FUNGAL UNSPECIFIC PEROXYGENASES: A NEW GENERATION OF OXYGEN-TRANSFERRING BIOCATALYSTS

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ABSTRACT

The oxygenation of organic molecules is a challenging task in synthetic chemistry and therefore biocatalytic approaches using oxygen-transferring enzymes have come into the focus of chemists and biotechnologists. Fungal peroxygenases represent a unique enzyme type that selectively transfers oxygen from peroxides (R-OOH) to numerous substrates such as benzene derivatives, polycyclic aromatic hydrocarbons, *N*- and *S*-heterocycles, linear and cyclic alkanes, alkenes as well as to complex drug molecules and pesticides. Peroxygenases are heavily glycosylated heme-thiolate proteins that are actively secreted by fungi. Over 1,000 putative peroxygenase-like sequences, which form at least two distinct clusters, can be found in genetic data bases indicating the widespread occurrence of such enzymes in the whole fungal kingdom including true fungi and fungus-like heterokonts. Thus, peroxygenases represent, on the phylogenetic level, a fungi-specific superfamily of hemeproteins.Their catalytic cycle combines the pathways of heme peroxidases and cytochrome P450 monooxygenases. Due to their high stability and the use of cheap peroxides as co-substrate, peroxygenases could become a powerful biocatalytic tool for applications in organic synthesis and other fields.

Keywords: EC 1.11.2.1, heme-thiolate, oxyfunctionalization, *Agrocybe*, *Marasmius*

INTRODUCTION

The term "peroxygenase" refers to enzymes that transfer a peroxide-borne oxygen atom to a substrate molecule. Biocatalysts preferably catalyzing such reactions are nowadays classified in a separate enzyme sub-subclass (EC 1.11.2) with five members that are all heme proteins (*www.chem.qmul.ac.uk/iubmb/enzyme/EC1/11/2/*). The name peroxygenase for an enzyme first appeared in the literature in 1977 in an article of Ishimarua and Yamazaki describing a hemeprotein that catalyzed the hydroperoxide-dependent hydroxylation of indole, phenol and aniline in microsomes of pea seeds [1]. Since the end of the 1980s, the term peroxygenase has been in use also for cytochrome P450 enzymes in the context of the so-called "peroxide shunt" pathway that is a side activity of some P450 monooxygenases [2]. The first fungal peroxygenase was discovered in the agaric mushroom *Agrocybe aegerita* by Upadhyay and co-workers in 1995 and described, for the time being, as an "alkaline lignin peroxidase" that oxidized veratryl alcohol to veratraldehyde [3].Ten years later, this enzyme was recognized as a heme-thiolate protein that acts on diverse aryl alcohols, halides [4] and simple aromatics such as toluene and naphthalene [5]. In 2011, after several renaming, this enzyme type was classified as unspecific peroxygenase (UPO) under EC 1.11.2.1 in the enzyme nomenclature system [6]. Nowadays, such enzymes of different fungal origin are usually abbreviated by using the first letter of the fungal genus, the first two letters of the specific epithet and the acronym UPO (e.g. *Aae*UPO = unspecific peroxygenase of *Agrocybe aegerita*).

OCCURRENCE OF UNSPECIFIC PEROXYGENASES

The UPO-model fungus *A. aegerita* (syn. *Agrocybe cylindracea*) belongs to the family Strophariaceae (formerly Bolbitiaceae) and is commonly known as the Black poplar mushroom. The fungus grows preferably on wood of poplars (*Populus* spp.) and other broad-leaved trees, causes an unspecific white rot and tolerates high amounts of phenols [7]. It is found in Europe, North America and Asia and prefers warm and mild climates. *A. aegerita* is a popular edible mushroom in Mediterranean countries, especially in Italy (ital. Pioppino or Piopparello), where it is also commercially cultured [8].The second UPO was described for the Ink-cap *Coprinellus* (*Coprinus*) *radians*, a wood- and mulch-dwelling fungus that

belongs to the family Psathyrellaceae closely related to the Strophariaceae [9]. As *Aae*UPO, *Cra*UPO was found to oxidize aryl alcohols, toluene, naphthalene and bromide [10]. The third enzyme of this type, *Mro*UPO, is produced by the boreo-subtropical Pinwheel mushroom (*Marasmius rotula*) that preferably colonizes twigs and belongs to the family of Marasmiaceae [11]. *Mro*UPO hardly oxidizes halides, exhibits a less pronounced oxygenating activity for aromatic rings but instead can oxidize bulkier substrates such as steroids [12]. In addition to these three well-studied UPO producers, we have identified several other mushroom species secreting UPOs, for example, *A.parasitica, A. chaxingu, A. alnetorum, Agaricus bisporus, Coprinus* sp. DSM 14545, *Coprinopsis verticillata, Auricularia auricula-judae, Mycena galopus*. Eventually, recombinant UPOs from *A. aegerita* (r*Aae*UPO) and the genome-sequenced model fungus *Coprinopsis cinerea* (r*Cci*UPO) have been recently expressed at laboratory scale in *Saccharomyces cerevisiae* [13] and *Aspergillus oryzae* [14].

More information on the occurrence of UPOs was obtained from genetic databases after the first UPO genes had been sequenced [15]. BLAST searches in Gene Bank (http://www.ncbi.nlm.nih.gov/nucleotide) and other public sequence databases revealed more than one thousand homologous nucleotide sequences encoding putative UPO proteins. The majority of these sequences belongs to the Basidiomycota (~35%) and Ascomycota (~60%) and the remaining 5% to the Mucoromycotina ("Zygomycota"), Chytridiomycota and Glomeromycota as well as to Oomycota of the genus *Phytophthora* (fungus-like heterokonts). No indication was found for the presence of UPO genes in plants including green

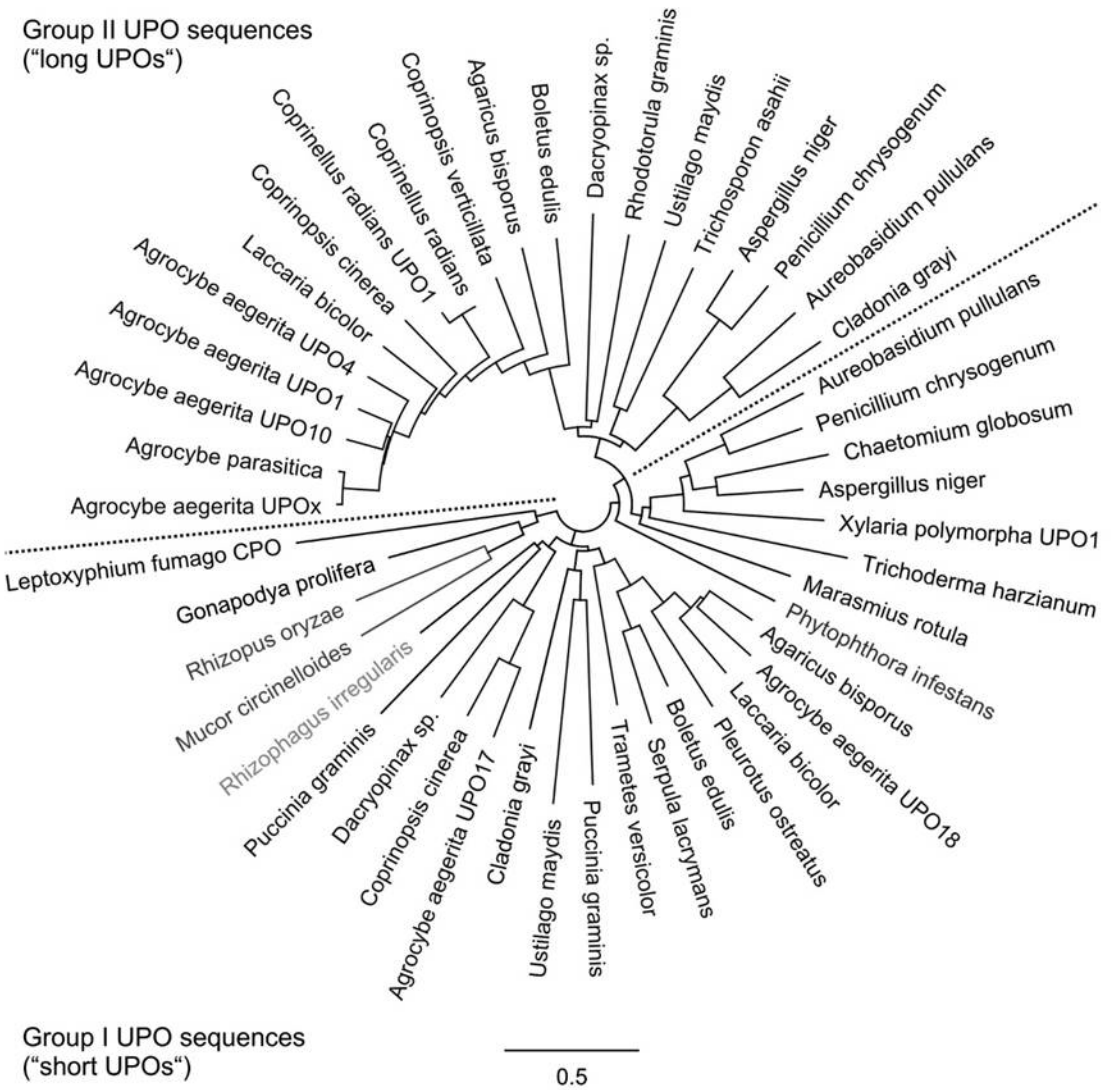

Figure 1. Neighbor-joining phylogentic tree based on 47 fungal UPO sequences from diverse fungi using Jukes-Cantor genetic distances. The dotted lines separate UPO sequences of groups I and II.

algae (Viridiplantae), animals (Metazoa), protists (Amoebae etc.) or prokaryotes (Eubacteria, Archaea). This indicates that UPOs are an evolutionary old, fungi-specific superfamily of proteins [15]. Furthermore, the presence of UPO genes in several *Phytophthora* species supports the hypothesis that an extensive horizontal gene transfer had taken place between phytopathogenic Ascomycota and Oomycota early in the evolution [16]. Fig. 1 illustrates the diversity of UPOs by a phylogenetic tree covering 47 sequences of 30 representative fungal species. The tree comprises examples of the above mentioned taxonomic entities as well as of different eco-physiological groups of fungi (litter decomposers, white-, brown- and soft-rot fungi, ectomycorrhizal fungi, phytopathogens, molds, yeast-like fungi and lichens). Last but not least, molecular screenings for UPO transcripts have indicated that these enzymes are ubiquitous in forest soils and leaf-litter [17].

PRODUCTION AND CHARACTERIZATION OF UPO PROTEINS

UPOs can be produced using wild-type strains of the above mentioned fungi (e.g. *A. aegerita* or *M. rotula*) in liquid culture (agitated flasks or stirred-tank bioreactors). Growth media must be rich in carbon and nitrogen and are usually based on complex substrates such as soybean flour, peptone, yeast extract or grass pellets. The exact composition of the growth medium and the growth conditions (agitation, aeration, pH control, etc.) must be optimized for each particular species/strain. For example, our model organism *A. aegerita* needs slurries of soybean meal and bactopeptone [4],while *C. radians* prefers mixtures of glucose and soybean meal [10] and *M. rotula* soluble soybean peptone, yeast extract and glucose [11]. UPO production in liquid culture starts when the fungus switches from primary to secondary metabolism between days 5 and 10. Currently, the highest yields of UPO protein are gained with *M. rotula* that produces more than 400 mg l^{-1}[11]. Since UPOs are extracellular enzymes, crude preparations can be obtained by ultrafiltration of the culture liquids. Further purification is achieved by multistep fast protein liquid chromatography (FPLC) using anion, cation and mixed-ion exchangers as well as molecular sieves [18]. Typically, several UPO forms are obtained that slightly differ in their physico-chemical properties. They can represent both true isoenzymes and differently glycosylated forms of the same protein.

The four UPOs characterized so far, i.e. *Aae*UPO, *Cra*UPO, *Mro*UPO and r*Cci*UPO, contain protoporphyrine IX (heme) as prosthetic group, which is linked via the iron to an exposed cysteine (proximal hemeligand). This specific structural feature is responsible for the characteristic UV-Vis spectra of native UPOs and their reduced carbon monoxide complexes, whose maxima (Soret bands) range from 415-420 nm and from 445-450 nm, respectively, and thus strongly resemble P450 enzymes [19]. UPOs are highly glycosylated proteins (10-40% sugars) with up to seven *N*-glycosylation sites of the high mannose type [6,15]. Their molecular masses and isoelectric points vary from 32 to 46 kDa and 3.8 to 6.1, respectively [6].

MOLECULAR PROPERTIES OF UNSPECIFIC PEROXYGENASES

The crystal structure of the major UPO form of *A. aegerita* was solved and reveals a compact globular shape with ten *alpha*-helices, five very short *beta*-sheets, a disulfide bridge in the C-terminal part as well as a magnesium in vicinity to a heme propionate residue [20] (Fig. 2, 3).

A more detailed analysis of all available UPO sequences, including that of chloroperoxidase (CPO, EC 1.11.1.10) from *Caldariomyces (Leptoxyphium) fumago* (a related heme-thiolate peroxidase with 27% sequence identity to *Aae*UPO), revealed that there are two large clusters of UPO-like proteins [15, 21]. They differ among others in size, which is why they have been designated as "short and long UPOs" (or group I and II UPOs) [15] (see also Fig. 1). The short UPOs of group I have a molecular mass around 30kDa and are found in all fungal phyla.The long UPOs of group II, with a mass around 45kDa,occur only in basidiomycetes and ascomycetes. *Mro*UPO[11] and CPO [22] belong to group I, and *Aae*UPO, *Cra*UPO and r*Cci*UPO to group II [6]. Both UPO groups bear a highly conserved cysteine exposed by two prolins (PCP-motif) as proximal heme ligand as well as a glutamate that acts as acid-base catalyst in peroxide activation/cleavage and two further acidic residues (ExD-motif) that are involved in the binding of stabilizing magnesium [15, 20]. Differences in the active sites of group I and II UPOs exist with respect to the alkaline amino acid that stabilizes the negative charge of the UPO intermediate "compounds zero"; it is histidine in the case of short UPOs and arginine in the long UPOs. Exemplarily, Fig. 2 shows the conserved amino acids in the active center of *Aae*UPO [6, 20].

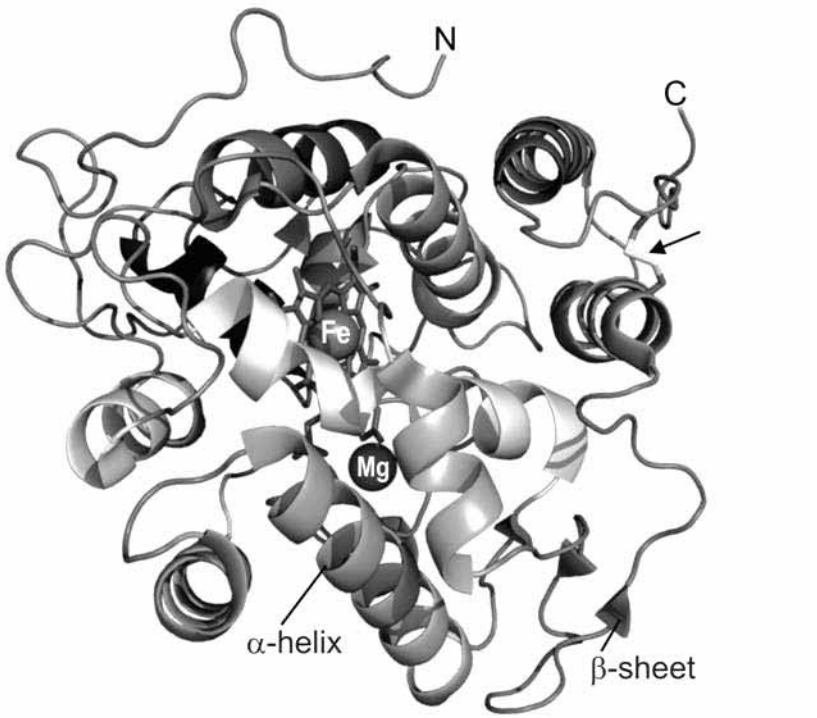

Figure 2. Ribbon model of *Aae*UPO. The black arrow indicates a disluphide bridge near the C-terminus (modelled by M. Pecyna based on the crystal structure data of Piontek *et al.* [20]).

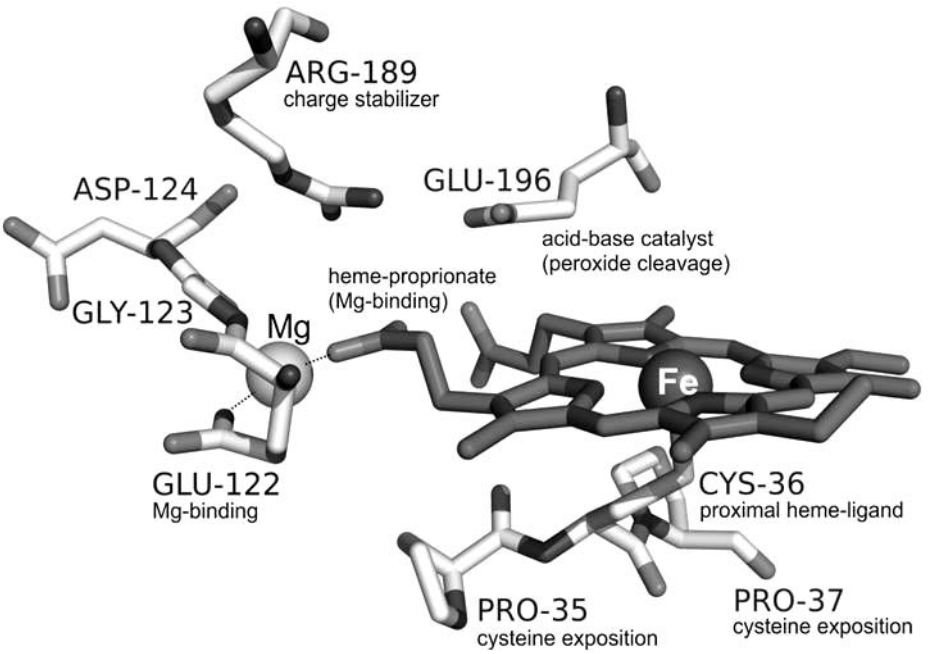

Figure 3. Arrangement of conserved amino acid residues and heme in the active site of *Aae* UPO (modified according to [6])

UPOs are organized in gene clusters (multigene families); ongoing own transcriptome studies on *A. aegerita* have indicated the presence of at least 16 UPO sequences, which probably include several gene variants, and in *M. rotula*, even over 50 UPO sequences have been found (MJ Pecyna and H Kellner 2014, unpublished results). In the genome of the common white button mushroom (*A. bisporus*), as much as 24 putative UPO sequences were identified and proposed to be related to the metabolism of humic substances [23].

CATALYTIC PROPERTIES OF UPOs

An overview on UPO-catalyzed reactions is given in Fig. 4. The portfolio of reactions includes alkane and alkyl hydroxylation, epoxidation of alkenes and aromatics, heteroatom oxygenation, *O*- and *N*-dealkylation as well as radical formation. All in all, the number of UPO substrates have been estimated to exceed 300 and it is expected that even more substrates will be found along with the discovery of new UPOs [6].

The reaction cycle of UPOs combines elements of the catalytic cycles of P450s and heme peroxidases, in which compounds I and II are the key reactive intermediates that catalyze either two-electron oxidations resulting in oxygen atom incorporation, or one-electron oxidations resulting in the formation of free substrate radicals [6, 19, 24]. In other words, UPOs oxygenate

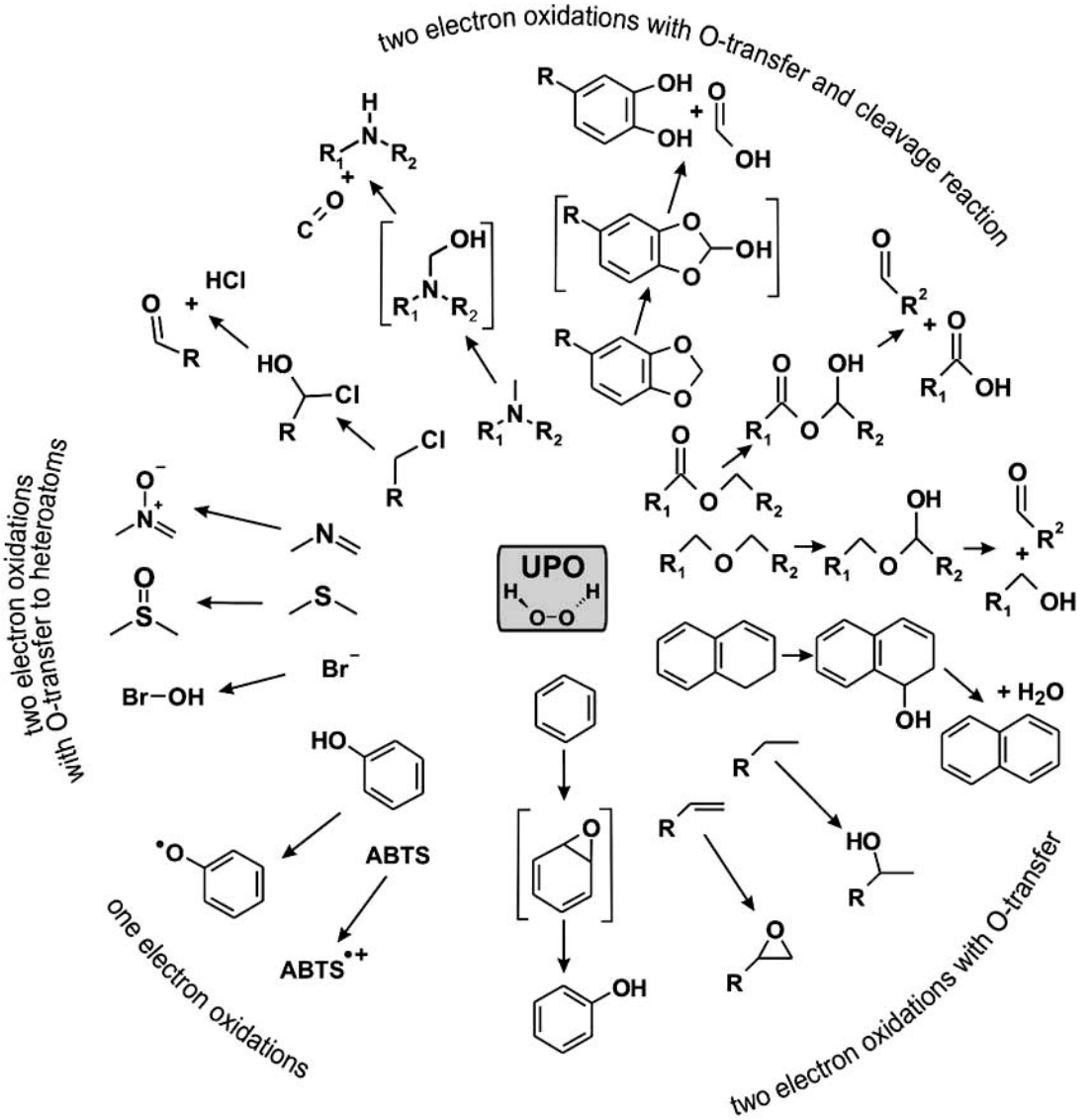

Figure 4. Summarizing overview on the reactions catalyzed by fungal UPOs

diverse substrates in a similar manner as P450s (mono-peroxygenase route) and oxidize phenolic compounds and ABTS (peroxidase route) like prototypical heme peroxidases [6, 25].

There are several spectrophotometric assays for the detection of the oxygenating activities of UPOs. They are based on the ability of UPOs to oxidize aryl alcohols to aldehydes, to *O*-dealkylate ethers or to oxygenate aromatic rings (Fig. 5). The oxidation of veratryl alcohol to veratraldehyde at neutral pH is used for routine measurements [3,4]. It starts with the

incipient hydroxylation of the benzylic carbon to give veratraldehyde hydrate (a *gem*-diol) that is in equilibrium with veratraldehyde absorbing at 310 nm. Veratraldehyde is also formed in the second assay that follows the cleavage of methyl veratryl ether (*O*-demethylation) leading to an unstable hemiacetal intermediate that spontaneously breaks down under release of methanol (CH_3OH) [26]. Demethylenation is a special case of *O*-dealkylation and performed with 5-nitro-1,3-benzodioxole, the oxidation of which results in the release of formic acid (HCOOH) and the formation of 4-nitrocatechol (followed at 425 nm) [27]. Aromatic ring oxygenation via initial epoxidation and subsequent spontaneous re-aromatization (phenol formation) can be monitored with naphthalene as a substrate at 303 nm [28]. One-electron oxidations catalyzed by UPOs are assayed with classical peroxidase substrates such as ABTS or 2,6-dimethoxyphenol [4]. It is also possible to determine UPO activities with chromatographic methods (HPLC, GC) as it was shown for the oxidation of pyridine, ethylbenzene, benzene, cyclohexane and methylbutene [29-33].

UPOs catalyze the hydroxylation of various linear, branched and cyclic alkanes as well as of alkyl groups (e.g. attached to aromatic rings). Due to the low solubility of alkane substrates, reactions are usually performed in the presence of co-

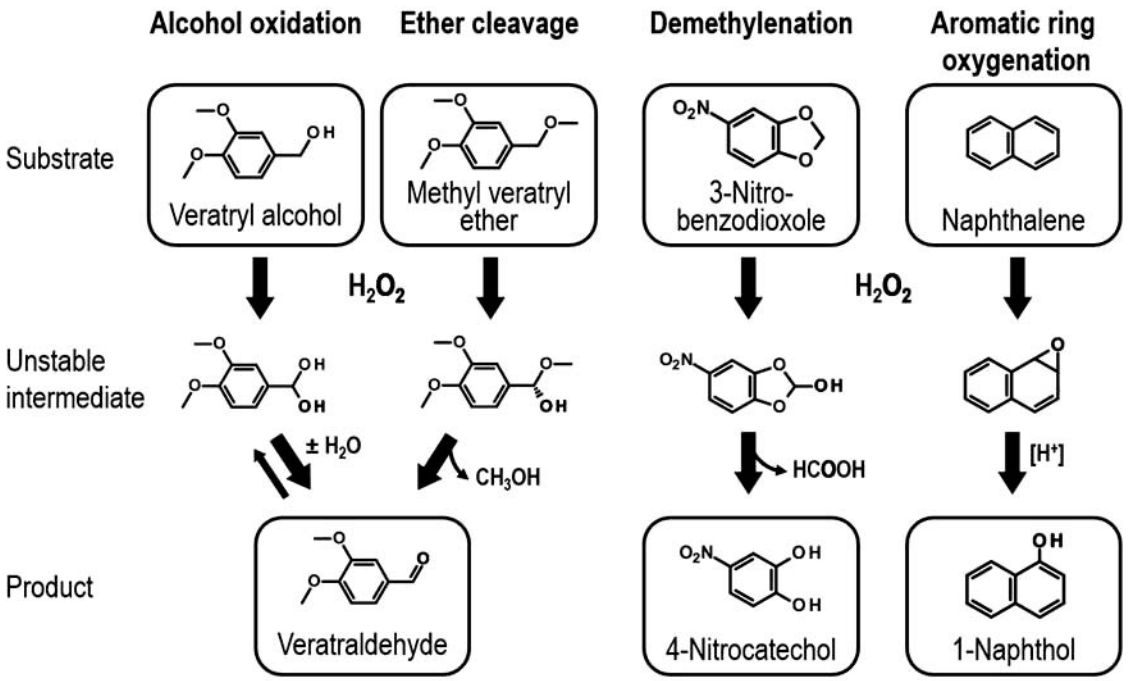

Figure 5. Reactions used to assay UPO activities [4, 26, 27, 28]

solvents (e.g. acetone 4% to 60% vol/vol). The size of linear alkane molecules that were found to be hydroxylated at *omega*-2 and -3 position ranges from gaseous propane to viscous *n*-hexadecane [32], and fatty acids were even oxidized up to a chain length of C_{20} (arachidic acid) [14]. Branched alkanes are hydroxylated by UPOs as well and preferably at the tertiary carbons. Cyclic alkanes from cyclopentane to cyclooctane are oxidized to form the corresponding cycloalkanols [32]. In general, alcohols formed can be subject to over-oxidation to the corresponding carbonyls (ketones, aldehydes), and aldehydes, in turn, can be oxidized to carboxylic acids (Fig. 4) [34].

UPOs oxidize alkenes and alkenyls, in which both epoxidation and hydroxylation of the double bond's adjacent carbons (allylic hydroxylation) occurs (Fig. 4). In a study comprising 20 alkenes, among them propene and linear 1-alkenes up to C_8, branched alkenes such as 2,3-dimethyl-2-butene, cyclohexene, butadiene and limonene, were oxidized by *Aae*UPO in that manner [33]. A special case of allylic hydroxylation is the formation of naphthalene hydrates (i.e. 1- and 2-hydroxy-1,2-dihydronaphthalene) during the UPO-catalyzed oxidation of 1,2-dihydronaphthalene. The arene hydrates formed decay into naphthalene via spontaneous aromatization (Fig. 4). This reaction sequence represents a simple pathway for the selective synthesis of aromatic hydrocarbons via arene hydrates of conjugated cyclic dienes or cycloalkenyl benzenes [35].

Aromatic oxygenation has been studied in detail with naphthalene and benzene as substrates [28, 31, 36]. Naphthalene is regioselectively epoxidized by different UPOs to form naphthalene 1, 2-oxide that hydrolyzes in the presence of protons (H^+) to 1-naphthol as the major product (Fig. 5). Other polycyclic aromatic hydrocarbons such as fluorene, anthracene, phenanthrene, pyrene and dibenzofuran were also found to be subject to UPO-catalyzed oxidation leading to mixtures of mono- and polyhydroxylated products [37]. Benzene oxidation proceeds via initial epoxide formation and subsequent re-aromatization to form phenol; further oxygenation is typical and gives mixtures of hydroquinone, catechol and 1,2,4-trihydroxybenzene [31]. Phenolic products formed can be in turn substrates of the peroxidative activity of UPOs (one-electron oxidation), which leads to undesired phenoxyl radicals (Fig. 4). This can be prevented by adding radical scavengers such as ascorbic acid to the reaction mixture. Re-reduction of phenoxyl radicals is of particular relevance when polyphenolic substrates such as flavonoids are attempted to peroxygenation [38].

UPOs catalyze *O*- and *N*-dealkylations of diverse ethers and secondary/tertiary amines, respectively. The mechanism involves, in both cases, initial hydroxylation of one of the heteroatoms' adjacent carbons (e.g. methyl or methylene groups) giving rise to unstable intermediates (hemiacetals, hemiaminals), which spontaneously cleave under release of water. Thus, hemiacetals yield alcohols/phenols and aldehydes, and hemiaminals generate primary or secondary amines and aldehydes. In both cases, the aldehydes indicative for this mechanism can be detected by their corresponding 2,4-dinitrohydrazone adducts [12, 26]. Ether cleavage occurs between aromatic and aliphatic molecule parts, e.g. in alkyl aryl ethers (e.g. 1,4-dimethoxybenzene) or in alicyclic and aliphatic ethers (e.g. tertrahydrofuran, diisopropyl ether). Substantial *N*-dealkylation (~60%) was observed during *N*-methylaniline oxidation by *Aae*UPO [39].

UPOs are also capable of transferring oxygen to organic heteroatoms such as sulfur and nitrogen (Fig. 4). For example, the heterocycledibenzothiophene is oxidized at the sulfur atom to form the corresponding sulfoxide and sulfone [44]. In a similar reaction, *Aae*UPO was found to enantio selectively oxidize the side chain of thioanisole into the corresponding (*R*)-sulfoxide with high efficiency [40]. Pyridine and halo-, nitro- and cyanopyridines are oxidized by *Aae*UPO exclusively at the nitrogen atom to form the respective pyridine *N*-oxides [29].

*Aae*UPO shows strong bromide oxidation but, in contrast to CPO, only very low chloride oxidation, even though (according to studies of compound I) its redox potential is higher than that of CPO [41]. The oxidation of halides (X) is actually also an oxygen transfer reaction yielding reactive hypohalites (OX) that in turn can halogenate organic substrates such as phenols [5, 19]. In contrast to *Aae*UPO, *Mro*UPO has almost no bromide oxidizing activity, indicating that not all peroxygenases have specific halide binding sites [11].On the other hand, halogens bound to carbon atoms undergoing hydroxylation are released as the corresponding halides, because the geminal halohydrins initially formedare unstable. The oxidation of benzylchloride by *Aae*UPO that yields benzaldehyde and chloride is an example of such a reaction (Fig. 4) [39].

The promiscuity of UPOs in oxyfunctionalization reactions becomes evident when the oxidation of pharmaceuticals and drugs is examined. All reactions mentioned above have been observed in this context, and more than 60 different pharmaceuticals and a number of illicit drugs were shown to undergo oxidative modification by UPOs [12, 42]. Examples are the painkillers diclofenac (phenyl hydroxylation) and ibuprofen (isopropyl hydroxylation), the antitussive dextromethorphan (*O*-demethylation), the *beta*-blocker propranolol (naphthyl hydroxylation), the K^+-channel blocker tolbutamide (benzylic hydroxylation), the anti-inflammatory aminophenazone (*N*,*N*-desmethylation) and the antiviral drug osaltamivir (ester cleavage). Among the drugs (of abuse) that are oxidized by UPOs are MDMA ("Ecstasy", demethylenation), LSD (aromatic hydroxylation), THC (methylcyclohexenyl hydroxylation) as well as cocaine and codeine (*N*-desmethylation). Last but not least, UPOs have also successfully been used to prepare specifically labeled human drug metabolites and drug-drug interaction probes [43].

CONCLUSION

Fungal unspecific peroxygenases can at least approach the catalytic versatility of cytochrome P450 enzymes and may suitably supplement existing oxyfunctionalization tools in biotechnological applications. Some examples that are currently

under development are biosensors for aromatic compounds [44] as well as new procedures for the synthesis of pesticide precursors [45], drug metabolites [46], chiral alcohols [47] and even bulk chemicals such as cyclohexanone [48].

Despite all the progress in understanding the catalytic mechanisms of UPOs and collecting their molecular data, the natural function of these enzymes in fungal organisms is not fully clear yet. Of course, the surpassing catalytic versatility may suggest their involvement in all kinds of detoxification reactions (e.g. of plant ingredients, phytoalexins, microbial toxins, xenobiotic compounds), but also other functions cannot be ruled out, e.g.,their involvement in lignin and humus modification or in biosynthetic pathways [49].

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