybenzoate and salicylate hydroxylases indicates that the corresponding region in salicylate dehydrogenase also binds the ADP portion of FAD. In the dichlorophenol and phenol hydroxylases, however, the amino terminal $\beta\alpha\beta$ ADP-binding fold may be involved in NADPH rather than FAD binding because highly conserved asparatate or glutamate residues associated with NADH or FAD binding are absent in these domains.

The second region of homology among these hydroxylases, which corresponds to the amino acid residues, Met276 to Ser329, in *p*-hydroxybenzoate hydroxylase, may be involved in FAD binding because, in the three-dimensional structures of *p*-hydroxybenzoate hydroxylase, this region contains a FAD-binding β-strand (19). The sequence homology among the flavoprotein monooxygenases extends beyond this β-strand. Except for the two indicated regions, the amino acid sequences of phenol and dichlorophenol hydroxylases are different from those of salicylate and *p*-hydroxybenzoate hydroxylases. Possible evolutionary implications of the observed local sequence similarities in these enzymes are discussed below.

Multicomponent Phenol and Toluene-4 Monooxygenases

Two multicomponent aromatic-ring monooxygenases catalyze the first hydroxylation step in the degradation of phenol by *Pseudomonas* species CF600, and of toluene in *Pseudomonas mendocina* KR1 (93, 143). Phenol hydroxylase converts phenol into catechol, while toluene-4 monooxygenase

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catalyzes the insertion of one hydroxyl group into the *para* position on the aromatic ring. These reactions are similar to those catalyzed by flavoprotein monooxygenases, but unlike the single-component flavoproteins, these multi-component enzymes are structurally related to methane monooxygenase, which contains a catalytic binuclear iron center (see below).

Genetic and biochemical analysis indicated that five different polypeptides are required for the phenol hydroxylase activity in vitro, whereas six polypeptides are required for the growth on phenolic substrates (108). One of these polypeptides has been purified and was characterized as an electron-transport component (see below). Genetic complementation and DNA sequencing analysis showed that, at least five, and probably six polypeptides are required for the activity of toluene-4 monooxygenase. Two of these, encoded by *tmoA* and *tmoE*, are presumably part of the terminal oxygenase (148), while the product of *tmoC* may be an electron-transport component (see below). The functions of two other polypeptides have not yet been identified. Interestingly, three components of toluene-4 monooxygenase were found to share significant sequence identity with three components of phenol hydroxylase (148).

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AROMATIC-RING DIOXYGENASES

Bacterial aromatic-ring dioxygenases catalyze the dihydroxylation of the aromatic ring (30). In this reaction, both atoms of dioxygen are incorporated into substrates, and two hydroxyl groups are formed on the aromatic ring. The product is (substituted) *cis*-1,2-dihydroxycyclohexadiene, which is subsequently converted to (substituted) benzene glycol by a dehydrogenase (93). Components of bacterial aromatic-ring dioxygenases fall into two different functional classes: hydroxylase components and electron-transport components. In this section, the hydroxylase components are described; their electron-transport components are discussed separately in a later section.

All hydroxylase components of aromatic-ring dioxygenases are oligomers composed of either a single or two different tightly associated subunits in an α_n or $(\alpha\beta)_n$ configuration (Table 1). They possess two common cofactors, a [2Fe-2S] iron-sulfur center and one mononuclear nonheme iron, both of which are associated with the α-subunit in the $(\alpha\beta)_n$-type enzymes (21, 128, 147). The [2Fe-2S] iron-sulfur center has the spectral properties of the so-called Rieske iron-sulfur center that is found in respiratory and photosynthetic electron-transport proteins. In contrast to plant-type ferredoxins, in which four cysteine residues coordinate a [2Fe-2S] redox center, one iron of the Rieske-type [2Fe-2S] center coordinates with two cysteines while the other coordinates with two histidine residues. Upon reduction of the

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Rieske-type [2Fe-2S] center, the iron coordinated with histidine is reduced (38).

In the $(\alpha\beta)_n$-type hydroxylases, the sizes of the two subunits, α and β, are roughly 50 and 20 kDa, respectively. A comparison of the amino acid sequences of the α- and β-subunits of benzoate, toluate, benzene, toluene, and naphthalene dioxygenases suggests that the α- and β-subunits are each derived from common ancestors (94). Alignment of the five α-subunits revealed five invariant histidines, two invariant cysteines, and two invariant tyrosines. The Rieske-type [2Fe-2S] cluster in the α-subunits may be coordinated with the two invariant cysteines and two of the invariant histidines. This [2Fe-2S] redox center probably accepts electrons from an electron-transport component. The binding of dioxygen is believed to be mediated by a mononuclear nonheme iron associated with the α-subunit (147). Some of the invariant tyrosines and histidines that are not involved in the [2Fe-2S] binding may coordinate the mononuclear iron.

The β-subunits share considerably less sequence similarity, although a global amino acid sequence pattern has been conserved. The lower degree of homology among the five β-subunits suggests that these subunits may not be directly involved in the common catalytic functions of the dioxygenases. Indeed, genetic studies indicate that the β-subunit may be important in the determination of substrate specificity (47). This observation suggests that the catalytic center of the hydroxylase component may be formed between the α- and β-subunits, with the α-subunit facing the oxidizable carbons of the substrate and the β-subunit recognizing the substrate structure (94).

The hydroxylase components of 4-sulphobenzoate-3,4-dioxygenase from *Comamonas testosteroni* (71), of phthalate dioxygenase from *Pseudomonas cepacia* (8), and of 4-chlorophenylacetate 3,4-dioxygenase from *Pseudomonas* sp. CBS (80) are all $(\alpha)_n$-type proteins with a subunit of approximately 50 kDa, similar to the α-subunits of the above-mentioned $(\alpha\beta)_n$ type enzymes. Unfortunately, no amino acid sequence information is available for these enzymes.

The hydroxylase component of 4-methoxybenzoate monooxygenase (putidamonooxin, see discussion below) also contains a Rieske-type [2Fe-2S] center and one nonheme iron (20).

AROMATIC-RING-CLEAVAGE DIOXYGENASES

Nearly all bacterial pathways for the degradation of aromatic compounds transform initial substrates into intermediates that carry two or more hydroxyl groups on the aromatic ring. These intermediates are substrates of aromatic-ring-cleavage dioxygenases. If two of the hydroxyl groups on a substrate are in the *ortho* position relative to each other, the ring fission by the aromatic-

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ring-cleavage dioxygenases occurs either between the two hydroxyl groups (intradiol cleavage) or at a bond proximal to one of the two hydroxyl groups (extradiol cleavage). For gentisate and homogentisate that carry two hydroxyl groups in the *para* position relative to each other, the ring cleavage occurs between the carboxyl or acetyl substituent and the proximal hydroxyl group.

Extradiol Cleavage Enzymes

The first aromatic-ring-cleavage dioxygenase for which the primary structure was determined is catechol 2,3-dioxygenase encoded by the *xylE* gene on the TOL catabolic plasmid (88). This enzyme consists of four identical subunits of 32 kDa, and contains one catalytically essential Fe(II) ion per subunit. The reaction product, 2-hydroxymuconic semialdehyde (or a substituted derivative), is yellow. Because of the intense color production, the structural gene for this enzyme, *xylE*, has been used as a reporter gene in molecular biology (41). The enzyme reaction proceeds by an ordered bi-uni mechanism: catechol first binds to the enzyme followed by the binding of dioxygen to form a ternary complex; the aromatic ring is then cleaved to produce 2-hydroxymuconic semialdehyde (99). The substrate range of catechol 2,3-dioxygenase is relatively broad: this enzyme oxidizes 3-methyl-, 3-ethyl-, 4-methyl-, and 4-chlorocatechol. 3-Chloro- and 4-ethylcatechol, in contrast, are not efficiently oxidized by this enzyme. 4-Ethylcatechol is a suicide inhibitor: this compound inactivates the enzyme in a mechanism-based fashion by oxidizing the Fe(II) cofactor to Fe(III). Mutant catechol 2,3-dioxygenases able to metabolize 4-ethylcatechol were obtained, and single amino acid substitutions were responsible for this phenotypic change (113; A. Wasserfallen, P. Cerdan, M. Rekik, K. N. Timmis & S. Harayama, in preparation). 3-Chlorocatechol is also a suicide inhibitor of catechol 2,3-dioxygenase (6). One mutant enzyme resistant to inactivation by 3-chlorocatechol was isolated in which the affinity to 3-chlorocatechol decreased but that to 3-methylcatechol increased.

The electronic structure of Fe(II) in the active site of catechol 2,3-dioxygenase was calculated from the data of magnetic circular dichroism in the near-infrared spectrum of this enzyme (77). Fe(II) apparently has five-coordinate square-pyramidal geometry. Catechol may bind as a bidentate ligand, occupying the axial and one equatorial ligation positions in the active site of the enzyme-substrate complex. The binding of catechol triggers a conformational change such that azide, an analog of dioxygen, can bind to an equatorial ligation site.

The comparison of the amino acid sequences of four catechol 2,3-dioxygenases from *Pseudomonas*, one 1,2-dihydroxynaphthalene dioxygenase, and three 2,3-dihydroxybiphenyl dioxygenases has revealed that they are members of the same superfamily (45). Catechol 2,3-dioxygenase

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from *A. eutrophus,* however, has a primary sequence quite different from those of the above-mentioned family (60). Therefore, catechol 2,3-dioxygenases appear to have at least two independent origins.

Protocatechuate 4,5-dioxygenase catalyzes the extradiol cleavage of protocatechuate. The enzyme consists of an equal number of two different subunits, α and β, 18 and 34 kDa, respectively, and its quaternary structure may be $(\alpha\beta)_2Fe^{2+}$ (4). The amino acid sequences of these subunits are not related to those of the main catechol 2,3-dioxygenase family, but the β-subunit of protocatechuate 4,5-dioxygenase (97) exhibits sequence similarity to that of catechol 2,3-dioxygenase from *A. eutrophus*. The Fe(II) environment of protocatechuate 4,5-dioxygenase from *C. testosteroni* was investigated using EPR spectroscopy (4). In a hypothetical reaction sequence, electron delocalization in the ternary complex, enzyme-Fe(II)-O-O, is assumed to polarize dioxygen, preparing the distal oxygen atom for nucleophilic attack on the aromatic ring of the substrates. The iron-peroxy-substrate intermediate, enzyme-Fe(II)-O-O-S, thus produced initiates a reaction sequence resulting in the ring fission of the substrate.

Homoprotocatechuate dioxygenase is a third kind of extradiol cleavage enzyme whose sequence indicates that it constitutes a discrete class of the extradiol cleavage enzymes (114).

Intradiol Cleavage Enzymes

In contrast to the extradiol cleavage enzymes, which contain Fe(II) as a cofactor, the intradiol cleavage enzymes, catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase, contain a nonheme, non-iron-sulfur Fe(III) as a prosthetic group. Catechol 1,2-dioxygenases from many bacteria consist of nonidentical α- and β-subunits, $[\alpha\beta\text{-}Fe^{3+}]_n$, whereas those from other bacteria consist of single polypeptide species $[\alpha\alpha\text{-}Fe^{3+}]_n$. One *Pseudomonas* species produces two types of catechol 1,2-dioxygenase polypeptides, α and β, and three isozymes, $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ (87). Catechol 1,2-dioxygenase exhibits little or no activity towards chlorocatechols and is also called catechol dioxygenase I in order to distinguish it from chlorocatechol 1,2-dioxygenase (catechol dioxygenase II) found in degradative pathways for chlorinated aromatic compounds. The latter enzyme exhibits broader substrate specificity and cleaves both catechol and chlorocatechols. Type II catechol 1,2-dioxygenases have increased specificity for halogenated substrates. Only subtle differences in enzymatic activities have been found between type II catechol 1,2-dioxygenases (12, 137). The type I and II enzymes show a global sequence similarity (52).

Protocatechuate 3,4-dioxygenases thus far characterized contain equal numbers of two different subunits, α and β, and form different quaternary structures of $(\alpha\beta)_n$ (n = 3–12). The similarity in the primary sequences of

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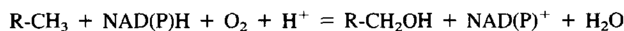
catechol 1,2-dioxygenases I and II and of the α- and β-subunits of protocatechuate 3,4-dioxygenases indicates that these enzymes are derived from a common ancestor. The three-dimensional structure of the $(\alpha\beta)_2$-type protocatechuate 3,4-dioxygenase has been determined (102). The active site of the enzyme is located at the interface of the two structurally related subunits. Ferric ion is coordinated in the β-subunit to two tyrosine and two histidine residues in a near trigonal bypyramidal configuration. The fifth iron-coordination position is occupied by a solvent molecule. The α-subunit does not bind iron, and only two of the residues corresponding to the iron ligands on the β-subunit have been conserved. Protocatechuate-3,4-dioxygenase from *Brevibacterium fuscum* was used to examine changes in the Fe(III) coordination environment in the course of catalysis, leading to conclusions similar to those from the X-ray crystallographic study (134). Four amino acid ligands and most probably one hydroxyl ion are coordinated with Fe(III) in the free enzyme. Upon binding of the substrate to the active-site Fe(III), the hydroxyl ion and one histidine ligand may be displaced. Dioxygen subsequently attacks the activated aromatic ring of the substrate leading to aromatic-ring fission and the introduction of two oxygen atoms into the substrate.

Gentisate 1,2-Dioxygenase

Gentisate is a metabolite formed from anthranilate, β-naphthol, 3- and 4-hydroxybenzoate, salicylate, flavonones, and naphthalene disulfonate. Its aromatic-ring-cleavage enzyme, gentisate 1,2-dioxygenase, has been purified from *Pseudomonas acidovorans* and *C. testosteroni* and consists of a single polypeptide species of about 40 kDa with a quaternary structure of $(\alpha Fe^{2+})_4$. This enzyme contains a Fe(II) cofactor. Results of EPR studies suggest that H_2O coordinates with Fe(II) and that gentisate binds directly to the iron cofactor through two coordination bondings using one oxygen atom of the carbon-1 carboxylate and the oxygen atom of the carbon-2 hydroxyl group (50, 51).

ALKYL-GROUP HYDROXYLASES

Hydroxylation of an alkyl group is often the first step in the complete degradation of organic compounds. Such reactions are usually catalyzed by monooxygenases.



$$R\text{-}CH_3 + NAD(P)H + O_2 + H^+ = R\text{-}CH_2OH + NAD(P)^+ + H_2O$$

Alkyl-group monooxygenases are usually multicomponent and consist of a hydroxylase component and one or two electron-transport component(s). The hydroxylation occurs on the hydroxylase component while two reducing

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equivalents, [H], required for the hydroxylation reaction are generated by the electron-transport component. One can classify alkyl-group monooxygenases in several distinct categories according to their primary structures and/or biochemical properties. Cytochrome P-450s (discussed in the next section) comprise one of these categories. In this section, we describe hydroxylase components of alkyl-group hydroxylases other than cytochrome P-450.

Xylene Monooxygenase and Alkane Hydroxylase

The TOL plasmid of *Pseudomonas putida* encodes a well-characterized catabolic pathway for the degradation of toluene/xylenes. The first enzyme of the pathway is xylene monooxygenase, which oxidizes toluene and xylenes to (methyl)benzyl alcohols. This enzyme is composed of two different polypeptides that are encoded by *xylM* and *xylA* (48, 129). Nucleotide sequence analysis of the *xylMA* genes indicated that one of the gene products (XylM) shares significant amino acid sequence identity (27%) with the membrane-associated hydroxylase component of the alkane hydroxylase from *P. putida* (*oleovorans*) (65). The sequence of XylA indicates that it plays a role in electron transport (see below).

Alkane hydroxylase from *P. putida* catalyzes the hydroxylation of the terminal carbon of alkanes and the omegahydroxylation of fatty acids. This enzyme consists of three different polypeptides: the membrane-bound hydroxylase component encoded by *alkB* and two other polypeptides that together constitute an electron-transport chain (see below). The AlkB hydroxylase component has been purified, and Fe(II) and phospholipids were found to be required for its activity (115). Similarly, the XylM protein, the hydroxylase component of xylene monooxygenase, is membrane-bound and requires Fe(II) for its activity (J. P. Shaw & S. Harayama, unpublished).

Despite the observed structural similarities between the hydroxylase components of both enzymes, alkane hydroxylase and xylene monooxygenase do not have any substrates in common. Alkane hydroxylase seems to have a broader substrate specificity than xylene monooxygenase (145). All substrates are hydroxylated at the terminal carbon atom (62).

Methane Monooxygenase

Soluble methane monooxygenases from two obligate methylotrophs, *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, convert methane to methanol at the expense of NADH. These enzymes exhibit broad substrate specificity; they can hydroxylate a variety of alkanes, haloalkanes, alkenes, and aromatic and heterocyclic compounds (14, 34). They consist of three components, A, B, and C, which have been purified. Component A, the hydroxylase component of the enzyme, comprises three subunits: α (54 kDa), β (44 kDa), and γ (20 kDa) with the configuration of $(\alpha\beta\gamma)_2$. The EPR

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spectrum of the partially reduced component showed that two irons are connected via a μ-oxo bridge (bridge made by a carboxylic group: Fe-O-C-O-Fe). Each subunit contains two binuclear iron clusters (27).

NADH oxidation could be achieved by combining component A and component C (the electron-transport component, see below) in vitro. This preparation, however, did not catalyze the hydroxylation of substrates. For the coupling between NADH oxidation and substrate hydroxylation, component B (16 kDa) is required. Addition of component B prevents uncoupled NADH oxidation in the absence of substrates. Component B has no redox-active metals or cofactors, and therefore is considered to be an effector of component A (28).

The reaction cycle of the (A+B+C) holoenzyme is initiated by substrate binding to component A. The substrate binding changes the environment of the iron center in component A, and hence its redox potential. The redox potential of the [Fe(II) Fe(II)/Fe(III) Fe(II)] couple in component A is $<$ -200 mV in the absence of the substrate, but becomes $>$150 mV in the presence of the substrate (70). The substrate binding therefore facilitates the reduction of the iron center by component C. A change in the redox potential of the catalytic iron after the substrate-binding is also observed in P-450_{cam}. After the reduction of the μ-oxo-bridged binuclear iron center by a single electron transfer, dioxygen binds to the reduced iron center. A second one-electron transfer to the iron-dioxygen complex then may yield an activated oxygen species: [Fe(II) Fe(II)-O-O-H]. This activated oxygen species is assumed to abstract a hydrogen on a methyl group of the substrate, forming a methyl-radical intermediate that will subsequently be oxygenated (70).

A μ-oxo bridged binuclear iron center is not only found in methane monooxygenase, but also in ribonucleotide reductase, hemerythrin, and purple acid phosphatase (11). The iron center of hemerythrin may be functionally different from those in other enzymes: hemerythrin reversibly binds dioxygen, whereas ribonucleotide reductase and methane monooxygenase bind and activate dioxygen irreversibly (76). Nevertheless, the studies on the binuclear iron centers in these enzymes may provide further information concerning the activity of methane monooxygenase.

The broad-substrate range of methane monooxygenase, like many cytochrome P-450s discussed below, is achieved at the expense of regiospecificity. Complex substrates are ususally converted into more than one oxidation product, some of which may be produced in excess over others. Such preferential hydroxylation at one position on the substrate molecule relative to another may result from either the architecture of the substrate-binding site, chemical constraints imposed by the reaction mechanism, or the combination of the two (34).

As mentioned earlier, some components of methane monooxygenase share

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sequence similarities with components of phenol hydroxylase, encoded by the *dmp* genes, and also with toluene-4 monooxygenase, encoded by the *tmo* genes (125). The sequences of the *tmoA* and *tmoE* and of the *dmpN* and *dmpL* gene products are similar to those of two subunits of methane monooxygenase encoded by the *mmoX* and *mmoY* genes. Because methane monooxygenase can hydroxylate the aromatic ring, it is not surprising to find that these three enzymes are derived from a common ancestor. In this respect, it would be interesting to determine whether the hydroxylase components of phenol hydroxylase and toluene-4 monooxygenase contain binuclear iron centers.

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4-Toluene Sulfonate Methyl Monooxygenase and 4-Methoxybenzoate Demethylase

4-Toluene sulfonate methyl monooxygenase from *C. testosteroni* catalyzes the hydroxylation of the alkyl groups on alkylphenylcarboxylates and alkylphenylsulfonates (72). This enzyme consists of a 36-kDa electron-transport component and a 43-kDa hydroxylase component. The hydroxylase component contains a Rieske-type iron-sulfur center. The best substrates of the enzyme are *p*-toluate and 4-toluene sulfonate, but 4-methoxybenzoate is also hydroxylated and yields 4-hydroxybenzoate and formaldehyde (72). This reaction resembles the oxidative demethylation of aliphatic substrates by alkane hydroxylase (62) as well as enzymatic demethylation of aromatic methyl ethers described below.

4-Methoxybenzoate demethylase from *P. putida* is a monooxygenase and hydroxylates the methyl group of the substrate. The intermediate thus formed is unstable and spontaneously transformed to 4-hydroxybenzoate and formate. This enzyme consists of two subunits: the hydroxylase component called putidamonooxin and the electron-transport component called NADH-putidamonooxin reductase. The enzyme resembles 4-toluene sulfonate methyl monooxygenase in many respects: the subunit structures, the presence of FMN and ferredoxin in the electron-transport components (see below), the sizes of the electron-transport and hydroxylase components, and the presence of Rieske-type iron-sulfur centers and of one essential mononuclear nonheme iron in the hydroxylase components. The mononuclear Fe(II) of putidamonooxin binds dioxygen. In uncoupled reactions of the enzyme in the presence of "bad" substrates, hydrogen peroxide forms. Therefore, iron-peroxo-complex, $(FeO_2)^+$, is inferred to be an activated oxygen intermediate (142).

Vanillate demethylase, which is composed of two polypeptides, catalyzes a reaction in a manner similar to 4-toluene sulfonate methyl monooxygenase and 4-methoxybenzoate demethylase. Although the amino acid sequences of these components are known, they have not been biochemically characterized (13). 2,4-Dichlorophenoxyacetate monooxygenase catalyzes oxidative removal of acetate by a mechanism that may be similar to that of demethylases.

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2,4-Dichlorophenoxyacetate monooxygenase, however, is composed of a single polypeptide species (127).

CYTOCHROME P-450 SYSTEMS

Cytochrome P-450 systems are multicomponent enzymes consisting of one terminal hydroxylase heme protein and one or two electron-transport component(s). They catalyze the hydroxylation of a variety of substrates (SH):

$$SH + O_2 + NAD(P)H + H^+ = SOH + H_2O + NAD(P)^+$$

The name cytochrome P-450 derives from the characteristic 450-nm absorption maximum of the enzyme bound to carbon monooxide. Many cytochrome P-450s have been identified from prokaryotes, yeast, fungi, plants, and insects, but most have been identified from mammals (92). Cytochrome P-450s are involved in the specific transformation of endogenous compounds, such as the biosynthesis of vitamin D and of sex-steroid and corticoid hormones (36, 101) or in the catabolism of a variety of natural and xenobiotic compounds. Enzymes that carry out the specific transformations have narrow substrate specificities, and their synthesis is tightly regulated. Enzymes that carry out the catabolic reactions are generally nonspecific, and these reactions protect mammals against the majority of chemicals they may encounter (36, 91, 106). Unfortunately, some chemicals are converted to toxic and/or carcinogenic compounds by several species of cytochrome P-450s (3).

Eukaryotic cytochrome P-450s located in the endoplasmic reticulum and mitochondria are membrane-bound, but bacterial P-450s are soluble (153). Membrane localization was once assumed to facilitate the binding of the typically water-insoluble substrates, but more recent research suggests that the substrate-binding site of membrane-bound enzymes faces the cytosol rather than the surface of the endoplasmic reticulum membrane (18). P-450_{cam}, the 414–amino acid bacterial enzyme that catalyzes the hydroxylation of camphor to 5-exo-hydroxycamphor has been crystallized and its three-dimensional structure determined (107, 109, 110). Amino acid sequence comparisons indicate that this enzyme diverged from other members of the cytochrome P-450 superfamily. Nevertheless, its three-dimensional folding pattern has been used to identify critical amino acid residues in other cytochrome P-450s, because all members of the cytochrome P-450 superfamily may have the same general folding pattern. The amino-terminal portions of the eukaryotic enzymes that mediate attachment to the microsomal or mitochondrial membrane (18, 153) are absent in cytochrome P-450_{cam}.

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Substrate Specificity

The natural substrate of P-450_{cam} binds relatively loosely to the enzyme by seven hydrophobic contacts and one hydrogen bond. Other substrates can be accommodated in the binding site, but most of them cannot hydrogen bond to the enzyme. The regiospecificity for the hydroxylation of these substrates is low, and hydrogen peroxide is produced as a byproduct because of an increased mobility of the substrates in the binding pocket that uncouples the electron transfer and the substrate hydroxylation (110). Considerable sequence variation is found in the central portion of cytochrome P-450s, corresponding to residues 180 to 320 in P-450_{cam}. This heterogeneity may reflect substrate-specificity variation among the cytochrome P-450s (91). Indeed, five of the substrate-binding residues of P-450_{cam} have been identified in this region. Using the P-450_{cam} structure as a model, investigators have tentatively identified substrate-binding residues in other P-450s (153).

The substrate specificity of two closely related P-450 enzymes, P-450_{cou7a} and P-450_{15a}, was systematically investigated (58, 69). P-450_{cou7a} hydroxylates coumarin, while P-450_{15a} hydroxylates testosterone. Despite their different substrate specificities, the amino acid sequences of these two enzymes differ only by 11 residues. An experiment in which specific amino acid replacements were used to alter P-450_{cou7a} substrate specificity revealed that one amino acid substitution was sufficient to convert the cou7a into 15a specificity.

Reaction Mechanisms

The active site of P-450_{cam} is located on the top of the iron porphyrin IX moiety. One axial iron coordination position points downwards to Cys357 and the other points upwards into the oxygen-binding site (109). The residue Cys357 is invariant among all P-450s, and its replacement by a histidine or alanine residue eliminated the enzyme activity, suggesting a specific role for this residue in catalysis (136).

The catalytic cycle is initiated by substrate binding to the enzyme, which facilitates the first one-electron reduction of heme-Fe^{3+} to heme-Fe^{2+}. The catalytic iron in the substrate-free enzyme exhibits a low redox potential (about -300 mV). Upon the binding of a substrate, however, this redox potential increases to -173 mV, enabling the catalytic iron to be reduced by its electron-transport component (36). Subsequently, dioxygen binds to the free axial coordination position of Fe^{2+}, *trans* to the cysteinyl residue. In this $(FeOO)^{2+}$ complex, dioxygen occupies a position very close to the substrate, which moves away from the catalytic iron–dioxygen complex by approximately 1 Å (109). A second one-electron reduction results in the formation of an activated peroxide species, $(FeOOH)^{2+}$. Thr252 may be in contact with the

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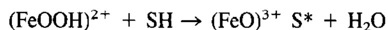
oxygen atoms because of its polar character, and it may stabilize this intermediate form. Site-directed mutagenesis of P-450_{cam} showed that the activation of dioxygen and the substrate hydroxylation are uncoupled in the absence of Thr252 (55). Thr252 may be a proton donor to $(FeOOH)^{2+}$, thereby facilitating the cleavage of the O-O bond. Another possibility is that the substitution of Thr252 changes the structure and/or the solvent channel inside the substrate-binding pocket (110). Hydroxylation is believed to proceed via a biradical recombination mechanism that results in substrate activation (S*) (37):



$$(FeOOH)^{2+} + SH \rightarrow (FeO)^{3+}\ S^* + H_2O$$

Less is known about catalysis by mammalian cytochrome P-450s, although some catalytically important residues have been identified (32). The monooxygenation reaction catalyzed by microsomal cytochrome P-450s involves the transfer of electrons from NADPH-P-450 reductase or from cytochrome b_5 (see below). Electrostatic interactions may be involved in the association of the hydroxylase and electron-transport components. Site-directed mutagenesis showed that seven lysine and arginine residues are, in fact, important in the interaction between the hydroxylase and electron-transport components of P-450_d (123).

Evolutionary Relationships

Comparison of the primary structures of 154 members of a cytochrome P-450 superfamily allowed the composition of an evolutionary tree representing the divergent evolution of these enzymes. Sequences that diverged recently are grouped together into 27 families. Interestingly, within families, intron-exon structures have been fully conserved, suggesting that rearrangement of introns occurred early in the stages of the divergence of the P-450 sequences (92).

Genes for eukaryotic P-450s are often organized in tandem, perhaps the result of gene amplification (91, 101). In such forms, gene conversion, in which a part of a gene is copied to the equivalent part of another gene, may occur. In fact, a gene-conversion mechanism has been inferred from genetic exchanges between the structural gene for a steroid hydroxylase and its pseudogene homologue (53).

AROMATIC AMINO ACID HYDROXYLASES

The mammalian aromatic amino acid hydroxylases, phenylalanine hydroxylase, tryptophan hydroxylase, and tyrosine hydroxylase, share many characteristics. All are homotetrameric and utilize the cofactor, tetrahydrobiopterin

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(16), as well as Fe(II) (24). This section describes the terminal hydroxylase components of these enzymes. Their electron-transport components, quinonoid dihydropteridine reductase, are described below. Some reaction steps of these enzymes are similar to those of the previously discussed flavoprotein monooxygenases.

Regulation of Enzyme Activity by Posttranslational Modification

Phenylalanine hydroxylase catalyzes the first step in the complete oxidation of phenylalanine. Disorders of phenylalanine catabolism, due to a mutation of either phenylalanine hydroxylase or a tetrahydrobiopterin synthetic enzyme, result in phenylketonuria or hyperphenylalaninemia (85). The two related tyrosine and tryptophan hydroxylases catalyze the rate-limiting steps in catecholamine and serotonin synthesis, respectively, thereby playing key roles in neural transmission and hormone regulation (79, 95). These enzymes are subject to strict regulatory controls, which reflects the importance of roles they play.

Tyrosine hydroxylase is activated by phosphorylation, heparin, salts, and phospholipids (152). It is also feedback inhibited by the binding of catecholamines to the oxidized form of the active-site iron. The binding blocks the reduction of this Fe(III) by tetrahydrobiopterin. The dissociation of catecholamines from the enzyme is promoted by phosphorylation of serine 40, and the release of catecholamine may be a major mechanism of phosphorylation activation of this enzyme (40).

The activity of phenylalanine hydroxylase is regulated by its substrate, phenylalanine, which binds to two different sites: an effector-binding site (the activation site) at which no hydroxylation occurs, and a substrate-binding site at which it is hydroxylated (122). Reversible phosphorylation further regulates phenylalanine hydroxylase, apparently by affecting the dissociation constant of the amino acid at the activation site (33). Proteolytic cleavage of the 52-kDa phenylalanine hydroxylase with chymotrypsin yields three peptide fragments. Of these, a 36-kDa fragment, corresponding to the central region of the enzyme, is enzymatically active, having a 30-fold higher specific activity than the native enzyme. An 11-kDa amino-terminal fragment containing the regulatory phosphorylation site may constitute a domain that represses enzyme activity (57).

Reaction Mechanisms

Kinetic experiments have shown that tetrahydrobiopterin binds first to the free hydroxylases, followed by the binding of dioxygen and an amino acid (25). The binding of dioxygen to the aromatic amino acid hydroxylases may occur in two steps. Dioxygen binding to the catalytic Fe(II) may activate dioxygen,

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which is then followed by binding to tetrahydrobiopterin to form 4a-peroxytetrahydropterin, the rate-limiting intermediate (39). This intermediate resembles 4a-hydroperoxy-flavin, an intermediate of flavoprotein monooxygenases. The nucleophilic attack on 4a-peroxytetrahydropterin by an aromatic-ring carbon may result in cleavage of the oxygen-oxygen bond and in hydroxylation of the substrate aromatic ring (26). This reaction mechanism is very similar to that of *p*-hydroxybenzoate hydroxylase. The iron cofactor of phenylalanine hydroxylase not only interacts with tetrahydrobiopterin but also with the substrate (phenylalanine), although the significance of this interaction is not yet known (81).

Phenylalanine hydroxylase converts the substrate analog L-[2,5-H_2] phenylalanine to the corresponding 3,4-epoxide (86), although the natural substrate, phenylalanine, is not necessarily hydroxylated via an epoxide intermediate (124). The tetrahydrobiopterin-dependent phenylalanine hydroxylase from *Chromobacterium violaceum* requires Cu(II) instead of iron for enzymatic activity (104). Mechanistically, this enzyme may be different from its eukaryotic counterpart. In the *C. violaceum* enzyme, dioxygen binds to a single Cu(II) center as the first substrate. The Cu(II) center may also be the site of tetrahydrobiopterin binding.

Primary Structure of Aromatic Amino Acid Hydroxylases

Homologous sequences are unevenly distributed along the polypeptide chains of aromatic amino acid hydroxylases. The amino-terminal 104 amino acid residues of rabbit tryptophan hydroxylase exhibit only 15% sequence identity with those of rat tyrosine hydroxylase, but show about 40% identity with those of human and rat phenylalanine hydroxylases. The sequence identity between these aromatic amino acid hydroxylases exceeds 60% in the carboxyl-terminal regions of the proteins (35). This strongly conserved part of the amino acid sequences corresponds to the region of the enzymatically active 36-kDa proteolytic fragment of phenylalanine hydroxylase (57). A monoclonal antibody was used to localize an amino acid segment of phenylalanine hydroxylase, residues 263–289, responsible for the binding of tetrahydrobiopterin (59). In tryptophan and tyrosine hydroxylases, the corresponding regions are strongly conserved. The amino-terminal regions that contain the regulatory phosphorylation site vary in size from 104 amino acid residues in rabbit tryptophan hydroxylase to 204 residues in human tyrosine hydroxylase (35, 63). The sequence heterogeneity observed in this region may reflect the different regulatory specificities of the three enzymes. Clearly phenylalanine, tyrosine, and tryptophan hydroxylases stem from a common ancestor. The amino-terminal sequences, encoded by exons one and two in human tyrosine hydroxylase, however, may have been acquired at a later stage in their evolutionary divergence.

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Mutant genes from patients with phenylketonuria allowed the identification of amino acid substitutions responsible for the phenylalanine hydroxylase deficiencies (10). In the near future, the three-dimensional structure of aromatic amino acid hydroxylases may be available (15), and crucial features of the aromatic amino acid hydroxylases will most likely then be elucidated.

ELECTRON-TRANSPORT COMPONENTS OF MULTICOMPONENT OXYGENASE SYSTEMS

With the exception of flavoprotein monooxygenases, oxygenases that introduce hydroxyl groups into hydrocarbon substrates require an electron-transport component. In this section, these electron-transport components are collectively discussed.

Dihydropteridine Reductase

As described in the previous section, tetrahydrobiopterin is a cofactor of phenylalanine, tyrosine, and tryptophan hydroxylases. Dihydropteridine reductase catalyzes the NADH-dependent reduction of quinonoid dihydrobiopterin to tetrahydrobiopterin, and, hence, is an essential component of the pterin-dependent aromatic amino acid hydroxylases. The reductases from human and rat are very similar 25-kDa polypeptides (73, 120). The preliminary X-ray diffraction pattern at 2.3-Å resolution was obtained (83). The amino-terminal region contains an ADP-binding $\beta\alpha\beta$-fold motif. This region may be the NADH-binding site because a mutation at Asp37 inside the motif reduced the affinity of the enzyme to NADH (82).

Electron-Transport Components of Cytochrome P-450s

Generally, a NADPH-P-450 reductase of approximately 80 kDa serves as the electron-transport component of microsomal cytochrome P-450s (9). This enzyme contains one mole each of FMN and FAD per subunit and is anchored to the endoplasmic reticulum by its hydrophobic amino-terminal region. The primary structures of these reductases from yeast, trout, rat, rabbit, and pig share significant homology (33%) (146). In these sequences, putative domains for the binding of FMN, FAD, and NADPH are conserved. Two separate regions that may be involved in FMN binding reside in the amino-terminal portion of the protein. Two possible FAD-binding domains, one for the binding of the pyrophosphate group of FAD, and the other for the binding of the isoalloxazine ring of FAD, are located in the middle part of the sequences, while the NADPH-binding site may reside at the carboxyl-terminal portion. Its 70-kDa hydrophilic domain is released from the membrane upon protease treatment. The soluble domain cannot interact with the

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hydroxylase components of cytochrome P-450s but does exhibit NADPH-cytochrome *c* reductase activity.

Some mitochondrial and bacterial P-450s utilize electron-transport systems consisting of two different polypeptide species, a [2Fe-2S] ferredoxin and a flavoprotein. In the P-450_{cam} system, these two polypeptides are called putidaredoxin and NADH-putidaredoxin reductase, respectively. Putidaredoxin shuttles electrons from NADH-putidaredoxin reductase to P-450_{cam}. This system is similar to that of the mitochondrial P-450 systems that are comprised of adrenodoxin and NADH-adrenodoxin reductase. NADH putidaredoxin reductase in the P-450_{cam} system is a member of the glutathione reductase family (19). This oxidoreductase family possesses two $\beta\alpha\beta$-type ADP-binding folds, one for FAD and the other for NAD(P)H binding (144).

P-$450_{Bm\text{-}3}$ from *Bacillus megaterium* catalyzes the hydroxylation of long-chain fatty acids and is inducible by barbiturates. This enzyme has a mass of 118 kDa and exhibits the activities of both P-450 hydroxylase and NADPH-P-450 reductase (68, 90). Such composite P-450 enzymes are not found in eukaryotes, but enzymatically active fusions between a eukaryotic P-450 and a NADPH-P-450 reductase have been constructed (121).

The electron-transport component of the P-450_{sca} system from *Streptomyces carbophilus,* NADH-P-450_{sca} reductase, is a single polypeptide containing FMN and FAD. This reductase thus resembles the eukaryotic reductases, but its size, 51 kDa, is smaller than the sizes of its eukaryotic counterparts (119).

In eukaryotic NADPH-P-450 reductases, reducing equivalents are transferred from NADPH, via the FAD and FMN redox centers, which are separated by about 20 Å (7), to P-450 hydroxylase components (91). In the absence of the P-450 hydroxylase components, the FAD and FMN redox centers of NADPH-P-450 reductase are reduced by NADPH to the semiquinone form of FAD and the fully reduced form of FMN, respectively. Full reduction is not accomplished because the NADP/NADPH couple is significantly more positive than the FADH/$FADH_2$ couple.

In the P-450_{cam} system, NADH reduces FAD to $FADH_2$, and $FADH_2$ subsequently reduces the [2Fe-2S] center of putidaredoxin. The reduction of the P-450_{cam} hydroxylase component is facilitated by the docking of reduced putidaredoxin on the surface of P-450_{cam}. This association is believed to be stabilized by electrostatic interactions between carboxylate groups on the surface of putidaredoxin and complementary arginine and lysine residues on the surface of the hydroxylase component (126). The two redox centers of putidaredoxin and of the P-450_{cam} hydroxylase cannot directly interact because the latter is buried in the hydrophobic core of the protein. The terminal tryptophan residue of putidaredoxin is assumed to be the first component of a long-range electron-transfer network (126).

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Electron-Transport Components of Aromatic-Ring Dioxygenases, Aromatic-Ring Hydroxylases, and Alkyl-Group Hydroxylases

Each of the electron-transport components of benzene, toluene, and naphthalene dioxygenases and of alkane hydroxylase is composed of two different proteins (42, 43, 56, 154). The first protein is a flavoprotein exhibiting an NAD(P)H-acceptor reductase activity, whereas the second protein is a ferredoxin. The flavoproteins of benzene and toluene dioxygenases contain two regions of approximatly 30 amino acids in length, one in the amino-terminal region and the other in the middle of the sequences, which fit the NADH- or FAD-binding $\beta\alpha\beta$ motif. Although the sequence of the equivalent component of naphthalene dioxygenase is not presently available, biochemical studies of this component (NADH-ferredoxin$_{NAP}$ reductase) suggest that it is a flavoprotein containing a chloroplast-type [2Fe-2S] cluster, hence different from the corresponding proteins in benzene and toluene dioxygenases. The ferredoxin components of the toluene, benzene, and naphthalene dioxygenases are structurally similar and contain one [2Fe-2S] cluster. Conserved sequence patterns of these ferredoxins suggest that their iron-sulfur clusters are Rieske-type (94).

In benzoate and toluate 1,2-dioxygenases that catalyze the dihydroxylation of the aromatic ring, and in xylene monooxygenase, multicomponent phenol hydroxylase, and methane monooxygenase that catalyze monohydroxylation of substrates, a single protein mediates the electron transfer from NADH to its hydroxylase partner (94, 98, 108, 129). In addition, the second component of naphthalene dioxygenase, NADH-ferredoxin$_{NAP}$ reductase (described above), may be structurally similar to these reductases. The primary structures of the electron-transport components of these enzymes that constitute a new flavoprotein family are similar to each other. Their amino-terminal sequences are similar to chloroplast-type ferredoxins while their carboxyl-terminal regions are similar to those of ferredoxin-NADP reductases (46, 94, 129). The sequence alignment of the electron-transport components of these oxygenases with the ferredoxin-NADP reductase whose three-dimensional structure has been determined (61) showed that strongly conserved sequences are located in the NAD(P)H- and FAD-binding regions. A domain of NADPH-P-450 reductase also appears to be related to this flavoprotein family (94). In the photosynthetic reactions, electrons are transferred from ferredoxin to FAD on ferredoxin-NADP reductase, then from reduced FAD to NADP to yield NADPH. In the oxygenase systems, however, electrons are transferred in the opposite direction: from NAD(P)H to FAD, then from FAD to the [2Fe-2S] cluster.

The *P. putida* (*oleovorans*) alkane hydroxylase is a three-component enzyme. The electron transfer from NADH to the active site of the membrane-

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bound hydroxylase component, AlkB, is achieved by the 41-kDa NADH-rubredoxin reductase (19) and the 18-kDa rubredoxin (64). NADH-rubredoxin reductase bears sequence similarity to NADH-putidaredoxin oxidoreductase of the P-450_{cam} hydroxylase and to several flavoprotein oxidoreductases such as glutathione reductase, *p*-hydroxybenzoate hydroxylase, and lipoamide dehydrogenase (19). The local peptide sequence of NADH-rubredoxin reductase could be superimposed upon the available three-dimensional structure of glutathione reductase using the WHATIF algorithm developed by Vriend (139). The primary structure of rubredoxin is related to rubredoxins from anaerobic bacteria (64), but not similar to those of other ferredoxins.

General Properties of Electron-Transport Components

The oxidation of NAD(P)H generates two electrons, but the reduction of hydroxylase components requires two one-electron transfer steps. The electron-transport components of the hydroxylase systems can convert a single two-electron transfer into two one-electron transfers. Two electrons in these systems are transferred from NAD(P)H to FAD (or FAD plus FMN in the case of NADPH-P-450 reductase). These flavins provide a reservoir for electrons, and their $2e^-$ forms (e.g. $FADH_2$) can transfer one electron to the [2Fe-2S] center, thereby becoming semiquinones (e.g. FAD*). The semiquinones can further transfer one electron to [2Fe-2S] and thus return to the $0e^-$ state (e.g. FAD).

The electron transfer in the hydroxylase systems can occur most efficiently when the midpoint potentials of electron acceptors are higher than those of electron donors. Where midpoint potentials are known, however, such as in the two redox centers of methane monooxygenase component C, electron acceptor potentials are not always higher than those of electron donors (74, 75). It is not evident why the array of the redox centers may be less than optimal. One possibility is that the electron-transfer reaction may be more efficient than the rate-limiting hydroxylation step, and therefore, an inefficient electron-transport system would not constitute a selective disadvantage. Many hydroxylase components change the midpoint potentials of their redox centers upon binding their substrates. Such a change could be an important mechanism to prevent undesirable uncoupling reactions between NAD(P)H oxidation and substrate hydroxylation. Some electron-transport components also change their midpoint potentials upon the formation of a complex with another component (67).

Cloning in *Escherichia coli* of an incomplete set, lacking one of the electron-transport components, of naphthalene dioxygenase genes, resulted in functional expression of this dioxygenase (66). This result suggests that the missing electron-transport component can be complemented by some un-

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identified *E. coli* electron-transport component(s). This nonspecific electron transfer is rather unexpected because interactions between unrelated electron-transport systems may cause undesirable short circuits. To what degree biological systems necessitate the specificity of a redox partnership is an interesting question. If a low degree of discrimination between correct and incorrect redox partners can be tolerated, then the evolutionary exchange of partnerships between hydroxylase and electron-transport components may occur frequently and may be the scenario of the natural evolution of multi-component oxygenase systems.

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CONCLUSIONS

Oxygenases from widely divergent organisms share many properties, structures, and reaction mechanisms. During the past decade, the isolation of many hydrocarbon-utilizing organisms combined with advances in recombinant DNA techniques allowed the rapid accumulation of primary sequences of many oxygenases. Sequence comparisons have enabled the classification of oxygenases into distinct families. Such data coupled with biochemical studies have provided important information concerning the structure-function relationships of these enzymes.

The grouping of proteins into related families is useful in several respects. Biochemical knowledge obtained from one protein may be applicable to another protein in the same family. Amino acid residues that are highly conserved among members of a gene family indicate residues essential for function. Furthermore, comparisons of related proteins may allow the reconstruction of genetic rearrangements that have taken place during the course of evolution and therefore suggest mechanisms of protein evolution (2). The oxygenases discussed here have been grouped into several families. Usually, those considered to be related show global sequence homologies: long stretches of amino acid sequences can be aligned without the introduction of many gaps, and overall sequence identities are more than 20%. Some regions of the aligned sequences show particularly high degrees of identity, and such patterns of conserved amino acids are noted, for instance, in regions that bind important cofactors. As discussed above, residues that bind NAD(P)H and FAD, and iron-sulfur centers, are highly conserved (94, 144).

Local sequence similarities are also observed among oxygenases from different families, especially in regions involved in cofactor binding. It is not known how the broad distribution of these coenzyme-binding domains arose. Similarities in the cofactor-binding regions of unrelated enzymes may reflect constraints imposed by the functioning of these cofactors. If this is the case, the observed sequence similarities may have arisen as the result of convergent evolution. Alternatively, common patterns in different oxygenases may be formed by the exchange and association of different protein domains. In

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eukaryotes, exons may code for compact protein structures called modules, and recombination events between the intervening introns may allow exon shuffling and thus creation of novel proteins (31). In bacteria, where introns do not generally exist, the propagation of protein-module-encoding DNA segments, if it does occur, should take place by a mechanism other than exon shuffling.

The electron-transport components of benzene dioxygenase from *P. putida* and the comparable component of benzoate dioxygenase from *A. calcoaceticus* do not appear to be evolutionarily related. The NADH- and FAD-binding regions of the electron-transport component of benzene dioxygenase are characterized by the ADP-binding $\beta\alpha\beta$ fold, whereas the equivalent cofactor-binding regions in the electron-transport component of benzoate dioxygenase resemble those of spinach ferredoxin-NADP$^+$ reductase, a member of a separate flavoprotein family (94). However, a comparison of the three-dimensional structures of the cofactor-binding regions in these two families shows that they are topologically similar (61). Whether similar cofactor-binding domains have been established in a process of divergent evolution or of convergent evolution is not clear.

Investigators have noticed genetic exchanges creating apparent protein fusions in some electron-transport components of oxygenases. The mammalian NADPH-P-450 reductases appear to be mosaic proteins, with distinct domains involved in the binding of different cofactors. In bacteria, the electron-transport component of benzoate dioxygenase and its family appear to be a fusion product of a chloroplast-type ferredoxin at the amino-terminal region with a ferredoxin-NADP reductase. A homologous chloroplast-type ferredoxin segment is found in the carboxyl-terminal region, rather than the amino-terminal region of the electron-transport component of vanillate demethylase (13).

In bacteria, catabolic genes, especially those for xenobiotic degradation, are often plasmid-born (5), and in some cases, these genes are integrated into transposons (135, 138). Chromosomally encoded catabolic genes often form supraoperonic structures. It is not known whether these genetic arrangements reflect requirements for gene expression, beyond transcriptional unity, or whether they reflect mechanisms of evolutionary change (103). Alternatively, they may reflect modes of genetic exchange. In the environment, certainly, genetic transfer could be mediated by plasmids, chromosomal gene mobilization, transduction, transformation, and transposition. Such mechanisms have also been suggested to mediate genetic exchange between prokaryotic and eukaryotic cells (54).

Several mechanisms may lead to the divergence of enzymes. The accumulation of point mutations and the power of natural selection have long been discussed. More recently, however, other kinds of DNA rearrangements have been suggested that may be responsible for dynamic changes in the

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primary structure of DNA (46). A variety of mutations in combination with gene amplification may provide additional possibilities of genetic evolution: a silent gene copy may accumulate mutations without imposing selective disadvantage to the host organism. In the process of gene conversion caused by DNA slippage, one or several of the acquired mutations may be integrated into the actively transcribed copy of the gene (23).

In the evolution of catabolic pathways, selective advantage may not be conferred until an entire suite of enzymes needed for substrate degradation is present. This situation may have created different pathways for the degradation of the same compound, which may reflect differences in evolutionary history. An example is provided by different pathways for the mineralization of toluene: in the TOL plasmid–encoded pathway, the methyl side chain of toluene is attacked by a monooxygenase, while in a chromosomally encoded pathway, the toluene ring is cleaved without the processing of this methyl side chain (148).

Enzymes may evolve by altering substrate specificity without changing catalytic mechanism. The plasmid-encoded *P. putida* alkane hydroxylase and xylene monooxygenase provide a naturally occurring example of structurally related but functionally distinct enzymes. Both enzymes catalyze the same kind of reaction, but they do not have a substrate in common. Mutations provoked by single amino acid substitutions can dramatically affect the substrate specificity of catechol 2,3-dioxygenase (113, 141). A wide spectrum of substrate specificity occurs naturally in enzymes of related gene families (12). Mechanisms of coupling substrate specificity and catalysis are largely unknown; often there is a trade-off between substrate specificity and catalytic efficiency. Oxygenases that can accept many substrates may be less efficient and may generate fewer specific oxidation products than their homologues that are more narrowly substrate specific (96).

The ability to engineer modifications in oxygenase function or specificity may be useful in environmental-protection efforts. Recalcitrant pollutants often resemble the natural substrates of microbial enzymes. The custom design of oxygenases may also be used in certain biotechnology processes in which biotransformations prove to be advantageous in the synthesis of useful chemicals. The accelerated evolution of catabolic pathways has been achieved in the laboratory, shedding light on the ways in which substrate specificity and regulation of existing enzymes may change (1). The biochemical studies of oxygenases will be relevant to these applications as well as to our general understanding of the function and structure of an important and interesting class of enzymes.

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