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Review

Enzymatic hydroxylation of aromatic compounds

René Ullrich and Martin Hofrichter*

International Graduate School of Zittau, Department of Environmental Biotechnology, Markt 23, 02763 Zittau, Germany, phone: +493583771521, fax: +493583771534, e-mail: ullrich@ihi-zittau.de, hofrichter@ihi-zittau.de

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Abstract. Selective hydroxylation of aromatic compounds is among the most challenging chemical reactions in synthetic chemistry and has gained steadily increasing attention during recent years, particularly because of the use of hydroxylated aromatics as precursors for pharmaceuticals. Biocatalytic oxygen transfer by isolated enzymes or whole microbial cells is an elegant and efficient way to achieve selective hydroxylation. This review gives an overview of the different enzymes and mechanisms used to introduce oxygen atoms into aromatic molecules using either dioxygen (O_2) or hydrogen peroxide (H_2O_2) as oxygen donors or indirect pathways via free radical intermediates. In this context, the article deals with Rieske-type and α-keto acid-dependent dioxygenases, as well as different non-heme monooxygenases (di-iron, pterin, and flavin enzymes), tyrosinase, laccase, and hydroxyl radical generating systems. The main emphasis is on the heme-containing enzymes, cytochrome P450 monooxygenases and peroxidases, including novel extracellular heme-thiolate haloperoxidases (peroxygenases), which are functional hybrids of both types of heme-biocatalysts.

Keywords. Dioxygenase, monooxygenase, peroxygenase, peroxidase, P450, tyrosinase, laccase, hydroxyl radicals.

Introduction

Hydroxylations belong to the oxygen transfer reactions introducing the hydroxyl group (-OH) into organic molecules, primarily via the substitution of functional groups or hydrogen atoms. From the point of view of an organic chemist, the direct and selective introduction of the hydroxyl group into aromatic rings is one of the most challenging fields in modern synthesis. Though progress has been reported in using hydrogen peroxide and metal catalysts (e.g., vanadium, palladium, TiO_2) for the oxidation of benzene, toluene, and xylene, the number of direct hydroxylations, as well as their selectivity is still limited [1, 2]. Similarly, this is also valid for direct chemical oxidations in supercritical carbon dioxide, where it is possible to oxygenate cyclic alkanes and alkenes, but not aromatic compounds [3]. Therefore, intricate, multi-step processes are used mostly for technical production of hydroxylated aromatics (e.g., Hock process catalyzing the conversion of *p*-cumene into phenol [4, 5]).

Biotransformations involving hydroxylation reactions have steadily gained attention since the first successful microbial steroid transformation in 1952 by the zygomycetous fungus *Rhizopus arrhizus*, which converts steroids, such as progesterone, into the corresponding 11α-hydroxy derivatives [6]. Three years later, two groups independently demonstrated by $^{18}O_2$ labeling that one or both oxygen atoms of dioxygen can be directly incorporated into aromatic molecules

* Corresponding author

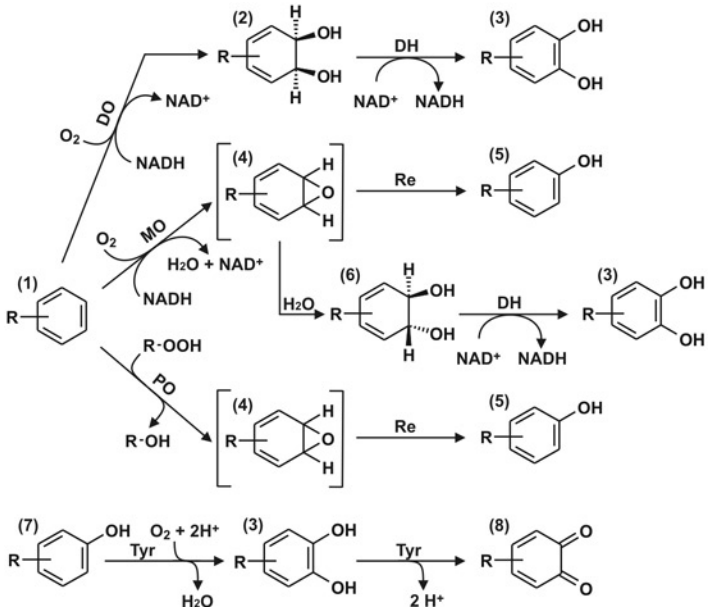

Figure 1. Basic routes of enzymatic hydroxylation of aromatic compounds. DO – dioxygenase, MO – monooxygenase, PO – peroxidase/peroxygenase, Tyr – tyrosinase, DH – dehydrogenase, Re – rearrangement. (1) aromatic substrate, (2) *cis*-dihydrodiol, (3) catecholic product, (4) epoxide intermediate, (5) phenolic product, (6) *cis*,*trans*-dihydrodiol, (7) phenolic substrate, (8) benzoquinone product.

following the enzymatic oxidation of 3,4-dimethylphenol by phenolase and catechol by pyrocatechase, respectively [7, 8]. It was also Hayaishi (1957) who designated these enzymes "oxygenases", which later turned out to occur throughout all living systems from archaea to mammals [9]. These ubiquitous enzymes are also of general interest in biotechnology since their specificity allows the selective oxygenation of organic molecules under environmentally friendly conditions [10]. Recent advances in oxygenase-catalyzed biotransformations with biotechnological background have been reviewed by Van Beilen et al. [11], Urlacher and Schmid [12], and Bernhardt [13].

Monooxygenases and dioxygenases can be distinguished from the introduction of either one or two oxygen atoms into the substrate [14]. The majority of "natural" oxygenases uses dioxygen (O_2, a stable diradical = triplet oxygen) as an oxygen source but there are also a few enzymes in plants and fungi, which can act as peroxygenases transferring peroxide-oxygen (from hydrogen peroxide or organic peroxides). Nomenclature of the Enzyme Commission (EC) distinguishes two major subclasses of oxidoreductases, which incorporate dioxygen into substrate molecules: EC 1.13 and 1.14. Enzymes of the former subclass do not need external hydrogen donors (e.g., NAD[P]H) for oxygenation and act on single substrate molecules, while the latter act on paired hydrogen donors. There are monooxygenases and dioxygenases in both subclasses and their sub-subclasses were re-classified in 1984 by the EC leading in both cases to the deletion of all sub-subclasses from 1 to 10, and hence the first sub-subclasses of oxygenases are now EC 1.13.11 and EC 1.14.11 (Enzyme nomenclature 1984 [15], Holland 1998 [14]; for details see: http://www.chem.qmul.ac.uk/iubmb/enzyme/ [the official page of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology; last update March 13, 2006]). Furthermore, there are enzymes showing hydroxylating side activities such as tyrosinase (EC 1.10.3.1) or certain peroxidases (1.11.1.–). The position of a group of membrane bound plant "peroxygenases", which hydroxylate fatty acids is uncertain and they have not yet been "officially" introduced into the EC classification (enzymatically, it is a matter of mixed co-oxidations initiated by lipoxygenases [EC 1.13.11.12] [16], peroxidase, and/or P450 enzymes [17], EC nomenclature 1998 [18]). Finally, some enzymes may incorporate oxygen indirectly via free radical mechanisms and/or addition of water (e.g., cellobiose oxidase, laccase). The basic routes of enzymatic hydroxylation are given in Figure 1. Most of these reactions occur intracellularly (mono- and dioxygenases), whereas only tyrosinase and peroxidases work extracellularly.

The present review gives a survey of the different types of oxygen incorporating enzymes. This approach, of course, cannot consider all aspects of this rapidly developing field of biochemical research and least of all, not all recent publications. One should keep in mind that in 2005 alone, more than 400 review articles, as well as 3300 original papers on cytochrome P450 enzymes (P450s) were published, according to a literature research in the PubMed database. Therefore, we focus our review on the basic aspects of aromatic hydroxylation, as well as on selected recent findings including our own results on fungal peroxidases, which hydroxylate aromatic compounds (per-

Table 1. Examples of enzymes catalyzing aromatic hydroxylation.

Enzyme	EC number	Organism	Active site	Aromatic substrate(s)	Major products	References
Naphthalene dioxygenase (NDO)	EC 1.14.12.12	*Pseudomonas putida*	Rieske-type [2Fe-2S]	Naphthalene, (indole, toluene, benzene)	cis-1,2-Dihydro-naphthalene, (*o*-dihydrodiols)	[23]
Fe^{2+}/α-keto acid dioxygenase (Fe^{2+}/α-KG DO)	EC 1.14.11.–	Aerobic organisms	[Fe^{2+}]-His1-X-Asp/Glu-Xn-His2	Flavonoids	Hydroxyflavonoids	[42]
4-Hydroxyphenol-pyruvate dioxygenase (HPPD)	EC 1.13.11.27	Aerobic organisms	[Fe^{2+}]-His1-X-Glu-Xn-His2	Hydroxyphenolpyruvate	Homogentisate, CO_2	[42]
p-Hydroxybenzoate hydroxylase (PHBH)	EC 1.14.13.2	Aerobic bacteria	Flavin (FAD) (metal-free)	p-Hydroxybenzoate	Protechatechuate	[52]
Phenylalanine hydroxylase	EC 1.14.16.1	Human liver *Chromobacterium violaceum*	[Fe^{2+}]-Tetrahydropterin	Phenylalanine	Tyrosine	[63]
Toluene monooxygenase (ToMO)	EC 1.14.14.1	*Pseudomonas stutzeri*	Carboxylate-bridged di-iron center [Fe^{3+}-OH-Fe^{3+}]	Toluene, (cresols, benzene, styrene, naphthalene)	*p*-Cresol, (*p*-hydroxyaromatics)	[68]
Camphor 5-mono-oxygenase (P450cam, CYP101)	EC 1.14.15.1	*Pseudomonas putida*	Ferric heme-thiolate [heme-Fe^{3+}]-Cys	Camphor, (naphthalene, pyrene, many more)	5-Hydroxycamphor, (1-naphthol, pyrene quinones)	[194]
Fatty acid hydroxylase (P450 BM3, CYP102A1)	EC 1.14.14.1	*Bacillus megaterium*	Ferric heme-thiolate [heme-Fe^{3+}]-Cys	Fatty acids, (aromatic substrates)	ω-hydroxy fatty acids, (hydroxyaromatic products)	[99]
Tyrosinase	EC 1.14.18.1	Aerobic organisms	Type-3 copper center [$Cu^{+/2+}$-$Cu^{+/2+}$]	Tyrosine, (phenolic compounds)	Dopachrome, dopaquinone, (*o*-diphenols)	[150]
Horseradish peroxidase (HRP)	EC 1.11.1.7	*Armoracia rusticana*	Ferric heme-histidyl [heme-Fe^{3+}]-His	Phenols (benzene)	Phenoxyl radicals, benzoquinones, (phenol)	[170, 176]
Agrocybe aegerita peroxidase (AaP)	EC 1.11.1.–	Agrocybe *aegerita* (mushroom)	Ferric heme-thiolate [heme-Fe^{3+}]-Cys	Naphthalene, (toluene, benzene, other aromatics)	1-Naphthol, (cresols and benzyl alcohol, *p*-benzo-quinone)	[122]
Microperoxidase-8 (MP8)	EC 1.11.1.–	Horse (partly digested heart cytochrome c)	Ferric heme-histidyl [heme-Fe^{3+}]-His	Anthracene, (naphthalene, aniline, phenol)	Anthraquinone, (1-naphthol, aminophenols, hydroquinone)	[182]

oxygenases). Table 1 lists representative biocatalysts of different enzyme families and subclasses, which incorporate oxygen into aromatic substrates, and Table 2 gives an overview of cofactors, prosthetic groups, and metals required, as well as selected activating compounds and inhibitors of the different groups of enzymes.

Dioxygenases

Arene dioxygenases (EC 1.14.12.–), which catalyze the conversion of simple aromatic compounds (e.g., benzene, naphthalene, biphenyl, phthalic and benzoic acids) into the corresponding enantiomerically pure, vicinal *cis*-dihydrodiols are intracellular biocatalysts exclusively produced by eubacteria (e.g., *Pseudomonas* spp., *Rhodococcus* spp., *Sphingomonas* spp.). They can initiate productive degradation pathways of aromatic hydrocarbons including a number of organopollutants [19–21]. These enzymes belong to the Rieske-type, non-heme oxygenases which bear a [2Fe-2S] cluster in the active site and have one or two electron transport proteins which precede the final oxygenase component (Fig. 2) [20, 22, 23]. For example, naphthalene dioxygenase (NDO; EC 1.14.12.12), the most studied enzyme of this group, consists of three proteins: the iron-sulfur flavoprotein reductase and the iron-sulfur ferredoxin are electron transfer proteins, which supply electrons derived from

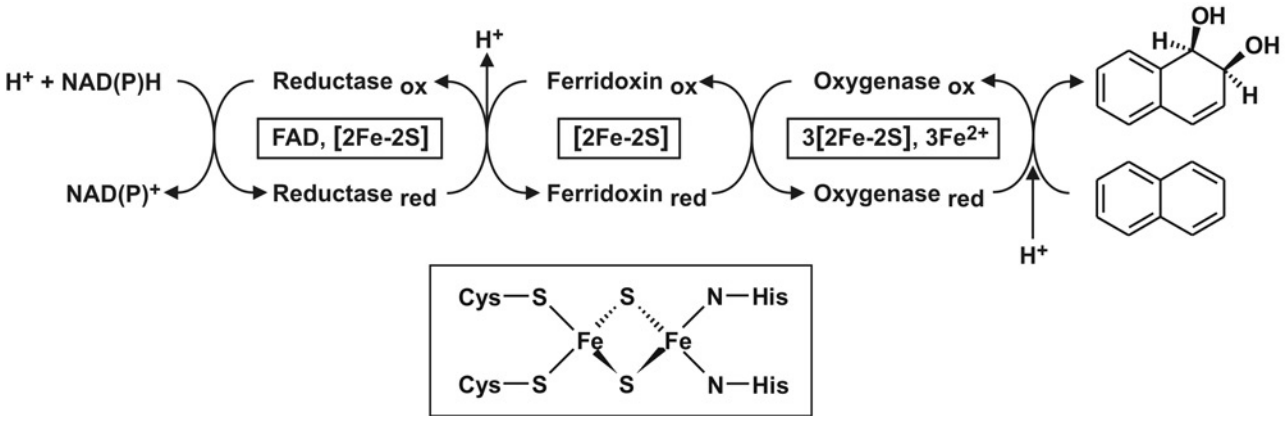

Figure 2. Electron transfer by Rieske-type dioxygenases (above) illustrated by the dihydroxylation of naphthalene by NDO (naphthalene dioxygenase) from *Pseudomonas putida*. Rieske-type [2Fe-2S] cluster in the active site of NOD (below).

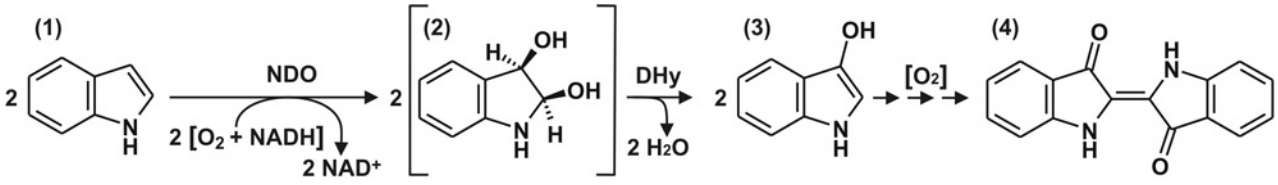

Figure 3. Whole-cell transformation of indole to indigo by *E. coli* host cells containing the NDO gene from *Pseudomonas putida* (modified according to [33]). NDO – naphthalene dioxygenase, DHy – spontaneous dehydratization of the indole oxygenation product, Ox – spontaneous oxidation of indoxyl and subsequent coupling to indigo in the presence of air. (1) indole, (2) instable *cis*-dihydrodiol intermediate, (3) indoxyl, (4) indigo.

NAD(P)H to the catalytic oxygenase with a mononuclear iron site (Fig. 2) [24, 25]. The catalytic cycle of arene dioxygenases proceeds in two steps, the activation of dioxygen (O_2) and its addition to the substrate. The reaction mechanism for O_2 activation is still elusive and there is a controversy on the oxidation states that iron goes through during catalysis [23, 26, 27]. Some reports suggest that O_2 activation at the oxygenase's active site happens through Fe^{4+} and Fe^{5+} oxo-states [R-$Fe^{4+/5+}$=O], however, more recent studies favor a Fe^{3+}-(hydro)peroxo complex [R-Fe^{3+}-O-OH]. Several experimental findings strongly support the latter assumption [23, 28].

According to the native substrate and sequence alignments, Gibson and Parales [20] distinguish four families of arene dioxygenases (toluene/biphenyl, naphthalene, benzoate, and phthalate families). In addition, there are several dioxygenases, which do not cluster with any of these families (e.g., enzymes for the oxygenation of aniline, dibenzodioxin, 3-phenylpropionate, salicylate, *o*-halobenzoate). Recently, the Gram-positive bacterium *Rhodococcus opacus*, which utilizes different polycyclic aromatic hydrocarbons (dibenzofuran and dibenzo-*p*-dioxin as carbon sources) was found to produce a unique arene dioxygenase catalyzing lateral dioxygenations [29].

NDO is relatively unspecific and also hydroxylates, in addition to naphthalene, benzene, toluene, and substituted phenols while incorporating dioxygen not only in *ortho*- but also in the *para*-position [30]. Moreover, NDO was shown to catalyze monohydroxylation, sulfoxidation, desaturation (formation of C=C bonds), and dehydrogenation, as well as O- and N-dealkylation and resembles in this respect cytochrome P450 monooxygenases [31, 32].

Arene dioxygenases are promising biocatalysts for biotechnological applications due to their versatility, but because of their complexity and the requirement of NAD(P)H, the focus remains on whole-cell biotransformations. The most well-known application is the biosynthesis of indigo, an aromatic compound used for denim dying [33]. It is produced from indole by engineered *E. coli* strains, possessing the NDO encoding genes from *Pseudomonas putida*, via *cis*-indole-2,3-dihydrodiol that dehydrates to form indoxyl, which in turn, is spontaneously oxidized to indigo in the presence of air (Fig. 3). [34, 35] Other specific applications of *cis*-dihydrodiols formed by arene dioxygenases have been reported in the synthesis of chiral precursors of drugs [36–38]. In this context, process parameters of whole-cell biotransformations were optimized using special bioreactors [39] and enzyme properties were improved by genetic engineering and directed evolution [40, 41].

Fe^{2+}/α-Keto acid-dependent dioxygenases (mostly α-ketoglutarate = α-KG is used; EC 1.14.11.–) represent

Table 2. Cofactors, prosthetic groups, and metals required, as well as selected activators and inhibitors of the different types of oxygenases (according to Springer Handbook of Enzymes; [215–217].

Enzyme group	Cofactors, prosthetic groups	Metals	Activators	Inhibitors
Intracellular bacterial arene dioxygenases	O_2, NAD(P)H, FAD or FMN, ferredoxin	Non-heme Fe^{2+}	Ferricyanide (NDO)	1,10-Phenanthroline, EDTA, NaN_3, 4-chloromercuribenzoate, H_2O_2
Intracellular flavonoids hydroxylating α-Keto acid dioxygenases	O_2, α-ketoglutarate	Non-heme Fe^{2+} (can be partially replaced by Co^{2+})	Ascorbate, catalase	Pyridine-2.4-dicarboxylate, EDTA, KCN, Fe^{3+}, Cu^{2+}, Zn^{2+}
Intracellular 4-hydroxy-phenylpyruvate dioxygenase	O_2	Non-heme Fe^{2+}, (Cu, Zn)	Organic solvents (e.g., tetrahydrofuran, acetone)	1,10-Phenathroline, EDTA, catechol, cupferron
Intracellular aromatic amino acid hydroxylases	O_2, tetrahydrobiopterin	Non-heme Fe^{2+} (Cu, Zn, Ca)	SDS, thiols, phospholipids, Mn^{2+}, NaCl	1,10-Phenanthroline, EDTA, H_2O_2, Co^{2+}, Ni^{2+} (competitive against Fe^{2+})
Intracellular flavin monooxygenases	O_2, NAD(P)H, FAD	Metal-free	Dihydroxyaromatic compounds	Halides, SO_4^{2-}, NO_3^-, 4-chloromercuribenzoate, Fe^{2+}, Hg^{2+}
Intracellular bacterial and fungal di-iron hydroxylases	O_2, NAD(P)H, FAD	Non-heme Fe^{3+} (Cu^{2+})	Thiols	1,10-Phenanthroline, halides, CN^-, H_2O_2
Intracellular cytochrome P450 monooxygenases	O_2 (H_2O_2), NAD(P)H, FAD/FMN or FAD/ferredoxin, proto-porphyrin IX (heme)	Heme Fe^{3+}	Tetrahydrofuran, K^+ (P450cam)	Pyridine and imidazole derivatives, methylenedioxy compounds, parathione, CN^-, NO, CO, Co^{2+}, Cd^{2+}, Mn^{2+}
Extracellular heme-thiolate haloperoxidases	H_2O_2, protoporphyrin IX	Heme Fe^{3+} (Mn^{2+})	Acetone	5-Vinyl-2-oxaolidinethione, ethanol, F^-, CN^-, NaN_3, NO_3^-, NO, CO
Extracellular histidyl-heme peroxidases	H_2O_2, protoporphyrin IX	Heme Fe^{3+} (Ca^{2+})	$CaCl_2$, ascorbate (<5 μM), polyvinyl-pyrrolidone	Benzhydroxamic acid, ascorbate, CN^-, NaN_3, $AlCl_3$, $Na_2S_2O_5$
Extracellular tyrosinases	O_2 (H_2O_2),	Type-3 Cu^{2+} (Ca^{2+})	3-Hydroxyanthtralinate, L-dopa, DMSO, Fe^{3+}, Mg^{2+}	1,10-Phenanthroline, 1-phenyl-2-thiourea, 4-nitrophenol, catechol, CN^-, NaN_3, CO

a second group of dioxygenases, which incorporate the two oxygen atoms of O_2 into two different substrates (one atom is transferred to the actual substrate, the second one to α-KG acting as the co-substrate). Some enzymes in this diverse group of biocatalysts catalyze the monohydroxylation of particular aromatic rings. Thus, plants synthesize a variety of compounds using Fe^{2+}/α-KG-dependent dioxygenases, among others flavonoids and alkaloids [42]. These biocatalysts contain ferrous iron (Fe^{2+}) in the active site of the resting enzyme, and an Fe^{4+}-oxo species, which is formed in the course of the complex catalytic cycle is likely to be the key intermediate for hydroxylation [43]. The function of α-KG as the co-substrate is to chelate Fe^{2+} while it is oxidatively decarboxylated to form CO_2, succinate and an activated oxo-ferryl intermediate [R-Fe^{4+}=O] that catalyzes the actual hydroxylation [42]. As an example, Figure 4 shows the hydroxylation of a methylated flavonol at the aromatic 6-position by a plant Fe^{2+}/α-KG hydroxylase from the saxifrage *Chrysosplenium americanum* [44]. A second example is naringenin 3-dioxygenase (EC 1.14.11.9), which specifically hydroxylates the 3-position of the non-aromatic ring of flavanones [45].

4-Hydroxyphenylpyruvate dioxygenase (HPPD; EC 1.13.11.27), which catalyzes the formation of 2,5-dihydroxyphenylacetate (homogentisate) is not related in sequence to the Fe^{2+}/α-KG hydroxylases, but exhibits a similar reaction mechanism [46, 47]. The enzyme, which is found in all aerobic forms of life and involved in tyrosine metabolism, also contains ferrous iron (Fe^{2+}) in the active site but the α-keto acid, which is decarboxylated is part of the substrate and the hydroxylation is associated with an "NIH" shift (migration of the acetyl group; [46]) (Fig. 5). Due to the involvement of HPPD in plant plastoquinone synthesis (starting from tyrosine), inhibitors of HPPD are used as herbicides, which uncouple photosynthesis [48]. Moreover, HPPD is currently subject of directed evolution studies to broaden its aromatic substrate spectrum [49].

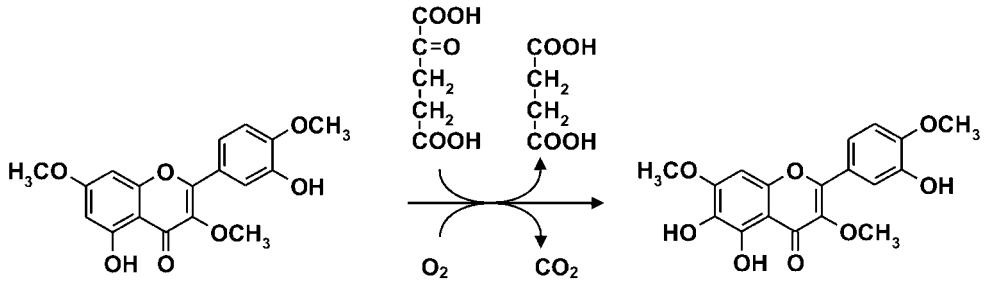

Figure 4. Specific 6-hydroxylation of 3,7,4′-trimethylquercitin by a Fe^{2+}/α-KG-dependent dioxygenase from the plant *Chrysosplenium americanum*. α-Ketoglutarate (α-KG) as the co-substrate is concomitantly oxidatively decarboxylated to succinate.

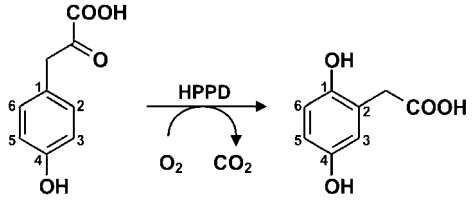

Figure 5. Hydroxylation and decarboxylation of 2,5-dihydroxyphenylacetate by HPPD (hydroxyphenolpyruvate dioxygenase) leading to homogentisate (2,5-dihydroxyphenylacetate). The reaction involves an NIH shift, in the course of which the acetyl rest migrates from the aromatic C_1 to C_2.

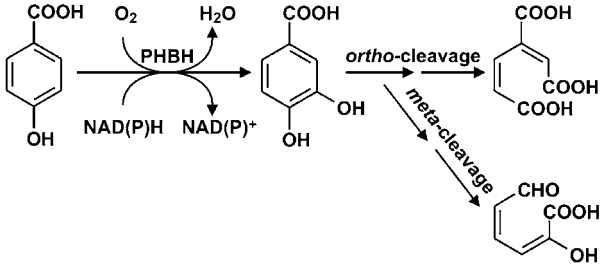

Figure 6. Hydroxylation of *p*-hydroxybenzoate in meta-position by microbial flavin-dependent PHBH (*p*-hydroxybenzoate hydroxylase). The product protocatechuate (3,4-dihydroxybenzoate) is the starting point for the further metabolism of aromatic carbon by *ortho*- or *meta*-ring cleavage leading to muconic acid or 2-hydroxymuconate semialdehyde derivatives, respectively.

Non-heme monooxygenases

Monooxygenases represent mixed-function oxidases which incorporate a single atom of molecular oxygen (O_2) into a substrate molecule while the other O-atom is concomitantly reduced to water. According to the prosthetic groups, four types of aromatic monooxygenases can be distinguished: heme-containing cytochrome P450 enzymes (P450s), di-iron hydroxylases, pterin-dependent monooxygenases, and flavin monooxygenases (FMO) [50]. Whereas the three former enzyme groups contain iron in the active site, FMOs are metal-free biocatalysts [51].

FMOs (EC 1.14.13.–) are involved in a variety of biochemical processes including microbial biodegradation of activated aromatic compounds, such as phenol, salicylic, or *p*-hydroxybenzoic acids [52]. *p*-Hydroxybenzoic acid hydroxylase (PHBH, EC 1.14.13.2) and phenol hydroxylase (EC 1.14.13.7) are thoroughly studied FMO enzymes, which occur in prokaryotic and eukaryotic microbes (e.g., *Pseudomonas putida*, *Trichosporon cutaneum*) and human liver, and introduce a second hydroxyl group in *ortho*-position to the existing one (Fig. 6) [53–56]. The second hydroxyl group activates the aromatic ring and facilitates its subsequent cleavage by specific dioxygenases (Fig. 6) [57]. In the course of the catalytic cycle, FMO protein and flavin moieties undergo significant dynamic changes, and a remarkable characteristic of these enzymes is their ability to catalyze both a reduction and an oxygenation in a single polypeptide [56]. The flavin component is reduced by NAD(P)H and the reduced flavin, in turn, reacts with O_2 to form a hydroperoxoflavin intermediate [R-(FAD)-O-OH], which is thought to act as the final oxygenating species [52, 58, 59]. A fundamental feature of PHBH is a network of H-bonds connecting the phenolic group of *p*-hydroxybenzoic acid in the buried active site to the surface of the protein. This network is involved in the regulation of the enzyme and promotes catalysis by protonating and deprotonating the substrate and product in the active site [56].

2-Hydroxybiphenyl-3-monooxygenase (EC 1.14.13.44), a specific FMO from *Pseudomonas azelaica*, was recently engineered by directed evolution to accept 2-*tert*-butylphenol as substrate, which is converted by *E. coli* host cells into 3-*tert*-butylcatechol, a costly synthon for pharmaceuticals and dye developers [60].

Pterin-dependent hydroxylases (EC 1.14.16.–) constitute a small family of monooxygenases, which catalyze the oxygenation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan, which results in the formation of tyrosine, 3,4-dihydroxyphenylalanine, and 5-hydroxytryptophan, respectively (Fig. 7). The enzymes are found in human liver and the central nervous system, as well as in a few bacteria (e.g.,

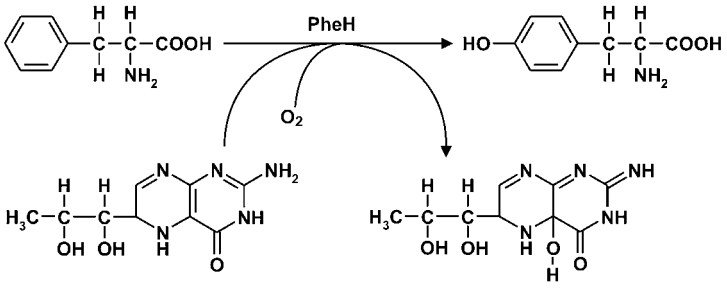

Figure 7. Hydroxylation of the amino acid phenylalanine in *para*-position yielding tyrosine by PheH (phenylalanine hydroxylase) (modified according to Fitzpatrick [63]). The reaction involves the conversion of the prosthetic group tetrahydropterin (left) to 4a-hydroxypterin (right). The oxygen atoms in both the amino acid and hydroxypterin come from dioxygen (O_2).

Chromobacterium violaceum, *Pseudomonas* spp.) [61–63]. They contain ferrous iron (Fe^{2+}) bound to two histidines and a glutamate at the active site, and utilize a tetrahydro(bio)pterin (Pte) as the co-substrate that is converted to 4α-hydroxy(bio)pterin during the catalytic cycle (see Fig. 5) [64]. The O-atoms in both the hydroxylated amino acid and the pterin products have been shown to originate from dioxygen. Although the ultimate hydroxylating intermediate of these enzymes has not been identified, the iron is very likely to be involved in the oxygen transfer; as promising reactive iron derivatives, ferric iron-(hydro)peroxo [R-(Pte)-O-OH–-Fe^{3+}] and high-valence iron-oxo intermediates [R-(Pte)-O=Fe^{4+}] are discussed [63, 64]. Recent studies have shown that pterin hydroxylases are relatively unspecific and can catalyze, in addition to aromatic oxygenation, also the benzylic hydroxylation of amino acids [65]. Phenylalanine hydroxylase (EC 1.14.16.1), which catalyzes the formation of the essential amino acid tyrosine, is the most important and thoroughly studied pterin monooxygenase since mutations in its sequence are responsible for the hereditary disease phenylketonuria [66, 67].

Bacterial multicomponent monooxygenases (BMMs) are soluble protein complexes, consisting of a hydroxylase, a reductase, and a small regulatory protein (coordinating electron consumption and substrate oxidation), which use a carboxylate-bridged di-iron center in their hydroxylase component to activate dioxygen for insertion into a C-H bond of the hydrocarbon substrate [68]. Though soluble methane monooxygenase (sMMO) may not catalyze aromatic hydroxylation under natural conditions, the enzyme, which converts methane to methanol, has been widely studied and is the model for the di-iron systems as shown in Figure 8A [69, 70]. Moreover, purified sMMO was found to have a broad substrate specificity and hydroxylates aromatic substrates in the laboratory [71]. Two groups of BMMS, alkene/aromatic monooxygenases (EC 1.14.14.1, EC. 1.14.13.–) and phenol hydroxylases (EC 1.14.13.7), incorporate one O-atom from dioxygen into aromatic substrates leading to the formation of monophenols or catechols, respectively (note that they are in the same subsubclass as many P450s and share with them EC 1.14.14.1 = unspecific monooxygenases; EC 1.14.13.7 is shared with FMO phenol hydroxylase) [72]. Aro-

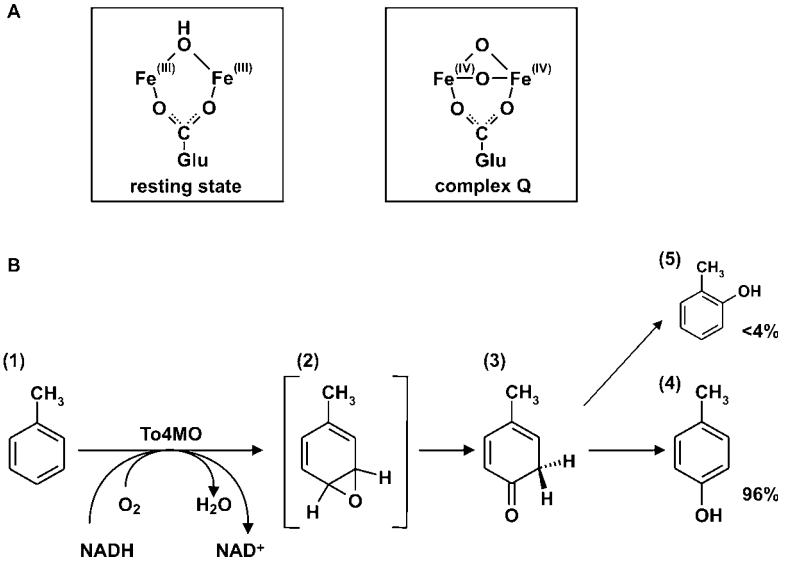

Figure 8. (A) Active site of toluene 4-monooxygenase (To4MO), a non-heme, bacterial multicomponent monooxygenase containing carboxylate-bridged di-iron, in its resting state (left) and the putative complex Q (right), which is thought to be the ultimate oxygenating species. (B) Selective hydroxylation of toluene (1) by To4MO via instable epoxide (2) and quinoide (2) intermediates. The reaction proceeds highly regioselectively and results in the formation of *p*-cresol (4) as major product and traces of *o*-cresol [50, 68, 77, 78].

matic BMMs are involved in the productive degradation and utilization of aromatic hydrocarbons in the environment (toluene, ethylbenzene, xylene) [73, 74]) and have phylogenetic and functional relations to methane monooxygenase and the Rieske-type dioxygenases, both mentioned above [68, 72, 75, 76]. The ferric resting di-iron(III) state [Fe^{3+}-OH-Fe^{3+}] is reduced by a 2-electron transfer from an NADH-dependent reductase to the active ferrous di-iron(II) state [Fe^{2+}-Fe^{2+}] that adds dioxygen to form, step-by-step, the so-called complex Q, a high-valent di-iron(IV) species [Fe^{4+}-O_2-Fe^{4+}] (Fig. 8A). The latter is thought to be the hydroxylating agent that introduces an oxygen atom to the aromatic ring via a postulated rebound mechanism and instable epoxide intermediates [68, 77, 78]. As an example, Figure 8B shows the regioselective hydroxylation of toluene by toluene 4-monooxygenase (To4MO) from *Pseudomonas mendocina* leading to the formation of *p*-cresol (yield 96%) [50].

Aromatic di-iron monooxygenases are versatile biocatalysts that hydroxylate, in addition to their "intrinsic substrate(s)", also other aromatic and even alicyclic compounds. Thus, To4MO regioselectively hydroxylates polycyclic aromatic hydrocarbons, in addition to toluene [79]. Toluene/*o*-xylene monooxygenase (ToMO) from *Pseudomonas stutzeri* oxidizes dimethylphenols, cresols, benzene, styrene, and naphthalene, which makes it an interesting candidate for bioremediation purposes. Recently, ToMO expression in the Antarctic cold-water bacterium *Pseudoalteromonas haloplanktis* has been reported. The aim of this study has been to develop specific degradative capabilities for the bioremediation of chemically contaminated marine environments characterized by low temperatures [80].

Heme monooxygenases (P450s)

While only a moderate number of enzymes and sequences of non-heme monooxygenases have been reported, the number of heme-containing monooxygenases is enormous and steadily increasing (at the moment, over 5500 sequences have been described; for details see http://drnelson.utmem.edu/P450.stats.2006.htm). Many of these enzymes, found in all kingdoms of life (archaea, eubacteria, eukaryota) [81–83], catalyze hydroxylations and epoxidations of a wide range of substrates including aromatic compounds from simple benzene to complex ring systems such as flavonoids or aromatic steroids [14]. In addition to oxygen transfers, P450s are responsible for at least 20 other chemical reactions including dealkylation, *S*- and *N*-oxidations, alcohol and aldehyde oxidations, as well as dehalogenation and denitration [84].

The first heme monooxygenase was discovered as a carbon monoxide-binding pigment in the microsomal fraction of rat liver cells in 1958 and named "cytochrome P-450" (P450) in 1962 due to the characteristic red shift of the Soret absorption peak of the carbon monoxide adduct [85, 86]. All P450s are heme-thiolate proteins whose prosthetic group is a protoheme (iron protoporphyrin IX) with a cysteine residue as the axial (= 5th) ligand. They constitute one of the two major groups of heme proteins found in nature (the other one, the heme-imidazole group, includes peroxidases and hemoglobin; see below) [87]. With the exception of soluble bacterial and fungal P450s, the majority of these enzymes is membrane-bound, being associated with either the inner mitochondrial or the microsomal (endoplasmatic reticulum) membranes [88]. Extracellular P450s have not been described so far. P450s are generally divided into two major groups (Class I and Class II) according to the different transfer systems they use for electron supply. Class I P450s include bacterial, mitochondrial, and fungal enzymes, which use a two-component electron transfer system consisting of ferredoxin and an FAD-dependent reductase. Class II enzymes are the microsomal monooxygenases receiving their electrons from membrane-bound FAD/FMN-dependent reductases (NADPH cytochrome P450 reductase) [89, 90]. According EC classification, P450 monooxygenases are grouped in following sub-subclasses EC 1.14.13.– (e.g., isoflavone hydroxylases: EC 1.14.13.52, EC 1.1.4.13.53), EC 1.14.14.– (unspecific monooxygenase(s): EC 1.14.14.1; largest group of P450s), EC 1.14.15.– (e.g., $P450_{cam}$: EC 1.14.15.1) and EC 1.14.99.– (miscellaneous monooxygenases including steroid 9a-monooxygenase: EC 1.14.99.24). P450s catalyzing aromatic hydroxylations are found within the first three sub-subclasses (for details see http://www.chem.qmul.ac.uk/iubmb/enzyme/).

The P450 enzyme superfamily is one of the largest and oldest enzyme gene families [91], whose internal classification is currently based on primary sequence homologies at the amino acid level (40% sequence identity = same family, 55% = same subfamily) [92]. The abbreviation for cytochrome P450 is *CYP* followed by a number denoting the family, a letter designating the subfamily (when two or more exist) and a number representing the individual gene within the subfamily (e.g., *CYP*1A1 = first gene in the subfamily A of the P450 gene family 1) [84, 93–95]. In addition, a number of common abbreviations exist, especially for the intensively studied enzymes such as $P450_{cam}$ (*CYP*101, camphor hydroxylating enzyme from Pseudomonas putida with a broad substrate specificity [96, 97] or P450 BM3 (*CYP*102A1; fatty acids and aromatic compounds hydroxylating enzyme from *Bacillus megaterium*; [98, 99].

Finally, due to their medicinal relevance (e.g., drug metabolism, carcinogenesis) and biotechnological significance (e.g., steroid transformation), P450s belong to the most intensively studied enzymes [100–103]. Excellent reviews on heme oxygenases, dealing with structural, mechanistic, genetic, metabolic, and historical aspects of cytochrome P450s, were published by Sono et al. [88], Werck-Reinhart and Feyereisen [104], Shou et al. [105], Guengerich [106], Lewis [84], Kirton et al. [107], Newcomb et al. [108], Estabrook [9], Hlavica [109] and Denisov et al. [110]; Table 3 lists a selection of other relevant recent publications on P450s published between 2004 and 2006.

Table 3. A selection of recent book publications and review articles on cytochrome P450 enzymes.

Topic	References
P450s as versatile biocatalysts with promising biotechnological potential	[13]
Essential techniques and methodologies for the investigation of P450s	[218]
Selective steroid hydroxylation by artificial P450 enzymes	[219]
Self-sufficient P450 monooxygenase from *Rhodococcus rubber*	[220]
Comprehensive survey on all aspects of P450s	[221]
Thermophilic cytochrome P450s	[222]
Biodiversity of cytochrome P450 redox systems	[223]
Molecular recognition of P450 proteins	[224]
Involvement of singlet oxygen in P450 catalysis	[225]
Intermediates in P450 catalysis	[226]
Structural biology of heme monooxygenases	[227]
Electrophilic oxidants in P450 catalysis	[228]
Flavocytochrome P450 BM3: Structure and mechanism of an biotechnologically important enzyme	[99]
Freeze-quenched, iron-oxo intermediates in P450s	[229]
Association of P450s and catalytic activity	[230]
Different pathways of monooxygenation by P450s	[231]
P450: Nature's most versatile biological catalyst	[232]
Mechanisms of P450 catalyzed oxidation reactions	[233]

The catalytic cycle of P450 has been studied over decades and there has been a long controversy about the ultimate oxygenating heme species [111–114]. Although it has not reliably detected as a "normal" intermediate, "an oxo-ferryl species (Fe^{4+}=O) is almost certainly responsible for the majority of the chemistry supported by P450 enzymes" [115]. A so-called consensus mechanism for the P450 reaction cycle has been proposed and can be summarized as follows (Fig. 9). The cycle starts with the concomitant binding of the (aromatic) substrate (R-H) and the release of the hydroxylated product from the P450-product complex (or a water molecule from the resting enzyme) leading to the ferric enzyme-substrate complex of P450 [(R-H)…heme(Fe^{3+})]. The latter accepts a first electron that is derived from NAD(P)H and supplied via flavin/ferredoxin or diflavin reductases to form the respective ferrous state of the enzyme [(R-H)…heme(Fe^{2+})] [13, 116]. The ferrous complex is reactive enough to add O_2 and the resulting ferrous dioxy-complex [(R-H)…heme(Fe^{2+}-O_2)] is capable of accepting a second electron from NAD(P)H via the electron transfer system to produce a ferric peroxy anion [(R-H)…heme(Fe^{3+}-O_2^-)] that is protonated to form the ferric hydroperoxy complex (also referred to as Compound 0 [(R-H)…heme(Fe^{3+}-O-OH)]. The hydroperoxy complex undergoes heterolytic cleavage between the oxygen atoms giving rise to the putative oxo-ferryl state [(R-H)…heme(Fe^{4+}=O)$^{\bullet+}$], that is an analog to Compound I of peroxidases and catalases. This highly reactive porphyrin species or a similar electrophilic complex, e.g., a protonated compound-II-substrate complex (protonated oxo-ferryl porphyrin [(R-H)…heme(Fe^{4+}-O-H)]), as recently shown for the heme-thiolate enzyme chloroperoxidase [117, 118], hydroxylates the (aromatic) substrate, which then dissociates and the cycle can start again [114–116].

The so-called "shunt" pathway is a remarkable side reaction of a number of P450s (but not of all), in the course of which the substrate is directly oxidized by hydrogen peroxide (H_2O_2 or an organic peroxide) to the hydroperoxo-ferryl state without the stepwise activation of dioxygen and electron requirement (i.e., without NADH). Under natural conditions (wild-type P450), this shunt pathway is an inefficient side activity but it offers an opportunity to create self-sufficient "P450 peroxygenases", which will be independent of NAD(P)H and auxiliary proteins [13, 119]. In fact, one promising approach in P450 research is the laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation (i.e., the improvement of the peroxide-shunt pathway) [120–122], which has already led to mutants of P450cam with 20-fold higher peroxygenase activity towards naphthalene as compared to the native enzyme [123, 124]. Other molecular approaches to improve the properties of P450s involve heterologous expression in microbial hosts, engineering using site-directed mutagenesis, laboratory evolution, and chimeragenesis. Besides enhanced peroxygenase activity, these investigations have the aim to extend the substrate spectrum of P450s and to improve the interaction of redox partners (electron transfer between reductases and hydroxylase) [13].

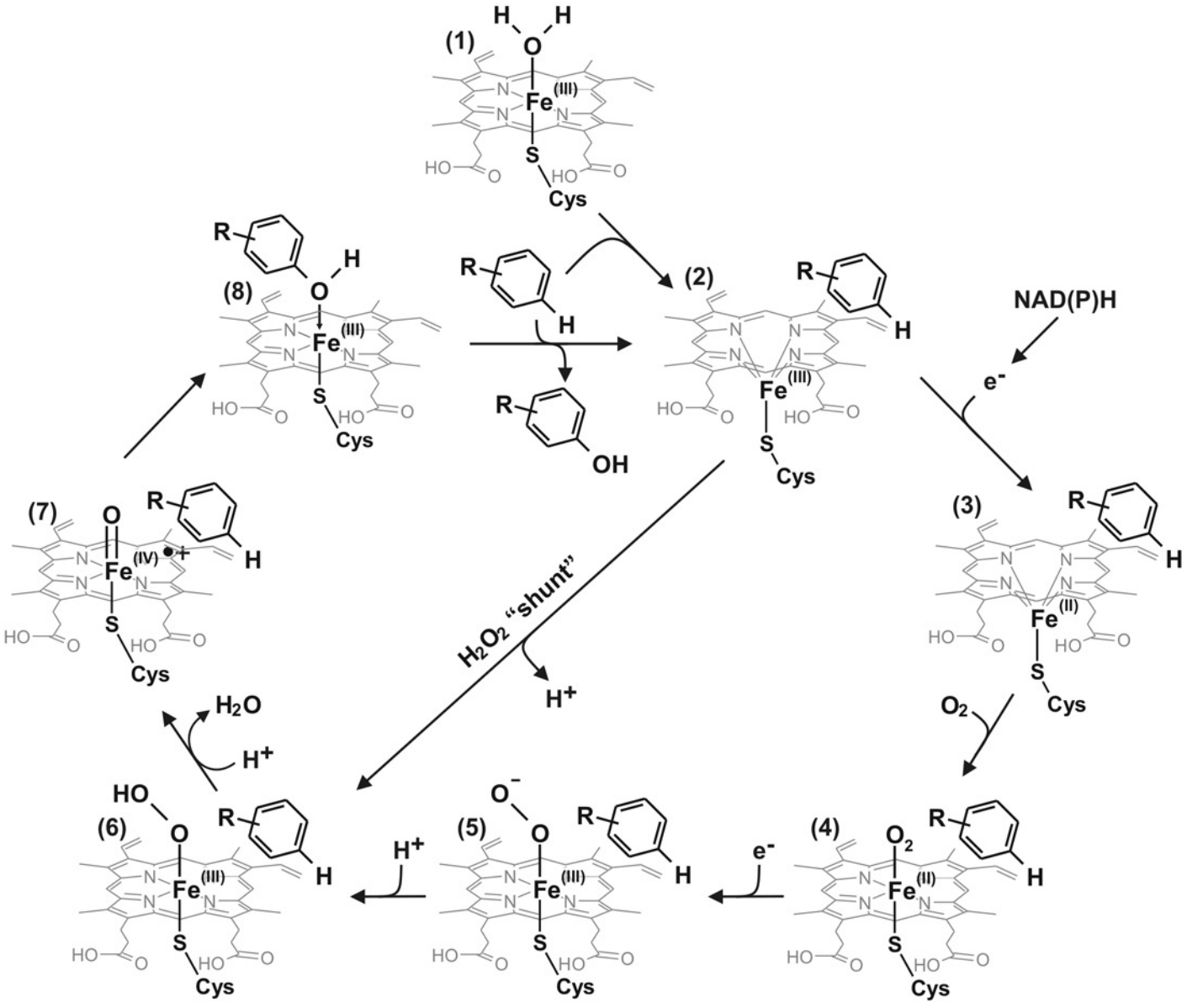

Figure 9. The consensus mechanism of cytochrome P450 catalysis (modified according to Sono et al. [88], Ortiz de Montellano and de Voss [115], Makris et al. [114], and Groves [116]). (1) native (hydro)ferric enzyme (resting state), (2) ferric heme-substrate complex, (3) ferrous heme-substrate complex, (4) ferrous-dioxygen complex, (5) ferric peroxy anion complex, (6) ferric hydroperoxy complex (Compound 0), (7) putative oxo-ferryl radical complex (compound I), (8) product-ferric enzyme complex (further explanations in the text).

It is impossible to mention all aromatic hydroxylations catalyzed by P450s. Therefore Fig. 10 depicts merely a selection of reactions and may simply demonstrate the versatility of this group of biocatalysts introducing oxygen into monoaromatic, polyaromatic, and heterocyclic compounds including organopollutants, plant ingredients, pesticides, and drugs [82, 84, 115, 125–128]. The broad substrate specificity of P450s, which all bear the same prosthetic group (cysteine-ligated heme) at the active site, can be attributed to different key amino acids, which are contact points for the binding of different substrates such that oxidation occurs in specific positions, being governed by the enzyme-mediated orientation of the substrate molecules relative to the heme moiety [129]. As a consequence, a set of moderately modified P450s enables an organism to hydroxylate almost any aromatic substrate.

The oxidation of aromatic rings by P450s involves oxidation of one of the π-bonds rather than direct insertion of the oxygen atom into one of the C-H bonds of the ring [115]. As a consequence, benzene oxide and similarly unstable arene oxides (epoxides) emerge and rapidly undergo heterolytic cleavage of

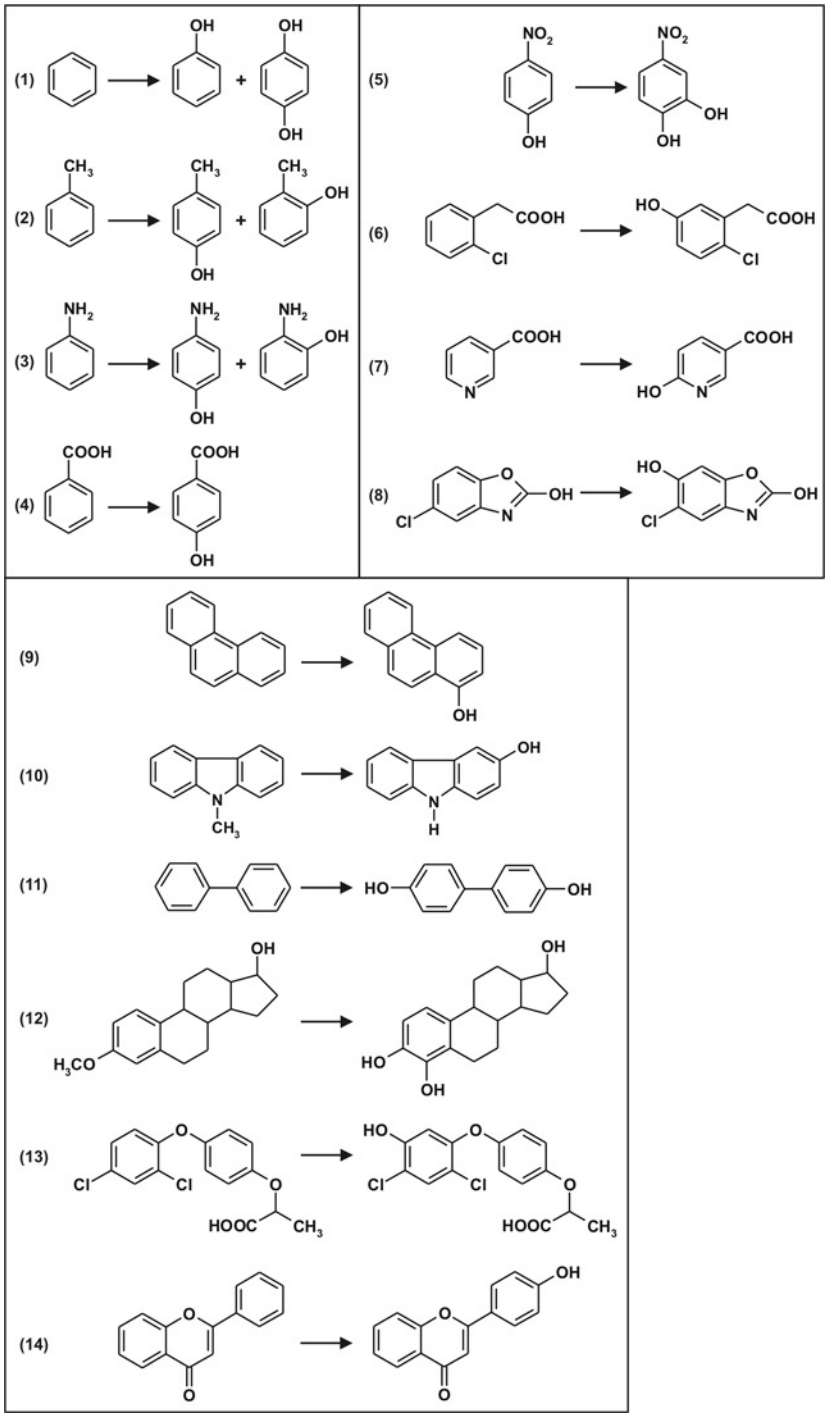

Figure 10. Selected aromatic hydroxylations catalyzed by P450s (according to review articles of Sono et al. [88], Holland [14], Lewis [84], and Ortiz de Montellano and Voss [115]). Conversion of monoaromatic compounds (1–7): (1) benzene hydroxylation yielding phenol and hydroquinone, (2) hydroxylation of toluene to *p*- and *o*-cresols, (3) aniline hydroxylation leading to 4- and 2-aminophenols, (4) benzoate hydroxylation at *para*-position, (5) hydroxylation of p-nitrophenol to 4-nitrocatechol, (6) *para*-hydroxylation of 2-chlorophenyl acetic acid, (7) specific hydroxylation of heterocyclic nicotinic acid to 6-hydroxynicotinic acid. Transformation of polycyclic and complex aromatics (8–14): (8) hydroxylation of the heterocyclic drug chlorzoxazone, (9) formation of 1-phenanthrol by hydroxylation of phenanthrene, (10) concomitant hydroxylation and *N*-demethylation of *N*-methylcarbazole to 3-hydroxycarbazole, (11) double hydroxylation of biphenyl leading to 4,4'-dihydroxybiphenyl, (12) steroid transformation: hydroxylation and *O*-demethylation of 3-O-methylestradiol, (13) hydroxylation of the herbicide dichlofop at position C_5, (14) flavone hydroxylation yielding 4'-hydroxyflavone.

one of the epoxide's C-O bonds, a process that is accompanied by migration of a hydride ion (H^-) from the carbon retaining the oxygen to the adjacent carbon cation (-C^+-) resulting in the formation of an also unstable ketone intermediate. Final tautomerization of this ketone yields the phenolic product (Fig. 11) [115]. The entire sequence of reactions is the so-called "NIH shift" (intramolecular hydrogen transfer = 1,2-hydride shift [130–132]); the name NIH shift arises from the U.S. National Institutes of Health, where studies were first reported on this type of reaction [133]).

Besides this generally accepted pathway, there is experimental and computational evidence for the oxidation of aromatic rings including benzene by a mechanism that does not involve formation of an epoxide intermediate [134–136]. The "non-epoxide" mechanism comprises a porphyrin proton shuttle and contributes, in addition to the non-enzymatic hydrolysis of arene oxides, to the formation of ketones and

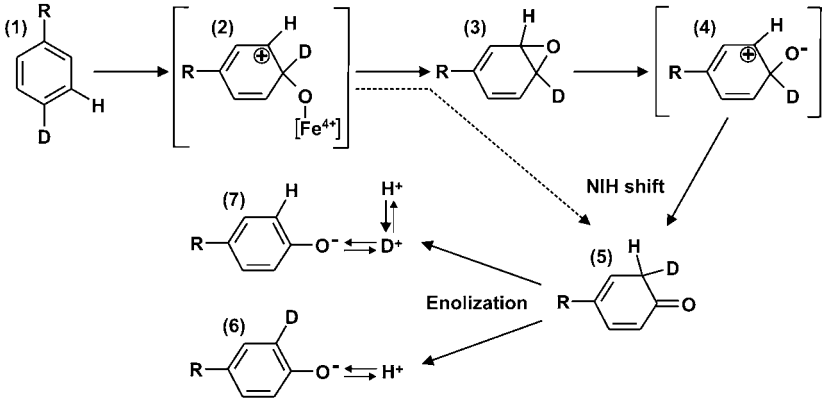

Figure 11. P450-catalyzed hydroxylation of deuterated aromatic substrates (1). The reaction proceeds via a putative enzyme-iron(IV)-substrate/product complex (2) and an unstable epoxide (3) to a carbocation intermediate (4) that undergoes NIH shift to give a ketone (5) tautomerizing to a deuterated (6) and a non-labeled (7) phenolic product. The partial loss of the deuterium (D) is a prove for the NIH shift (modified according to Ortiz de Montellano and Voss [115]). The dashed arrow indicates the possibility of the direct hydroxylation without epoxide intermediate [135].

phenols (Fig. 11) [113, 135]. This reaction type is especially favored with aromatic rings bearing electron-donating groups (e.g., phenols, anilines) [115].

Due to the almost unlimited catalytic potential of P450s, these enzymes have been subject of extensive studies in order to make use of them in biotechnological applications. Despite this fact, the use of P450s in industrial processes is still restricted to whole-cell oxidations due to the instability of cell-free P450 preparations and their complexity. Examples of technical applications are the biotransformation of steroid hormones by molds of the genus *Curvularia* [137] and recombinant *Saccharomyces cerevisiae* strains [138], the production of dicarboxylic acids from alkanes by the yeasts *Candida tropicalis* and *Yarrowina lipolytica* [139, 140], and the synthesis of aromatic precursors for agrochemicals by the entomopathogenic fungus *Beauveria bassiana* (Fig. 10) [141]. "Blue roses" certainly are the most famous example of a P450 whole-cell biotransformation. They contain the gene of *CYP*75A from *Petunia hybrida*, which is responsible for the synthesis of the blue blossom dye delphinidin [142, 143]. Comprehensive reviews on P450 in biotechnology including promising future applications and approaches to improve their catalytic properties and performance have been published by Schwanenberg et al. [144], Gillam and Guengerich [145], Guengerich [146], Urlacher et al. [101], Buchholz et al. [147], and Bernhardt [13].

Phenol oxidases

Tyrosinase (*o*-diphenol:dioxygen oxidoreductase) is found in almost all domains of life and belongs to a group of enzymes named type-3 copper proteins, which also include oxygen carriers (hemocyanins) from mollusks as well as plant catechol oxidases [148]. Excellent reviews on bacterial and fungal tyrosinases have recently been published [149, 150]. Among other functions, the extracellular enzyme is involved in the synthesis of melanins, where it is responsible for the first dioxygen-consuming steps of the pathway: the hydroxylation of L-tyrosine (monophenolase or cresolase activity) to DOPA and its subsequent oxidation to L-dopaquinone and L-dopachrome (diphenolase or catecholase activity) [151]. Subsequently, the reactive *o*-quinones formed tend to polymerize non-enzymatically to the high-molecular mass melanins. Due to these two functions, tyrosinase has been grouped into two enzyme subclasses (EC 1.14.18.1 and EC 1.10.3.1, respectively; for details see http://www.chem.qmul.ac.uk/iubmb/ enzyme/).

Tyrosinase bears two copper atoms in the active site, CuA and CuB, which are coordinated by three histidine residues ("type-3 copper center") [148]. Depending on the oxidation state of copper and the linking with dioxygen, the active site can exist in three different states: *deoxy*-, *oxy*-, and *met*-tyrosinase [(Cu^{+}-Cu^{+}), (Cu^{2+}-O_2-Cu^{2+}), (Cu^{2+}-OH^{-}-Cu^{2+}), respectively][151]. Figure 12 shows a simplified catalytic cycle of tyrosinase with focus on the hydroxylase activity [149, 152]. The *deoxy*-state reversibly adds dioxygen (O_2) leading to the *oxy*-state in which the oxygen is bound as a peroxide between the copper atoms. In the absence of any substrate, more than 80% of the protein is in the *met*-state that therefore represents the resting state of tyrosinase. In the *met*-form, the two copper atoms are bridged by hydroxo ions and it is formed from the *oxy*-state by binding a monophenol. The *meta*-form is converted back into the *deoxy*-state via different *meta*-intermediates by a two electron reduction, in the course of which *o*-quinones and *o*-diphenols are released. The intimate mechanism of tyrosinase reaction remains partially unclear. However, it was shown that the *o*-diphenol oxidation follows Michaelis–Menten kinetics, whereas monophenol hydroxylation shows a characteristic lag-phase [151]. The latter can be counteracted by addition of traces of *o*-diphenols [153]. An unusual

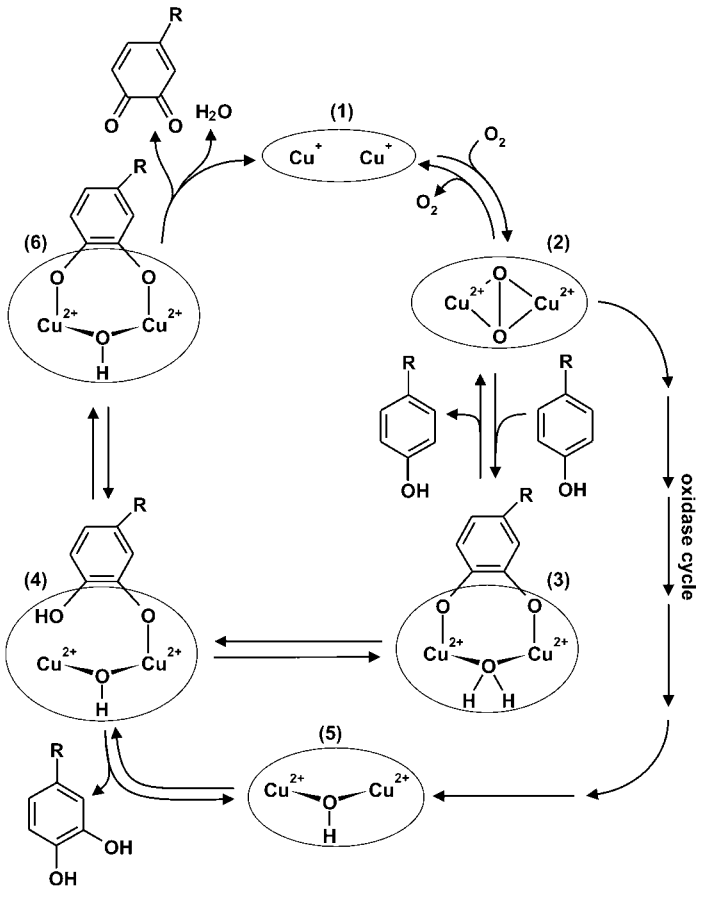

Figure 12. Catalytic cycle of tyrosinase yielding catechol and *o*-benzoquinones from monophenolic substrates (modified according to Fenoll et al. [152, 212]. (1) *deoxy*-tyrosinase, (2) *oxy*-tyrosinase, (3) hydroxylation complex of *met*-tyrosinase, (4) nucleophilic attack complex of the phenolic substrate and *met*-tyrosinase, (5) *met*-tyrosinase, (6) diaxial binding complex of *met*-tyrosinase.

tyrosinase with higher cresolase than catecholase activity has recently been found in the phytopathogenic bacterium *Ralstonia solanacearum* [154]. Furthermore, purified tyrosinase was shown to act as a peroxygenase incorporating oxygen from hydrogen peroxide (H_2O_2) into phenolic substrates [155]. There are several attempts to make use of tyrosinase-catalyzed hydroxylation. An example is the production of antioxidant *o*-diphenols with beneficial properties as food additives or pharmaceutical drugs [150, 156].

Unlike tyrosinase, laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2), a second widespread, extracellular phenol oxidase belonging to the blue-copper proteins [157, 158], cannot directly introduce oxygen into phenolic rings but it is able to initiate a cascade that can lead indirectly, via phenoxyl radicals, cyclodienone cations, and the addition of H_2O to the formation of *p*-quinones and *p*-hydroquinones (Fig. 13). Analogous reactions have been proposed for phenol-oxidizing peroxidases (e.g., horseradish peroxidase) [159–164].

Peroxidases

Heme-peroxidases (EC 1.11.1.–) using hydrogen peroxide as oxidant are ubiquitous in nature. The active site of these enzymes contains iron heme (protoporphyrin IX) as a prosthetic group, which is mostly linked to a proximal histidine (5th ligand). Exceptions to this structural rule are chloroperoxidase and *Agrocybe aegerita* peroxidase, where the 5th ligand is, as in case of P450s, a cysteine (heme-thiolate proteins) [87, 122]. In their natural function, heme-peroxidases generally perform one-electron, rather than two-electron oxidations. Most microbial peroxidases are extracellular enzymes and involved in the degradation of recalcitrant aromatic polymers (lignin, humic materials) and in the oxidative detoxification of plant ingredients and organopollutants. Typical peroxidase substrates are phenolic compounds, methoxylated aromatics (e.g., veratryl alcohol), as well as manganese(II) ions (Mn^{2+}) [165–167]. Plant peroxidases are involved in cell-wall synthesis (lignification, formation of suberin), phytohormone metabolism, as well as infection response [168, 169]. Animal/human peroxidases play a defensive role against microbial infection and thyroid peroxidase is essential for the synthesis of the hormone thyroxine. A comprehensive

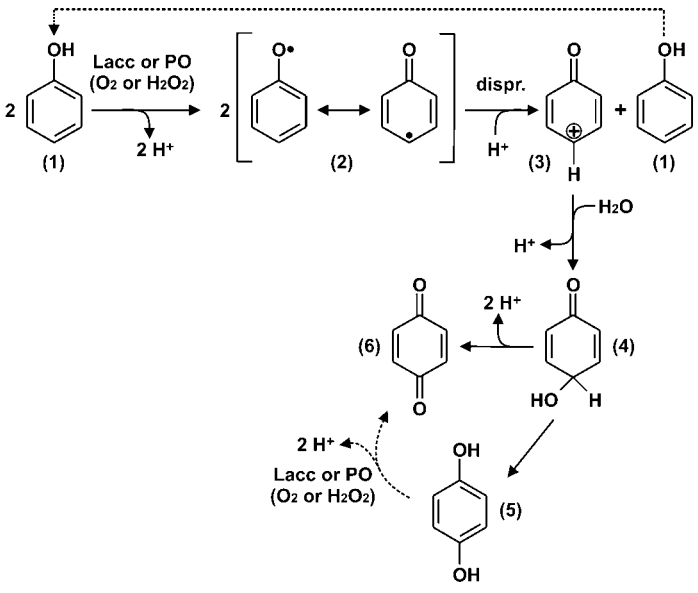

Figure 13. Indirect hydroxylation of phenol by laccase (Lacc) or phenol-oxidizing peroxidases (PO). (1) phenol, (2) two mesomeric forms of the phenoxyl radical that can disproportionate to a cyclodienone cation (3) and a phenol molecule (1), (4) instable hydroxycyclodienone rearranging to hydroquinone (5) or *p*-benzoquinone (6).

book survey on heme peroxidases featuring plant, fungal, bacterial, and animal enzymes was published by Dunford [170].

The catalytic cycle of heme-peroxidases illustrated by the oxidation of a phenolic substrate is given in Figure 14 (modified according to Dunford [170], van Rantwijk and Sheldon [171], Veitch [169, 172]). It shows similarities to the P450 cycle and passes through following intermediates: the native (hydro)ferric peroxidase (=resting enzyme) [heme(Fe^{3+}-H_2O)] binds H_2O_2 to form an extremely short-lived iron(-III)-peroxide complex [heme(Fe^{3+}-O-OH)] ("Com-

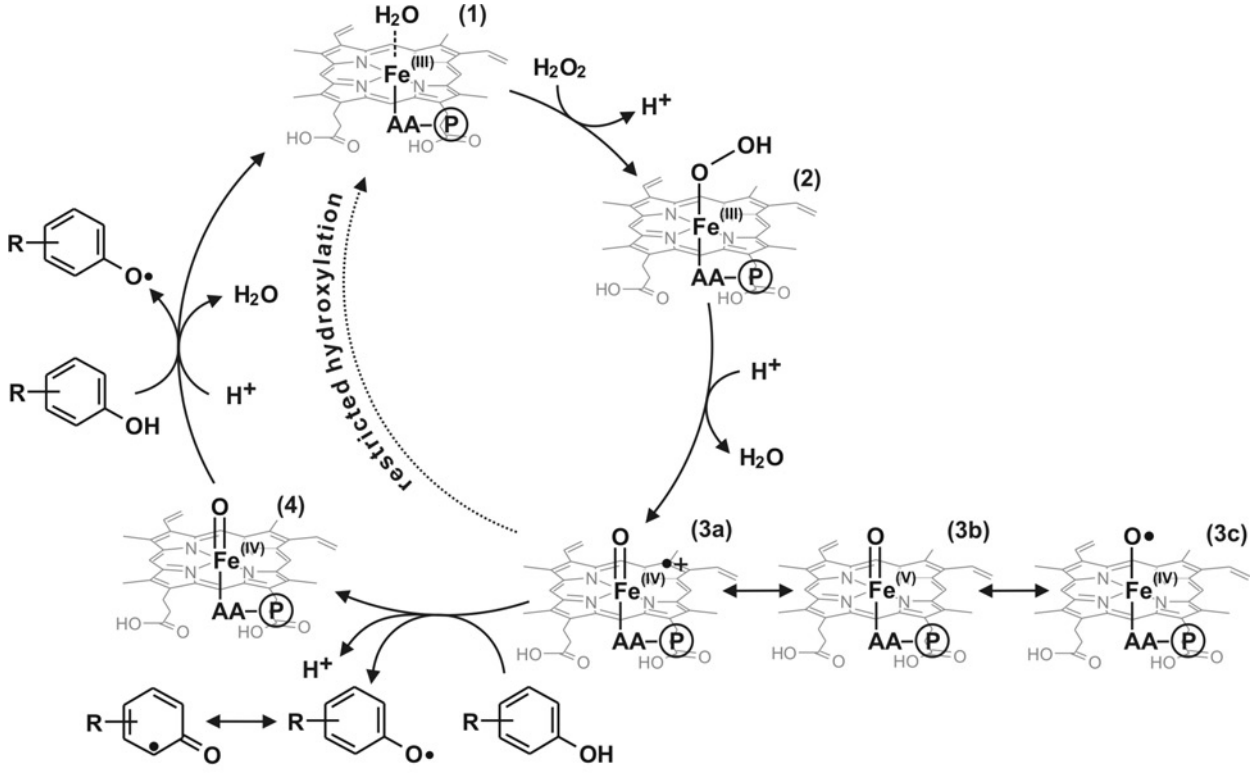

Figure 14. The catalytic cycle of heme peroxidases illustrated by the oxidation of a phenolic substrate. The phenoxyl radicals formed may undergo the cascade described in Fig. 13. The dotted arrow indicates the capability of a few peroxidases to transfer the oxygen from Compound I to a substrate (see text for further details). (1) native (hydro)ferric enzyme, (2) iron(III)-peroxide complex (analogous to Compound 0 of P450s), (3a) peroxidase Compound I (oxo-ferryl radical complex) that exists in different mesomeric forms: (3b) oxo-iron(V) complex, (3c) oxo-radical ferryl complex, (4) Compound II (oxo-ferryl complex).

pound 0" of P450s), that is heterolytically cleaved between the oxygen atoms by a two-electron transfer from the heme. As the result, a water molecule is expelled and Compound I, an oxo-ferryl, heme-radical cation complex [heme(Fe^{4+}=O)$^{\bullet+}$], emerges and can react with a first phenolic molecule to give a phenoxyl radical and Compound II. The latter is an oxo-ferryl heme [heme(Fe^{4+}=O)] and reacts with the second substrate molecule resulting in the formation of a second phenoxyl radical and the native ferric enzyme. In summary, within the typical heme-peroxidase cycle, two substrate molecules are oxidized by one-electron abstraction (but without oxygen transfer) while one molecule of hydrogen peroxide is consumed and two water molecules are produced.

It is assumed that Compound I (Fig. 14, dashed arrow) or a derived protonated oxo-ferryl species (Fig. 14) is the active intermediate in oxygen-transfer reactions of peroxidases, and the same assumption has been made regarding P450s [117, 173]. But unlike P450s, only a few examples of such reactions, in the course of which peroxidases act as peroxygenases, have been reported in the literature. The main obstacles in this respect are the preference of one-electron versus two-electron transfers by most peroxidases and the sterically restricted active site, which limits the access of the substrate to the heme iron and ferryl oxygen [171, 174]. An excellent review comparing the structures of the high-valent, metal-ion, heme-oxygen intermediates in peroxidases, oxygenases, and catalases has been published recently [175].

Concerning oxygen transfer, four peroxidases are of particular interest: horseradish peroxidase (HRP, EC 1.11.1.7), chloroperoxidase (CPO, 1.11.1.10), and the recently discovered *Agrocybe aegerita* peroxidase (AaP, 1.11.1.–), as well as the "artificial" microperoxidases (MPs, 1.11.1.–). Commercial HRP from *Armoracia rusticana* was shown to hydroxylate benzene to phenol when benzene was used as the solvent with just 0.1–5% phosphate buffer, whereas in aqueous buffered media benzene was inert to HRP attack [176]. Oxygen, in this reaction, came from hydrogen peroxide which was demonstrated using $H_2{}^{18}O_2$. Klibanov et al. [177] reported the efficient hydroxylation of aromatic substrates (phenolic compounds, phenylalanine) by HRP and O_2 in the presence of dihydroxyfumaric acid as hydrogen donor. This reaction, however, was later shown to be radical-mediated and the last step non-specifically catalyzed by hydroxyl radicals, and thus, independent of the pure catalytic cycle of the enzyme [178]. HRP is currently the subject of genetic engineering to improve the oxygen transfer potential and variants have been obtained, which have at least some of the key functional properties of a cytochrome P450. [171, 179].

Microperoxidase-8 (MP-8) is a "minienzyme" that consists of an octapeptide obtained after proteolytic digestion of cytochrome *c* from horse heart and is covalently bound to heme [180]. Upon reaction with peroxides, it forms high-valency, oxo-ferryl intermediates that are analogous to Compounds I and II and can take effect in peroxidase or P450 modes [181, 182]. With respect to the latter mode, MP-8 was shown to hydroxylate aniline, phenol, and anthracene, as well as to some extent naphthalene, but not benzene [183, 184]. Hydroxylation was favored over peroxidation after addition of ascorbate to the reaction mixture preventing the coupling of phenoxyl radicals [183]. Later, other microperoxidases have been prepared (e.g., MP-9 and MP-11 from horse-heart cytochrome *c*, MP-5 from cytochrome c_{552} of *Marinobacter hydrocarbonoclasticus*) and they are thought to have a promising potential as a new generation of biocatalysts and/or biomimics in biotechnological applications [182, 185, 186]. However, to achieve this goal it will be necessary to improve the catalytic properties of MPs, particularly with respect to turnover numbers and enzyme-substrate ratios [184, 187].

CPO is an extracellular enzyme that was discovered by Hager and co-workers in the 1950s in *Caldariomyces fumago*, an ascomycete belonging to the so-called sooty molds [188, 189]. As P450s, it is a heme-thiolate protein with versatile catalytic properties. Thus, CPO chlorinates, brominates, and iodates organic compounds including aromatic substrates and catalyzes a series of non-halogenating oxidations, among others, epoxidation and hydroxylation of activated C-H bonds, as well as selective sulphoxidations [171]. Aromatic rings, however, are not susceptible to oxygen transfer by CPO. Merely cyclic dienes, such as 1,2-dihydronyphthalene or indene, can be epoxidized by CPO and subsequently undergo spontaneous transformation to the respective oxides or dihydrodiols [190, 191]. Furthermore, CPO catalyzes benzylic hydroxylation, e.g., of *p*-xylene or toluene [192, 193].

We have recently reviewed the reactions catalyzed by heme-thiolate haloperoxidases [122] and described, in this context, a second enzyme of this type. The haloperoxidase of the agaric mushroom *Agrocybe aegerita* (AaP) is an extracellular peroxygenase that can be regarded as a true functional hybrid of heme-thiolate haloperoxidases and cytochrome P450s. Thus, as the latter and unlike CPO, AaP selectively hydroxylates aromatic substrates such as toluene or naphthalene [122] and recently, even benzene and chlorobenzene have been found to be subject of AaP-catalyzed oxygen transfer (Ullrich 2006, unpublished results). In case of naphthalene hydroxylation, almost the same ratio of 1-naphthol versus 2-naphthol (36:1) was observed as for a $P450_{cam}$ mutant [194]. The

resting state of AaP has its Soret absorption maximum at 420 nm, which is also characteristic for P450s (416–420 nm) and differs from CPO (401 nm) (interestingly, recent studies with CPO indicate that, in the presence of 100 mM formic acid, the CPO spectrum shifts and becomes almost identical to those of P450s and AaP [195]). On the other hand, the N-terminus of AaP shows 36% sequence identity (5 out of 14 amino acids) with that of CPO, and AaP brominates (but hardly chlorinates and iodates) aromatic substrates, which is typical for peroxidases, but has not been reported for P450s [122, 196–198]. The hypothetical catalytic cycle of AaP combining both own observations and recent findings on CPO and P450s, is given in Figure 15. It considers, inter alia, the fact that hydroxylation products of AaP have never been observed as sole reaction products but always together with varying amounts of their oxidation products (quinones) [198]. Furthermore, it should be mentioned that AaP catalyzes, in addition to hydroxylations and halogenations, the oxidation of aromatic alcohols and aldehydes, *O*-dealkylation, phenol oxidation, and the catalase-like destruction of H_2O_2 [122, 197, 198].

Data in the literature and own recent findings indicate that extracellular peroxygenases of the AaP-type do also occur in other organisms. Thus, we found AaP-like activities in other *Agrocybe* strains, as well as in the coprophilous ink caps *Coprinus radians* and *C. verticillatus* ([122] and Dau, et al., unpublished results). An unusual peroxidase, that oxidized the methyl group of different toluenes via benzyl alcohols into the corresponding aldehyde, a reaction that was also reported for AaP and CPO, was described for a not further characterized Coprinus sp. strain [199] and the aromatic hydroxylation of 4-hydroxybenzyl alcohol by a heme-bromoperoxidase of the red alga *Cystoclonium purpureum* was already reported in 1976 [200]. Unfortunately, more detailed information on these enzymes is lacking since their purification and characterization is still pending. Maybe, a recently developed hydroxylation assay, which uses naphthalene as target substrate and directly follows its conversion to 1-naphthol, will help to find more fungal species producing AaP-like peroxygenases (Kluge et al., unpublished results).

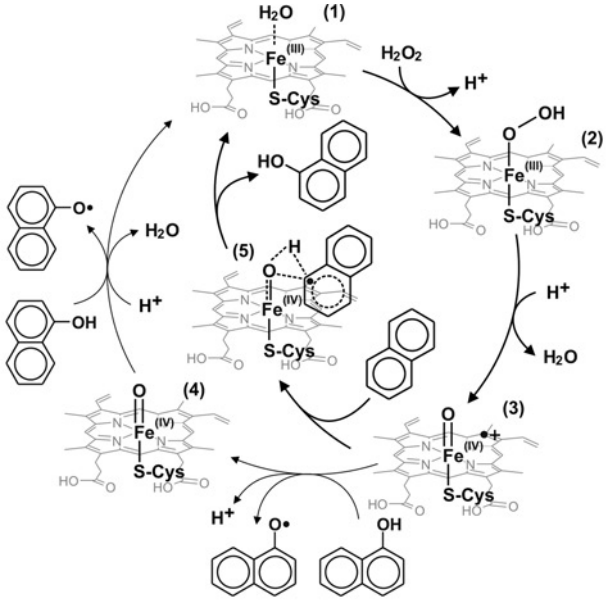

Figure 15. Hypothetical catalytic cycle of AaP (*Agrocybe aegerita* peroxidase) illustrated by the hydroxylation/oxidation of naphthalene and calculated on the basis of recent findings on CPO (Green et al. [117, 213]), P450s (Wong [214], Makris et al. [114]), and our own studies on AaP (Ullrich et al. [197], Ullrich and Hofrichter [198], Hofrichter and Ullrich [122], Kluge et al., unpublished results). (1) native (hydro)ferric enzyme, (2) iron(III)-peroxide complex (Compound 0), (3) Compound I, (4) Compound II, (5) putative transition state of a protonated Compound I/II-substrate complex.

Aromatic hydroxylation by hydroxyl radicals

Several biochemical mechanisms are considered to facilitate extracellular aromatic hydroxylation in vivo by means of hydroxyl radicals (·OH). The ability of ·OH to hydroxylate aromatic compounds has been known for a long time and used to follow the formation of this extremely reactive oxygen species [203]. Among microorganisms, brown-rot fungi belonging to the basidiomycetes (e.g., *Gloeophyllum* spp.) are regarded as the most potent producers of ·OH, and they use the radicals in vivo to decompose recalcitrant crystalline cellulose and modify aromatic compounds such as lignin [167, 202]. Two main pathways of the formation of ·OH by Fenton's reagent chemistry ($Fe^{2+} + H_2O_2 \rightarrow OH^- + {}^{\cdot}OH$) have been proposed: quinone redox cycling and cellobiose dehydrogenase (CDH) turnover [202]. The former reactions are based on the fungal metabolite dimethoxy-*p*-benzoquinone (DMBQ) that is secreted and reduced by a mycelial reductase to the corresponding hydroquinone (DMHQ). DMHQ, which in turn, reduces Fe^{3+} while it is converted to a semiquinone radical that reacts with O_2 to give H_2O_2 and DMBQ [203]. A number of xenobiotic aromatics (halogenated phenols, fluoroquinolone) were found to be subject of rapid oxidation and decomposition by the *Gloeophyllum* Fenton system leading to a series of hydroxylated products [204, 205].

CDH (EC 1.1.99.18) is an interesting extracellular enzyme found in various wood-degrading fungi (brown-, white-, and soft-rot fungi) and contains both heme and FAD. It oxidizes soluble cellodextrins

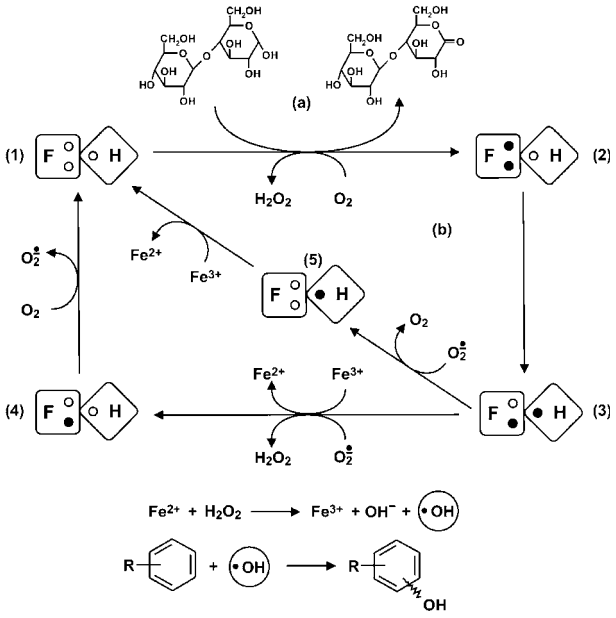

Figure 16. Reaction scheme for the formation of hydroxyl radicals (•OH) by CDH (cellobiose dehydrogenase) (modified according to Mason et al. [211]). (a) enzymatic oxidation of cellobiose to cellobionolactone by CDH and concomitant production of H_2O_2, (b) iron reduction cycle ($Fe^{3+} \rightarrow Fe^{2+}$). (c) H_2O_2 and Fe^{2+} make Fenton chemistry possible resulting in the formation of hydroxyl radicals (•OH), which unspecifically hydroxylate aromatic compounds. F – flavin, H – heme, ○ – empty electron sites, • – filled electron sites.

and lactose to lactones while using a wide spectrum of electron acceptors including dioxygen (that is reduced to H_2O_2), quinones, phenoxyradicals, and Fe^{3+} (Fig. 16) [206]. In the presence of Fe^{3+} and O_2, the CDH-producing white-rot fungus *Phanerochaete chrysosporium* efficiently hydroxylated salicylic acid to 2,3- and 2,5-diydroxybenzoic acids, which was attributed to •OH [207]. Hildén et al. [208] demonstrated that the CDH-dependent Fenton system hydroxylates methoxylated aromatics (lignin models) and cooperated, in this way, with manganese peroxidase (a key enzyme of lignin degradation). Other fungal enzymes which have been proposed to generate hydroxyl radicals are the couple laccase and aryl alcohol oxidase [209] and lignin peroxidase [210]. Though Fenton's reaction is an efficient catalytic system, its hydroxylating potential may be relevant only for degradative activities of wood-rotting fungi due to a lack of specificity and selectivity [211].

Outlook

Regarding the enormous biocatalytic potential of oxygenating enzymes (regardless of which group they belong to) and the fact that most of them are still little used in industry, it is to be expected that the following scientific and technical approaches will be increasingly pursued in the near future:

- further development of whole-cell transformations with genetically engineered organisms (preferably fast-growing bacteria and fungi)
- site-directed mutagenesis, laboratory evolution, and chimeragenesis to improve catalytic enzyme properties with respect to cofactor requirement, electron transfer, catalytic constants, substrate binding sites, and stability (pH, temperature, H_2O_2)
- search for novel (per)oxygenases using molecular and chemical methods (specific DNA probes, specific colorimetric tests, high-throughput screenings)
- process optimization of enzyme catalysis in special enzyme reactors (membrane techniques, non-ionic fluids, micelles)
- construction of "artificial" biocatalysts ("enzyme mimics") by chemical methods

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