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Unspecific Peroxygenases - Functional Hybrids between P450 Monooxygenases and Heme Peroxidases

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Abstract

Unspecific peroxygenases (UPOs) secreted by fungi represent an intriguing enzyme type that selectively transfers peroxide-borne oxygen with high efficiency to diverse substrates including unactivated hydrocarbons. They contain a cysteine-ligated heme and catalyze hydroxylation, epoxidation, dealkylation, deacylation as well as hetero atom, halide and one-electron oxidations. Substrate spectra of UPOs resemble both those of P450 monooxygenases and heme peroxidases.

Non-specific peroxygenases (UPOs, EC 1.11.2.1) belong to the heme-thiolate proteins and behave "promiscuously" with respect to demanding oxygen transfer reactions. The first UPO was discovered in 2004 in the Southern field mushroom (*Agrocybe aegerita*), a hardwood-colonizing edible mushroom from the wider mushroom family (order Agaricales) [1]. Further enzymes of this type were found in cultures of other fungi (e.g. *Marasmius rotula*, *Chaetomium globosum*) [2,3] and the long-known chloroperoxidase (CPO, EC 1.11.1.10) turned out to be a special case of UPOs [4]. The heterologous expression of UPOs is associated with major difficulties and has so far only been successful in a few cases (e.g. in *Sacharomyces*, Pichia); the complex folding and the formation of disulfide bridges may play a role in this [3,6]. The crystal structures of the UPOs of *A. aegerita* and *M. rotula* have been solved and reveal a compact spherical shape dominated by alpha-helices and containing a heme-stabilizing magnesium and a highly conserved PCP motif. The latter ideally exposes a cysteine as proximal heme ligand towards the iron. The heme access channels of UPOs are lined with hydrophobic amino acid residues (Phe or Leu/Ile/Val) and their molecular architecture is of crucial importance for the substrate specificity of the respective enzyme [3,6].

UPO reactions and Mechanism

Functionally, UPOs are monooxygenases acting outside the fungal hyphae, which transfer a peroxide-borne oxygen atom (H-O-O-R) to various organic substrates. The target substrates are subject to hydroxylation, epoxidation, dealkylation, deacylation and heteroatom oxygenation; in addition, one-electron oxidations are catalyzed analogous to classical peroxidases (Figure 1) [4,7]. The product spectra of UPOs are often similar to those of cytochrome P450 monooxygenases, which are active as universal detoxification enzymes in the human liver [8,9,10], e.g. with regard to the conversion of active pharmaceutical ingredients or xenobiotics. Figure 2 illustrates the mechanisms of UPO-catalyzed conversions using the substrates *p*-cresol, 1,4-dimethoxybenzene and styrene. The former is both benzylically hydroxylated and oxidized to the corresponding phenoxy radical, the aromatic diether is *O*-dealkylated and styrene is epoxidized. In each case, the

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key intermediate is the so-called UPO compound I (Cpd-I), an oxoferryl cation radical complex of heme, which is formed after binding and heterolytic cleavage of H_2O_2 [4,11]. Cpd-I is an extremely strong oxidizing agent that attacks C-H bonds, double bonds and phenolic OH groups, among others, forming radicals. Subsequently, rebound mechanisms lead to the transfer of oxygen (peroxygenation of a C atom or epoxide formation) or to a second radical bond (phenol oxidation). The reactions that take place thus correspond to a combination of the so-called "peroxide shunt" of certain P450 enzymes and the catalytic cycle of classic heme peroxidases [4].

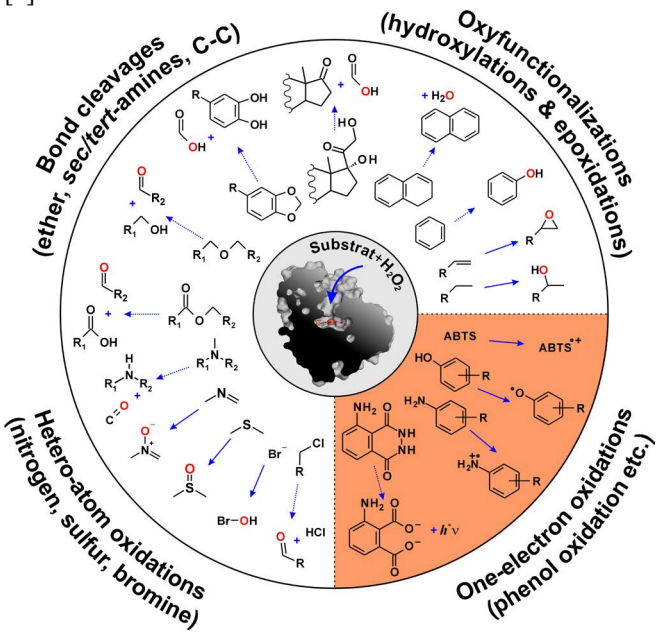

Figure 1: Reactions of non-specific peroxygenases (UPOs). The reactions in the lower right quarter are one-electron oxidations, while all other reactions are oxygen transfer reactions. In the center of the figure, the UPO from *Agrocybe aegerita* (*Aae*UPO) is shown in cross-section; modified after [4,12].

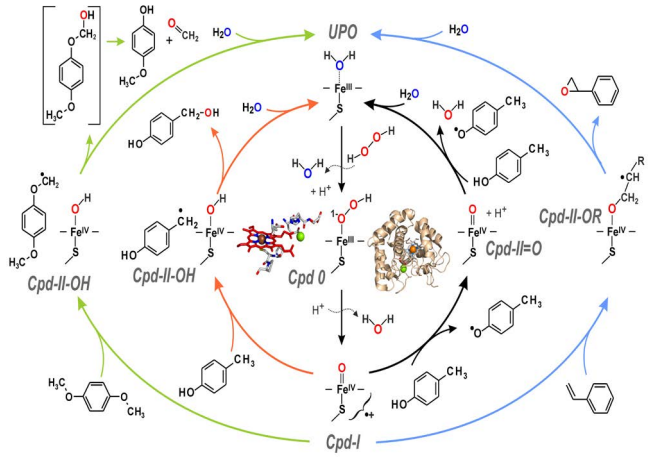

Figure 2: Catalytic cycles of unspecific peroxygenases (UPOs) with four possible routes that branch off at the UPO compound I (Cpd I) stage depending on the substrate. The inner cycles describe the oxygenation (red arrows) and oxidation (black arrows) of *p*-cresol, the outer cycles the epoxidation of styrene (green arrows) and the *O*-dealkylation of 1,4-dimethoxybenzene (blue arrows); modified and rearranged according to [4,12].

Distribution in the fungal kingdom and phylogeny

Several thousand putative UPO sequences have now been found in fungal genomes. They suggest the wide distribution of these enzymes throughout the fungal kingdom, including all strains of true fungi (Eumycota) and some fungus-like Stramenopiles [4,12]. Within the basal sister group of the other fungi, the Cryptomycota, a UPO gene was detected in *Rozella allomycis*. Other basal fungal groups (Microsporidia, Neocallimastigomycota, Blastocladiomycota, Kickxellomycotina) lack UPO genes; the same applies to the sister group of fungi, the Holozoa, which comprises the animals (Metazoa) and their closest relatives (Choanoflagellata, Ichthyosporea). Representatives of the Chytridiomycota (flagellate fungi), on the other hand, possess up to seven UPO genes; their enzymes may have been the starting point for the evolutionary development of the UPO multigene family, which ultimately led to the possession of 70 and more UPO genes in individual species of higher fungi. Apart from the Mortierellomycotina, representatives of the other groups of polyphyletic Zygomycota and Glomeromycota possess one or two UPO genes.

Phylogenetically, the UPO sequences can be divided into two families. Family I - the "short" UPO sequences - comprises representatives of all the above-mentioned UPO-positive fungal groups. Family II contains the "long" UPOs, whose occurrence is restricted to the Ascomycota and Basidiomycota (Fig. 3). Some distinct basal subgroups in family I contain UPO sequences without recognizable signal peptides, which is why it seems plausible that the UPO archetype was a similar intracellular enzyme. This applies to about a quarter of the UPO sequences analyzed. Whether these enzymes work freely in the cytosol or are active in specific hyphal compartments is unclear. Since UPOs are found exclusively in the fungal kingdom, including the basal Cryptomycota, and no evidence of their presence in the Holozoa has been found to date, it can be postulated that they arose over 600 million years ago, after the separation of fungi and animals. It is possible that the drastic conditions of the developing oxygen atmosphere, similar to the case of the P450 enzymes, played an evolutionary role. Whether UPOs and P450s have a common origin is still unclear. Although both enzyme types have structural and catalytic similarities (cysteine as proximal heme ligand, compounds 0, I, II), there is no homology at the sequence level.

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Outlook

Despite their high relevance for enzyme technology applications, only a few UPOs are currently available and almost nothing is known about their physiological significance. Considering their wide distribution in the fungal kingdom, their high number in the genomes of some fungi and their versatility in terms of catalyzed reactions, various functions are conceivable. First and foremost, detoxification reactions should certainly be mentioned, as UPOs attack structures that frequently occur in plant and microbial secondary metabolites and environmental pollutants [9,12]. Considering the high diversity of UPO genes, an involvement in pathogenicity or in lignin and humus transformation should not be excluded. The latter would require a molecular architecture that allows aromatic polymer structures to be attacked superficially via heme access channels that are as broad and shallow as possible. In this context, it should be noted that the crystalline cellulose-cleaving LPMOs (lytic polysaccharide monooxygenases) discovered only a few years ago are, according to recent publications, also surface-active, extracellular peroxygenases (albeit based on copper-dependent catalysis) [13].

Modern molecular techniques such as genome editing using CRISPR/Cas could help to answer the question of the function of UPOs. To this end, UPO knock-out mutants of suitable model organisms would have to be generated and subsequently tested physiologically. Molecular and enzymatic field studies (e.g. in the context of the Biodiversity Exploratories, DFG SPP-1374) could also help to clarify the functions of UPOs (e.g. in a similar way to what has recently been achieved in the case of manganese peroxidases [14]). In addition to the further development of microbial expression systems (yeasts, molds, bacteria), cell-free expression with isolated ribosomes could be an innovative approach to accelerate the production of recombinant UPOs [15].

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