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MINI-REVIEW

Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation

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Abstract Aryl-alcohol oxidase (AAO) is an extracellular flavoprotein providing the H_2O_2 required by ligninolytic peroxidases for fungal degradation of lignin, the key step for carbon recycling in land ecosystems. O_2 activation by *Pleurotus eryngii* AAO takes place during the redox-cycling of *p*-methoxylated benzylic metabolites secreted by the fungus. Only *Pleurotus* AAO sequences were available for years, but the number strongly increased recently due to sequencing of different basidiomycete genomes, and a comparison of 112 GMC (glucose–methanol–choline oxidase) superfamily sequences including 40 AAOs is presented. As shown by kinetic isotope effects, alcohol oxidation by AAO is produced by hydride transfer to the flavin, and hydroxyl proton transfer to a base. Moreover, site-directed mutagenesis studies showed that His502 activates the alcohol substrate by proton abstraction, and this result was extended to other GMC oxidoreductases where the nature of the base was under discussion. However, in contrast with that proposed for GMC oxidoreductases, the two transfers are not stepwise but concerted. Alcohol docking at the buried AAO active site resulted in only one catalytically relevant position for concerted transfer, with the pro-*R* α-hydrogen at distance for hydride abstraction. The expected hydride-transfer stereoselectivity was demonstrated, for the first time in a GMC oxidoreductase, by using the *(R)* and *(S)* enantiomers of α-deuterated *p*-methoxybenzyl alcohol. Other largely unexplained aspects of AAO catalysis (such as the unexpected specificity on substituted aldehydes) can also be explained in the light of the recent results. Finally, the biotechnological interest of AAO in flavor production is extended by its potential in production of chiral compounds taking advantage from the above-described stereoselectivity.

Keywords Aryl-alcohol oxidase · Fungal enzymes · GMC oxidoreductases · Lignin biodegradation · Reaction mechanism · Stereoselectivity

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Importance of lignin in natural recycling and industrial use of plant biomass

Lignin is an essential constituent of the cell wall of vascular plants and the second most abundant biopolymer in earth (after cellulose) representing around 20% of the total carbon fixed by photosynthesis in land ecosystems. The main role of lignin is to protect the cell-wall polysaccharides cellulose, and hemicelluloses (the latter being formed by different pentosans and hexosans) against hydrolytic attack by saprophytic and pathogenic microorganisms. Additionally, it provides to plant stems the rigidity required for growth on land and waterproofs vascular tissues for sap circulation. The recalcitrance of lignin against biodegradation is due to its bulky and aromatic nature that limits the accessibility of microbial enzymes and decreases its biodegradability. Lignin is formed from three main *p*-hydroxycinnamyl alcohols and their acylated forms, collectively known as monolignols (Martínez et al. 2008; Ralph et al. 2004). Although the lignin building blocks are phenolic compounds, the resulting polymer is non-phenolic. During lignin biosynthesis,

monolignols are oxidized to their phenoxy radicals by plant peroxidases (Higuchi 1997). Due to the prevalence of certain radical resonant forms, and the different stability of the coupling products, ether linkages between the phenolic position and the side-chain β-carbon of the *p*-hydroxyphenylpropenoid precursors are strongly predominant in the growing polymer, resulting in its non-phenolic nature.

Microbial degradation of lignin represents a key step for carbon recycling in land ecosystems since removal of the lignin barrier enables the subsequent use of plant carbohydrates by microorganisms (Kersten and Cullen 2007; Martínez et al. 2005). Lignin removal is also a central process for the industrial utilization of plant biomass as a source of renewable chemicals, materials, and fuels for sustainable development in future lignocellulose biorefineries (Himmel et al. 2007; Ragauskas et al. 2006). The growing demand for fossil fuels has increased the interest on alternative, environmentally friendly, and renewable energy sources. In this context, renewable biomass sources are generally viewed as important contributors to the development of a sustainable industrial society and the reduction of net greenhouse gas emissions from petrochemical sources. For bioethanol production from lignocellulosic biomass, the lignin barrier limits the accessibility to fermentable sugars (derived from cellulose and hemicelluloses). Disruption of this barrier can be achieved through traditional methods, including high-temperature and strong chemical reagents, or by sustainable and environmentally friendly bio-pretreatment (Salvachúa et al. 2011). In the future, biorefinery processes will extract first high-value chemicals present in the biomass, such as fragrances, flavoring agents, food-related products, high-value nutraceuticals, and other fine chemicals. Later, plant polysaccharides and lignin will be processed into feedstocks for bio-derivate materials, bulk chemicals, and fuels (Ragauskas et al. 2006).

Overcoming the lignin barrier: two different strategies based on H_2O_2

Wood-rotting basidiomycetes are the most efficient degraders of lignocellulose. Based on macroscopic and chemical degradation patterns, they are classified as white-rot or brown-rot fungi (Martínez et al. 2005; Schwarze et al. 2000; Zabel and Morrell 1992). White-rot fungi are able to degrade lignin and hemicelluloses. According to their ability to degrade lignin selectively or simultaneously with cellulose, two white-rot patterns have been described, the former having the highest interest for delignification biotechnological applications (Otjen and Blanchette 1986). A different group of basidiomycetes called brown-rot fungi can degrade wood polysaccharides after only partial modification of lignin, resulting in a brown material consisting of modified lignin (Martínez et al. 2011; Yelle et al. 2011).

Two of the most extensively investigated white-rot fungi, *Phanerochaete chrysosporium* and *Pleurotus eryngii*, evolved different extracellular enzymatic machineries to degrade lignin. *P. chrysosporium* secretes peroxidases, called lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) (Hammel and Cullen 2008), and the copper-radical glyoxal oxidase (EC 1.1.3.-) (Kersten 1990), while *P. eryngii* produces manganese peroxidase, and versatile peroxidase (EC 1.11.1.16) instead of lignin peroxidase (Ruiz-Dueñas et al. 2009), together with laccase (EC 1.10.3.2) (Muñoz et al. 1997) and aryl-alcohol oxidase (AAO, EC 1.1.3.7) (Guillén et al. 1990). White-rot fungi also secrete different aryl metabolites involved in lignin degradation (de Jong et al. 1994b; Gutiérrez et al. 1994). The above enzymes cannot penetrate the compact cell-wall structure in sound wood due to their molecular size; therefore, small chemical oxidizers—including activated oxygen species, metal cations, and aromatic radicals—are probably involved in the initial steps of fungal decay of wood (Evans et al. 1994). The mechanism of lignocellulose degradation by brown-rot fungi is still poorly understood. These fungi are able to access and degrade wood polysaccharides without removing the lignin. It is believed that they generate extracellular Fe^{2+} and H_2O_2, which are small enough to attack the wood cell wall by Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + H_2O + OH\bullet$) generating hydroxyl free radical (OH•) that oxidatively cleaves cellulose and hemicelluloses. Brown-rot basidiomycetes produce extracellular hydroquinones (Hammel et al. 2002) that reduce Fe^{3+} and O_2 to drive Fenton chemistry (Kerem et al. 1999). Comparison of the transcriptomes and secretomes of the model fungi *P. chrysosporium* (white-rot) and *Postia placenta* (brown-rot) reflects the above differences in lignocellulose degradation (Martínez et al. 2009; Vanden Wymelenberg et al. 2009). Comparison of these genomes (Table 1) indicates that brown-rot fungi evolution concentrated or lost gene families that are important in typical white-rot fungi, such as copper-radical oxidases from one side and LiPs, MnPs, and pyranose oxidase (EC 1.1.3.10) from the other. Additionally, the origin of the H_2O_2 involved in oxidative degradation of lignin and cellulose differs in white-rot and brown-rot basidiomycetes. In the former fungi, H_2O_2, for extracellular peroxidases, is generated by secreted oxidases in which flavin or copper act as cofactors for *P. eryngii* AAO and *P. chrysosporium* glyoxal oxidase, respectively (Guillén et al. 1992; Kersten 1990). In brown-rot decay of wood, methanol from lignin attack (demethoxylation) is proposed to serve as substrate for methanol oxidase (EC 1.1.3.13) to provide the H_2O_2 for hydroxyl radical generation in Fenton reaction (Daniel et al. 2007; Martínez et al. 2009).

Table 1 Differences in genes of enzymes potentially involved in lignin degradation in the white-rot (*P. chrysosporium*) and brown-rot (*P. placenta*) fungal genomes[a]

	White-rot fungus	Brown-rot fungus
Peroxide production:		
Methanol (alcohol) oxidase	1	1
Aryl-alcohol oxidase	4	3
Glucose oxidase	1	5
Pyranose oxidase	1	0
Glyoxal oxidase and other copper-radical oxidases	7	3
Lignin modification:		
Lignin peroxidase	10	0
Manganese peroxidase	5	0
Generic peroxidase	1	1
Laccase	0	3
Iron reduction:		
Quinone reductase	4	1
Cellobiose dehydrogenase	1	0

[a]Adapted from Martínez et al. (2009)

AAO and other H_2O_2-producing enzymes involved in lignin degradation

H_2O_2 plays a central role in wood attack by white-rot fungi as the oxidizing substrate of ligninolytic peroxidases (Ruiz-Dueñas and Martínez 2009). Moreover, it was postulated, first in *P. chrysosporium* (Faison and Kirk 1983; Forney et al. 1982) and later in *P. eryngii* (Gómez-Toribio et al. 2009; Guillén et al. 2000), that H_2O_2 in white-rot decay could also act as the precursor of highly reactive OH• being able to depolymerize lignin (and polysaccharides). It was early demonstrated that H_2O_2 is formed simultaneously with the ligninolytic system and, when it is destroyed by catalase addition to the fungal culture, the lignin-degrading capacity is reduced (Faison and Kirk 1983). Several extracellular enzymes have been postulated to be involved in the extracellular production of H_2O_2 in white-rot fungi including, among others, the above-mentioned glyoxal oxidase (Kersten and Kirk 1987) and AAO (Guillén et al. 1990), and pyranose oxidase (Daniel et al. 1994). H_2O_2 generated by intracellular enzymes—e.g., glucose oxidase (EC 1.1.3.4) (Eriksson et al. 1986) or fatty-acyl-CoA oxidase (EC 1.3.3.6) (Greene and Gould 1984)—requires an exportation system that has not been reported to date. Interestingly, it has been shown that methanol (alcohol) oxidase (EC 1.1.1.1), also reported in white-rot fungi (Nishida and Eriksson 1987), is secreted by the brown-rot fungus *Gloeophyllum trabeum* although its sequence does not include a typical signal peptide (Daniel et al. 2007). Another intracellular enzyme, the bicupin oxalate oxidase (EC 1.2.3.4), has been reported as responsible for the initial supply of H_2O_2 in the selective lignin degrader *Ceriporiopsis subvermispora* (Aguilar et al. 1999).

In *P. chrysosporium*, both glyoxal oxidase (Kersten and Kirk 1987) and pyranose oxidase (Daniel et al. 1994) have been reported under ligninolytic conditions. For glyoxal oxidase, a temporal relationship between activity, production of glyoxal/methylglyoxal, and lignin peroxidase was found, supporting its involvement in lignin degradation as a source of H_2O_2. In a similar way, pyranose oxidase shows a (hyphal and periplasmic space) distribution similar to those reported for the H_2O_2-dependent ligninolytic peroxidases in *P. chrysosporium* (Daniel et al. 1994). AAO activity was found in different fungi, including *Pleurotus* species (Bourbonnais and Paice 1988; Guillén et al. 1992; Sannia et al. 1991), *Trametes versicolor* (Farmer et al. 1960), *Fusarium solani* (Iwahara et al. 1980), *Rigidoporus microporus* (syn. *Fomes lignosus*) (Waldner et al. 1988), *Bjerkandera adusta* (Kimura et al. 1990; Romero et al. 2009, 2010), and *Botrytis cinerea* (Goetghebeur et al. 1993). The simultaneous production of AAO and lignin peroxidase in *B. adusta* (Muheim et al. 1990), and AAO and versatile peroxidase in *Pleurotus* cultures (Camarero et al. 1996) supports AAO involvement in lignin degradation. AAO localization was investigated during wheat straw degradation by *P. eryngii* under solid-state fermentation conditions, resembling those of natural degradation of lignocellulose by fungi (Barrasa et al. 1998). The enzyme was found in the hyphal sheath (formed by secreted polysaccharide) (Fig. 1), as reported for lignin peroxidase and laccase in other basidiomycetes (Gallagher et al. 1989; Green et al. 1992). In spite of its initial location on the hyphal surface, AAO can penetrate degraded cell walls of phloem and parenchyma, and also the more lignified sclerenchymatic tissues, as shown by both immuno-gold and immuno-fluorescence microscopy (Fig. 1).

AAO substrates in lignin degradation can include both lignin-derived compounds and aromatic fungal metabolites. The former would be phenolic aromatic aldehydes and acids (Kirk and Farrell 1987; Shimada and Higuchi 1991) being reduced to alcohol substrates by aryl-alcohol dehydrogenases (EC 1.1.1.90) (Muheim et al. 1991) and aryl-aldehyde dehydrogenases (E.C.1.2.1.29) (Lundell et al. 1990), respectively.

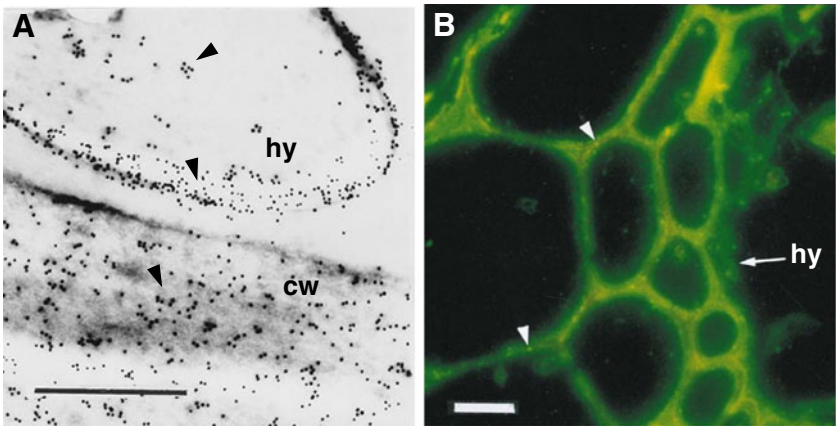

Fig. 1 AAO immunolocalization during wheat-straw degradation by *P. eryngii*. **a** Transmission electron microscopy localization of AAO by immuno-gold labeling (*black spheres*, *arrowheads*) in fungal hypha (*hy*) and partially degraded straw cell wall (*cw*). **b** Fluorescence microscopy localization of AAO by immuno-fluorescence labeling (*green-yellow spots*, *arrowheads*) in sclerenchymatic cell walls and fungal hyphae (*hy*). Adapted from Barrasa et al. (1998) (*bars* represent 1 μm in **a** and 10 μm in **b**)

Non-phenolic aromatic metabolites would be preferred taking into account the substrate specificity of *Pleurotus* AAO (Ferreira et al. 2005; Guillén et al. 1992), but the *Bjerkandera* AAO also efficiently oxidizes phenolic benzylic alcohols (Romero et al. 2009). Studies by Guillén and Evans (1994) and Guillén et al. (1994) using different aromatic (benzylic, *p*-methoxybenzylic, veratrylic, and vanillylic) compounds have demonstrated that *P. eryngii* AAO provides a continuous supply of H_2O_2 by redox cycling the above compounds, in collaboration with mycelium dehydrogenases. This was concluded after observing an equilibrium between oxidative and reductive reactions on benzylic alcohol, aldehydes, and acids that maintain the continuous H_2O_2 production at expenses of intracellular reducing power (Guillén et al. 1994). At least three enzymatic activities are involved in this process: extracellular AAO, which oxidizes aryl alcohols to aldehydes and eventually to acids (Ferreira et al. 2010; Guillén et al. 1992), and intracellular aryl-alcohol and aryl-aldehyde dehydrogenases, reducing them back to the aldehydes and alcohols (Gutiérrez et al. 1994). Interestingly, *p*-methoxybenzaldehyde (*p*-anisaldehyde) is the main extracellular aromatic metabolite in *Pleurotus* species (Gutiérrez et al. 1994) and its reduced form, *p*-methoxybenzyl alcohol, is one of the best substrates of AAO in these fungi (see Table 2) (Ferreira et al. 2005; Guillén et al. 1992). Therefore, most probably its physiological role is to maintain the redox cycle performed by aryl-alcohol dehydrogenase and AAO to supply H_2O_2 (Guillén and Evans 1994; Gutiérrez et al. 1994). The same function was suggested for chlorinated aryl alcohols in AAO-producing *Bjerkandera* species (de Jong et al. 1994a).

A simplified scheme for lignin degradation by white-rot fungi, based on *P. eryngii* studies (Ruiz-Dueñas and Martínez 2009), is presented in Fig. 2. Ligninolytic peroxidases (versatile peroxidase in this case) oxidize the lignin polymer, thereby generating aromatic radicals that evolve in different non-enzymatic reactions including Cα–Cβ (and C4–Cβ ether) linkage breakdown. As described above, the aromatic aldehydes from the latter reaction or synthesized *de novo* by the fungus are the substrates for H_2O_2 generation by AAO in redox cycle reactions after their reduction by mycelium dehydrogenases. Phenoxy radicals from C4–ether breakdown can also repolymerize if they are not first reduced, and AAO contribution to this reaction has also been suggested (Marzullo et al. 1995).

AAO: a member of the GMC oxidoreductases superfamily

AAO, a secreted monomeric enzyme, is a member of the glucose–methanol–choline oxidase (GMC) oxidoreductase superfamily, which is composed of a variety of prokaryotic and eukaryotic enzymes containing FAD as cofactor. This superfamily was created by Cavener (1992) including *Drosophila melanogaster* glucose dehydrogenase (EC 1.1.5.2), *Aspergillus niger* glucose oxidase, *Hansenula polymorpha* methanol (alcohol) oxidase, and *Escherichia coli* choline dehydrogenase (EC 1.1.99.1). Later, additional flavoenzyme sequences have been reported containing the typical signatures of GMC oxidoreductases. More recently, this number has strongly increased in the course of numerous genome sequencing projects (although many putative GMC open reading frames encode unknown proteins). The conserved sequences of all the GMC superfamily members (from N terminus to C terminus) include (Kiess et al. 1998) (1) The FAD-binding domain composed of four separate subregions, containing the ADP-binding βαβ motif that is not only characteristic for the GMC oxidoreductases but conserved among many FAD-binding proteins (Wierenga et al. 1983), and the GMC pattern-2 (PROSITE PS00624)

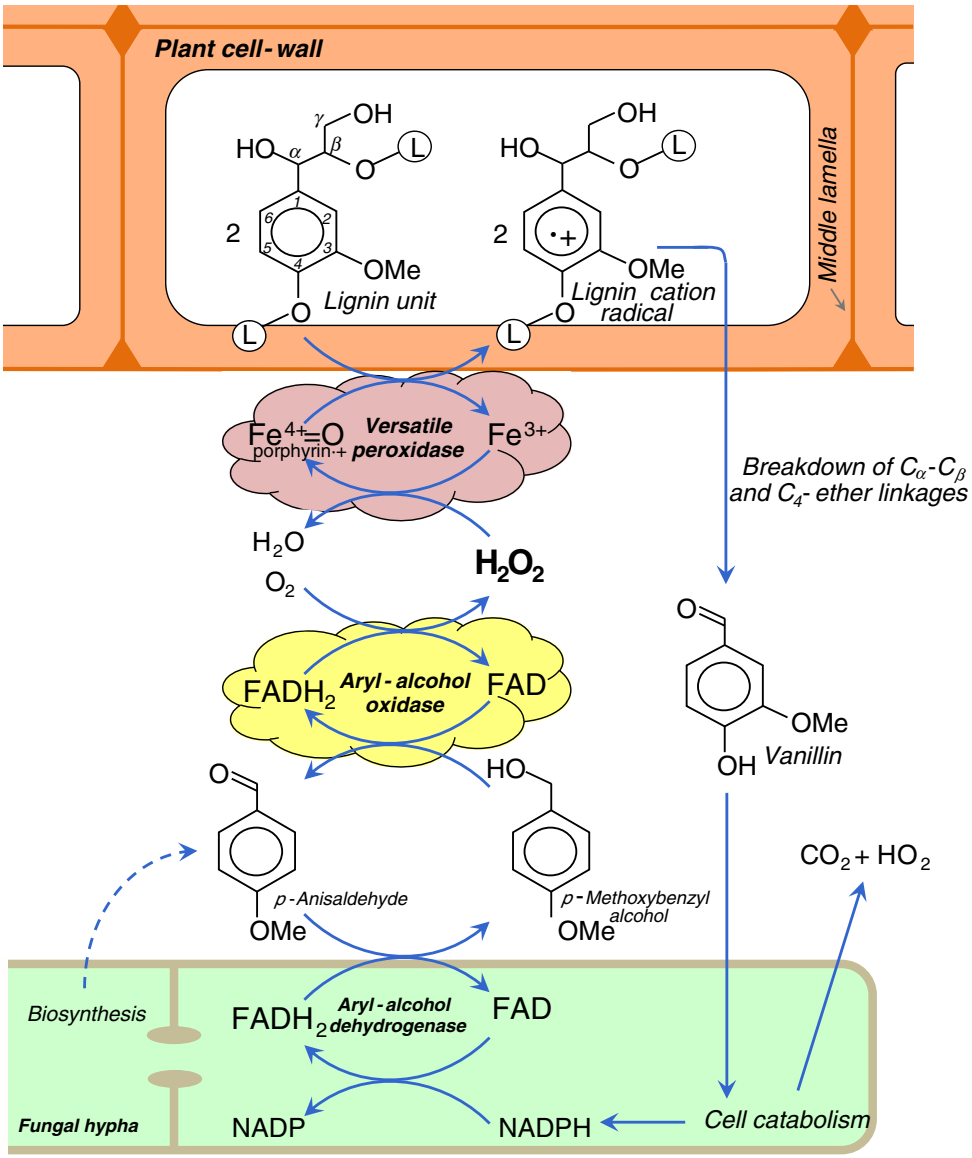

Fig. 2 Scheme for the enzymatic degradation of lignin by *Pleurotus* species. Versatile peroxidase attacks cell-wall lignin substructures (*L-containing circles* represent the remaining lignin polymer, which is guaiacyl-type in the scheme) with participation of extracellular AAO generating H_2O_2 during redox cycling of aromatic aldehydes also involving intracellular aryl-alcohol dehydrogenase (the stoichiometry of the reaction is one molecule of NADPH reduces one molecule of *p*-anisaldehyde, that generates one molecule of peroxide, and finally oxidizes two lignin subunits). See text for details. Adapted from Ruiz-Dueñas et al. (2009)

suggested to be implicated in binding of the FAD adenine moiety; (2) the GMC oxidoreductase pattern-1 (PROSITE PS00623), involved in both stabilization of the FAD-covering lid and formation of the FAD-attachment loop; and (3) the C-terminal region, including the substrate-binding domain and the catalytic region, the most variable in both sequence and number of elements.

For many years, the only AAO sequences available were those from *P. eryngii* and *Pleurotus pulmonarius* reported by Varela et al. (1999, 2000a) showing ~33% amino acid identity with *A. niger* glucose oxidase (Varela et al. 2000b). With the recent interest on white-rot fungi in lignocellulose biorefineries, an increasing number of basidiomycetes genomes are being sequenced at the DOE Joint Genome Institute (http://www.jgi.doe.gov) including around 50 sequences annotated as putative AAO. Those from the genomes of *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *Fomitiporia mediterranea*, *Fomitopsis pinicola*, *Gloeophyllum trabeum*, *P. chrysosporium*, *P. ostreatus*, *P. placenta*, *Punctularia strigosozonata*, *Stereum hirsutum*, and *Trametes versicolor* are included in the dendrogram shown in Fig. 3, after manual curation by the authors, together with the two *Pleurotus* AAO cited above, and the *B. adusta* AAO (Romero et al. 2010). The dendrogram also includes the sequences from all the other members of the GMC superfamily currently available from basidiomycetes (up to a total of 112 sequences from the above species, plus *Agaricus xanthodermus*, *Athelia rolfsii*, *Auricularia delicata*, *Coniophora puteana*, *Dacryopinax* sp., *Grifola frondosa*, *Irpex lacteus*, *Leucoagaricus meleagris*, *Lyophyllum shimeji*, *Myriococcum thermophilum*, *Pycnoporus cinnabarinus*, *Phlebiopsis gigantea*, *Schizophyllum commune*,

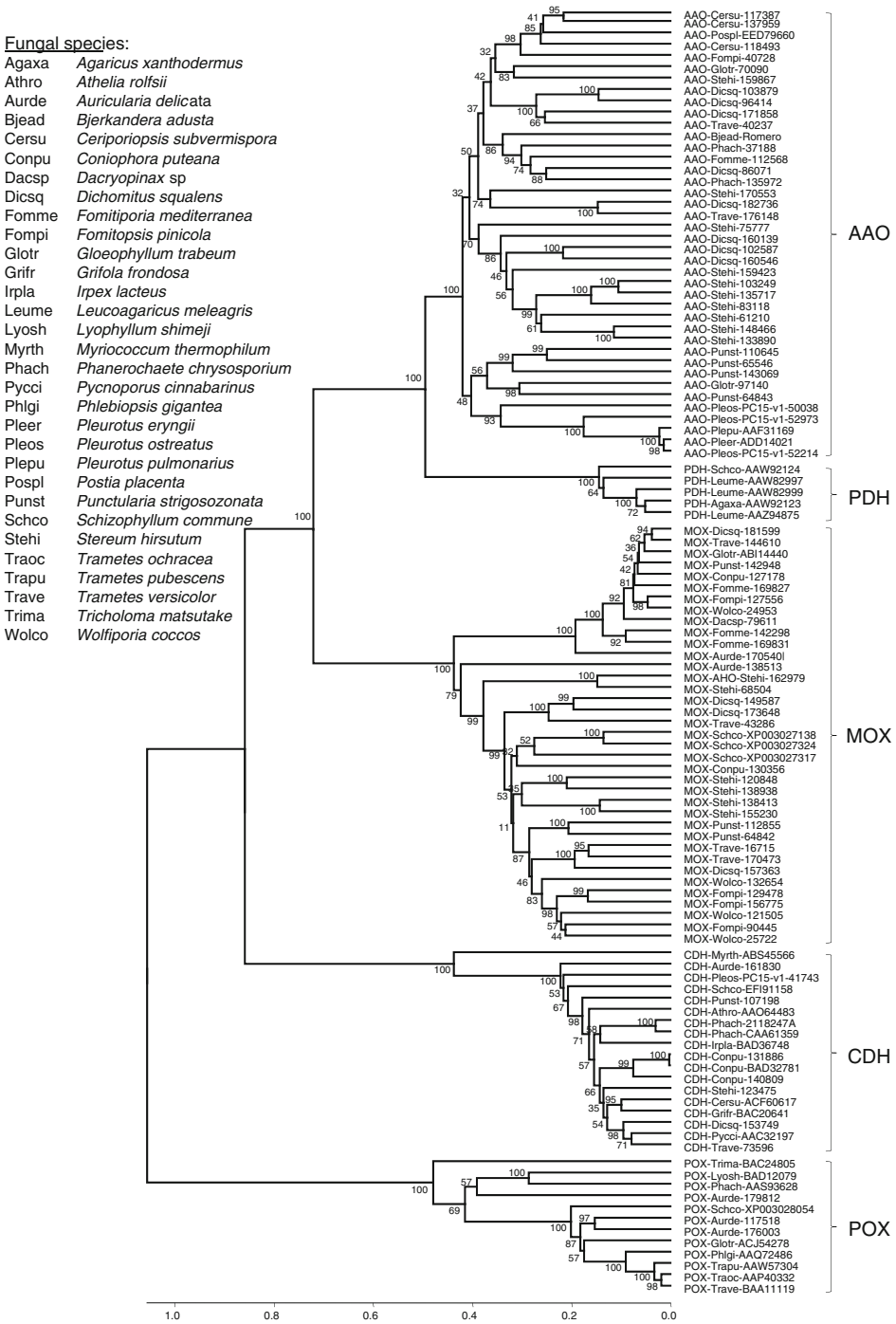

Fig. 3 Comparison of 112 GMC oxidoreductases from basidiomycetes (31 species) including AAO (40 sequences), pyranose dehydrogenase (*PDH*; 5), methanol oxidase (*MOX*; 37), cellobiose dehydrogenase flavin domain (*CDH*; 18), and pyranose oxidase (*POX*; 12) sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the already released JGI (http://www.jgi.doe.gov) genomes (of *C. subvermispora*, *D. squalens*, *F. mediterranea*, *F. pinicola*, *G. trabeum*, *P. chrysosporium*, *P. ostreatus*, *P. placenta*, *P. strigosozonata*, *S. hirsutum*, and *T. versicolor*). The dendrogram was obtained with MEGA5 (Tamura et al. 2011) using Poisson distances and UPGMA clustering (pairwise deletion). *Numbers* on the *branches* represent bootstrap values after 1,000 replications

Trametes ochracea, *Trametes pubescens*, *Tricholoma matsutake*, and *Wolfiporia coccos*).

Five clusters corresponding to the different GMC types found in basidiomycetes—namely AAO, cellobiose dehydrogenase (EC 1.1.99.18), methanol oxidase, pyranose dehydrogenase (EC 1.1.99.29), and pyranose oxidase—are clearly delimited in the dendrogram. The AAO and methanol oxidase clusters are the largest ones, while only a small number of basidiomycete pyranose dehydrogenase sequences (from four different species) are available. Interestingly, the above clusters were fully homogeneous in the sense that each of them exclusively included sequences from one GMC type, revealing phylogenetic distance and clear delimitation between the different enzyme types in this superfamily. In the AAO cluster, a first subcluster including the *Pleurotus* sequences from one side and the *P. strigosozonata* sequences (together with one *G. trabeum* sequence) from the other side is observed, together with a second large subcluster including AAO sequences from a variety of basidiomycete species. In agreement with other phylogenetic

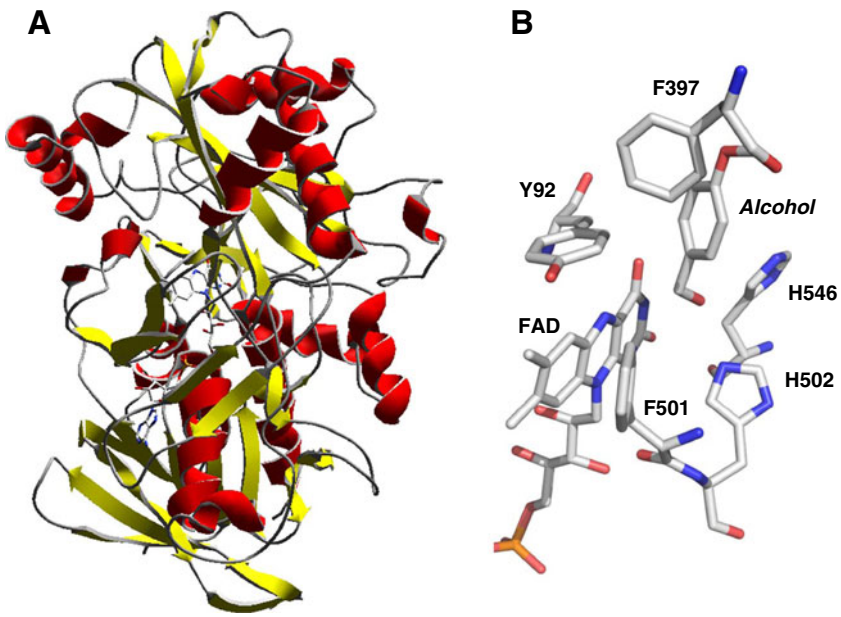

Fig. 4 AAO molecular structure. **a** Secondary structure represented as ribbon diagram, α-helixes in *red* and β-sheets in *yellow*, with the FAD cofactor represented as Corey–Pauling–Koltun (CPK) colored sticks, as shown in crystal structure (PDB entry 3FIM). **b** Active-site detail showing five residues (Tyr92, Phe397, Phe501, His502, and His546), the FAD cofactor flavin ring, and *p*-methoxybenzyl alcohol substrate docked by PELE as described by Hernández-Ortega et al. (2011a) (CPK colors)

analyses of GMC oxidoreductases (Kittl et al. 2008; Zámocký et al. 2004), basidiomycete pyranose dehydrogenases were the closest to the AAO cluster, and methanol oxidases were the first joining the two above clusters followed by cellobiose dehydrogenases (flavin domain), and pyranose oxidases formed the more distant cluster. Three well-known GMC types, namely glucose oxidases, cholesterol oxidases (EC 1.1.3.6), and choline oxidases (EC 1.1.3.17), are not included in the present comparison since they have been only rarely reported from basidiomycetes (the two latter being predominantly reported from bacteria).

General aspects of AAO structure and function compared to other GMC flavoproteins

The recently solved crystal structure (Fernández et al. 2009) of the *P. eryngii* AAO expressed in *E. coli* (Ruiz-Dueñas et al. 2006) shows the highest similarity to the crystal structures of choline oxidase from *Arthrobacter globiformis* (Quaye et al. 2008) and *A. niger* glucose oxidase (Hecht et al. 1993), the first GMC oxidoreductase whose structure was solved. Low structural similarities were found with other GMC oxidoreductases, as white-rot fungal pyranose oxidase (Bannwarth et al. 2004) and flavin domain of cellobiose dehydrogenase (Yoshida et al. 2001), and bacterial cholesterol oxidase (Lario et al. 2003). Based on overall topology and function, two different domains could be defined in the AAO structure: FAD-binding domain and substrate-binding domain (Fig. 4a). The FAD-binding domain is a novel variation of glutathione–disulfide reductase (EC 1.8.1.7) fold (Dym and Eisenberg 2001; Fernández et al. 2009). The FAD molecule interacts non-covalently with the protein through a network of hydrogen bonds involving the main chain NH and CO groups of residues located at the N terminus. Additionally, the N-terminal region structure, which primary sequence corresponds to a conserved GMC signature, is adopting the βαβ fold in which the most important residues involved in cofactor stabilization are located. On the other hand, AAO crystal structure revealed a funnel-shaped channel that connects the solvent with the flavin cofactor, in contrast to that observed in glucose oxidase (Hecht et al. 1993) and pyranose oxidase (Bannwarth et al. 2004), where the active site of the monomeric form is solvent exposed. Substrate diffusion in AAO is limited by three aromatic residues—Tyr92, Phe397, and Phe501 (Fig. 4b)—that form a bottleneck limiting the free access to the active-site cavity (Fernández et al. 2009). Using the PELE software for prediction of ligand diffusion in proteins (Borrelli et al. 2005), it was shown that these residues establish interactions with the alcohol substrate during its access to the buried AAO active site (Hernández-Ortega et al. 2011a), where it adopts a final position with the α-carbon near the FAD flavin ring and the side chains of His502 and His546 (Fig. 4b).

Crystal structures of AAO, glucose oxidase (Wohlfahrt et al. 1999), cholesterol oxidase (Lario et al. 2003), choline oxidase (Quaye et al. 2008), and the flavin domain of cellobiose dehydrogenase (Hallberg et al. 2002) share a highly conserved catalytic site, suggesting a similar oxidation mechanism (Fig. 5a, b, d–f). Hydroxynitrile lyase (EC 4.1.2.10), an enzyme with fully different activity, also shares the general folding and flavin pocket architecture with AAO (Fig. 5c) although the latter is not involved in catalysis (Dreveny et al. 2009). The oxidation mechanism proposed for GMC oxidoreductases is a hydride transfer to flavin assisted by a catalytic base that activates the alcohol substrate. The nature of the catalytic base that abstracts the hydroxyl proton from the substrate has been largely

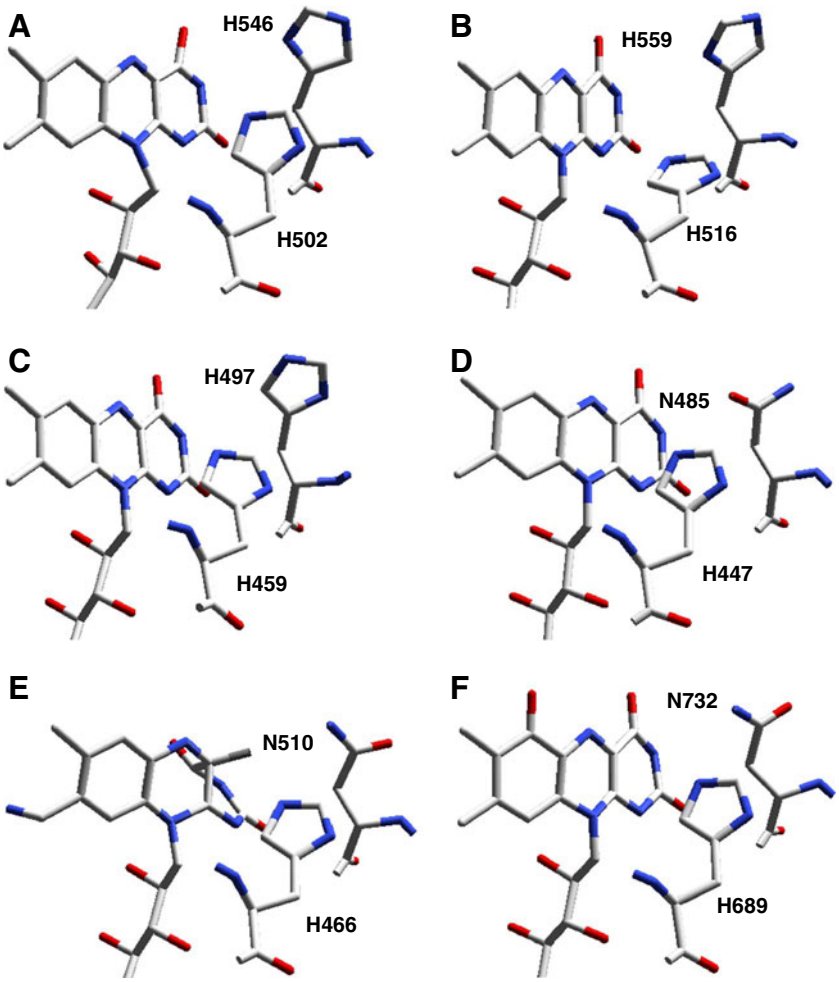

Fig. 5 Conserved active-site residues among GMC superfamily members: *P. eryngii* AAO (**a**), *A. niger* glucose oxidase (**b**), *Prunus amigdalus* hydroxynitrile lyase (**c**), *Brevibacterium sterolicum* cholesterol oxidase (**d**), *A. globiformis* choline oxidase (**e**), and *P. chrysosporium* cellobiose dehydrogenase (**f**). From PDB entries 3FIM, 1CF3, 1JU2 1COY, 2JBV, and 1KDG, respectively

discussed in these GMC oxidoreductases. As described in more detail in the next section, two different residues were investigated as catalytic bases in AAO: (1) His502, fully conserved among GMC superfamily proteins; and (2) His546, that is spatially conserved in AAO and glucose oxidase (as well as in hydroxynitrile lyase) while it corresponds to an asparagine residue in choline oxidase, cholesterol oxidase, and cellobiose dehydrogenase (Fig. 5). Different site-directed mutagenesis studies have shown that both residues are involved in catalysis and/or substrate binding in AAO (Ferreira et al. 2006; Hernández-Ortega et al. 2011a), glucose oxidase (Witt et al. 2000), choline oxidase (Ghanem and Gadda 2005; Rungsrisuriyachai and Gadda 2008), cellobiose dehydrogenase (Rotsaert et al. 2003), and cholesterol oxidase (Yue et al. 1999). The fully conserved histidine was proposed as catalytic base in *A. niger* glucose oxidase (Wohlfahrt et al. 2004), cellobiose dehydrogenase (Rotsaert et al. 2003), and pyranose oxidase (Wongnate et al. 2011). However, a cholesterol oxidase crystal structure at atomic resolution showed that the homologous histidine (His447) is protonated, suggesting a different role (maybe acting as a hydrogen bond donor that would assist during catalysis) (Lyubimov et al. 2006). Additionally, other studies challenged the nature of His466 as catalytic base in choline oxidase (Ghanem and Gadda 2005). The role of these conserved histidine residues in GMC catalysis is discussed below based on AAO recent results.

Recent answers to some old questions on AAO catalysis

Recent studies on *P. eryngii* AAO structure–function relationships and catalytic properties (by a combination of kinetic, isotope labeling, crystallographic, directed mutagenesis, chromatographic, spectroscopic, and computational techniques) have contributed to solve some questions on the catalytic mechanisms of AAO that remained unsolved for years, such as (1) the final nature of the catalytic base contributing to alcohol substrate oxidation and (2) the apparently inverted substrate specificity of AAO when oxidizing substituted aromatic aldehydes, with respect to aromatic alcohols.

Nature and role of the postulated catalytic base in AAO (and other GMC oxidoreductases) AAO crystal structure (Fernández et al. 2009) suggested catalytic features in common with glucose oxidase. Two possible mechanisms for

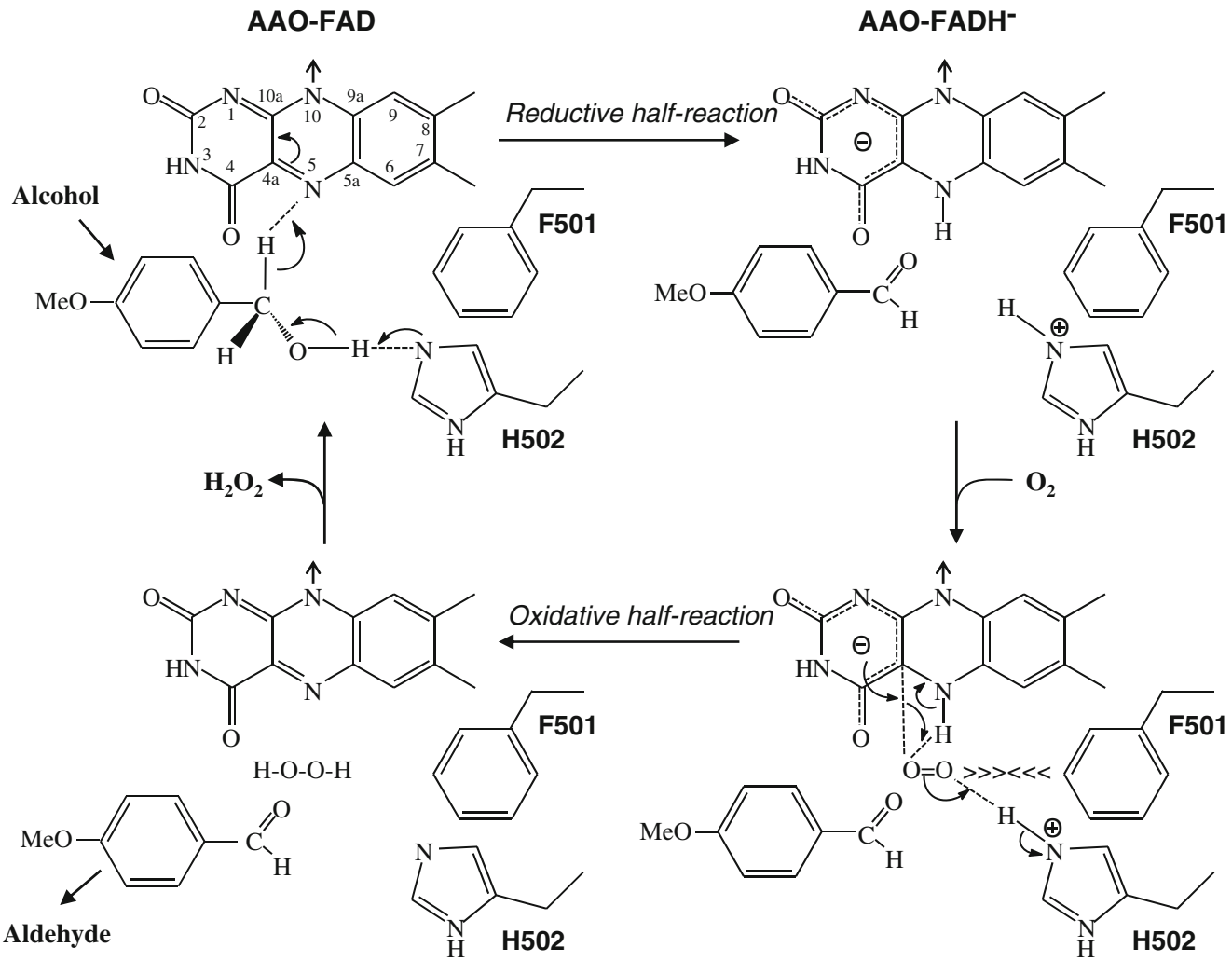

Fig. 6 Scheme for AAO catalytic cycle including two half-reactions. In the reductive half-reaction (*top*), the alcohol substrate (here *p*-methoxybenzyl alcohol) is two-electron oxidized by the flavin N5, which simultaneously receives one of the alcohol α-hydrogens, in a hydride transfer reaction concerted with proton transfer to His502 acting as a base (Hernández-Ortega et al. 2011a) that yields the aldehyde product and the reduced flavin. In the oxidative half-reaction (*bottom*), the O_2 substrate is two-electron reduced by the flavin (C4a) with contributions of Phe501, which forces O_2 to approach the flavin C4a (Hernández-Ortega et al. 2011d), and His502 (the nature of the catalytic acid has not been determined although this role is assigned here to the protonated histidine) yielding hydrogen peroxide and the reoxidized flavin. Adapted from Hernández-Ortega et al. (2011d)

glucose oxidation by the enzyme have been suggested: (1) direct hydride transfer from glucose C1 to flavin, aided by the removal of the C1-hydroxyl proton by a basic group on the enzyme (Bright and Appleby 1969); and (2) nucleophilic addition by glucose C1 hydroxyl to flavin C4a, followed by proton abstraction from C1 (Weibel and Bright 1971). Both mechanisms are expected to be assisted by a general catalytic base, with His516 or His559 acting as potential proton acceptors (Witt et al. 2000). However, the nucleophilic addition mechanism seemed to be less probable, as the covalent intermediate enzyme–glucose has never been detected (Leskovac et al. 2005).

In a similar way, the AAO catalytic mechanism has been discussed during recent years (Ferreira et al. 2005; 2009; Guillén et al. 1992; Hernández-Ortega et al. 2011a; Varela et al. 2000b). First, sequence alignment and homology modeling (using glucose oxidase crystal structure as template) showed that AAO His502 and His546 correspond to glucose oxidase His516 and His559, respectively (Fig. 5a, b), and could be playing similar roles in both enzymes (Varela et al. 2000b). Later, the AAO mechanism for alcohol oxidation (reductive half-reaction) was established by kinetic studies, including substrate and solvent kinetic isotope effects (KIE), and was defined as a hydride transfer from substrate Cα to flavin N5 concerted with proton abstraction from α-hydroxyl by a catalytic base (Ferreira et al. 2009). This was confirmed by QM/MM studies that did not predict the existence of a reaction intermediate, in agreement with the

experimental results (Hernández-Ortega et al. 2011a). When AAO was compared with choline oxidase, the QM/MM energy profile for the latter enzyme was different, showing a stable intermediate after proton abstraction, in agreement with the two-step process mechanism reported and the lack of solvent KIE during choline oxidation (Fan and Gadda 2005; Gadda 2003).

Simultaneously, directed-mutagenesis and computational studies provided an unambiguous answer on the nature of the catalytic base in AAO (Hernández-Ortega et al. 2011a). His546 plays a role in alcohol binding, while His502 is the catalytic base as revealed by the 3,000-fold and 1,800-fold decreased k_{cat} and k_{red} in the H502A variant, respectively (additionally, His502 participates in alcohol binding as shown by the increased K_m and K_d values in this variant). When the nature of choline oxidase catalytic base was simultaneously investigated by QM/MM, the results obtained pointed to His466 as the base. The AAO His502 and choline oxidase His466 are conserved among different GMC flavoenzymes and occupy structurally homologous positions in front of the flavin *re*-side, as shown by the crystal structures of both enzymes (Fig. 5a and e, respectively). Although the studies from Gadda and co-workers (Ghanem and Gadda 2005; Rungsrisuriyachai and Gadda 2008) have not definitively concluded the nature of the choline oxidase catalytic base, they showed that mutation of His466 caused the main effect on k_{cat}. Therefore, most probably the histidine residue conserved in all GMC oxidoreductases (such as choline oxidase His466 and glucose oxidase His516) is the one acting as the catalytic base in substrate oxidation, as shown by the authors for AAO His502 (Hernández-Ortega et al. 2011a). A scheme of the reductive half-reaction in AAO oxidation of aryl alcohols is shown in Fig. 6 (top) including hydride abstraction by flavin N5 and proton abstraction by His502 acting as the catalytic base. Fig. 6 also shows (bottom) the second (oxidative) half-reaction that closes the AAO catalytic cycle, where O_2 is reduced to H_2O_2 with contributions of Phe501, which forces O_2 to attain the catalytic C4a of flavin (Hernández-Ortega et al. 2011d), and the same His502 involved in the previous half-reaction, which also contributes to the H_2O_2 formation reaction (Hernández-Ortega et al. 2008).

Inverse AAO specificity on substituted aromatic aldehydes (with respect to alcohols) AAO substrate specificity has been extensively studied for the *P. eryngii* enzyme (Ferreira et al. 2005; Guillén et al. 1992). The rates of oxidation of different aromatic alcohol substrates by AAO are compared in Table 2, as percentages of the activity observed with benzyl alcohol. β-Naphthylmethanol is the most readily oxidized substrate, indicating that extension of the aromatic system favors the action of the enzyme. The oxidation rates for benzylic alcohols are strongly affected by the nature, position, and number of the aromatic-ring substituents. In general, electron donor substituents (as methoxy groups) promote alcohol oxidation by AAO, whereas electron-withdrawing substituents (as nitro groups) produce the opposite effect, as expected.

As already reported by Guillén et al. (1992), AAO also shows some activity on aromatic aldehydes, the highest activity (on 4-nitrobenzaldehyde) being ~5% the activity for benzyl alcohol (Table 2). Surprisingly, electron-withdrawing substituents promoted aldehyde oxidation by AAO, while electron donors caused the opposite effect, in contrast to that observed with alcohols. This suggested a reaction mechanism different from that described for alcohol oxidation. However, recent work by Ferreira et al. (2010) showed that the enzyme activity on these aldehydes is in general terms correlated with their hydration degree in water media (forming the corresponding *gem*-diols) as estimated by ^{1}H NMR. This fact, together with the absence at the AAO active site of a residue that could be responsible for aldehyde activation, as reported in aldehyde dehydrogenase where a cysteine residue forms a thiohemiacetal adduct with the substrate (Marchal and Branlant 1999), suggest that water is the activating molecule in aldehyde oxidation by AAO. Oxidation of aldehyde hydrated forms has also been described for *D. melanogaster* alcohol oxidase (Eisses 1989) and bacterial choline oxidase (Fan et al. 2006).

The reactions shown in Fig. 7b are suggested for AAO oxidation of aromatic aldehydes: first the aldehyde would be hydrated to the *gem*-diol form, and then the *gem*-diol would be oxidized by AAO by a mechanism analogous to that described for alcohol oxidation (Fig. 7a). Site-directed mutagenesis and KIE studies in *p*-nitrobenzaldehyde oxidation by AAO supported proton transfer to His502 (the same active-site base activating simple alcohols) being concerted with hydride transfer to the flavin (with similar solvent KIE in both alcohol and aldehyde oxidations) (Ferreira et al. 2010). Therefore, we can conclude that the catalytic mechanism for AAO oxidation of aromatic aldehydes is similar to that operating for the alcohol substrates, but it requires their previous hydration to the *gem*-diol forms. The substrate specificity differences observed (in terms of AAO activity on aldehyde substrates bearing electron donor or withdrawing substituents) are not related to the catalytic oxidation reaction but to the previous hydration step that is favored by the presence of electron-withdrawing substituents promoting incorporation of the water hydroxyl to the aldehyde α-carbon.

Biotechnological interest of AAO: flavor synthesis and enzyme stereoselectivity

Customer demands for products with natural origin have produced a new interest on the biotechnological production

Table 2 AAO activities on different aryl alcohols (left) and the corresponding aldehydes (right) relative to benzyl alcohol[a]

Benzyl alcohol	100	Benzaldehyde	0.86
3-Methoxybenzyl alcohol	100	3-Methoxybenzaldehyde	0.85
4-Methoxybenzyl alcohol	571	4-Methoxybenzaldehyde	0.03
2,4-Dimethoxybenzyl alcohol	178	2,4-Dimethoxybenzaldehyde	nd
Veratryl alcohol	326	Veratraldehyde	0.01
4-Hydroxybenzyl	<5	4-Hydroxybenzaldehyde	nd
4-Hydroxy-3-methoxybenzyl alcohol	<5	4-Hydroxy-3-methoxybenzaldehyde	nd
4-Nitrobenzyl alcohol	9	4-Nitrobenzaldehyde	4.77
β-Naphthylmethanol	746	β-Naphthaldehyde	nd
Cinnamyl alcohol	451	Cinnamaldehyde	0.38
4-Methoxycinnamyl alcohol	<5	4-Methoxycinnamaldehyde	0.01

nd not detected

[a]From Guillén et al. (1992) (estimated as O_2 consumption)

of natural flavors and aromas (Krings and Berger 1998). White-rot fungi are among the most versatile potential aroma producers (Fraatz and Zorn 2011; Lapadatescu et al. 2000). These fungi are able to produce two of the most important aroma chemicals: vanillin and benzaldehyde. Another aromatic flavor, *p*-anisaldehyde, and its halogenated derivatives, are also synthesized by *Pleurotus* and *Bjerkandera* species being involved in H_2O_2 supply (Fig. 2) by the concerted action of AAO and mycelium-associated aromatic dehydrogenases (de Jong et al. 1994a; Guillén and Evans 1994; Gutiérrez et al. 1994).

Vanillin is mainly produced nowadays by chemical synthesis from petrochemical precursors since its production from alkaline depolymerization of lignosulfonates (Wünning 2001) is decreasing due to environmental reasons (resulting in worldwide use of kraft pulping) and natural vanillin from *Vanilla planifolia* only provides a very small percentage of the world consumption. However, environmentally friendly alternatives are available (Priefert et al. 2001) including the possibility to using white-rot organisms or their enzymes. As illustrated in Fig. 2, vanillin is the first product from the enzymatic (Cα–Cβ) breakdown of gymnosperm (guaiacyl-type) lignin substructures by the synergistic action of lignin-degrading (lignin peroxidase and versatile peroxidase) and peroxide-producing (AAO and other oxidases) produced by white-rot fungi (while angiosperm lignin would yield both vanillin and syringaldehyde) (Kirk and Farrell 1987). Several patents have been deposited on these biotransformations (e.g., Gross et al. 1993), although the processes are still to be optimized. Additionally, ferulic acid and specially eugenol, as a lower cost compound, have been suggested as vanillin precursors of renewable origin among others (Priefert et al. 2001). Two different strategies, using modified organisms or enzymes, have been recently proposed to improve the

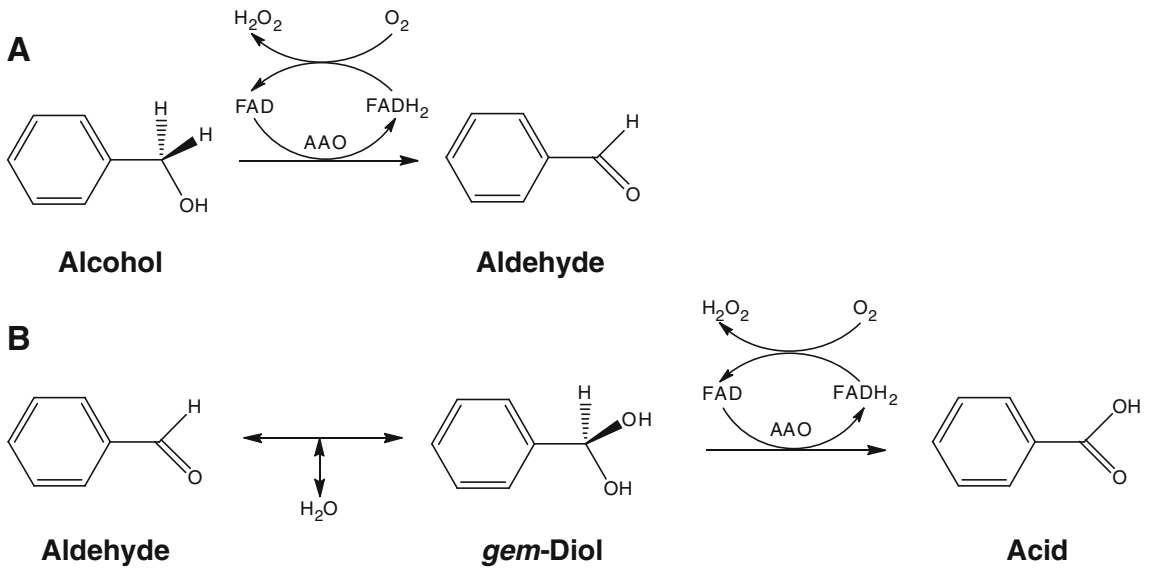

Fig. 7 Scheme for reactions catalyzed by AAO. AAO typically oxidizes aromatic alcohols to the corresponding aldehydes (**a**) but, additionally, it can oxidize aromatic aldehydes to their corresponding acids (**b**). The second reaction occurs via the *gem*-diols formed by aldehyde hydration (Ferreira et al. 2010). After alcohol (or *gem*-diol) oxidation by oxidized FAD, H_2O_2 is generated from O_2 reduction (during FAD regeneration) in both (**a**) and (**b**)

biotechnological production of vanillin. The first strategy involves the genetic modification of *Rhodococcus* strains being able to initiate the eugenol transformation, by introducing genes of ferulic acid metabolism (Plaggenborg et al. 2006) or the *Pseudomonas fluorescens* modification by inactivating the genes involved in vanillin oxidation (Di Gioia et al. 2011). The second strategy consisted on the usage of natural or evolved variants of vanillyl-alcohol oxidase, an enzyme sharing catalytic properties with AAO although belonging to a different family, for creosol or vanillylamine oxidation, as cheap vanillin precursors of petrochemical or renewable origin, respectively (van den Heuvel et al. 2001, 2004). Interestingly, *B. adusta* AAO can oxidize vanillyl alcohol to vanillin as efficiently as vanillyl-alcohol oxidase, although their activities on other substrates are different (Fraaije et al. 1995; Romero et al. 2009). An additional advantage of vanillin from some of the above processes is that it could be considered as a "nearly natural" flavor, obtained from natural raw materials by a biological (instead of chemical) natural process.

Additionally, fungal transformation of L-phenylalanine leads to the above-mentioned benzaldehyde, together with a wide spectrum of phenolic and non-phenolic aryl-metabolites of industrial interest, as shown for the AAO-producing fungus *B. adusta* (Lapadatescu et al. 2000). After *trans*-cinnamic acid formation by L-phenylalanine ammonia-lyase, two metabolic pathways for benzylic compound appear involving (1) α-oxidation in which benzaldehyde and benzyl alcohol are the major benzyl metabolites, and (2) β-oxidation leading to benzoic acid formation. Benzoic acid, benzaldehyde, and benzyl alcohol can be later hydroxylated and methylated to give different methoxybenzyl metabolites whose redox state is affected by the AAO activity level. In the above L-phenylalanine transformations for flavor production, aryl alcohols are often the main aromatic metabolites produced, as reported for *B. adusta* (benzyl alcohol) (Lapadatescu et al. 2000) and *P. chrysosporium* (veratryl alcohol) (Jensen et al. 1994) among other fungi. Therefore, these microbial transformations could be combined with AAO treatment to obtain benzaldehyde or other aromatic aldehydes. As an alternative, the fungal growth conditions can be manipulated to increase the aldehyde levels, as described for *B. adusta* growing on L-phenylalanine in the presence of lecithin that simultaneously promotes the AAO and benzaldehyde production by the fungus (Lapadatescu et al. 1999). Interestingly, the *B. adusta* AAO has activity on (*para*) phenolic and non-phenolic benzyl alcohols (Romero et al. 2009) and it can, therefore, be of interest in the production of both phenolic (e.g., vanillin) and non-phenolic (e.g., benzaldehyde) aromatic aldehydes.

Enantioselective biotransformations, including the use of oxidative enzymes, are being actively investigated for a variety of asymmetric reactions of high commercial interest (e.g., more than half of drug candidates have chiral centers) (Carey et al. 2006; Matsuda et al. 2009). Chiral secondary alcohols (including benzylic alcohols) are widely used as synthetic intermediates, chiral auxiliaries, and analytical reagents. Very recent studies using α-monodeuterated *p*-methoxybenzyl alcohol (*R* and *S* enantiomers) have shown that hydride abstraction from alcohol substrates by AAO (see the AAO oxidation mechanism described above) is stereoselective (Hernández-Ortega et al. 2011b, c). This result is in agreement with the concerted hydride and proton transfer mechanism found in AAO (Ferreira et al. 2009), in contrast with the stepwise process reported for other GMC oxidoreductases (Gadda 2008; Sucharitakul et al. 2010), as well as with the position of *p*-methoxybenzyl alcohol after docking at the active site of the enzyme (Hernández-Ortega et al. 2011a). Asymmetric redox reactions have been reported for other flavoenzymes, such as stereoselective vanillyl-alcohol oxidase (van den Heuvel et al. 2000, 1998), but as far as we know this is the first time that enantioselective alcohol oxidation is reported for a member of the GMC oxidoreductase superfamily.

Interestingly, very recent studies (Hernández-Ortega et al. 2011b, c) have also shown that the AAO stereoselectivity in hydride abstraction from primary (non-chiral) alcohol substrates is maintained when secondary (chiral) aromatic alcohols are used, although the AAO activity on these substrates is several orders of magnitude lower. AAO crystal structure after molecular docking with *p*-methoxybenzyl alcohol showed why AAO is unable to bind secondary alcohols efficiently. The small space available at the bottom part of the active site cavity is restricting large substituents at the Cα position. Therefore, for better accommodating a secondary alcohol, the active site should be enlarged. In this way, engineered AAO could be used in the future for deracemization of secondary alcohols mixtures, enabling purification of the enantiomer that is not oxidized by the enzyme. Stereoselective galactose oxidase (Minasian et al. 2004) has also been studied for chiral alcohol deracemization and, after several rounds of directed evolution, an improved variant was obtained (Escalettes and Turner 2008). AAO is highly stereoselective on primary alcohols; therefore, its eventual use for enzymatic deracemization would not require the introduction of stereoselectivity, as often intended engineering other industrial biocatalysts, but to extend its activity to secondary alcohols by rational design using site-directed mutagenesis, without modifying stereoselectivity.

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