Aromatic stacking interactions govern catalysis in aryl-alcohol oxidase

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Short title

Substrate stacking interactions in aryl-alcohol oxidase

Keywords

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Abbreviations

AAO, aryl-alcohol oxidase; AAO_{ox}, oxidized AAO; AAO_{red}, reduced AAO; CTC, charge-transfer complex; KIE, kinetic isotope effect; ^D(^{app}*k*_{cat}), KIE for apparent *k*_{cat} estimated under O₂-saturation conditions; ^D(^{app}*K*_{m(Al)}), KIE for apparent *K*_{m(Al)} estimated under O₂-saturation conditions; GMC, glucose-methanol-choline oxidase (oxidoreductase superfamily); *k*_{cat}, catalytic constant (turnover number); *K*_d, dissociation constant; *K*_{m(Al)}, Michaelis constant for alcohol substrate; *K*_{m(Ox)}, Michaelis constant for O₂ substrate; ^{app}*k*_{ox}, apparent reoxidation constant; *k*_{red}, reduction constant; *k*₁ to *k*₇, rates for AAO catalytic cycles/reactions; MM, molecular mechanics; Alc, alcohol; Ald, aldehyde; QM, quantum mechanics

Abstract

Aryl-alcohol oxidase (AAO) generates H₂O₂ for lignin degradation at expenses of benzylic (and other π -system-containing) primary alcohols, which are oxidized to the corresponding aldehydes. Ligand diffusion on *Pleurotus eryngii* AAO showed a T-shaped stacking interaction between Tyr92 side-chain and the alcohol substrate at the catalytically-competent

position for concerted hydride/proton transfer. Bi-substrate kinetics revealed that reactions with 3-chloro- or 3-fluorobenzyl alcohols (halogen substituents) proceed *via* a ping-pong mechanism. However, mono- and dimethoxylated substituents (in 4-methoxybenzyl and 3,4-dimethoxybenzyl alcohols) switched the mechanism and a ternary complex is formed. This correlated with fully-oxidized AAO during turnover with the above halogenated alcohols, while a reduced fraction (up to ~40%) was found for other substrates. Tyr92 contribution to AAO reaction mechanism was investigated by calculation of stacking interaction energies and site-directed mutagenesis. Electron-withdrawing substituents resulted in lower QM stacking-energies between aldehyde and tyrosine side-chain, which would contribute to product release, in agreement with the ping-pong mechanism observed in 3-chloro- and 3-fluorobenzyl alcohol kinetics. In contrast, the higher stacking-energies when electron-donor substituents are present would result in O₂ reaction with the flavin through a ternary complex, in agreement with the kinetics of methoxylated alcohols. Tyr92 replacement by phenylalanine does not alter the AAO kinetic constants (on 4-methoxybenzyl alcohol), most probably because the stacking interaction is still possible. However, introduction of a tryptophan residue at this position strongly hinders the affinity for the substrate (pre-steady state K_d and steady-state K_m increase by 150-fold and 75-fold, respectively) and, therefore the steady-state catalytic efficiency, suggesting that proper stacking is impossible with this bulky residue. The above results confirm the role of Tyr92 in substrate binding, governing the kinetic mechanism in AAO.

Introduction

Aryl-alcohol oxidases (AAO, EC 1.1.3.7) are extracellular flavoproteins that typically catalyze the oxidative dehydrogenation of polyunsaturated alcohols using molecular oxygen as final electron acceptor and producing hydrogen peroxide [1]. This activity was first reported in *Trametes versicolor* [2] and has been later described in other white-rot basidiomycetes, mainly responsible for lignin degradation in nature, such as *Pleurotus eryngii*, *Bjerkandera adusta* and *Phanerochaete chrysosporium* [3-7]. Lignin removal is a rate-limiting step for carbon recycling in land ecosystems, playing also a central role in paper pulp manufacture and in the production of chemicals and biofuels from renewable lignocellulosic biomass [8]. The physiological role of AAO in wood-rotting basidiomycetes is to provide a continuous supply of extracellular H₂O₂, required as substrate for ligninolytic peroxidases (in white-rot fungi) and as precursor of hydroxyl radical depolymerizing plant polysaccharides (in brown-rot fungi) [1, 9].

AAO from *Pleurotus eryngii* has been intensively investigated [10-14]. This enzyme contains one molecule of non-covalently bound FAD which acts as two-electron acceptor during the oxidation of a wide range of benzylic and other aromatic and aliphatic polyunsaturated primary alcohols (reductive half-reaction). The oxidative half-reaction, where the FAD hydroquinone is oxidized by O₂, closes the catalytic cycle [13-15]. AAO also oxidizes some aromatic aldehydes *via* their hydrated (*gem*-diol) forms, suggesting similar mechanisms for alcohol and aldehyde oxidation [16].

The AAO crystal structure (PDB entry 3FIM) confirmed that it shares similar fold topology with other members of the glucose-methanol-choline oxidase (GMC) oxidoreductase superfamily [17], and the role in catalysis of two conserved active-site residues (His502 and His546 in *Pleurotus eryngii* AAO) was postulated [18]. Reducing substrate diffusion simulations towards AAO active site, previously identified in the crystal structure, suggested an entrance channel next to the Gln395-Thr406 loop [19]. These studies additionally suggested that the enzyme-substrate complex formation, on its catalytically competent form, requires significant displacements of side-chains in the active site environment (including Phe397), as well as the substrate π - π stacking interaction with Tyr92

(**Fig. 1**) [19]. On the contrary, the oxidative substrate migration did not require the above structure reorganization, despite Phe501 was shown to be essential for correct O₂ positioning [13].

A hydride transfer reaction assisted by a conserved active site base is the consensus catalytic mechanism in the GMC superfamily [20-26]. An initial mechanistic study on AAO, including analysis of substrate and solvent kinetic isotope effects (KIE), suggested a concerted proton abstraction from the alcohol hydroxyl and hydride transfer from the alcohol α -carbon to FAD, without formation of a stable alkoxide intermediate [14]. Later research combining site-directed mutagenesis and quantum mechanics/molecular mechanics (QM/MM) calculations, confirmed the role of His502 as AAO catalytic base [19]. Moreover, the combination of QM/MM profiles and solvent KIE data showed that the O-H bond cleavage for proton abstraction from the alcohol hydroxyl precedes the C α -H bond cleavage for hydride transfer, although both processes are highly coupled. These observations revealed a non-synchronous concerted mechanism for alcohol oxidation by AAO [19]. This finding contrasts with the non-concerted mechanism previously reported for choline and methanol oxidases, which include a stable alkoxide intermediate, and provides an alternative mechanism for alcohol oxidation in the GMC superfamily [27, 28].

In the present study, different aromatic alcohol substrates have been used to complete the description of the AAO overall catalytic cycle and to evaluate the influence of the aromatic-stacking interactions on catalysis. This later aim has also been addressed by using some mutants at position Tyr92 to investigate its contribution to the AAO reaction mechanism.

Results

Influence of alcohol type on AAO kinetic mechanism: Steady-state results

Double reciprocal plots of the initial rates of AAO reaction with different types of substituted benzyl alcohol substrates at different O₂ concentrations yielded two different kinetic patterns. For 3,4-dimethoxybenzyl and 3-chloro-4-methoxybenzyl alcohols, plots were linear and intersected to the left of the Y-axis (below zero with respect to X-axis) (**Fig. 2A**), as previously reported for 4-methoxybenzyl alcohol and 2-4-hexadien-1-ol [14]. This is indicative of a sequential reaction mechanism involving a ternary complex between AAO and its reducing/oxidizing substrates (**Scheme 1**, top). On the contrary, a parallel line pattern was obtained for the two other substrates investigated, 3-chlorobenzyl and 3-fluorobenzyl alcohols (**Fig. 2B**), indicating a ping-pong mechanism (**Scheme 1**, bottom).

The steady-state kinetic parameters obtained by fitting the experimental data of bi-substrate kinetics to either Eq. 1 or Eq. 2 (describing ternary or ping-pong mechanisms, respectively; see Materials and Methods), are summarized in **Table 1**. Turnover numbers (k_{cat}) calculated under substrate (alcohol and O₂) saturation conditions showed a similar dependence on the electronic nature of the substituents on the benzenic ring to those previously reported under air atmosphere [15]. The highest (129 s⁻¹ for 4-methoxybenzyl alcohol) and lowest (13 s⁻¹ for 3-fluorobenzyl alcohol) k_{cat} values were observed in the presence of electron donor and withdrawing substituents, respectively. However, the type of alcohol substrate also had a marked influence on $K_{\text{m(O}_2\text{)}}$, therefore on the AAO affinity for O₂. In this way, the enzyme exhibits a considerably higher affinity for O₂ when the reaction takes place with alcohols showing a ping-pong mechanism in comparison with those forming a ternary complex: $K_{\text{m(O}_2\text{)}}$ for 3-chlorobenzyl and 3-fluorobenzyl alcohols are up 35 times lower than for 4-methoxybenzyl alcohol. As a consequence, the catalytic efficiency for O₂, $k_{\text{cat}}/K_{\text{m(O}_2\text{)}}$, is 3 times higher in the presence of alcohol substrates leading to a ping-pong

mechanism. Nevertheless, the catalytic efficiency regarding the alcohol substrate, $k_{\text{cat}}/K_{\text{m(Al)}}$, is considerably decreased in the ping-pong mechanism due to both a reduction in k_{cat} and an increase in $K_{\text{m(Al)}}$ values (**Table 1**).

AAO redox state during turnover

To further investigate the rate-limiting step during oxidation of the different alcohol substrates, we analyzed the redox state of the FAD cofactor during the steady-state turnover of AAO. **Fig. 3** compares the time course of absorbance changes at the 462 nm maximum during AAO reaction after mixing with an equal volume of each alcohol at saturation concentration under air atmosphere conditions in the stopped-flow equipment. The flavin redox state during the initial steady-state phase, whose time duration varies with the different alcohol substrates, shows the relative rates of AAO reduction (by each alcohol substrate) and its oxidation (by O_2). For all the assayed substrates AAO is predominantly in the oxidized form during turnover, and the reduced fraction percentage depends on the alcohol type. For 3-chloro-4-methoxybenzyl, 3-chlorobenzyl and 3-fluoro-benzyl alcohols, the AAO oxidized form is highly predominant (100-95 %) at the onset of the steady state, indicating that the oxidative-half reaction is much faster than the reductive one. Similar results have been previously observed for 4-methoxybenzyl alcohol (~80 % of oxidized form) [14]. By contrast, when 3,4-dimethoxybenzyl alcohol was assayed, the percentage of reduced AAO during turnover increased, up to ~40%, indicating that the rates for the reductive and oxidative half-reactions are in this case almost balanced, in agreement with the similar values observed for alcohol and O_2 catalytic efficiencies (**Table 1**).

Regarding the spectral changes during AAO turnover, the first spectra obtained after mixing showed a displacement of the main flavin band (462 nm) and an absorbance increase, reflecting the formation of the oxidized enzyme complex with the alcohol substrate ($\text{AAO}_{\text{ox}}\text{-Alc}$). After this initial kinetic event, the results with 3-chloro-4-methoxybenzyl, 4-methoxybenzyl and 3,4-dimethoxybenzyl alcohols best fitted a two-step process (**Fig. 4A**). The first step observed reflects the flavin reduction with concomitant formation of a charge-transfer complex (CTC) between the reduced enzyme and the aldehyde product of the reaction ($\text{AAO}_{\text{red}}\text{-Ald}$), characterized by a broad band centered at ~550-650 nm. The second observed step involves the spectral perturbation in the initially formed $\text{AAO}_{\text{red}}\text{-Ald}$ complex with the appearance of a new peak at 490 nm. These events (CTC formation and spectral perturbations) can be related with the presence of O_2 , since AAO anaerobic reduction by these substrates prevents its formation, as described below (**Fig. 5**). However, a one-step process is observed with 3-chlorobenzyl and 3-fluorobenzyl alcohols, leading directly from oxidized to fully reduced flavin without stabilizations of any CTC intermediate (**Fig. 4B**). These turnover results using different substrates, together with the obtained steady-state kinetic constants, provide valuable mechanistic information, which can be correlated with the pre-steady-state studies as described below.

Influence of alcohol type on AAO reductive and oxidative half-reactions

The time course of AAO reduction by the five alcohol substrates studied was monitored using a stopped-flow spectrophotometer in the 350-900 nm range under anaerobic conditions. All assayed substrates fully reduced AAO without appearance of any FAD semiquinone intermediate (**Fig. 5**), consistent with a hydride transfer mechanism as previously reported for 4-methoxybenzyl alcohol and 2,4-hexadien-1-ol [14].

For 3,4-dimethoxybenzyl and 3-chloro-4-methoxybenzyl alcohols the evolution of spectral changes upon AAO reduction was consistent with a two-steps kinetic model ($A \rightarrow B \rightarrow C$) (**Fig. 5A**), as previously reported for 4-methoxybenzyl alcohol [14]. The first process ($A \rightarrow B$) was fast and hyperbolically dependent on substrate concentration, accounting more than the 80%

of the reaction amplitude. This step is assigned to substrate oxidation and formation of an AAO_{red}-P complex. The second process ($B \rightarrow C$) was concentration-independent and too slow ($3\text{--}5\text{ s}^{-1}$) to be relevant for overall turnover (**Table 1**). This step might be related to a slow release of the aldehyde product from the reduced AAO active site in the absence of O₂ (k_3 in **Scheme 1**). Formation of such unstable enzyme-product complexes have also been reported in other flavin-dependent oxidases showing a biphasic reaction course [29, 30]. The observed reduction rates (k_{obs}) exhibit a hyperbolic substrate dependence that upon fitting to Eq. 3 or Eq. 4 (see Materials and Methods) are consistent with an essentially irreversible flavin reduction ($k_{\text{rev}} \sim 0$). The calculated reduction constants (k_{red}) for the AAO reduction by 3,4-dimethoxybenzyl and 3-chloro-4-methoxybenzyl alcohols were up to 3-fold faster than their turnover rates, suggesting that the reductive half-reaction is not the limiting step when these alcohols are oxidized.

On the contrary, a one-step model ($A \rightarrow B$) was the most satisfactory to describe the irreversible AAO reduction by 3-chlorobenzyl and 3-fluorobenzyl alcohols after the initial formation of the AAO_{ox}-Alc complex (**Fig. 5B**). The k_{red} values for these two alcohols (8 ± 1 and $6 \pm 1\text{ s}^{-1}$, respectively) were similar to the above reported turnover rates (**Table 1**). Clearly indicating that for these substrates the reductive half-reaction is the limiting step in catalysis.

Similary AAO oxidative half-reaction was studied in this case by following the flavin absorbance increase at 462 nm after mixing the reduced enzyme with buffer containing known O₂ concentrations. Independently of the method used to reduce AAO, the rates of flavin reoxidation were linearly dependent on O₂ concentration (**Fig. S1**). When AAO was photo-reduced, the determined second-order rate constant ($^{\text{app}}k_{\text{ox}}$ of $1.9 \times 10^4\text{ M}^{-1}\text{ s}^{-1}$) was much higher than the expected by reoxidation of free reduced flavins but clearly inconsistent with the AAO turnover rates. Therefore, we studied the AAO reoxidation reaction after its anaerobic reduction *via* incubation with a slight excess of different alcohol substrates. For all reducing substrates, the rates of flavin reoxidation were in the range of those reported for others oxidases. The values for second-order rate constants obtained were 7.0×10^5 , 8.0×10^5 and $8.4 \times 10^5\text{ M}^{-1}\text{ s}^{-1}$ for 3-chlorobenzyl, 3-chloro-4-methoxybenzyl and 3,4-dimethoxybenzyl alcohol, respectively, similar to those previously reported for 4-methoxybenzyl alcohol ($6.7 \times 10^5\text{ M}^{-1}\text{ s}^{-1}$) [13]. Therefore rates of flavin reoxidation are neither dependent on the nature of the alcohol substrate or on the kinetic mechanism. However, the experimental conditions used do not guarantee the presence of the aldehyde product at the active site during flavin reoxidation (although a slow dissociation is suggested by previous reductive-half reaction experiments).

Stacking stabilization of the substrate in the AAO active site

AAO oxidizes mainly aromatic and other π -systems containing conjugated primary alcohols. The aromatic nature of the AAO alcohol substrates pointed to the importance of π - π stacking in substrate stabilization by AAO. Ligand migration studies showed a T-shaped interaction between Tyr92 and the alcohol substrate in the catalytically-active poses [19]. Starting from this active complex, the Tyr-substrate binding energy was evaluated using *ab initio* quantum chemistry.

Table 2 shows the T-shaped interaction energies between a tyrosine side-chain and five AAO substrates: 4-methoxybenzyl, 3-chloro-4-methoxybenzyl, 3,4-dimethoxybenzyl, 3-chlorobenzyl and 3-fluorobenzyl. For each substrate we modeled both the alcohol and the aldehyde forms. The first clear result is that all the interaction energies are stabilizing, with similar values to those obtained by others [31]. In all cases the stabilization energy is slightly higher for the alcohol reactants than for the aldehyde products. This difference will facilitate the release of the products. Furthermore, important differences are observed for 3-chlorobenzyl and 3-fluorobenzyl alcohols. First we observe a lower stacking stabilization for

the alcoholic form and, second, a larger decrease in the interaction energy with the aldehyde. The significant lower stabilization for the aldehyde product for these two substrates, also establishes a direct correlation between lower π - π stacking stabilization energies and the ping-pong mechanism.

Kinetic properties for Tyr92 variants

Several site-directed variants of AAO at Tyr92 were prepared to investigate its role on catalysis. The Y92L, Y92F and Y92W variants were purified as oxidized holoproteins, showing an A_{280}/A_{463} ratio (~ 10) similar to the native recombinant enzyme. The absorption spectra of Y92L and Y92F were basically similar to that of the native enzyme, with absorption maxima at 387 and 463 nm, while maxima for the Y92W variant were slightly displaced at 384 nm and 457 nm (**Fig. S2**).

The catalytic properties of the Y92L, Y92F and Y92W AAO variants were determined using 4-methoxybenzyl alcohol as substrate (**Table 3**). While mutating the tyrosine to phenylalanine only causes a slight increase in $K_{m(O_2)}$, its replacing with a leucine produces a decrease in catalytic efficiency for the alcohol substrate (2.6-fold lower) accompanied by ~ 2 -fold increases in both $K_{m(Al)}$ and K_d . Finally, the incorporation of a bulkier residue, by the Y92W mutation, causes a strong decrease in catalytic efficiencies for both O_2 (6-fold lower) and, particularly, 4-methoxybenzyl alcohol (860-fold lower). Since for the Y92W variant the turnover rate was 10-fold lowered, we conclude that the main effect of the mutation concerns the availability of the alcohol substrate at the AAO active site (with 75-fold higher K_m values). Likewise, the Y92W mutation prevents CTC formation during the enzyme turnover with 4-methoxybenzyl alcohol, also suggesting that substrate accommodation is affected by this mutation (**Supplemental Fig. S3**).

Fast reaction of the tyrosine variants with 4-methoxybenzyl alcohol was also investigated by anaerobic stopped-flow techniques. The spectral evolution obtained for all of them indicated complete (two electron) enzyme reduction, in agreement with a hydride transfer reaction (**Fig. 6**). The calculated k_{red} and K_d agreed with the steady-state k_{cat} and K_m values (**Table 3**), indicating that the reductive half-reaction is the rate limiting step in catalysis for these variants.

To investigate whether Tyr92, establishing a stacking interaction with the docked 4-methoxybenzyl alcohol (**Fig. 1**), might contribute to the hydride transfer selectivity previously reported for native AAO [19], we measured the KIE values on apparent steady-state kinetic constants for the Y92L variant using three different isotopically-labelled preparations of this alcohol: (*R*)-[α - 2H]-, (*S*)-[α - 2H]- and [α - 2H_2]-4-methoxybenzyl alcohol (**Table 4**). The KIE on the apparent turnover values, $D^{(app)}k_{cat}$, slightly decreased ($\sim 20\%$) for the (*R*) and dideuterated forms, being unaffected for the (*S*) form. However, the KIEs on the apparent catalytic efficiency, $D^{(app)}k_{cat}/K_{m(Al)}$, for the (*R*) and, especially, for the dideuterated alcohols were significantly higher for the Y92L variant and, therefore, the $D(K_{m(Al)})$ values were significantly lower than those of the native AAO. In fact, the small KIE value for $D(K_{m(Al)})$ for native AAO (deuteration mainly affects the $^1H/2H$ abstraction ratio) was absent for the Y92L variant (despite the KIE on turnover was not modified) indicating that Tyr92 contributes to 4-methoxybenzyl alcohol binding.

Discussion

Ping-pong vs ternary mechanism (in AAO and other flavooxidases)

Comparison with other GMC oxidoreductases, which catalyze the oxidation of alcohol groups in relatively specific reactions, AAO reveals a broad electron donor substrate specificity of AAO oxidizing aromatic and other π -system substrates with conjugated primary alcohols

(including benzylic, naphthyl, and aliphatic polyunsaturated alcohols) [5, 15, 32]. These conjugated double bond systems increase the electron availability at the benzylic position enabling hydride abstraction by flavin N5. Moreover, the architecture of the AAO active site prevents the oxidation of secondary alcohols that cannot be accommodated at adequate distances of the catalytic histidine and the above-mentioned flavin N5 atom due to the presence of Phe501 [33].

Here we have performed a detailed study on AAO kinetic mechanism using different alcohol substrates. In all cases the AAO catalytic reaction can be divided into a reductive half-reaction, in which two electrons are transferred via a hydride ion to the oxidized FAD, and an oxidative half-reaction, in which two electrons are transferred from the reduced flavin to O₂ yielding hydrogen peroxide. However, based on the results of bisubstrate kinetics analysis with different benzylic alcohols, the overall AAO catalytic cycle seems to be highly influenced by the chemical nature of the substituents in the substrate benzenic ring. For electron-withdrawing substituents (in 3-chloro- and 3-fluorobenzyl alcohols), both half-reactions are independent, where the aldehyde product dissociation is taken place before the O₂ (the second substrate) reaction, in a ping-pong steady-state kinetic mechanism (**Scheme 1**, bottom). On the contrary, a sequential mechanism operates for alcohols with electron-donor substituents (methoxylated benzyl alcohols) in which O₂ reacts with the AAO_{red}-Ald complex, forming a ternary complex prior to aldehyde product release (**Scheme 1**, top).

Vainillyl alcohol oxidase, another versatile flavoenzyme which is able to oxidize aromatic alcohols as AAO, although belonging to a different superfamily, also exhibits a substrate-dependent overall catalysis [34]. This behavior differs from other fungal GMC oxidoreductases, such as glucose oxidase, pyranose 2-oxidase, cholesterol oxidase and cellobiose dehydrogenase, where ping-pong was reported as the general mechanism [24, 35-37]. Interestingly, detailed investigations on pyranose 2-oxidase recently indicated that its steady-state mechanism switches as a function of pH [38].

Stacking interactions govern AAO catalytic mechanism

Classical force fields semiquantitatively reproduce T-shape stacking dispersion forces [39]. Moreover, high level quantum mechanistic studies pointed out at the importance of T-shaped interactions in molecular stacking, for example in benzene dimer [40]. In AAO, previous ligand migration simulations (using classical force fields) [19] suggested a T-shape substrate-Tyr92 interaction. Our QM calculations (table 2) confirm such stabilizing interactions for the different substrate (Alc)/product (Ald) benzenic rings. In addition, stacking energies estimated for the different substrate/product correlate fairly well with their electron donating/withdrawing properties (to the ring π -cloud) of their different substituents. Methoxybenzene nucleophilicity is stronger than benzene due to resonance, increasing the electronic density in the ring. These effects explain why dimethoxylated alcohols/aldehydes have the largest stacking interaction energies. On the other side, for 3-chloro and 3-fluoro substituted alcohols/aldehydes, the halogen acts as an electron withdrawer (inductive effect), resulting in the lowest stabilization energies. Moreover, the slightly different interaction energies for these two halogenated compounds follow the expected trend for the increased electronegativity of the fluoride compared to chloride and thus, stronger inductive electron withdrawing effect. Thus, the lower interaction energies for the two halogenated compared with the methoxylated aldehydes, together with the higher alcohol-aldehyde differential interaction energies, should contribute to the release of the aldehyde product, favoring the ping-pong mechanism. The stacking interactions were intermediate for the 3-chloro-4-methoxybenzyl alcohol/aldehyde due to the combination of electron donor and withdrawing substituents.

Taking together the kinetic data, the stacking energy calculations and the available structural and computational information reported previously, the overall turnover of AAO is consistent with the proposed kinetic model of **Scheme 1**. Substrate diffusion studies had shown that alcohol and O₂ molecules access the AAO active site through the same hydrophobic channel [13, 19]. The alcohol diffusion pathway requires, however, chain displacements and interaction with residues Phe397, Tyr92 and Phe501 to attain the AAO active site, while O₂ diffusion does not need any rearrangement. This continuous O₂ supply supports the fact that for the sequential mechanism, where the aldehyde product is still bound at the active site upon O₂ arrival, some arrangement of the aldehyde product for flavin reoxidation must be required (for more detail see supplemental movie S1 in [13, 19]). However, the O₂ affinity increases when the reaction takes place with alcohols leading to a ping-pong mechanism. This may be caused by prior dissociation of product aldehyde leaving more space at the active site for proper O₂ binding.

Rate-limiting step/s in AAO catalysis

Rapid kinetic experiments provided separate information on the reductive and oxidative half-reactions for each alcohol substrate. For 3-chlorobenzyl and 3-fluorobenzyl alcohols, the reductive half-reaction is the rate determining step in catalysis and responsible of the low efficiency in substrate oxidation.

However, for 3,4-dimethoxybenzyl and 3-chloro-4-methoxybenzyl alcohols, flavin reduction rate is far from being the limiting state. In the first case, the apparent second-order constant estimated from pre-steady kinetic data ($k_{\text{red}}/K_d=156 \pm 46 \text{ s}^{-1} \text{ mM}^{-1}$) is in agreement with the steady-state alcohol catalytic efficiency obtained ($105 \pm 8 \text{ s}^{-1} \text{ mM}^{-1}$). In the ternary complex mechanism shown in **Scheme 1** (top), k_{cat} is a combination of catalytic steps in which the 3,4-dimethoxybenzyl alcohol is oxidized to 3,4-dimethoxybenzaldehyde ($k_2 \approx k_{\text{red}}$) and the product is released (k_5) ($k_{\text{cat}}=k_2k_5/(k_2+k_5)$). The estimated value for k_5 (101 s^{-1}) would be indicative of a partially rate-limiting step for catalysis, suggesting that other kinetic step (after hydride transfer and before product release) also contributes to limiting the reaction rate. This step could be the flavin reoxidation, as suggested by the 40% AAO reduction during enzyme turnover when this substrate was assayed, showing similar values for alcohol and O₂ catalytic efficiencies. Finally, for 3-chloro-4-methoxybenzyl alcohol oxidation, the estimated k_5 value (42 s^{-1}) suggests that product release could be the rate-limiting step (**Scheme 1**, top). In fact, previous studies on 3-chloro-4-methoxybenzyl alcohol oxidation by AAO indicate that a fraction of the aldehyde formed was oxidized to the corresponding acid, rather than being released from the active site [16].

Regarding the oxidative half-reaction, reoxidation of AAO, previously reduced by different alcohols, yielded bimolecular rate constants (7.0×10^5 – $8.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) in the typical range described for other flavooxidases, ~3 orders of magnitude larger than the values reported for non-enzymatic reoxidation of free flavins ($250 \text{ M}^{-1} \text{ s}^{-1}$), and ~40-fold faster than found for photo-reduced AAO [41, 42]. The latter observation is consistent with enhanced O₂ reactivity of AAO when the conserved active site His502 is protonated during the oxidative half-reaction, reducing the singlet/triplet energy gap [43]. The importance of a positively charged group for O₂ activation has been reported for flavoprotein oxidases [41, 44].

Tyr92 role in AAO catalysis

Finally, the kinetic data obtained in the present work for the Tyr92 variants strongly suggest that this residue is involved in the alcohol substrate stabilization at AAO active site. The Y92F variant exhibits similar rates for 3-methoxybenzyl alcohol oxidation than the native enzyme, while the leucine and tryptophan substitutions mainly alter alcohol substrate affinity. In the case of the Y92W variant, substrate binding results significantly weakened with 76 and

150 fold higher K_m and K_d , respectively, while the changes on the affinity constants for the Y92L variant were modest (suggesting that some stacking is still possible). In a previous study, Tyr92 substitution with alanine (fully removing the stacking interaction) did not yield active enzyme, supporting the involvement of the above-mentioned π - π interaction in AAO catalysis [18]. Interestingly is that Tyr92 is not conserved in the GMC oxidoreductase superfamily. However, tyrosine, phenylalanine and leucine residues are frequently found at this position in the putative AAO sequences from different basidiomycetes genomes (**Fig. 7**). This suggests that stacking might be a common catalytic strategy for alcohol substrate oxidation in AAO enzymes.

In conclusion the study of the overall catalytic cycle of AAO presented herein shows that stacking stabilization interaction of the aromatic substrate/product by active-site Tyr92 governs AAO catalysis, switching between the ping-pong and the ternary mechanism depending on the stacking stabilization energies. The importance of Tyr92 for alcohol substrate binding was also evidenced by site-directed mutagenesis, kinetics and KIE studies suggesting a common role in other AAO proteins where this residue is conserved or replaced by others which are able to establish similar stacking interactions.

Materials and methods

Chemicals

4-Methoxybenzyl, 3,4-dimethoxybenzyl (veratryl), 3-chlorobenzyl, and 3-fluorobenzyl alcohols were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-Chloro-4-methoxybenzyl alcohol, (*R*)-[α - 2 H]-4-methoxybenzyl alcohol, (*S*)-[α - 2 H]-4-methoxybenzyl alcohol and [α - 2 H₂]-4-methoxybenzyl alcohol were synthesized at the Instituto de Ciencia de Materiales de Aragón (CSIC-UZ, Zaragoza, Spain).

Protein production and purification

Native AAO from *P. eryngii* was obtained by expression in *E. coli* of the mature AAO cDNA (GenBank AF064069), followed by *in vitro* activation in the presence of the FAD cofactor, and purification by ion-exchange chromatography as described previously [45]. Mutated variants were prepared using the QuikChange site-directed mutagenesis kit (Stratagene). For the PCR reactions, the cDNA cloned into the pFLAG1 vector was used as template, and the following oligonucleotides (direct sequences) bearing mutations (italics) as primers: **i**) Y92L, 5'- GGG TCT AGC TCT GTT CAC *CTC* ATG GTC ATG ATG CG-3'; **ii**) Y92F, 5'- GGG TCT AGC TCT GTT CAC *TTC* ATG GTC ATG ATG CG-3'; and **iii**) Y92W, 5'-GGG TCT AGC TCT GTT CAC *TGG* ATG GTC ATG ATG CG-3'. Mutations were confirmed by sequencing (GS-FLX sequencer from Roche) and the mutated variants were obtained as described for recombinant AAO. Naturally-oxidized AAO concentration was determined using the molar absorbances of native AAO and of its Y92L, Y92F and Y92W variants (ϵ_{463} 11050 M⁻¹ cm⁻¹, ϵ_{463} 11240 M⁻¹ cm⁻¹, ϵ_{463} 10044 M⁻¹ cm⁻¹, ϵ_{457} 10693 M⁻¹ cm⁻¹, respectively) calculated by heat denaturation and estimation of the free FAD released (ϵ_{450} 11300 M⁻¹·cm⁻¹) [45].

Steady-state kinetic measurements

Steady-state kinetics measurements were spectrophotometrically monitored by following the oxidation of the alcohol substrate to the corresponding aldehyde as previously described [15]. Two-substrate steady-state kinetics measurements were performed by simultaneously varying the concentrations of alcohol substrate and O₂ in 0.1 M phosphate buffer, pH 6, at 12 °C. The reactions were carried out in a screw-cap cuvette where the buffer solution was first equilibrated at the desired concentration of O₂ (61, 152, 319, 668 and 1520 μ M at 12 °C) by

bubbling with the appropriate O₂/N₂ gas mixture for 10-15 min. Then, reactions were started by the addition of the alcohol substrate (around 5-10 µL) and AAO (5 µL, 0.03 µM final concentration) into a reaction mixture with a final volume of 1.5 mL. Initial rates were calculated during the linear phase of alcohol oxidation to corresponding aldehyde, and analyzed by fitting to Eq. 1 or Eq. 2 describing ternary complex and ping-pong mechanisms, respectively:

$$\frac{v}{e} = \frac{k_{cat}AB}{K_{m(Ox)}A + K_{m(Al)}B + AB + K_{i(Al)}K_{m(Ox)}} \quad (\text{Eq. 1})$$

$$\frac{v}{e} = \frac{k_{cat}AB}{K_{m(Ox)}A + K_{m(Al)}B + AB} \quad (\text{Eq. 2})$$

where v represents the observed initial rate, e is the enzyme concentration, k_{cat} is the maximal turnover, A is the alcohol substrate concentration, B is the O₂ concentration, $K_{m(Al)}$ and $K_{m(Ox)}$ are the Michaelis constants for alcohol and O₂, respectively, and $K_{i(Al)}$ is the alcohol dissociation constant.

Stopped-flow measurements: Enzyme turnover and pre-steady-state kinetics

Stopped-flow experiments were carried out on an Applied Photophysics SX17. MV spectrophotometer (Applied Photophysics Ltd.) using the SX18.MV or the Xscan softwares for experiments with single wavelength or photodiode-array detection, respectively. For enzyme-monitored turnover experiments, air-saturated enzyme and substrate solutions were mixed, and the evolution of the flavin redox state was monitored in the range of 350-900 nm. Reductive half-reaction studies were performed under anaerobic conditions. Tonometers containing enzyme and substrate solutions were made anaerobic by successive evacuation and flushing with argon. These solutions also contained glucose (10 mM) and glucose oxidase (10 U·mL⁻¹) to ensure anaerobiosis. Drive syringes in the stopped-flow apparatus were made anaerobic by sequentially passing a sodium dithionite solution and O₂-free buffer [46]. Measurements were carried out in 0.1 M phosphate buffer, pH 6, at 12 °C. All given concentrations are those after mixing an equal volume of substrate and enzyme (i.e. final concentrations). Spectral evolution was studied by global analysis and numerical integration methods using the Pro-K software (Applied Photophysics Ltd.). Observed rate constant (k_{obs}) from traces recorded at 462 nm were calculated from exponential fits. Rate constants were obtained by a nonlinear fitting of k_{obs} at different substrate concentrations to either Eq. 3 or Eq. 4:

$$k_{obs} = \frac{k_{red}A}{K_d + A} \quad (\text{Eq. 3})$$

$$k_{obs} = \frac{k_{red}A}{K_d + A} + k_{rev} \quad (\text{Eq. 4})$$

where k_{obs} is the observed rate for reduction of the enzyme at a given alcohol concentration, k_{red} and k_{rev} are the limiting rates for hydride transfer from the substrate to the flavin and for the reverse reaction, respectively, at saturating substrate concentrations and K_d is the dissociation constant for the enzyme-substrate complex.

Rate constants for the oxidative half-reaction were measured at 12 °C in 0.1 M phosphate buffer by monitoring the absorbance increase at 462 nm upon mixing the previously anaerobically reduced enzyme with buffer equilibrated at different O₂ concentrations. The enzyme was reduced either by mixing the oxidized protein under anaerobic conditions with a 1.2 fold-excess of the corresponding alcohol substrate or by photoreduction in the presence of 2 mM 5-deazariboflavin and 3 mM EDTA [15]. The

apparent second-order rate constants for the oxidative half-reaction (k_{ox}) were determined as a function of the O_2 concentrations and calculated using Eq. 5, where the k_{obs} is the experimentally observed rate constant associated for flavin oxidation at any given O_2 concentration

$$k_{\text{obs}} = {}^{\text{app}} k_{\text{ox}} [\text{O}_2] \quad (\text{Eq. 5})$$

KIE in *p*-methoxybenzyl alcohol oxidation

The substrate KIEs due to *p*-methoxybenzyl alcohol α -deuteration (*R*, *S* and dideuterated forms) on apparent steady-state kinetic constants were measured in O_2 -saturated (0.1279 mM) 0.1 M phosphate buffer, pH 6.0 at 25°C, were and calculated by fitting the initial rates to Eq. 6, which describes a mechanism with separate isotope effects on k_{cat} and $k_{\text{cat}}/K_{\text{m}}$; where S is substrate concentration, F_i is the atom fraction of deuterium label in the substrate (0.98 in the present case) and $E_{k_{\text{cat}}/K_{\text{m}}}$ and $E_{k_{\text{cat}}}$ are the isotope effect minus 1 in the two kinetic constants [47].

$$\frac{v}{e} = \frac{k_{\text{cat}} S}{K_{\text{m}} (1 + F_i E_{\frac{k_{\text{cat}}}{K_{\text{m}}}}) + S (1 + F_i E_{k_{\text{cat}}})} \quad (\text{Eq. 6})$$

QM calculations

Second-order Moller-Plesset (MP2) [48] QM calculations were performed using the Gaussian03 program [49]. Triple-zeta split-valence basis sets with polarization orbitals (6-311G*) were used throughout. The structures of the T-shaped complexes between a model tyrosine side-chain and the 4-methoxybenzyl alcohol were optimized using MP2 in gas phase and the most stable structure was selected. To isolate the stacking interactions and avoid distortions from the gas phase minimization for the remaining complexes, the H atom in meta position of the methoxybenzyl structure was replaced by a chloride or a methoxy group. The 3-chlorobenzyl alcohol was obtained by removing the *para* methoxyl from the 3-chloro-4-methoxybenzyl while the 3-fluorobenzyl alcohol structure was obtained by a chloride to fluoride change in 3-chlorobenzyl alcohol. At this point, the benzene rings are maintained frozen and all substituents are optimized. This procedure was done for both the alcohol and aldehyde forms. Binding energies were approximated from the interaction energies: AB-(A+B) for all complexes and basis-set-superposition-error (BSSE) corrections [50] were taken into account at the same level of theory. Several studies on substituted benzene dimers have shown that this level of theory is capable of a qualitative description of differences in stabilization energies [51].

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Author contributions

All of the authors performed the research and discussed the results obtained. P. Ferreira conceived the study, performed some kinetics studies, produced figures, and wrote the paper, A. Hernandez-Ortega designed and constructed the mutations and performed some kinetic studies. F. Lucas, B. Kenneth and V. Guallar performed the computational part of the work and wrote the corresponding sections of the paper. J. Carro produced and characterized the

protein variants and produce figures. B. Herguedas performed some kinetic studies. AT Martinez and M Medina contributed to integrate the study and write the paper.

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Table 1. Kinetics parameters for the steady-state reaction and for the pre-steady-state reductive half-reaction of AAO with five different alcohol substrates

Alcohol substrate	Steady-state turnover					Reductive half-reaction	
	k_{cat} (s ⁻¹)	$K_{\text{m(Al)}}$ (μM)	$k_{\text{cat}}/K_{\text{m(Al)}}$ (s ⁻¹ mM ⁻¹)	$K_{\text{m(Ox)}}$ (μM)	$k_{\text{cat}}/K_{\text{m(Ox)}}$ (s ⁻¹ mM ⁻¹)	k_{red} (s ⁻¹)	K_{d} (μM)
4-Methoxybenzyl ¹	129 ± 5	25 ± 3	5160 ± 650	348 ± 36	371 ± 41	115 ± 3	31 ± 2
3-Chloro-4-methoxybenzyl	29 ± 1	8 ± 1	3630 ± 470	214 ± 30	136 ± 20	95 ± 3	71 ± 10
3,4-Dimethoxybenzyl	57 ± 1	543 ± 39	105 ± 8	119 ± 11	479 ± 45	131 ± 10	838 ± 242
3-Chlorobenzyl	13 ± 1	62 ± 1	210 ± 4	10 ± 1	1300 ± 130	8 ± 1	58 ± 2
3-Fluorobenzyl	9 ± 1	164 ± 3	56 ± 1	7 ± 1	1400 ± 160	6 ± 1	180 ± 7

Measurements were performed in 0.1 M phosphate buffer, pH 6, at 12 °C. Steady-state kinetic constants were determined by varying the concentrations of both alcohol and O₂, and calculated by fitting to Eq. 1 (4-methoxybenzyl, 3-chloro-4-methoxybenzyl and 3,4-dimethoxybenzyl alcohols) or Eq. 2 (3-chlorobenzyl and 3-fluorobenzyl alcohols) describing either a ternary or ping-pong mechanism, respectively (see Materials and Methods). The pre-steady state observed reduction rate constants were fitted to Eq. 3. Means and standard deviations are provided ¹Data from Ferreira et al. [14]

Table 2: T-shaped stacking energies between five AAO alcohol substrates and their corresponding aldehydes with a tyrosine side-chain, and differences between the two calculated energies (in Kcal·mol⁻¹)

	Alcohols (Alc)	Aldehyde (Ald)	Difference (Alc-Ald)
4-Methoxybenzyl	-3.27	-2.83	-0.44
3-Chloro-4-methoxybenzyl	-3.16	-3.00	-0.16
3,4-Dimethoxybenzyl	-4.11	-3.62	-0.49
3-Chlorobenzyl	-2.77	-1.96	-0.81
3-Fluorobenzyl	-2.67	-1.81	-0.86

T-shaped stacking energies between Tyr and alcohol (Alc) or aldehyde (Ald) were calculated as described in the Materials and Methods section. Stacking energy difference between alcohol and substrate (Alc-Ald) is shown. Energies (in Kcal·mol⁻¹)

Table 3. Kinetic parameters for steady-state reaction and for the pre-steady-state reductive half-reaction of native AAO and three Tyr92 variants in the oxidation of 4-methoxybenzyl alcohol

	Steady-state turnover					Reductive half-reaction	
	k_{cat} (s ⁻¹)	$K_{\text{m(Al)}}$ (μM)	$k_{\text{cat}}/K_{\text{m(Al)}}$ (s ⁻¹ mM ⁻¹)	$K_{\text{m(Ox)}}$ (μM)	$k_{\text{cat}}/K_{\text{m(Ox)}}$ (s ⁻¹ mM ⁻¹)	k_{red} (s ⁻¹)	K_{d} (μM)
AAO ¹	129 ± 5	25 ± 3	5160 ± 650	348 ± 36	371 ± 41	115 ± 3	31 ± 2
Y92F	120 ± 1	30 ± 1	4450 ± 120	147 ± 4	814 ± 21	119 ± 9	42 ± 12
Y92L	100 ± 2	51 ± 2	1940 ± 4	348 ± 13	286 ± 12	95 ± 2	52 ± 4
Y92W	11 ± 0.3	1890 ± 70	6 ± 0.3	181 ± 7	60 ± 3	14 ± 1	4630 ± 520

Measurements were carried out in 0.1 M phosphate buffer, pH 6, at 12 °C. Bisubstrate steady-state constants were determined by varying the concentrations of both alcohol and O₂, and fitting the data to Eq. 1 (native AAO) or Eq. 2 (Y92F, Y92L and Y92W variants) describing ternary and ping-pong mechanisms, respectively (see Materials and Methods). Means and standard deviations are provided. ¹Data from Ferreira et al. [14]

Table 4. KIE on apparent steady-state parameters for the oxidation of three α -deuterated *p*-methoxybenzyl alcohols by native AAO and its Y92L variant estimated under O₂-saturation conditions

	$D(^{app}k_{cat})$	$D(^{app}K_{m(Al)})$	$D(^{app}k_{cat}/K_{m(Al)})$
<i>Native AAO¹:</i>			
(<i>R</i>)-[α - ² H]-4-Methoxybenzyl alcohol	5.0 ± 0.0	1.3 ± 0.1	3.8 ± 0.1
(<i>S</i>)-[α - ² H]-4-Methoxybenzyl alcohol	1.3 ± 0.0	1.0 ± 0.1	1.3 ± 0.1
[α - ² H ₂]-4-Methoxybenzyl alcohol	7.6 ± 0.1	1.9 ± 0.1	4.1 ± 0.1
<i>Y92L variant:</i>			
(<i>R</i>)-[α - ² H]-4-Methoxybenzyl alcohol	4.0 ± 0.0	1.0 ± 0.1	4.3. ± 0.1
(<i>S</i>)-[α - ² H]-4-Methoxybenzyl alcohol	1.3 ± 0.0	1.1 ± 0.1	1.2 ± 0.1
[α - ² H ₂]-4-Methoxybenzyl alcohol	6.2 ± 0.1	1.1 ± 0.1	5.8 ± 0.1

Steady-state constants were estimated in O₂-saturated (1.279 mM O₂) 0.1 M phosphate, pH 6, at 25°C. KIE values are the ratio between the activity on protiated and deuterated substrate calculated by fitting constants to Eq. 6. Means and standard deviations are provided. ¹Data from Ferreira et al. and Hernandez-Ortega et al. [14, 33]

Scheme 1. Ternary and ping-pong mechanisms in AAO catalysis. Ternary and ping-pong mechanisms are described by the upper and lower loops, respectively, while the right reaction corresponds to aldehyde release in anaerobic experiments.

Figure 1. Detail of the AAO active site after alcohol substrate migration. Position of 4-methoxybenzyl alcohol after migration to the AAO active site using the PELE software [52] (on 3FIM crystal structure). The alcohol situates in the environment of two conserved histidines, three aromatic residues, and the FAD isoalloxazine ring.

Figure 2. Bisubstrate steady-state kinetics for AAO. Double reciprocal plot for the oxidation of 3-chloro-4-methoxybenzyl (A) and 3-chlorobenzyl (B) alcohols by native AAO, measured in 0.1 M phosphate buffer, pH 6, at 12 °C, as a function of the alcohol substrate concentration at fixed O₂ concentrations: 61 μM (x), 152 μM (▲), 319 μM (○), 668 μM (●) and 1520 μM (□). Direct plots are included in the corresponding insets.

Figure 3. AAO redox state during turnover with five alcohol substrates. The enzyme (~16 μM) was mixed in the stopped-flow spectrophotometer with equal volumes of 0.1 M phosphate buffer, pH 6, containing 2 mM 3-chlorobenzyl alcohol (trace 1), 5 mM 3-fluorobenzyl-alcohol (trace 2), 1 mM 3-chloro-4-methoxybenzyl alcohol (trace 3), 2 mM 4-methoxybenzyl alcohol (trace 4), 4 mM 3,4-dimethoxybenzyl alcohol (trace 5). The time course of the reaction (25 °C) was monitored at 462 nm.

Figure 4. AAO spectral changes during turnover. The AAO spectral changes during turnover experiments described in Fig. 3 with 1 mM 3-chloro-4-methoxybenzyl alcohol (A) and 3 mM 3-chlorobenzyl alcohol (B), are here shown. The oxidized enzyme spectrum before mixing is shown as a dashed line. Spectra after mixing are indicated at 0.003, 0.011, 0.08, 0.15, 0.3, 0.45, 0.60, 0.75, 1, 2, 10, 20 and 40 s in B, and at 0.16, 2.7, 4.3, 5, 10, 20, 30, 40, 50 and 60 s in A. The corresponding insets show the spectral species (A, B and C) obtained after global analysis of the reactions. **Revisar si estan cambiados los tiempos entre A y B**

Figure 5. Pre-steady-state kinetics for the reductive half-reaction of AAO. Spectral time-course of the anaerobic reduction of native AAO (7.5 μM) by 3-chloro-4-methoxybenzyl (A) and 3-chlorobenzyl (B) alcohols. The spectrum of the oxidized enzyme before mixing is shown as a dashed line. Spectra after mixing are shown at 4, 19, 39, 80, 330 and 500 ms in A, and at 2, 39, 78, 129, 219, 500 ms in B. The corresponding insets show the absorbance spectra for the two-three kinetically distinguishable spectroscopic species (A, B and C) obtained by global analysis of the spectral evolution. The second inset shows the evolution of these species along the reaction course.

Figure 6. Pre-steady-state kinetics for the reductive half-reaction of three Tyr92 AAO variants when using as substrate 4-methoxybenzyl alcohol. Spectral evolution of the anaerobic reduction of Y92F (A), Y92L (B), and Y92W (C) AAO variants (9 μM) by 4-methoxybenzyl alcohol. Spectra for the oxidized enzyme before mixing are shown by dashed lines. Spectra after mixing are shown at 0.004, 0.006, 0.009, 0.0115, 0.0141, 0.0192 and 0.032 s in A, 0.004, 0.006, 0.012, 0.017, 0.029, 0.099, 0.25 s in B, and 0.004, 0.006, 0.03, 0.11, 0.18, 0.44, 0.98 s in C. The corresponding insets show the spectra for the kinetically distinguishable species (A and B) obtained by global analysis of the spectral evolution. Dependences of observed rates on the concentrations of 4-methoxybenzyl alcohol by native

AAO (●), Y92F (○), Y92L (▲) and Y92W (△) AAO variants are shown in **D** and fitted to Eq. 5. Assays performed at 12 °C.

Figure 7. Logo of sequence of 70 putative AAO sequences from different basidiomycetes in positions 83-97 of mature *P. eryngii* AAO. The compared sequences were taken from the following genomes available at JGI (www.jgi.doe.gov); the numbers of AAO sequences are indicated in parentheses): *B. adusta* (11), *Dichomitus squalens* (9), *Fomitiporia mediterranea* (1), *Fomitopsis pinicola* (1), *Ganoderma* sp. (7), *Gelatoporia subvermispora* (4), *Gloeophyllum trabeum* (2), *Laccaria bicolor* (1), *P. chrysosporium* (3), *Phlebia brevispora* (3), *Punctularia strigosozonata* (6), *Rhodonina placenta* (2), *Stereum hirsutum* (15) and *Trametes versicolor* (3). Numbers under each stack indicate the position in the mature *P. eryngii* AAO sequence; the overall height of the stack reflects the sequence conservation at that position; and the height of each symbol in the same column indicates the relative frequency of each amino acid.