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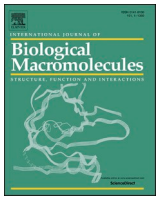

Review

Molecular diversity of unspecific heme peroxygenases with promising applications in sustainable oxyfunctionalizations

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ABSTRACT

Unspecific peroxygenases (UPOs) are highly versatile biocatalysts capable of removing various persistent environmental contaminants and performing sustainable chemical transformations. These oxidoreductases contain heme *b* as their prosthetic group. As all classical peroxidases, they are activated by the molecules of hydrogen peroxide to incorporate the oxygen atom into numerous organic molecules. Alternatively, they can use ascorbate as a cosubstrate. In sequence databases an ever-increasing number of their DNA and protein sequences occurs. Reconstructed molecular phylogeny of the corresponding peroxidase-peroxygenase superfamily reveals a high diversity of gene distribution for UPOs in the whole kingdom of fungi. A majority of identified UPO sequences stems from numerous species of Dikarya. Although members of this superfamily were recently detected also in early diverging fungal lineages, UPOs from the phyla of Mucoromycota, Glomeromycota and Chytridiomycota remain not sufficiently investigated. Moreover, newly discovered genes coding for UPOs were recently identified also among early diverging eukaryotic lineages of amoebas and green algae in various biotops. With a large palette of potential substrates these oxidoreductases serve as a versatile tool in enzyme catalysed synthetic reactions, but their real physiological substrates need to be recognized in the future. Most important among self-sufficient UPO-catalysed reactions are oxyfunctionalizations of various aliphatic and aromatic molecules. In this critical review an outlook is given for investigation and engineering of novel UPO variants including products of directed evolution. Future research on UPOs shall be mainly focused on basal fungal and emerging non-fungal sources for their promising applications in environmentally friendly technologies.

1. Introduction

Unspecific peroxygenases (UPOs; EC 1.11.2.1), previously referred to as chloroperoxidases or more generally as haloperoxidases, are predominantly microbial heme-*b* enzymes that exhibit remarkable reactivity and functional diversity. They play an important role in both natural processes and industrial applications. As an abundant subclass of oxidoreductases, UPOs catalyze efficient oxygenation reactions by selectively transferring an oxygen atom from hydrogen peroxide to a wide range of organic substrates. Owing to this unique capability, they have been described as “dream catalysts” for organic synthesis. Thus far, research has mainly focused on UPOs from a limited number of fungal pathogenic and non-pathogenic species [1]. Recently, emerging evidence revealed their presence in certain non-fungal single cell eukaryotes. Corresponding genes were detected in Oomycetes (belonging to the kingdom *Stramenopila*), amoebas [2] and even in some green algae of the genus *Closterium* [3]. Therefore, it is evident that a vast diversity of unexplored UPOs originating from ancestral fungi and other eukaryotic lineages with ancestral origin remains to be discovered. Unlocking such untapped reservoir of heme-thiolate enzymes will enable deeper insights

Abbreviations: 2LPS, Two-Liquid Phase System; AA, amino acid; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; APO, aromatic peroxygenase; BIC, Bayesian Information Criterion; CpdI, compound I – a reactive intermediate of heme peroxidases and peroxygenases; CPO, chloroperoxidase; CYP450, cytochrome P450; DES, Deep Eutectic Solvent; HGT, horizontal gene transfer; HTP, heme-thiolate peroxidase; IDR, intrinsically disordered region in the protein; IL, Ionic Liquid; LG, Le-Gascuel model of amino acid substitutions; HMM, Hidden Markov model; MCD, monochlordimedon; ML, maximum likelihood method of phylogeny reconstruction; ORF, open reading frame; UPO, unspecific peroxygenases.

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into their substrate scope and selectivity. This systematic research will ultimately position UPOs as versatile tools for advanced organic synthesis [4,5]. Robust phylogenomic approach on the whole peroxidase-peroxygenase superfamily is based on collected data from numerous eukaryotic genomes. It systematically compares the changing architecture of genes coding for heme peroxygenase. Updated phylogenetic reconstruction discovers yet unknown representatives mainly in ancestral fungal and non-fungal evolutionary clades. We try to uncover typical features of a highly conserved structural fold spread throughout the entire protein superfamily allowing the important reaction resulting in oxyfunctionalization of numerous organic substrates. At the same time, envisioning the industrial implementation of novel UPOs requires early and critical assessment of the environmental impact of UPO-catalysed processes. The mere fact that a reaction is enzymatic does not automatically guarantee its environmental sustainability. Multiple factors of the planned catalysis must be carefully evaluated. Insights into the long-term evolutionary trajectories of unspecific peroxygenases offer a valuable framework for guiding sustainable synthetic biocatalytic processes. This contribution aims to inspire young researchers to address described challenges and to help overcome the current limitations in the field of UPO research with a real multidisciplinary approach.

2. Types of possible reactions catalysed by unspecific peroxygenases

Although the enzyme classification for all unspecific heme peroxygenases is clearly defined as EC 1.11.2.1 in Brenda database [6] there are several types of reactions that can be considered as possible in connection with this group of metalloproteins that act as flexible oxidoreductases. Following four simple reaction schemes (where A is an aromatic compound, R is an organic radical and X a halide) describe the potential reaction versatility of unspecific peroxygenases:

$$H_2O_2 + 2\,AH_2 \rightarrow 2\,H_2O + 2^{\bullet}AH \qquad \text{[Reaction 1]}$$

$$H_2O_2 + X^- + H^+ \rightarrow H_2O + HOX \qquad \text{[Reaction 2]}$$

$$H_2O_2 + RH \rightarrow H_2O + ROH \qquad \text{[Reaction 3]}$$

$$2\,H_2O_2 \rightarrow 2\,H_2O + O_2 \qquad \text{[Reaction 4]}$$

The most general type of specifically catalysed reaction that can be assigned to majority of well-known peroxidases is Reaction 1. It describes the reduction of hydrogen peroxide to water with concomitant oxidation of various substrates. In the case of classical heme peroxidases such substrates were mostly described as aromatic 1-electron or more rarely also as 2-electron donors. Therefore, the appropriate abbreviation for this substrate is AH_2. The reaction product is not only water from the cleaved molecule of hydrogen peroxide but more importantly a reactive aromatic radical. This reactive product obtained after one-electron oxidation from the heme enzyme can be a starting point for a consecutive polymerization reaction [7]. This is true for many heme peroxidases by using various aromatic 1-electron donors giving colored products that can easily be followed and quantified spectrophotometrically. Reaction 2 is a typical haloperoxidase reaction. Those peroxidases that perform it efficiently by oxidation of halides can form corresponding hypohalous acids with proven antimicrobial properties [8]. Such enzymes are regular components of innate immunity in vertebrate animals but this specifically catalysed reaction can apparently be attributed also to certain fungal enzymes annotated as chloroperoxidases [9]. In contrast, reaction 3 shall be typical only for the enzyme subclass of peroxygenases by introducing an atom of peroxide-born oxygen into numerous organic molecules here generally abbreviated as RH. Finally, reaction 4 is simply a hydrogen peroxide dismutation into water and molecular oxygen that is mainly the property of monofunctional catalases but shall also be possible to some extent for various heme-thiolate enzymes [10,11].

All known unspecific heme peroxygenases (UPOs) that are heme-thiolate enzymes, use hydrogen peroxide (H_2O_2) as a co-substrate to drive oxygen transfer reactions. Mechanistically, UPOs activate H_2O_2 by heterolytic O–O bond cleavage at the heme cofactor to generate the reactive Compound I species (an oxoferryl heme π-cation radical) [4]. This high-valent intermediate is capable of electrophilic oxygen-transfer. In fact, this mechanism is well-known from phylogenetically unrelated CYP450 monooxygenases as “peroxide shunt” avoiding the reductive activation of molecular oxygen [12,13]. Importantly, the H_2O_2-driven catalysis allows all naturally occurring UPOs to be self-sufficient monooxygenases, in contrast to P450 enzymes that require auxiliary redox proteins (Fig. 1). Once formed, Compound I of UPOs (CpdI) is capable of various decay reactions including the oxyfunctionalisation of (inert) C-H- and C=C-bonds, heteroatoms (including halides), single electron- and hydrogen atom-abstractions (Fig. 2). In addition, catalase-activity of peroxygenases (i.e. the decomposition of hydrogen peroxide to oxygen and water) is also known and well documented [14]. The practical simplicity of peroxygenase reactions and their catalytic versatility have inspired researchers to explore peroxygenases as catalysts for selective oxyfunctionalization reactions [15,16]. Therefore, it is important to evaluate the extent of natural diversity and distribution of these attractive enzymes with a phylogenetic approach followed by genomic analysis of their gene architecture and proteomic aspects of corresponding UPO proteins. This finally leads to considerations about their specific applications in sustainable biotechnological processes.

3. Reconstruction of the evolutionary relationships in the peroxidase-peroxygenase superfamily

Detailed phylogenetic reconstructions of the evolutionary history for UPOs were performed previously with various approaches. They were chosen mostly from diverse taxonomic phyla of fungi. The appreciated catalytic performance of these highly versatile oxidoreductases underlines the importance of this task. Main attempt was focused on a systematic classification of diverse UPO sequences based on observed sequence homologies between fungal genomic DNA. First, it needs to be noted that all known heme-thiolate peroxidases that can act as real peroxygenases belong to one of the previously phylogenetically defined heme peroxidase superfamilies [17]. All fungal and also later discovered non-fungal enzymes that can be described as heme-thiolate peroxidases always contain cysteine as their proximal heme ligand. This leads to their typical reaction specificity by incorporating peroxide-derived oxygen in numerous organic molecules. According to this significant reactivity they were classified into peroxidase-peroxygenase superfamily with typical conserved sequence signatures along the whole protein. However, in the InterPro protein database [18] this superfamily is still annotated as chloroperoxidase with the accession code IPR000028. Additionally, there exists also a related chloroperoxidase-like superfamily having a recent accession code IPR036851. In both cases, fungal secretory representatives are predominant members. A phylogeny based on comparison of chloroperoxidases with UPOs but only from dikaryal species of Basidiomycota and Ascomycota was published later [19]. These authors screened in 812 fungal genomes available at that time in Ensembl database and found 113 putative UPO-encoding sequences distributed in only 35 different fungal species. This clearly demonstrates the presence of multiple paralogs of this superfamily spread in fungal genomes and the question remains which of these multiple forms was optimal for reconstructing the superfamily phylogeny as there exist at least the previously defined short (Family I) and long (Family II) UPO variants [5]. The main aim of these authors was to distinguish between evolutionary clades of chloroperoxidase(s) and clades of unspecific peroxygenases. Chloroperoxidases could be better named as haloperoxidases because e.g. the well-known counterpart from *Marasmius rotula* can besides its peroxygenase activity only oxidize iodide [20]. It is not surprising that within this reconstructed phylogeny some of the

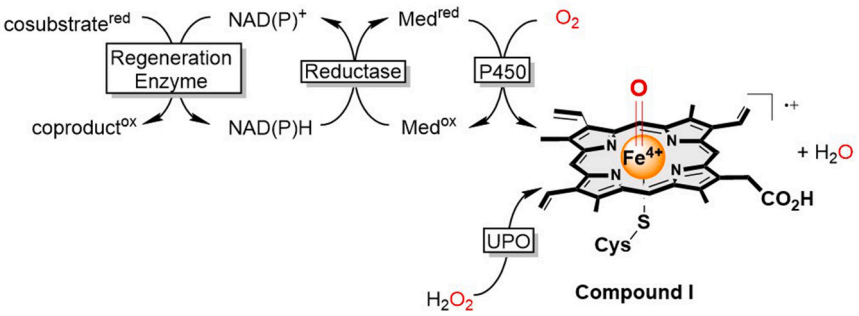

Fig. 1. Comparison of the Compound I-formation mechanisms of P450 monooxygenases and peroxygenases. The catalytic mechanism of P450 monooxygenases comprises the reductive activation of molecular oxygen via two subsequent single electron transfers to the heme Fe-ion. The mechanistic inability of NAD(*P*)H to function as single electron donor necessitates a relays enzyme (reductase) transforming the hydride transfer from NAD(*P*)H into two single, mediated, electron-transfer steps. Additionally, in situ regeneration of NAD(*P*)H also demands a regeneration enzyme. In contrast, peroxygenases rely on already reduced oxygen species (H_2O_2) thereby avoiding the above-mentioned electron-transport chain.

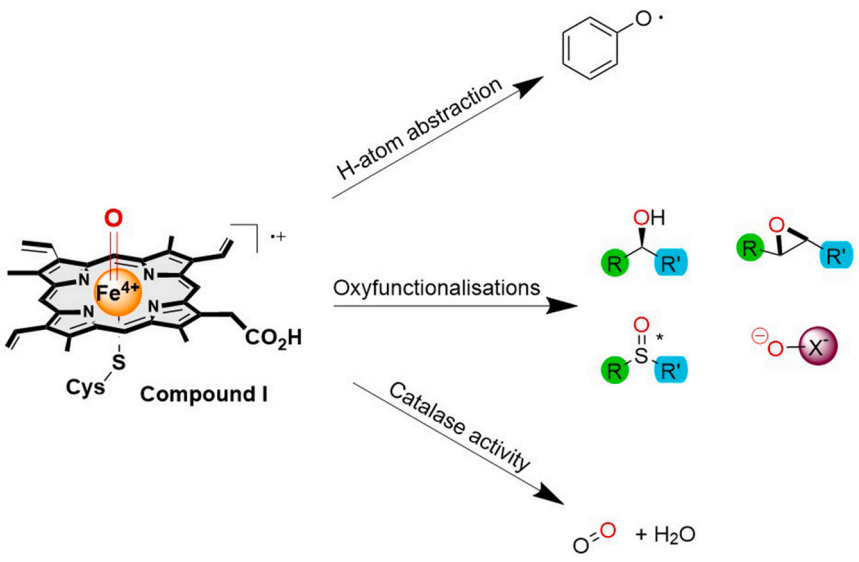

Fig. 2. Fates of CpdI in heme peroxygenases.

previously named CPO (chloroperoxidase) protein sequences were placed among UPO (unspecific peroxygenase) clades. However, without a clear experimental evidence such simplified division between CPOs and UPOs cannot be supported. Presented evolutionary tree shows in a small detail the occurrence of few chloroperoxidase genes also among Mucoromycota that are evolutionary seen as basal fungi and significantly different from Dikarya [21] due to their lower genetic complexity and simpler ecological niches. Apparently, such gene name nomenclature (prevalently "CPO") comes just from automatic annotations of ORFs in GenBank and no functional verification from experimental evidence was provided yet.

We have recently published a more abundant phylogeny with 174 complete protein sequences from the same superfamily showing the occurrence of corresponding HTP/UPO genes besides Dikarya also in Mucoromycota, Chytridiomycota and in Stramenopiles (Oomycetes) [2]. Additionally, novel peroxygenase genes were detected in few amoebas that were not presented in the previous phylogenetic work. They unambiguously contain all sequence patterns and features typical for this conserved metalloprotein superfamily. Our currently updated Maximum Likelihood phylogenetic reconstruction (Figs. 1 and 2) of the peroxidase-peroxygenase superfamily containing all types of known UPOs now comprises up to 280 full length sequences. It is important to notice that no prokaryotic member of this superfamily was detected in any sequence database yet. This is in striking contrast with all 3 remaining heme peroxidase superfamilies [17]. A nice example is the larger peroxidase-catalase superfamily where bifunctional enzymes are found in bacteria, archaea [22] and fungi [23,24] but their monofunctional enzyme counterparts are present selectively either in fungal [25] or in plant lineages [26]. Within here investigated peroxidase-peroxygenase superfamily we cannot find any bacterial or archaeal sequence that would reveal a significant homology with proven eukaryotic members. The updated robust evolutionary tree of this superfamily was reconstructed in MEGA X suite [27] and is presented in rectangular form in Fig. 3 and in circular form with details in Fig. 4. Le-Gascuel model of amino acid substitutions with a discrete gamma distribution and the presence of invariant sites was selected according to achieved lowest BIC score (among 56 tested models). For statistical purposes 1000 bootstrap replications were used on a set of 280 Muscle-aligned protein sequences. Optimized alignment parameters were gap open penalty −0.8, gap extend penalty −0.05 and hydrophobicity multiplier 0.9. In this overview we focus mainly on the basal/ancestral fungal clades and on apparent occurrence of several non-fungal evolutionary branches to explore the real extent of natural gene distribution for the whole superfamily. Fig. 3 depicts schematically the division in 14 main well-resolved UPO clades available in various eukaryotic, mainly fungal taxa. Few non-fungal UPO clades could probably emerge with horizontal gene transfer events from more ancestral fungal UPO gene predecessors (Fig. 4). We can observe this rare phenomenon with a

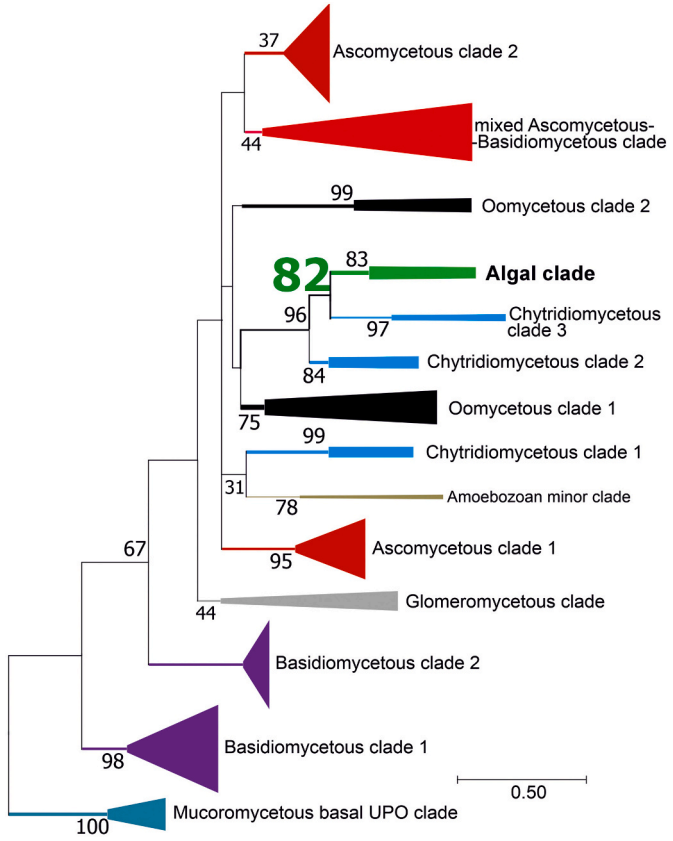

Fig. 3. Schematic representation of reconstructed phylogenetic tree for the whole peroxidase-peroxygenase superfamily. Only main clades are shown in this projection. Obtained bootstrap values after 1000 replications are presented in nodes of resolved clades. Only bootstrap support values ≥30 are shown. This graph was rendered with MEGA-X phylogeny package [27]. Detailed presentation of UPO clades is presented in Fig. 4.

bootstrap support achieving the value of 82 in the node between the *Globomyces* branch (which belongs to Chytridiomycota fungi) and *Closterium* branch (the desmid group of charophyte green algae). Obtained high bootstrap support is necessary but not sufficient evidence for a HGT. Possible ancestral presence of UPO genes already in unicellular flagellated eukaryotes supposed as direct algal predecessors followed by probable UPO gene loss in majority of their descendants needs to be considered as another alternative. However, the fact that chytridiomycetous fungi and green algae inhabit similar environment of freshwater biotops where they could come into parasitic or saprotrophic contact represents further point of indirect evidence. More sequence data from ongoing algal and chytridiomycetous sequencing projects need to be included in future approach to resolve this issue. Few other potential HGT events in the robust phylogenetic tree cannot be ruled out but they did not achieve a sufficient bootstrap support in corresponding nodes and need to be resolved in more detail in future projects. In next sections we describe details and typical features among well resolved UPO clades.

3.1. Basal fungal clades

The basal mucoromycetous clade contains representatives mainly from the genera Absidia, Cunninghamella, Rhizopus and Mucor. Typical for them is the saprotrophic way of life where yet undiscovered heme peroxygenases can find their physiological importance. Rare exception is the representative from *Cokeromyces recurvatus* which is considered as a coinfecting pathogen of humans [28]. Putative heme peroxygenase CrecUPO1 could serve as a potential biomarker to distinguish this fungus from other pathogenic fungi. The neighbouring basidiomycetous clade 1 in the resolved phylogenetic tree contains several distinct sequences of UPOs from ectomycorrhizal fungi e.g. from the genera *Suillus, Rhizopogon* or *Candolleomyces*. These club fungi were shown to form symbiotic associations mainly with roots of coniferous trees [29]. Although there are numerous indications on the presence of UPO gene paralogs among these forest fungi e.g. [30–32] the extent of their natural expression needs to be resolved in detail with the transcriptomic approach. Besides mainly ectomycorrhizal fungi also several paralogs of UPOs from brown rot fungi (e.g. *Postia placenta)* or other wood decaying fungi are present in this clade that could have occurred with frequent gene duplications within already established genomes. Next well resolved clade of UPOs occurs selectively in the genomes of Glomeromycetous fungi that also grow predominantly in association with the roots of various trees and many other plants. Presented in this clade are peroxygenase enzymes mainly from the genera *Rhizophagus* and *Gigaspora* which are among typical arbuscular mycorrhizal fungi forming symbiotic relationships with wide variety of plant species, namely gymnosperms, angiosperms, hornworts and ferns e.g. [33–34]. A possible involvement of here presented UPOs in symbiotic events of arbuscular mycorrhizas between Glomeromycota and selected plants need to be verified experimentally.

Ascomycetous clade 1 contains several UPO genes from the genus *Penicillium* originating from various regions and locations (*P. brasilianum, argentinense, expansum* and *oxalicum*). It would be interesting to investigate the potential involvement of corresponding UPO proteins in biosynthesis pathways of secondary metabolites, in particular several diverse antibiotics and related bioactive compounds typical for this genus. Further, many UPO genes from pathogenic *Aspergilli* and *Fusaria* are also included in this clade and the potential involvement of such UPO proteins in their phytopathogenicity needs also to be addressed. Part of this clade is formed by peroxygenases from nematode-trapping fungi (*Arthrobotrys* or *Dactylella*). Their UPOs also remain on the level of just putative proteins.

3.2. Amoebozoan UPOs

Amoebozoan unspecific heme peroxygenases are currently represented by only two unique sequences detected in the genome of *Planoprotostelium fungivorum* strain Jena. Interestingly, a member from another peroxygenase gene family was analysed in this slime mold before [35]. However, the caleosin-related peroxygenase family with Intepro identification IPR007736 is phylogenetically and structurally different from here presented superfamily. This phagocytosis-capable amoeba is known to feed on soil fungi including mainly species of *Candida* and *Aspergillus*. Regarding its mycophagous evolutionary adaptation, it can be deduced that from some ancestral fungal genome the corresponding UPO gene could be incorporated to its own genome by yet unclear mechanism. The bootstrap support in the node connecting chytridiomycetous UPOs with PfungUPOs (Figs. 3 and 4) reveals a value below 50. Thus, many more amoebozoan genomic UPO sequences including their regulatory regions from ongoing genomic projects need to be included for a more robust and well supported analysis of this part of the evolutionary tree. Only afterwards, it could be decided about a possible horizontal UPO gene transfer between an ancestral water fungus and its predatory amoeba.

3.3. Chytridiomycetous clades

Chytridiomycetous heme peroxygenases are presented by three dissipated clades phylogenetically located between bigger Ascomycetous and Oomycetous clades. Within chytridiomycetous clade 1 representative UPOs from the genus *Spizelomyces* are present that are important soil fungi in various terrestrial ecosystems [36]. This indirectly underlines the significance of their potential heme peroxygenase paralogs. Next small clade of UPOs from Chytrids contains

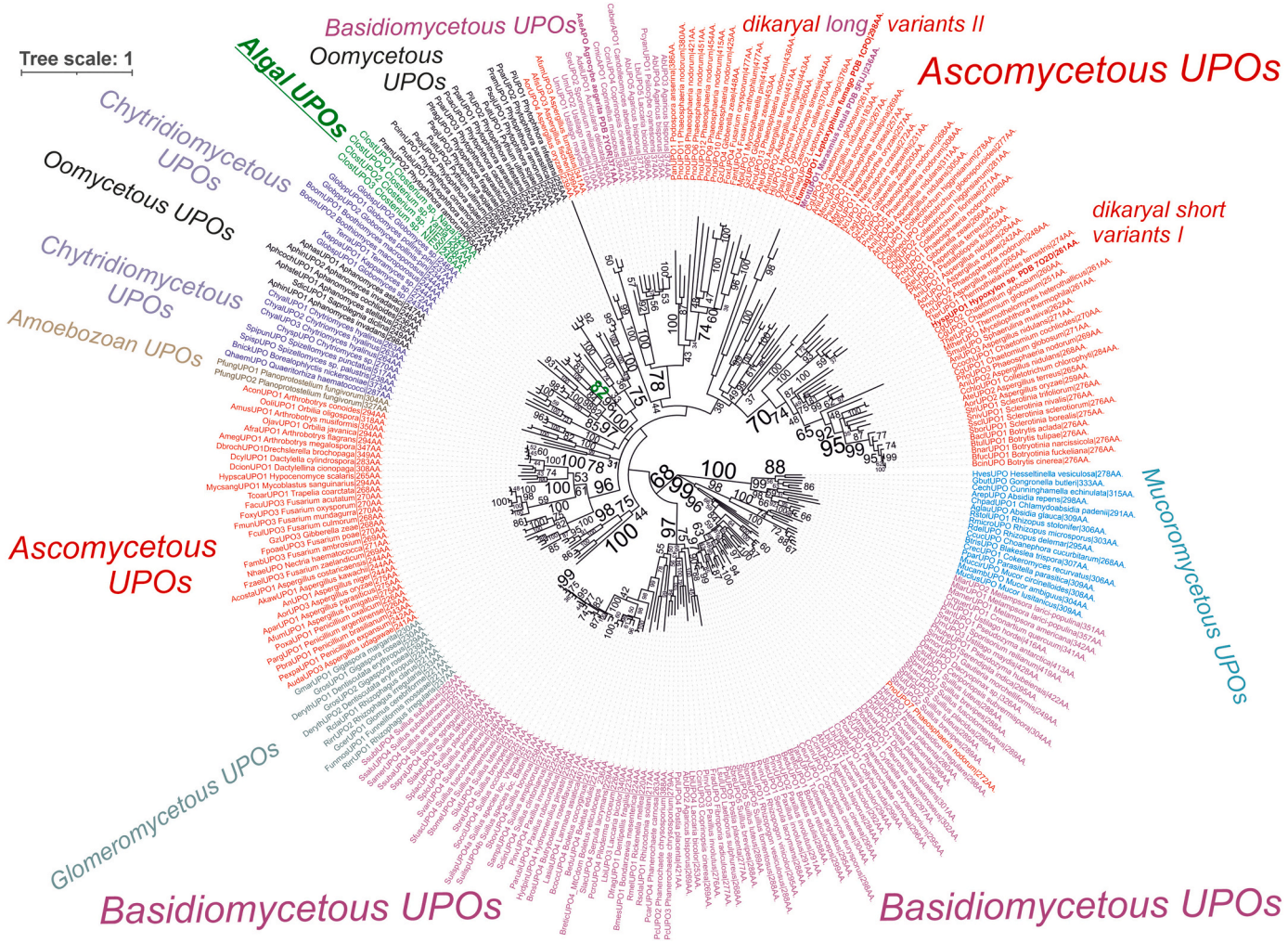

Fig. 4. Reconstructed phylogenetic tree showing details for 280 complete UPO protein sequences. This reconstruction was obtained with MEGA X package [27] by applying the Maximum Likelihood method, LG model of amino acid substitutions and 1000 bootstrap replications. Numbers in nodes represent obtained bootstrap values and only values ≥30 are shown. Graph was rendered with iTOL [111]. Colour scheme red – sequences originating from Ascomycota, purple - from Basidiomycota, blue - from Chytridiomycota, forest green - from Glomeromycota, light blue - from Mucoromycota, black - from Stramenopiles/Oomycota, brown - from Amoebozoa and light green - from Algae. Sequences in bold letters possess an experimentally resolved crystal structure. Used abbreviations of sequence names correspond with their description in Suppl. Table 1.

representatives from the genus *Chytriomyces* known for their ecological decomposer properties in aquatic and moist terrestrial biotops [37]. This also implicates that their UPO genes need to be expressed intensively dependent on various environmental stimuli to perform the essential decomposing reactions. In the third small chytrid UPOs clade representatives from the genus *Globomyces* are present [38]. It is interesting to note that they are primarily found in aquatic habitats and not in soils as compared with related chytrid genera e.g. *Spizelomyces*.

3.4. Algal UPOs

Unique algal heme peroxygenases were detected recently in the genome of *Closterium* sp. [3]. They are positioned in a close proximity with two clades of UPO genes from Globomyces chytrids in the reconstructed phylogenetic tree. A statistically robust bootstrap support of 82 is observed for branch location of these genes from the order *Desmidiales* (in Figs. 3 and 4 labelled in green colour) with fungal genes positioned in neighbouring clades. This observation would suggest a potential HGT event, but a high statistical support is necessary but not sufficient evidence. The comparison of detailed gene architectures, comparable G + C content and presence of similar regulatory elements need to be performed from further genomic analyses within ongoing sequencing projects. Interestingly, certain green algae were detected in the aquatic habitats that are typical for *Globomyces* chytrids mentioned above [38]. Although desmids are an order with a high diversity currently counting over 6000 species the putative genes for heme peroxygenases were detected just in the genus *Closterium* pointing to a relative recent metagenomic event that would be favourable for the recipient. Potential involvement of such UPOs in the physiological processes of this freshwater algae needs to be investigated also in connection with other related enzymes as the research on algal enzymology is still underrepresented in the scientific literature [39].

3.5. Oomycetous clades

Oomycetous UPOs are distributed in two distinct clades located between ascomycetous and chytridiomycetous clades. In the oomycetous clade 1 members from the genus *Aphanomyces* are presented that are known as dangerous pathogens of both plants and animals. Also, a putative heme peroxygenase from *Saprolegnia* infecting amphibians is present in this clade and the involvement of this type of UPOs in the pathogenesis of the early developmental stages of various frogs remains open [40]. The second oomycetous clade contains numerous peroxygenase representatives from the genera *Phytophtora* and *Pythium*. Also,

in this case it can be suggested that ancestral UPO genes were transferred to Oomycota that belong to the large kingdom of Stramenopiles via a possible HGT event from ancestral chytrid fungi. But a higher bootstrap support is necessary to substantiate such hypothesis. Alternatively, a loss of internal UPO gene in certain predecessors of extant Stramenopiles needs also to be considered. Comparison of gene architecture in oomycetous and chytridiomycetous genes including a detailed location of introns would further support the final interpretation of resolved tree topology at the node leading to oomycetous UPOs. Both genera *Phytophtora* and *Pythium* represent important plant-damaging parasitic water molds [41]. Therefore, the involvement of presented UPOs in phytopathogenesis needs to be evaluated in detail with the aim of designing potent inhibitors of the growth of these destructive pathogens in agricultural applications.

3.6. Dikaryal fungal clades

In the reconstructed tree the next position is occupied by a mixed basidiomycetous-ascomycetous clade. However, it contains ascomycetous UPO representatives as a majority. Some important basidiomycetous peroxygenases are also present in this clade e.g. UPO from *Agrocybe aegerita* with known crystal structure [42] that was first described as aromatic peroxygenase with the corresponding abbreviation APO. For historical reasons we use this abbreviation also in Fig. 4 as this was among the first ever discovered heme thiolate peroxygenases capable of oxidizing aromatic compounds [43]. Later experiments have shown that this HTP exhibits typical properties of the unspecific peroxygenase [44]. Other basidiomycetous UPO representatives of this clade are mostly paralogs from the mushroom genera *Agaricus*, *Laccaria* and *Coprinopsis*. Also, representatives from the phytopathogen *Ustilago maydis* are present here with yet unknown physiological function. Ascomycetous representatives of this mixed clade are also dominated by UPOs from diverse phytopathogenic fungi. Although several diverse enzymes have been already studied in the genera of *Phaeosphaeria* and *Fusarium* [45] that cause significant agricultural losses, all detected UPO sequences remain at the level of putative proteins. This clade was classified as „long UPO variants“in older phylogenetic analysis based on a simpler neighbour-joining approach [1]. Based on their calculated average size of 44.4 kDa this would correspond to 404 amino acids long proteins which agrees well with values in this part of the phylogenetic tree shown in Fig. 4.

Large ascomycetous clade 2 is the last detected clade in the robust phylogenetic tree presented in Figs. 3 and 4. It contains UPO isozymes mostly from diverse phytopathogenic but also from numerous saprophytic fungi. This clade was previously classified as „short UPO variants“[5] and in contrast to „long UPO“counterparts it has the size between 29 and 32 kDa that corresponds to 264–291 amino acids which again corresponds well with the size of proteins given in this clade of the reconstructed tree shown in Fig. 4. Among long-known representatives the chloroperoxidase from *Caldariomyces fumago* currently taxonomically classified as the saprotrophic fungus *Leptoxyphium fumago* with known crystal structure is present [46]. Interestingly, this ascomycete contains also a second paralog, namely LfumagUPO2, that reveals only 77 % sequence identity with the crystallized variant pointing to an older gene duplication event within this subclade. Further in this last part of the evolutionary tree numerous paralogs of *Aspergilli* and *Chaetomia* UPOs are present with yet unknown physiological functions. Important is the *Hypoxylon* sp. representative where the crystal structure was determined recently [47]. This endophytic fungus can protect mainly diverse trees offering them through symbiotic interactions enhanced stress tolerance and protection against certain pathogens. For this purpose, the already investigated HyspUPO1 can serve as a versatile catalyst with a variety of possible performed reactions [47] but its real physiological function remains largely unknown. Just few heme peroxygenases from thermophilic or thermotolerant saprophytic fungi are present in this large ascomycetous clade. It will be interesting to compare their structure with mesophilic counterparts.

3.7. Directed evolution of fungal UPO genes

As unspecific heme peroxygenases are very versatile biocatalysts there were already attempts to evolve their genes also in vitro. This procedure can be million-fold faster than the natural evolution of corresponding mostly introns containing genes (cf. Fig. 5) within e.g. fungal genomes. Important preconditions for directed evolution of enzyme-encoding genes are optimal selection of the “parental” version and targeted high-throughput assay for specific aims of requested catalysis [48]. After discussing long-lasting natural pathways of UPO gene evolution in previous sections it is logical to add what was already achieved by the means of directed evolution in corresponding engineered peroxygenase genes. The approach of error-prone PCR and DNA shuffling routinely used in directed evolution methodology was applied first on the *AaeUPO* gene. Several generations of mutated libraries were consecutively screened for the output yielding mutated variants of 5th generation with 162 fold improved activity if compared with the parental type [49]. This catalytic improvement was detected with ABTS and NBD as routinely used substrates colorimetric substrates [50]. Finally, a “mature” PaDa-I mutant with 9 substitutions was obtained in the last generation that was already used in many further experiments of catalytic oxyfunctionalizations (see Chapter 8 for details). Further specific aim of directed UPO evolution was to engineer the secretory machinery of AaeUPO in the yeast secretion system and this succeeded in 1114-fold increased secretion of mutated peroxygenase from the host yeast *S. cerevisiae* giving very high production yields [51]. PaDa-I mutant was further subjected to directed evolution rounds and in already 7th generation a novel variant named JaWa was obtained with two-fold increased peroxygenase activity and diminished peroxidase activity [52]. Another round of directed evolution was performed again on AaeUPO but with a special MORPHING method in *S. cerevisiae* [53] yielding thermostable substitution mutants in structural loops that were subjected to combinatorial saturation mutagenesis. These results helped to differentiate regions responsible for peroxidase and peroxygenase activity of basidiomycetous UPOs. Finally, AaeUPO was subjected to directed evolution by combination of neutral genetic drift with targeted selection to obtain variants with increased stability in organic solvents [54]. From presented overview it is obvious that among a natural diversity of hundreds of UPO genes just one was subjected to a comprehensive directed evolution approach. This situation definitely needs more effort in future projects. Although it is pragmatic to further evolve a stable peroxygenase variant with already well studied properties it would be stimulating to access also ancestral UPO genes with directed evolution to resolve a paradigm of rather unknown ancestral reaction mechanisms of this protein family. When a series of new random evolution rounds will produce numerous novel UPO variants their more systematic nomenclature would be highly recommended.

4. Typical architecture of UPO genes with detection of transcription regulatory regions

Comparison of typical gene primary structures including the promoter sites and other transcription regulatory regions in eukaryotic genomes with open reading frames coding for unspecific peroxygenases is presented in Fig. 5. This comparison was obtained with HMM-based FGENESH genomic prediction with ab initio approach [55] and drawn to scale. Presented are typical examples chosen from available sequence data of corresponding fungal and also rare algal genomic DNA regions. Ascomycetous and basidiomycetous genes shown in Fig. 5a and b contain on average 1 to 3 short introns distributed along the whole coding region (as calculated from totally 20 diverse fungal genomic regions with UPO genes - not shown here). During recent genomic searches it was detected that distantly related UPO genes from green algae can exist as variants with no introns but also few counterparts with

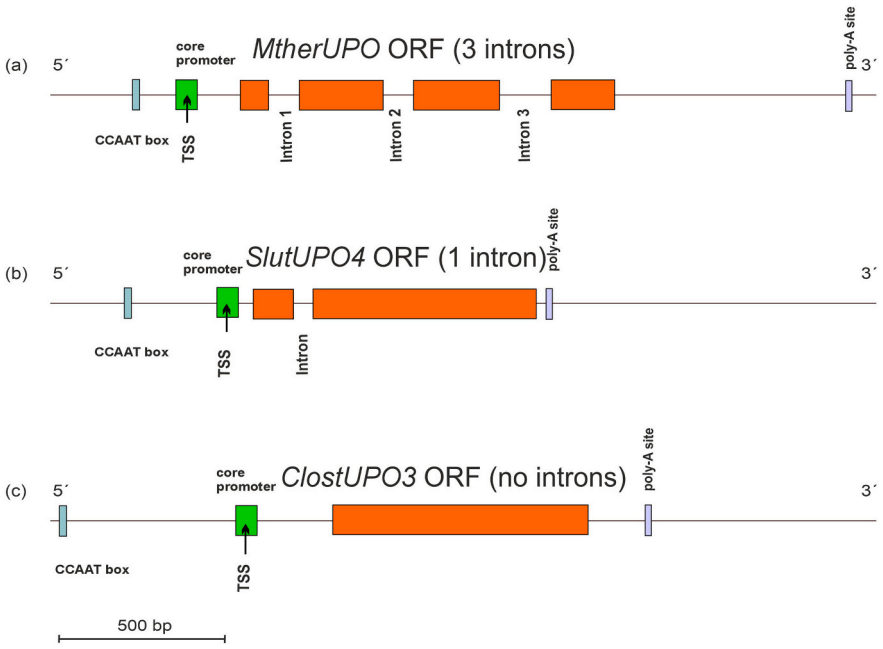

Fig. 5. Gene architecture for three typical UPO genes: a) ascomycetous b) basidiomycetous and c) a distinct representative from a green algae. Presented regions were detected with FGENESH suite [55] or in the case of CCAAT boxes they were obtained with pairwise global alignment of the consensus sequence to particular UPO DNA sequences. Drawn to scale. Colour scheme: green – core promoter region, orange – exons coding for UPO proteins, cyan - regulatory CCAAT box, violet – polyA site.

up to 2 short introns could be located in algal genomes. However, the detection of more novel algal UPO genes is necessary to evaluate the extent and occurrence of introns more comprehensively. Obviously, all genomic regions comprising described peroxygenase genes contain the conserved CCAAT box responsible for binding of transcription factors thus regulating the specific gene expression. These short boxes are located at the 5termini of analysed UPO genomic regions (Fig. 5) with slightly different distances to core promoter regions that include their transcription start sites. Also, the poly-A site typical for eukaryotic mRNA processing is located at the 3termini of all here analysed UPO genomic DNA regions but again with a variable distance after the respective stop codons coding for heme peroxygenase open reading frames.

Our knowledge about the topological location and genomic organization of UPO genes in particular chromosomes still remains rather limited although hundreds of heme peroxygenase genes from very diverse fungi and recently also from non-fungal unicellular eukaryotes were already identified and deposited in sequence databases [1]. Just for few basidiomycetous representatives the clusters containing several UPO genes combined with genes of yet unknown function were described in detail for some sequenced contigs [56]. These can be probably the products of relative recent gene duplication events within already formed *Agaricales* genomes. A different situation occurs when inspecting ascomycetous genomes in those locations where UPO gene paralogs were found e.g. in the well documented genome of *Chaetomium globosum* for the reference genome assembly ASM14336v1. In this case four UPO gene paralogs are located in different contigs far away from each other and in their genomic vicinity genes with completely different function appear. For example, CgUPO1 (with no surrounded duplicate) has in its proximity a gene coding for putative fungal transcription factor (cf. GenBank accesion NT_165981). A similar situation is seen in phylogenetically older basal fungi where, as a rule, fewer UPO gene paralogs (mostly a single one) occur in different locations of their genomes embedded by genes with different functions.

Whether UPO gene paralogs of a particular genome are naturally expressed in a similar or different way to each other and to functionally related peroxidases remains to be detected. Transcriptomic analysis focused on thermotolerant and symbiotic basidiomycetes from the genus *Termitomyces* revealed a differentiating expression of UPOs in connection with Fenton-based degradation pathways for biomass conversion [57]. Such comprehensive RNA-seq analysis ultimately needs to be extended for many other fungal species including primarily the basal fungal lineages. It can demonstrate possible conservations or modifications of UPO-mRNA transcription during the evolution of these genes within fungal kingdom and other eukaryotic lineages.

5. Subcellular location of diverse unspecific peroxygenases

Although at the beginning of the discovery of this superfamily it was supposed that most of identified UPO proteins are targeted for the secretion outside of fungal cells [58] with rapidly accumulating novel peroxygenase sequences deposited in databases it could be deduced that there are several alternatives for subcellular locations of unspecific peroxygenase variants. Surely, also this phenomenon depends on the evolutionary history of identified UPO clades. It was even hypothesized that the UPO archetype could be an intracellular enzyme [1]. Functionally related but evolutionary diverse cytochrome P450 enzymes [5] that can also incorporate oxygen into organic molecules are predominantly membrane bound. Besides the supposed extracellular presence of extant unspecific peroxygenases also intracellular forms can be detected among the members of the large peroxidase-peroxygenase superfamily. Prediction of the subcellular location for selected UPO sequences from all here resolved evolutionary clades (shown in Fig. 3) was performed with SignalP 6.0 methodology [59] and obtained output is given in Table 1.

From Table 1 it is obvious that a significant portion of identified heme peroxygenase sequences does not contain any signal for extracellular secretion over the classical Sec/SPI pathway. Those representatives that contain a signal peptide with a high prediction probability differ in its length. The average length calculated from all UPOs presented in Table 1 is 23 amino acids that are cleaved away from the nascent protein. This is in good agreement with previous general analysis of signal peptides in phytopathogenic fungi revealing the most common length of 19 amino acids [60]. Larger discrepancies from this

Table 1
Prediction of N-terminal signal peptide Sec/SPI in protein sequences of unspecific heme peroxygenases for the Sec pathway over the endoplasmic reticulum. Performed with SignalP 6.0 [59] by using the default parameter setting given for eukaryotic proteins and a slow model mode for the prediction of accurate region borders.

Sequence abbreviation*	Length of signal sequence (AA)	Probability of prediction
AbUPO1 (B)	0	0.0001
AbUPO3 (B)	20	0.9997
AnUPO1 (A)	0	0.0004
BcinUPO (A)	19	0.9993
BeduUPO1 (B)	31	0.6078
BfucUPO1 (A)	19	0.9993
CcinUPO4 (B)	25	0.9992
ChyalUPO3 (C)	18	0.9996
ClostUPO1 (Al)	0	0
ClostUPO2 (Al)	0	0
ClostUPO3 (Al)	21	0.9985
ClostUPO4 (Al)	44	0.5728
GcerUPO1 (G)	0	0
GrosUPO2 (G)	0	0.0007
GzUPO3 (A)	0	0
GzUPO5 (A)	20	0.9995
HvesUPO (M)	0	0.1653
MlarUPO2 (B)	0	0
MoUPO1 (A)	17	0.9994
MtherUPO (A)	17	0.9992
PcinnUPO1 (O)	20	0.7203
PexpaUPO1 (A)	0	0
PfungUPO2 (Am)	0	0.0003
PparUPO2 (O)	17	0.9995
PultUPO1 (O)	25	0.9996
RdelUPO (M)	39	0.9641
SbovUPO4 (B)	0	0.0001
SpipunUPO (C)	0	0
SreUPO2 (B)	22	0.9997
TerraUPO1 (C)	14	0.9994

* For abbreviations of all here presented protein sequences, refer to Table S1. Assignment to taxonomic phyla: (A)-Ascomycota, (Al)-Algae, (Am)-Amobeozoa, (B)-Basidiomycota, (C)-Chytridiomycota, (G)-Glomeromycota, (M)-Mucoromycota, (O)-Oomycota.

average length are rarely seen in Mucoromycota and among green algae that tend to have slightly longer signal sequences for their extracellular secretion. The amino acid composition of detected signal peptides reveals significant diversity if aligned. Just at the C-terminus near the KEX2 protease cleavage site one or two alanines embed an arginine very frequently and one of them is sometimes replaced by a valine.

Several of those UPOs that do not have a signal peptide for extracellular secretion probably contain a transmembrane helix as predicted with Deep TMHMM algorithm [61]. This would mean that an internal part of such peroxygenases is capable of spanning a membrane. In cases like ClostUPO1, ClostUPO2, MlarUPO2, PfungUPO2 and SpipunUPO (all listed in Table 1) it can be a membrane of certain organelle such as the mitochondria, endoplasmic reticulum, Golgi apparatus or even plastids in the case of algal representatives. In the case of basidiomycetous representative BretiUPO4 a hypothetical fusion was detected. This complex protein consists of a mitochondrial carrier domain and a heme peroxygenase domain (cf. Suppl. Table 1 for details). Predicted transmembrane helix is located in the part preceding the C-terminal heme peroxygenase domain. However, such possible but still hypothetical fusion needs an experimental verification.

6. Conserved regions of UPOs in aligned protein sequences with functional impact

The multiple protein sequence alignment of UPO proteins was performed with the Muscle program [62]. Output is presented in three conserved regions of Fig. 6 where twenty selected representatives from all evolutionary clades resolved in Fig. 4 are shown. Complete alignment for all 280 UPO sequences used for phylogeny reconstruction in Fig. 4 is available in Suppl. Material 1. In selected parts of the alignment we can detect and distinguish important amino acid sequence motifs that are responsible for the overall and typical reactivity of unspecific heme peroxygenases as well as for their highly conserved architecture in formed active centres. Namely, there are invariantly conserved amino acid positions serving as ligands for a correct coordination of iron cation in the heme prosthetic group and also for the ligation of a second metal cation. It is important to note that non-fungal members such as UPOs from the amoeba *Planoprotostelium* [35] and UPOs from green algae *Closterium* contain the same conserved regions along the entire protein sequence.

First, the proximal side of the prosthetic heme group is formed by a short but highly conserved sequence motif with the consensus sequence -DXRXPCPXLN- as obvious in Fig. 6A. In this exceedingly preserved part of the whole enzyme from the scope of long-term evolution mainly two flanking proline residues hold an exposed cysteine in an optimal spatial position to serve as a strong thiolate ligand for the iron cation of the prosthetic heme group. This corresponds to AA residues 35–37 in the 3D structure of AaeUPO located in the upper part of presented protein alignment. Besides this essential triad several other polar amino acid residues in the vicinity of heme are also quite decidedly conserved forming the UPO-typical architecture of the proximal heme side (Fig. 6A). Second highly conserved motif is located further towards C-terminus and is represented by amino acids with a shorter consensus -EHDXSL- (Fig. 6B). This is the location for the binding of the second cation of presented metalloenzyme. In most cases magnesium ion was found here but rarely also manganese ion can be present [46] at the same position. For efficient binding of this second cation on the heme periphery mainly conserved glutamate, aspartate and serine are responsible with the AA numbering E122, D124 and S126 in the upper AaeUPO sequence. Third motif in connection with the catalytic iron cation apparently not so highly conserved can be found at the distal side of prosthetic heme group. Just one moderately conserved glutamate corresponding to position E196 in AaeUPO can be located here (Fig. 6C). In some cases it is mutated to N. Rather far away, also a conserved arginine corresponding to the position R172 of AaeUPO can be detected with yet unknown function. In contrast with other heme peroxidase superfamilies this rather mutable region allows enough space and variability on the distal side of prosthetic heme that is typical for unspecific peroxygenases. There are few other short stretches of conserved amino acid positions down to the C-terminus but generally the N-terminal part is more conserved in comparison with the more variable C-terminal UPO region (cf. Suppl. Material 1.).

In Table 2 the pI values for all 20 here aligned UPO sequences are given to distinguish possible differences. Obviously, presented heme peroxygenases fall into two distinct groups. Either they have their pI values in the acidic region (between pI 5–6), or the pI values are shifted towards the alkaline region, mostly pronounced for the algal UPO representative (up to pI of 10). This can be connected with their physiological conditions and subcellular location (as discussed in chapter 5) and the distantly related pI values can be also associated with rather distant evolutionary position of respective UPOs as can be inspected in Fig. 4 for representatives of Table 2. From obtained alignment of 20 selected protein sequences involved in the reconstruction of the evolutionary tree we have produced also a sequence logo [63,64] to see the level of amino acid conservation or possible variations in important regions of heme peroxygenases. It is presented in Fig. 7. Here, both highly conserved protein regions around the prosthetic heme group are clearly discernible. At the proximal side the two flanking prolines embedding a reactive cysteine are obvious with highest bit values. Mainly the proximal Cys is by far the position with highest value in the whole UPO sequence. However, it is also important to see that several other amino acids on both sides of this invariantly conserved proximal triad also reveal rather high bit values underlying also their importance for the typical architecture. At the place for location of a second metal

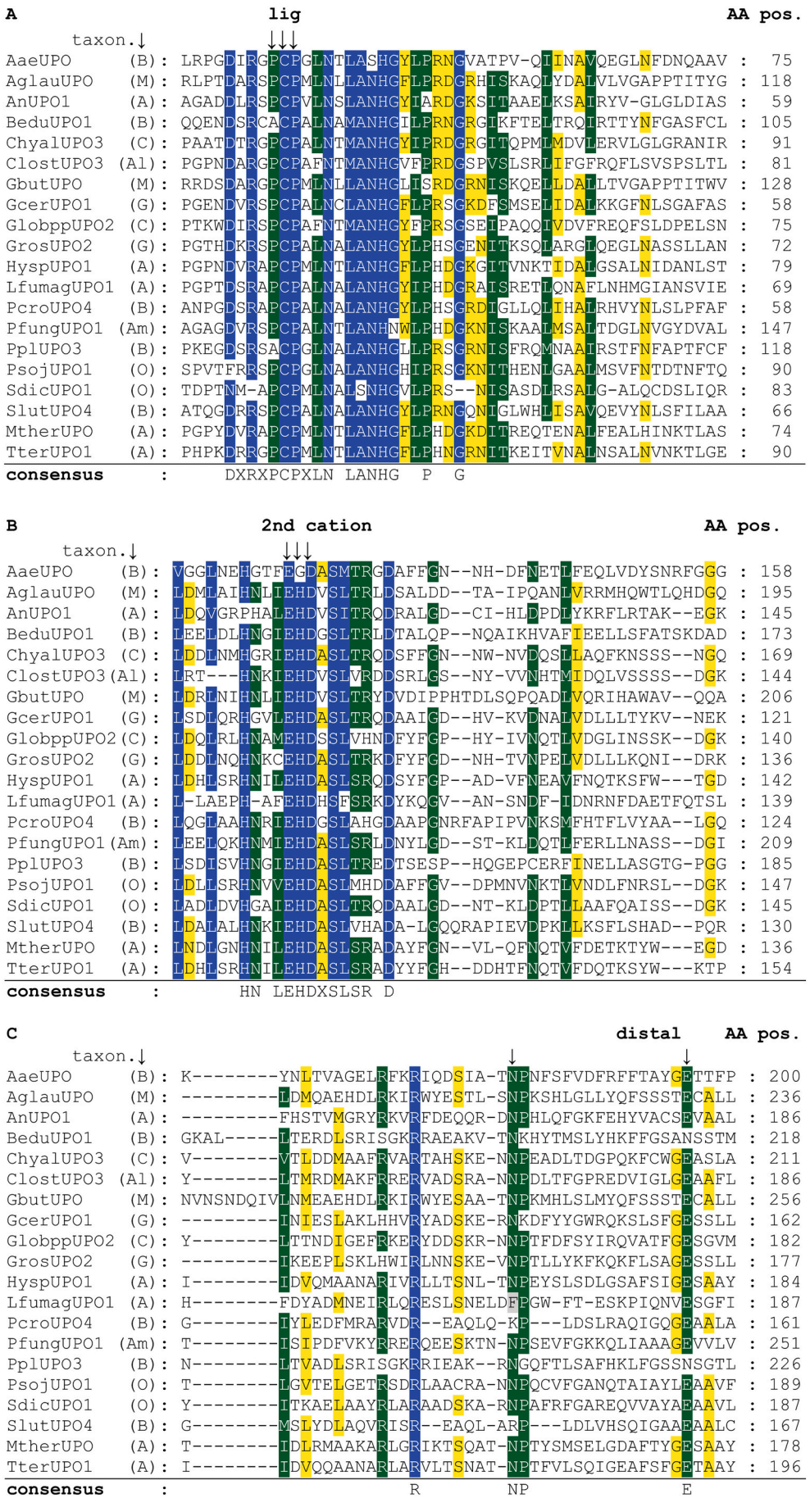

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Fig. 6. Multiple protein sequence alignment of unspecific heme peroxygenases. Sequences of 20 selected UPOs from all phyla of Fig. 1 were collected and aligned with Muscle program [62]. This selection includes representatives from all resolved clades with focus on those that are also mentioned in further analyses. Presented are only essential conserved parts of obtained alignment that is completely available in Suppl. Material 1. A) Sequence region at the proximal side of heme, B) sequence motif around binding site of the second cation near heme, C) region at the distal side of heme conserved moderately. Colour scheme: blue >95 % overall sequence conservation, green >75 % conservation, yellow >60 % conservation. Assignment to taxonomic phyla: (A)-Ascomycota, (Al)-Algae, (Am)-Amobeozoa, (B)-Basidiomycota, (C)-Chytridiomycota, (G)-Glomeromycota, (M)-Mucoromycota, (O)-Oomycota. Abbreviations of presented sequences are explained in Suppl. Table 1.

Table 2
Calculated pI values of unspecific heme peroxygenases aligned in Fig. 4. Average values were obtained from the application Compute pI/Mw at server Expasy https://web.expasy.org (accessed 20th August 2025).

Sequence abbreviation, phylum	Calculated pI value
AaeUPO (AaeAPO)	5.42
AglauUPO	9.08
AnUPO1	8.37
BcinUPO1	5.64
BeduUPO1	8.72
ChyalUPO3	5.08
ClostUPO3	10.23
GbutUPO	6.06
GcerUPO1	7.06
GlobppUPO2	5.97
GrosUPO2	9.18
HyspUPO1	5.28
PcroUPO4	8.47
PfungUPO1	5.73
PplUPO3	7.72
PsojUPO1	5.70
SdicUPO1	5.95
SlutUPO4	8.58
MtherUPO	5.86
TterUPO1	7.94

ion binding another amino acid triad, namely the motif EHD with highest bit value for the histidine, is apparent. In this case few other amino acids towards the C-terminus also reveal increased bit values, among them one highly conserved aspartate. This also underlines the significance of particular amino acids near the binding site of magnesium or rarely manganese for the architecture of unspecific peroxygenases active site.

7. Structural comparison of unspecific heme peroxygenases from various biotopes

For search of all available experimentally determined 3D structures of various UPO proteins the HHpred tool (https://toolkit.tuebingen.mpg.de/tools/hhpred) from the server of MPI Bioinformatics Toolkit [65] was used. In PDB structural database up to 86 entries of various crystal structures presenting unspecific heme peroxygenases are currently deposited (annotated in some older cases as chloroperoxidases). However, this corresponds to only eight unique peroxygenase proteins and the rest is represented by numerous protein variants in complexes with substrates or ligands or selected point mutations that were also crystallized to comparable resolution. Unique 3D structures from all available natural basidiomycetous or ascomycetous UPOs are summarized in Table 3. In comparison with other heme peroxidase superfamilies this amount of experimentally determined crystal structures is still rather underrepresented. Such limited number of PDB structures from few fungal proteins can be connected with troubles of their large-scale homologous or heterologous expression in suitable hosts [66] and their sufficient purifications. Recently, a novel expression system based on modular Golden Gate-based secretion was established [67]. This can stimulate the production of crystals and novel 3D structures of further interesting heme peroxygenases. It will be interesting to focus mainly on yet unknown representatives from non-dikaryal i.e. basal fungal clades but moreover also from various non-fungal ancestral UPO clades (cf. Fig. 4 for their detailed distribution). Mainly the insight into 3D structures of still rare basal fungal UPOs are strongly awaited to get integrated findings on the conservation of the typical structural fold among phylogenetically distantly related fungal species with consequences for their expected function. The detailed structures of still putative UPOs from green algae or from amoebozoa can also give important insights into their potential significance for the specific physiology of the algal or protist organism that can be different from previous results obtained for saprotrophic or lignolytic fungal UPOs. Structural comparison of experimentally solved UPO structures from diverse fungal sources is presented in Fig. 8 together with one structural prediction obtained with Alpha Fold server. Confidence scores calculated for this model (cf. Fig. 8D – legend for details) indicate that a reliable high-quality prediction was obtained for the whole catalytic domain of ClostUPO3. The spatial orientation of an almost invariantly conserved proximal amino

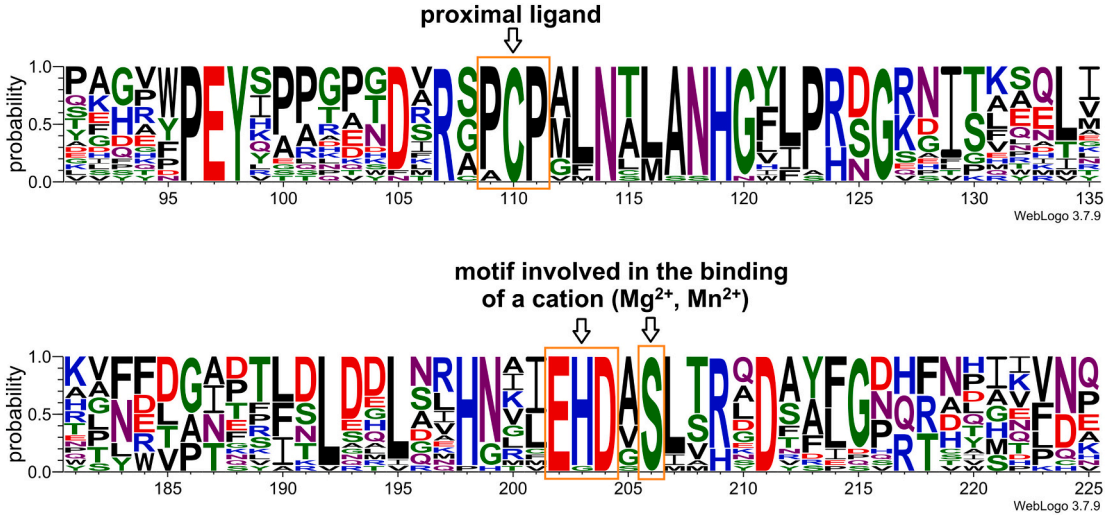

Fig. 7. Sequence logo output [64] reconstructed for 20 aligned UPO representatives of Fig. 4 focused on the A) proximal side of the prosthetic heme group and B) location for binding of the second metal ion in the active centre.

Table 3
Overview on unique experimentally determined 3D structures available for diverse unspecific heme peroxygenases. Presented are their PDB codes with links and systematic names of organisms from which corresponding proteins originate. Contents of the secondary structure is provided for protein regions with regular secondary structure elements.

PDB code	Organism – systematic name	Taxonomy	Length [AA]	Secondary structure contents	Publication
7ZCL	*Achaetomiella virescens*	Ascomycota	226	α-helix 45 % 3–10 helix 2 % β-strand 2 %	[107]
9HE6	*Candolleomyces aberdarensis*	Basidiomycota	335	α-helix 39 % 3–10 helix 2 % β-strand 4 %	[108]
2YOR	*Cyclocybe aegerita* (formerly Agrocybe aegerita)	Basidiomycota	324	α-helix 40 % 3–10 helix 2 % β-strand 3 %	[42]
8IAG	*Daldinia caldariorum*	Ascomycota	222	α-helix 46 % 3–10 helix 4 % β-strand 4 %	[109]
7O1R	*Hypoxylon* sp. EC38	Ascomycota	227	α-helix 44 % 3–10 helix 4 % β-strand 3 %	[47]
1CPO	*Leptoxyphium fumago* (formerly *Caldariomyces fumago*)	Ascomycota	299	α-helix 34 % 3–10 helix 3 % β-strand 3 %	[46]
7ZBP	*Marasmius rotula*	Basidiomycota	234	α-helix 41 % 3–10 helix 5 % β-strand 3 %	[107]
8RNJ	*Marasmius wettsteinii*	Basidiomycota	240	α-helix 41 % 3–10 helix 5 % β-strand 3 %	[110]

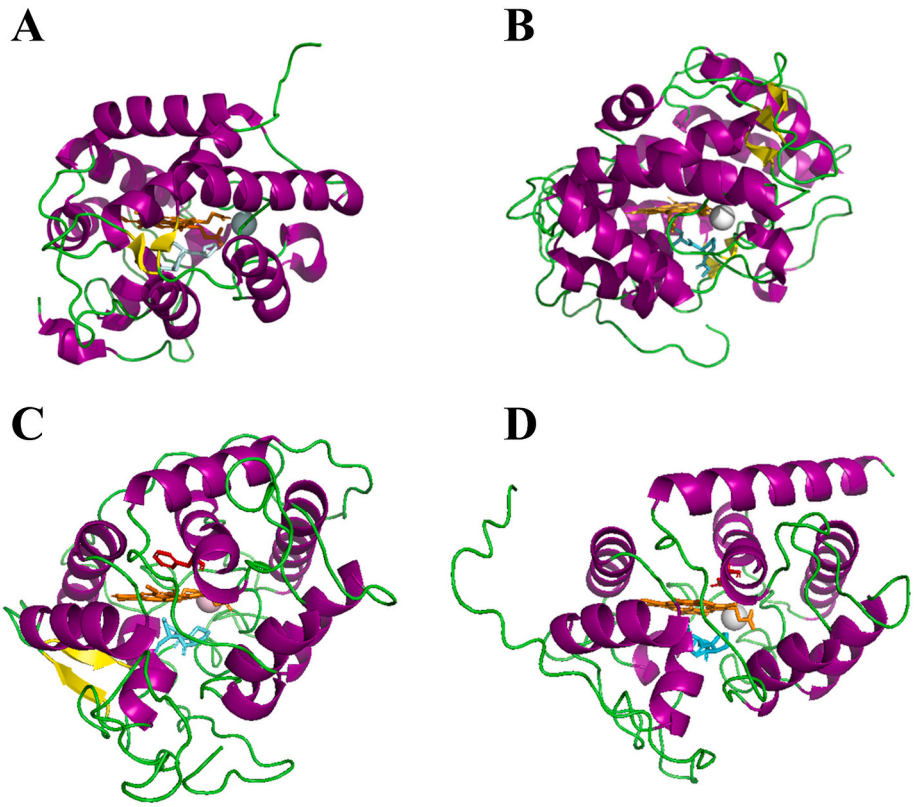

Fig. 8. Structural comparison of unspecific heme peroxygenases from various microbial sources. A) typical representative of ascomycetous UPOs with PDB code 7ZCL representing the heme enzyme from *Achaetomiella virescens*, B) typical representative of basidiomycetous UPOs with PDB code 2YOR representing the heme enzyme from the mushroom *Cyclocybe aegerita,* C) Chloroperoxidase (original annotation) from the ascomycete *Leptoxyphium fumago* (previously known as *Caldariomyces fumago*) with PDB code 1CPO. D) Homology model of algal ClostUPO3 with heme and magnesium as ligands obtained with Alpha Fold 3 server. Prediction confidence scores for this model are: ipTM = 0.95, pTM = 0.89 and pLDDT>90 for the whole catalytic domain (only the short N-terminal loop revealed pLDDT<50). Prosthetic heme groups are colored orange in all presented structures, proximal amino acid residues are cyan and distal residues are red, a-helices are purple and b-strands yellow. Rendered with PyMOL.

acid triad in the vicinity of heme discussed already in section 6 is in comparable position in all here presented structures. Also, the presence of the second cation near heme edge appears as highly conserved in all available UPO structures. Interestingly, only in the *Leptoxyphium fumago* enzyme previously described as chloroperoxidase manganese ion is found instead of magnesium at this site (cf. PDB code 1CPO). The distal side of heme is apparently a less conserved region. Two neighbouring positions of the distal glutamate (E196 in AaeUPO) are occupied by a tandem of conserved asparagine and a proline (cf. Fig. 6C). Surprisingly, in LfumagUPO1 there is a rare substitution of bulky phenylalanine at position 169 in above mentioned *Leptoxyphium* enzyme. Therefore, a question remains whether certain UPOs previously described as haloperoxidases possess fine but important differences in their amino acid composition in comparison with strictly defined peroxygenases?

In all currently available globular UPO structures α helices are the dominant secondary structure elements as obvious from all proteins presented in Fig. 8. This mainly α helical subunit structure resembles the solved crystal structures of numerous peroxidases from other three heme peroxidase superfamilies. The calculated α-helical content varies among eight available UPO structures between 34 and 46 % (Table 3). If we include also the 3–10 helical regions (that differ in geometry and hydrogen bonding from α-helices) the total amount of helices present in UPO structures grows up to 37–50 %. This is in significant contrast with β-strand regions that cover only 2–4 % (Table 3) of the total composition in solved UPO structures. Such high helical content can contribute to the overall stability, compactness and efficient enzymatic activity of investigated unspecific peroxygenases. However, it is known that catalytically related Cytochrome P-450 proteins contain intrinsically disordered regions in the middle or within the C-terminal portion of their protein sequences as can be detected with the IDEAL database [68]. Therefore, it is questionable, whether some UPOs also contain intrinsically disordered regions. Selected UPO structures presented in Fig. 8 and Table 3 were subjected for this purpose to IDR prediction server [69] but no disordered regions were found in the whole protein structures of 7ZCL (*Achaetomiella virescens* UPO) and 2YOR (*Cyclocybe aegerita* UPO).

8. Potential environmental impact of UPOs in technological applications

8.1. Environmental considerations for the use of heme peroxygenases in chemical synthesis

Biocatalysis is widely regarded as an environmentally friendly technology. Frequently cited arguments include mild reaction conditions (aqueous reaction media, low reaction temperatures, etc.), purported sustainability of the catalysts, as well as reduced generation of hazardous waste. However, unless substantiated quantitatively through comparative life-cycle analyses, these arguments remain insufficiently robust. Heme-containing peroxygenases are no exception in this regard. To attain a balanced view on the real environmental impact of UPO production and purification, the following considerations should not be neglected.

8.1.1. Environmental impact of UPO catalyst preparation

Enzyme production in general requires carbon, nitrogen, minerals, and energy inputs, many of which have significant environmental footprints. In the special case of heme-dependent peroxygenases a sufficient source of iron ions needs to be supplied as an essential microelement during the cultivation to boost their biosynthesis. Fig. 9 visualises the complexity of the feedstocks used for a typical heterologous UPO production.

Renewable carbon sources like crop-derived sugars are often used, but they still demand land, water, and energy. Nitrogen sources (e.g., ammonia) are energy-intensive to produce, while minerals like phosphate and potassium are finite and mined. Trace metals are required in small amounts but can have sourcing concerns. Additional inputs like antifoams, pH adjusters, and steam also contribute to the footprint. Recently, the energy required for the laboratory-scale (10*L*) heterologous expression of peroxygenase from *Agrocybe aegerita* (rAaeUPO) was evaluated, revealing an alarmingly high carbon footprint of approximately 111 t of CO_2 per kilogram of crude enzyme produced [70]. This value was higher than corresponding value for formate oxidase that does not require heme. Even when accounting for economies of scale, at least 100 kg of CO_2 per kilogram of recombinant UPO can be expected. Therefore, caution is advised before designating any produced enzyme as 'green' and this needs to be evaluated for each particular UPO to be produced heterologously in future projects.

However, enzymes are generally not the final product but serve as

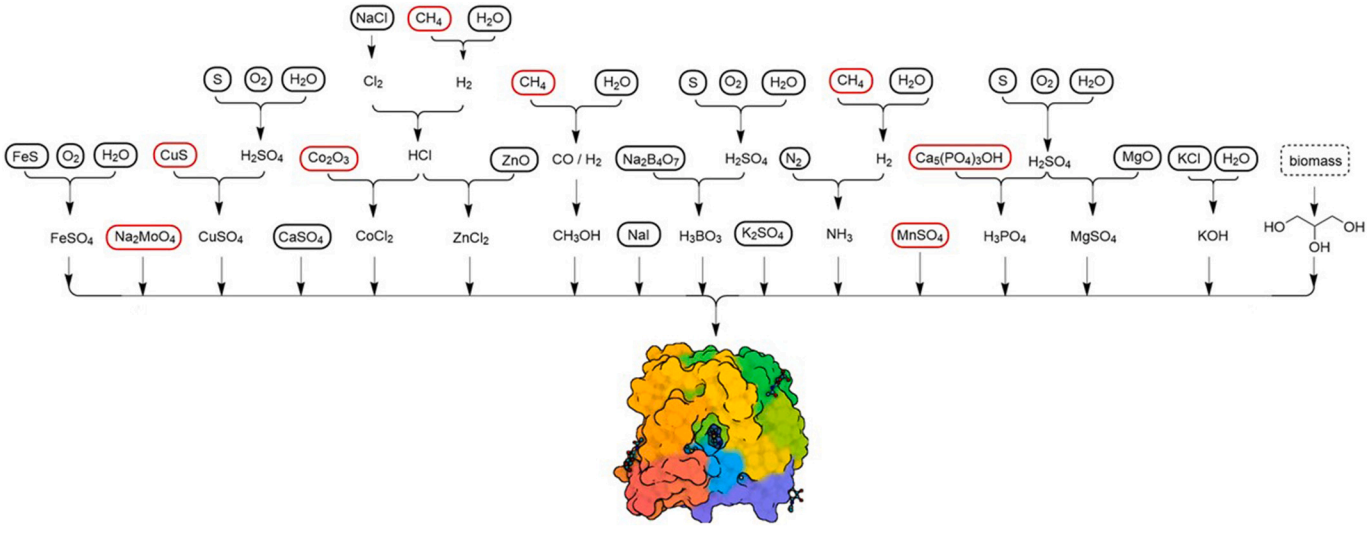

Fig. 9. Production scheme of a model peroxygenase tracing back the nutrients used for fermentation of the original compounds (extracted from the ground, air, or sea). This methodological approach was routinely applied to most already heterologously produced UPOs as discussed in [58] and needs renewable media components and reduced energy input to achieve sustainability goals in future projects. Therefore, alternative hosts and production media need to be considered.

catalysts facilitating chemical transformations. Thus, the critical factor is not the CO_2 footprint of the engineered peroxygenase itself but rather its contribution to the overall footprint of the final product. A highly active and stable oxidoreductase will perform effectively as a catalyst, requiring only minimal amounts for the desired reaction. In other words, the higher an enzyme's turnover number in a given transformation, the lower its relative CO_2 contribution to the final product. In Fig. 10 a typical example for a long variant II of fungal UPO oxidoreductase is presented (cf. Fig. 4 for overview on available UPO variants) and it will be interesting to compare such footprints with mainly fungal non-dikaryal or even non-fungal heme peroxygenases in future projects.

8.1.2. *Environmental impact of the transformation driven by UPOs*

Clearly, the chemical transformation itself also contributes to environmental impact. Various factors, including reaction temperature, reaction duration, product titer, and solvent choice, influence the economic and environmental outcomes of any given transformation.

Among these factors, reagent concentration is particularly significant in determining environmental impact. Energy consumption, associated CO_2 emissions, and waste generated during reactor operation are typically evaluated per unit of product produced. Consequently, higher product concentrations lead to reduced environmental impacts per unit of product, thereby simultaneously enhancing process sustainability and economic attractiveness.

Fig. 11 illustrates the relationship between product concentration and the specific environmental impact using stirring-related CO_2 emissions as an example. As the product concentration increases, the environmental burden per unit of product is proportionally reduced. In the case of most UPOs mainly Reaction 3 described in chapter 2 is relevant for the evaluation of such environmental impact producing selectively oxyfunctionalized organic molecules. The situation can be different for Reaction 4 where molecular oxygen is released but it needs to be systematically determined which UPO variants and to which extent can perform this peroxide dismutation reaction.

Sheldon and Woodley noted a clear correlation between higher substrate concentration and a lower *E*-factor (the mass of waste per mass of product) [71]. They illustrated this effect on numerous enzymes including also a vanadium peroxidase that belongs to a different metalloprotein family. It will be interesting to evaluate such correlation also for biocatalysis with involvement of unspecific peroxygenases that reveal also a remarkable substrate promiscuity in here analysed phylogenetic clades (cf. chapter 3). Achieving a high product titer (concentration) is not only economically beneficial but also crucial to ensure that environmental metrics are favourable. Having this in mind, it is astonishing that 90 % of reported enzymatic reactions used substrate loadings below 100 mM (roughly 20 g L^{-1}). For currently mostly investigated fungal UPOs a typical example was described for facile functionalization of fatty acids [72]. Numerous other lab-scale oxidoreductase reactions are routinely run in very dilute solutions. The consequence is that a reactor containing ca. 20 g L^{-1} of substrate also contains approximately 980 g L^{-1} of water; 98 % of the mixture is solvent, which the equipment must heat, cool, and stir.

8.1.3. *Solvent systems*

Aqueous buffers are traditionally used in enzymatic oxidations due to their excellent compatibility with enzymes. Typical buffers include phosphate, Tris-HCl, HEPES, and MES at concentrations of 50–200 mM to ensure pH stability. However, hydrophobic substrates have limited solubility in water, restricting substrate loading and product yields. This leads to increased solvent volumes and higher environmental impacts

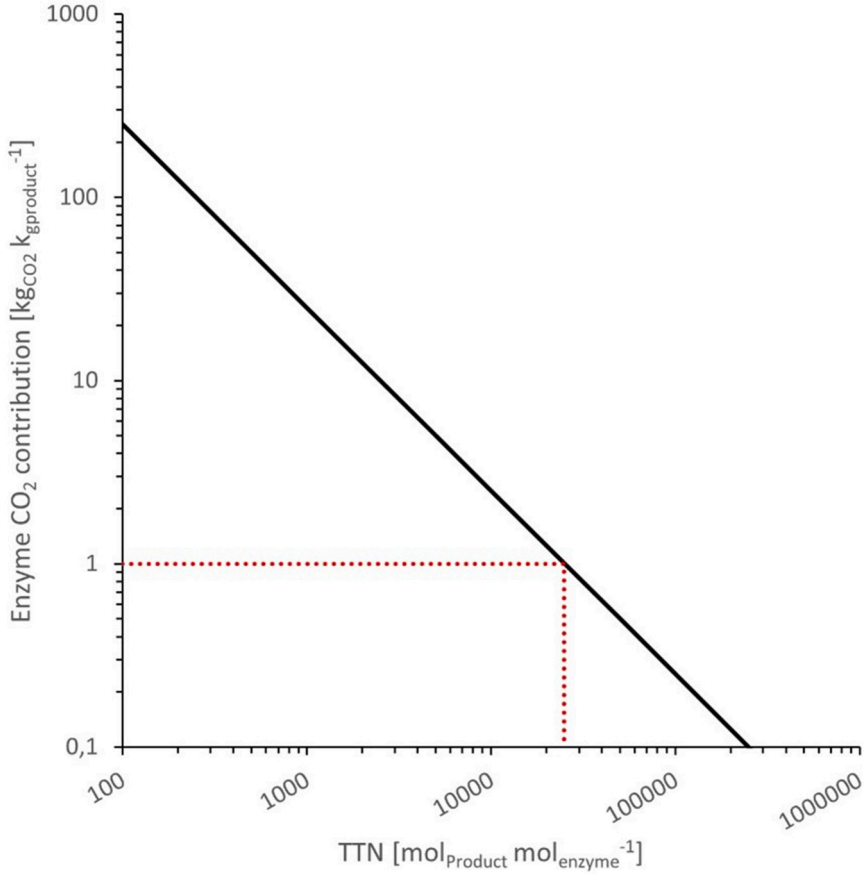

Fig. 10. Contribution of CO_2 footprint for a typical dikaryal long variant II UPO enzyme to the final product. Assumptions: M_w(Enzyme) = 45 kDa, M_w(Product) = 200 g mol^{-1}. Calculated CO_2 footprint of this oxidoreductase represents 100 kg (CO_2) per kg (Enzyme).

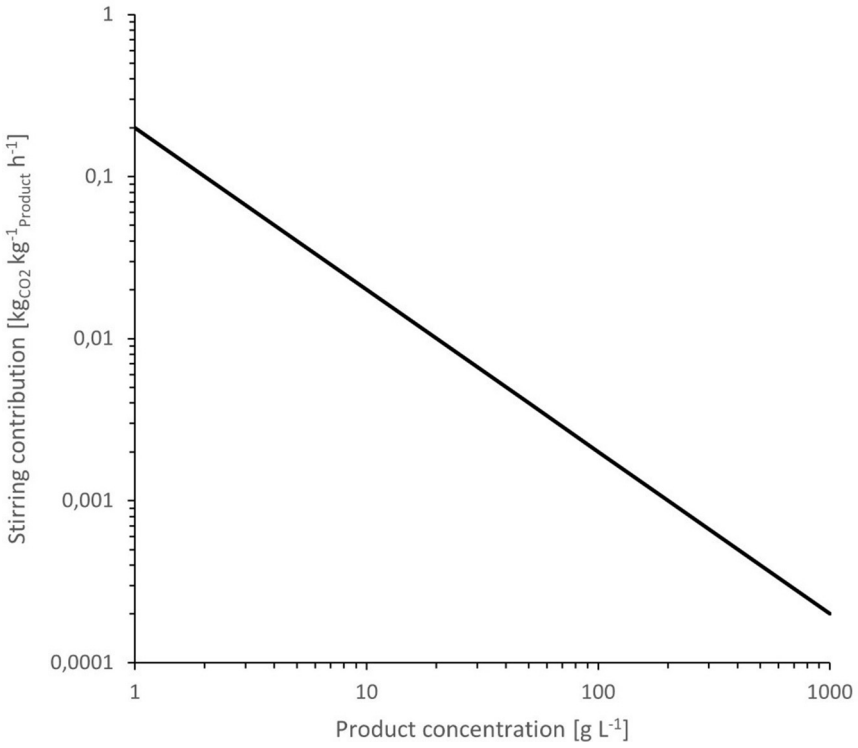

Fig. 11. Exemplary effect of product concentration on the stirring CO_2 footprint. Assumptions: power input 0.5 W L^{-1}, CO_2 intensity of electricity: 0.4 kg $_{CO2}$ kWh^{-1}.

due to energy-intensive downstream processing, particularly solvent extraction and waste management. A specific approach of neat substrate system to reduce solvent waste was already applied on basidiomycetous peroxygenase AaeUPO [73]. For this purpose, UPOs need to be immobilized on suitable carriers or beads. Water-miscible organic solvents (DMSO, acetonitrile, THF, alcohols) enhance substrate solubility and process efficiency for immobilized variants. However, peroxygenase stability often declines due to certain protein structural disruptions and hydration shell loss that can be experimentally verified. Prior to large-scale application, each UPO needs to be evaluated under diverse solvent conditions for its performance. Organic solvents that are used for some engineered peroxygenases [54] can also complicate downstream processing due to azeotrope formation with water, increasing energy demands and solvent recovery issues.

Two-Liquid Phase Systems consisting of an aqueous enzyme-containing phase and a second hydrophobic organic phase, significantly enhance substrate loadings and simplify product isolation through phase separation. 2LPS approach was already applied for the immobilized AaeUPO revealing a significantly prolonged production period [74]. The organic phase functions as a substrate reservoir, alleviating heme peroxygenase inhibition and shifting reaction equilibria favourably.

Slurry-to-Slurry biocatalysis involves running reactions with solid substrates and products suspended in minimal aqueous media, maximising substrate concentration and minimising solvent use. It simplifies downstream processing through direct product filtration. However, control and monitoring challenges limit its widespread application. This approach has not been reported for any UPO yet but it represents at least a promising alternative for recently discovered non-fungal peroxygenase variants because many of them have a different subcellular location in comparison with classical fungal secretory variants (cf. Table 1).

Ionic Liquids and Deep Eutectic Solvents represent another interesting alternative to classical organic solvents due to negligible vapour pressures and tunable properties. However, most ILs pose sustainability challenges due to their synthesis, environmental persistence, and toxicity risks [75]. Although monitored peroxidases from a different protein family revealed significantly increased activities [76] the usage of IL for peroxygenases in large scale assays is discouraged for environmental reasons. DESs, formed from biodegradable and inexpensive components, present lower toxicity and better environmental profiles but suffer from high viscosity, potentially increasing energy requirements for mixing [77]. Special DES was already applied for rAaeUPO driven hydroxylation and epoxidation [75] but its broader applicability for numerous novel peroxygenases described in chapter 3 currently remains questionable and needs to be tested.

8.1.4. H_2O_2 sources

H_2O_2 plays a pivotal role in peroxygenase reactions. On the one hand, H_2O_2 acts as a stoichiometric oxidant, while on the other, it serves as a potent enzyme inhibitor. Even slightly elevated H_2O_2 concentrations can dramatically decrease peroxygenase activity within minutes. This issue can be addressed either by controlled dosing of H_2O_2 or by in situ generation of H_2O_2 [78]. The first method stands out due to its simplicity. Indeed, the highest reported productivity of peroxygenase reactions, reaching 157 mM h^{-1} (approx. 15 g L^{-1} h^{-1}), was achieved by this approach [79]. However, not all reactor configurations and industrial infrastructures are suitable for handling concentrated H_2O_2 solutions. Additionally, dilution poses another significant challenge. If a commercially standard (30 % *w*/w) H_2O_2 solution is employed, approximately 7 mol water (1 mol originating from the reaction itself and 6 mol from the aqueous H_2O_2 solution) are introduced per mol of product formed. In the case of a 1 M substrate solution, this corresponds to approximately 12 % dilution, which naturally increases further with decreasing H_2O_2 concentration.

In situ generation of H_2O_2 represents a promising alternative. The underlying principle involves the catalytic reduction of molecular oxygen to H_2O_2, enabling precise control over the generation rate by adjusting the concentration of the reduction catalyst. Various approaches have been developed in recent years, each offering specific advantages and disadvantages. The glucose/glucose oxidase system was established early on and its widespread use persists in laboratory practice to this day [78]. However, this system suffers from poor atom

efficiency, producing 198 g of gluconate by-product per mol of H_2O_2 formed. Additionally, it is important to consider that increasing glucose concentrations result in elevated viscosity of the reaction mixture, approximately doubling the viscosity of an aqueous buffer when glucose concentration reaches 1 M. More attractive alternatives would be in situ H_2O_2 generation systems offering higher atom efficiencies, such as the triple oxidation of methanol to CO_2, generating three equivalents of H_2O_2 [80–82]. Another smart and essentially waste-free method is based on electrochemical in situ generation of H_2O_2, provided that renewable electricity sources are utilised. Recently, this methodology was successfully employed for the selective oxidative activation of C–H bonds catalysed by UPOs [83]. Presented study showed that gold-palladium nanoparticles supported on TiO_2 or carbon have sufficient activity at ambient temperature and pressure to generate H_2O_2 from H_2 and O_2 and supply the oxidant to the engineered peroxygenase variant PaDa-I (which is a mutated form of already frequently analysed and used AaeUPO cf. section 3.7).

8.1.5. Alternative small-molecule reductants for peroxygenase catalysis

Finally, the potential of H_2O_2-independent (per)oxygenase catalysis discovered recently also merits a brief discussion. By employing small reductants such as ascorbic acid [84], it was possible to mimic the catalytic cycle of P450 monooxygenases in unspecific heme peroxygenases while avoiding the inactivation issues commonly associated with H_2O_2 [85] and heme. Although this strategy is still in its early stages of development, it may evolve into a promising route towards more robust alternative UPO catalysis that will then apparently turn to a mono-oxygenase mechanism. Besides ascorbate also dehydroascorbate and two phenolic substances were identified as efficient co-substrates for PaDa-I variant of AaeUPO, for non-mutated MroUPO1 and LfumagUPO1. All these enzymes belong to fungal dikaryal heme peroxygenase representatives (Fig. 4) and no information in this respect is available for remaining UPO clades yet. It is also interesting to note that the catalytic efficiency was much higher in aerobic than in anaerobic conditions [84]. This can raise the question whether under aerobic conditions micromolar traces of reactive oxygen species can be present in heme microenvironment that can influence the progress of monitored peroxygenase catalysis with involvement of described small-molecule reductants. Mainly ascorbate is known as a radical scavenger and this can influence the active centre of heme enzymes. In this context it is interesting to mention another protein family, namely ascorbate peroxidases, that use both H_2O_2 and ascorbate as substrates in their catalytic cycle [17]. The approach of addition of small-molecule cosubstrates was verified with a recent report on oxyfunctionalisation of anisole by several UPOs with and without ascorbic acid [86]. The observed product palette was different dependent on the presence of hydrogen peroxide or ascorbate. Apparently, the detailed architecture of heme active site in diverse UPOs is important for the progress of the catalytic reaction with various small cosubstrates and the specificity of obtained products.

8.2. Peroxygenase-catalysed oxyfunctionalisation reactions – Some case studies

The reactions catalysed by peroxygenases have been extensively reviewed in recent years [87–90]. Therefore, in this section, we will focus on selected case studies that exemplify both the potential and the current challenges associated with peroxygenases in chemical synthesis.

8.2.1. Reactions in non-aqueous media

A significant advantage of peroxygenase-catalysed oxyfunctionalisation reactions, compared to those catalysed by P450 monooxygenases, is that aqueous reaction media are not required to facilitate diffusion of the nicotinamide cofactor between the regeneration enzyme and the catalytic enzyme. This advantage was first demonstrated by Klibanov and co-workers as early as the 1980s [91]. Recently, interest in peroxygenase catalysis within non-aqueous media has increased. Currently, successful application of this approach typically requires two prerequisites: (1) immobilisation of the peroxygenase enzyme and (2) the use of organic hydroperoxides [92].

Immobilized *Aae*UPO has been employed for the enantioselective oxyfunctionalisation of various aryl thioethers [93], achieving exceptionally high product concentrations of over 600 mM. Other examples comprise (cyclo)alkane and -ether hydroxylation [94,95]. Similarly, selective benzylic hydroxylation of several toluene derivatives has also been successfully accomplished using immobilized *Aae*UPO [96]. Intriguingly, in this study, the hydrophobic medium favoured further oxidation of initially formed benzyl alcohols—typically the dominant products under aqueous conditions [97]—to the corresponding aldehydes, possibly due to an increased affinity of the enzyme for the polar alcohol intermediate. This observation underscores the potential for reaction engineering in peroxygenase catalysis.

Another notable finding was that ring substituents on the toluene derivatives significantly influenced the regioselectivity of the peroxygenase reactions. While unsubstituted toluene underwent hydroxylation with relatively low selectivity between ring and side-chain positions, the presence of substituents resulted in exclusive ring hydroxylation. This may be explained by a reduced number of productive substrate-binding modes relative to Compound I.

8.2.2. Overoxidation and multistep syntheses

Considering the hydroxylation of non-activated C–H bonds (particularly methylene C–H bonds), the most commonly targeted products are enantiomerically pure alcohols. However, this emphasis tends to overlook the versatility of achiral carbonyl compounds (aldehydes and ketones). Due to their extensive reactivity, these carbonyl products serve as valuable intermediates in organic synthesis. For example, coupling peroxygenase-catalysed ketone formation with alcohol dehydrogenase-catalysed enantioselective reduction enables access to both enantiomers of the corresponding alcohol via a two-step oxyfunctionalisation and reduction cascade [98]. Similarly, combining peroxygenase-catalysed oxidation with reductive amination provides an efficient route to chiral amines directly from alkanes (Fig. 12) [99]. Further elaborations of such strategies allow access to even more structurally complex products [100–102]. The mild reaction conditions typical of peroxygenase-catalysed transformations facilitate their integration into multi-step processes, either concurrently or sequentially with other chemical transformations. Notably, this approach eliminates the necessity for isolating and purifying intermediates, thereby reducing environmental impacts associated with downstream processing and enhancing infrastructure utilisation [95]. For instance, a chemoenzymatic cascade has recently been reported in which peroxygenase-catalysed aldehyde formation was combined with an organocatalytic aldol reaction (Fig. 12) [96].

8.2.3. Peroxygenases in environmental technology

Peroxygenases have demonstrated activity towards numerous pharmaceuticals, agrochemicals, and environmental pollutants. *Aae*UPO, by far mostly applied representative, has been shown to hydroxylate the β-blocker propranolol to its primary human metabolite, 5-hydroxypropranolol, as well as to convert the anti-inflammatory drug diclofenac into 4′-hydroxydiclofenac [103–105]. Recently, oxidative degradation of antibiotics found in surface wastewaters has also been reported [106]. In this case a comparison of AaeUPO with a different chloroperoxidase from a filamentous Ascomycete was investigated in a reactor. Interestingly, AaeUPO was more stable in wastewaters and removed more rapidly the contaminant but its catalase activity was posing a problem. Due to here presented recent knowledge, this issue can be overcome by using alternative small molecule reductants (section 8.1.4).

One of the most promising applications of peroxygenases is in the degradation of environmental pollutants, including micropollutants and recalcitrant organic compounds found in wastewater. Peroxygenases have proven effective in oxidizing many of the EPA's priority pollutants.

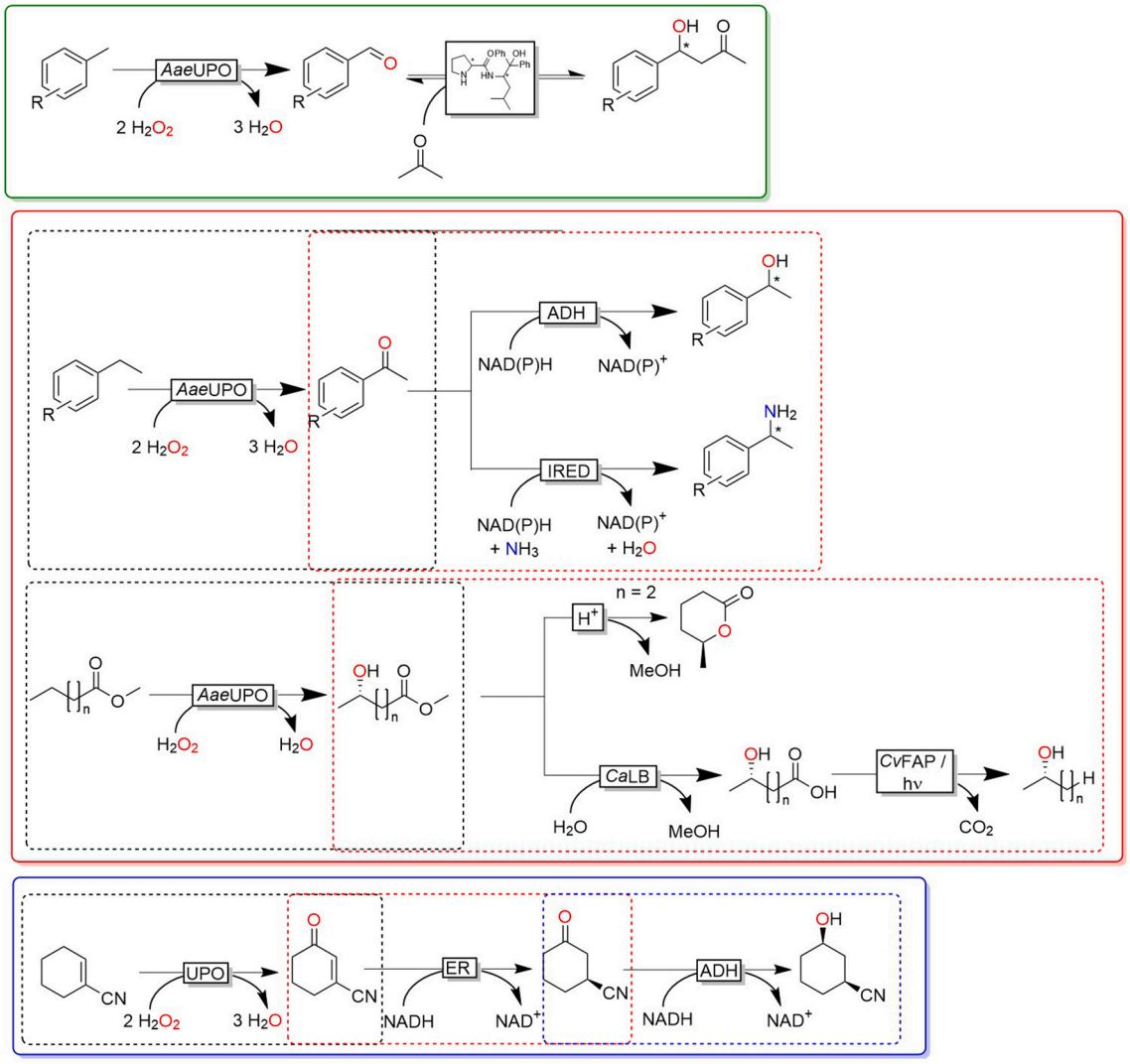

Fig. 12. Examples for peroxygenase reactions as key steps for multistep cascades. Green: one pot – one step; red: one pot – two steps and blue: one pot -three steps.

For instance, a 2017 study demonstrated that *Aae*UPO successfully oxidised 32 out of 38 tested priority pollutants, including polyaromatic hydrocarbons, chlorinated benzenes, nitrophenols, among others [10]. According to newly discovered UPO clades containing representatives from basal fungal lineages and from non-fungal eukaryotes it is strongly recommended to replace the almost solely used *AaeUPO* with at least few new candidates from Mucoromycota, Glomeromycota or from green algae. It will be interesting to evaluate in trial experiments their productivity in oxyfunctionalizations with potential environmental impact.

9. Concluding remarks and outlook for future research directions

Heme containing peroxygenases were subjected to intensive investigations worldwide in last two decades. From here presented phylogenomic analyses it is however obvious that only a small part of the large and abundant peroxidase-peroxygenase superfamily was examined on the protein level to comprehensive biochemical and biophysical details and still rather small amount of experimentally determined 3D UPO structures is available for detailed molecular comparisons. Vast majority of cloned and heterologously expressed UPOs are directly used for a palette of synthetic or model substrates. An important aspect remains a systematic search for their real natural substrates that have very probably influenced the specific UPO evolution among diverse cells and microorganisms. This knowledge gap can be a stimulating research challenge for the future. The physiological role of UPOs present in various lignolytic fungi can be supposed to be a yet hypothetical and auxiliary part of the lignocellulose degradation or modification machinery. To verify this possibility a focused transcriptomic and proteomic research is indispensable for selected, already well documented lignolytic fungi. The precise role of heme peroxygenases originating from phytopathogenic fungi and water molds in cellular physiology is completely unknown and needs a focused -omics research and direct comparison with their non-pathogenic mostly saprotrophic counterparts. Moreover, the investigation of numerous recently discovered UPO sequences from non-dikaryal fungi needs much more attention because in this case the real physiological function cannot be simply connected with lignocellulose metabolism. It can more likely be oriented in some yet unknown detoxification pathway probably connected with oxidative stress response. A possible discovery of natural substrates for UPOs from Mucoromycota, Glomeromycota and Chytridiomycota can give valuable hints for the evaluation and comparison of physiological substrates for their evolutionary descendants in the clades of Ascomycota and Basidiomycota. Finally, the discovery of natural substrates for yet rare unspecific heme peroxygenases from alternative non-fungal sources like amoebas or green algae can give valuable hints for future nature-inspired organic synthesis. Recently discovered alternative small reductants like ascorbate or dehydroascorbate can indeed be promising

candidates of real physiologically occurring secondary metabolism pathways driven by non-fungal unspecific peroxygenases. They may serve as key modules in the design of alternative biocatalytic strategies. Protein engineering and directed evolution of selected heme peroxygenase genes that will be focused mainly on their ancestral variants can open new horizons with the perspective of constructing robust and multistep catalytic transformations for sustainable and environmentally acceptable products driven by highly efficient unspecific heme peroxygenases even in non-aqueous media.

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CRediT authorship contribution statement

Marcel Zámocký: Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Bohuš Kubala:** Formal analysis, Data curation. **Barbora Zámocká:** Investigation, Formal analysis, Data curation. **Juraj Kronek:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Frank Hollmann:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors have no potential conflict of interest to disclose.

Data availability

Data will be made available on request.

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