Expression and structural determination of an alcohol oxidase from *Phanerochaete chrysosporium*

Quoc-Thai Nguyen, Willem P. Dijkman, Elvira Romero, Suzan Pantaroto de Vasconcellos, Claudia Binda, Marco W. Fraaije, and Andrea Mattevi

The recently described alcohol oxidase from the white-rot basidiomycete *Phanerochaete chrysosporium* (PcAOX) was heterologously expressed in *Escherichia coli* as a fusion protein. The recombinant enzyme was obtained with high yield and displayed high thermostability in commonly used buffers. Steady-state kinetics revealed that the enzyme is highly active towards methanol and ethanol ($k_{cat} = 18$ and $19 \text{ s}^{-1}$, respectively), but showed very limited activity towards glycerol. The crystal structure of the native homo-octameric PcAOX was determined at 2.40 Å resolution. The subunit arrangement and the secondary architecture of each monomer is highly conserved in PcAOX as compared to its homolog AOX1 from *Pichia pastoris* (PpAOX1). Similar to PpAOX1, the catalytic center is a remarkably solvent-inaccessible cavity located at the si site of the flavin cofactor. The active site is intriguingly small in size, which could well explain the observed preference for methanol and ethanol as best substrates. With the available expression system and structural information, PcAOX represents a potential candidate for protein engineering towards more industrially attractive substrates, for instance, glycerol—a major byproduct of biodiesel production.

Manuscript in preparation
3.1. Introduction

Flavin-containing oxidases are gaining attention and popularity in biotechnological applications because of their ability to catalyze oxidations of alcohols and amines with exquisite chemo-, regio- and/or enantioselectivity (Hollmann et al. 2011; Dijkman et al. 2013; Romero and Gadda 2014; Pickl et al. 2015). Part of the attraction also stems from their dependence on molecular oxygen—a cheap and environmentally benign oxidant—as electron acceptor, which is typically reduced to hydrogen peroxide (H$_2$O$_2$). The most well-known examples include glucose oxidase (Bankar et al. 2009), D-amino acid oxidase (Pollegioni et al. 2008), and monoamine oxidase (Turner 2011), which have been widely used for decades in bioanalytical, agrochemical, and pharmaceutical applications.

Alcohol oxidases (AOX, EC 1.1.3.13), also known as methanol oxidases, belong to the glucose–methanol–choline (GMC) oxidoreductase superfamily and contain a non-covalently bound FAD cofactor. AOXs catalyze the oxidation of primary alcohols into aldehydes and H$_2$O$_2$ in specialized organelles, the peroxisomes, where the latter product can be decomposed by co-compartmentalized catalase. AOXs have been described in numerous yeasts and filamentous fungi such as Candida biodinii, Hansenula polymorpha, Pichia pastoris and have been extensively studied (Bringer et al. 1979; Kato et al. 1976; Vonck and van Bruggen 1992; Menon et al. 1995). In addition to the preferred substrate methanol, other short aliphatic primary alcohols of up to four carbons are also accepted by AOXs. Although being discovered in the 70s of the last century, only recently was the first AOX structure determined (from P. pastoris; Koch et al. 2016; Vonck et al. 2016).

Recently, a novel AOX from the white-rot basidiomycete Phanerochaete chrysosporium was identified and isolated (PcAOX) (Linke et al. 2014). In contrast to AOX from P. pastoris (PpAOX1), which harbors a peroxisomal targeting signal at the C-terminus, no hypothetical signal sequencing was identified for PcAOX, suggesting a cytosolic localization of the enzyme. Furthermore, the preliminary substrate profiling data indicated that the enzyme accepts aliphatic primary alcohols of up to five carbon atoms. Interestingly, glycerol, a polyol currently accumulated as an excessive side-stream of biodiesel manufacture, was described as a good substrate for PcAOX. Therefore, PcAOX represents an attractive target for further investigations for its potential in biotechnology.

The aim of the present study is two-fold. First, we focused on establishing an effective recombinant expression system for producing PcAOX in Escherichia coli (rather than in yeast). This would enable more detailed characterizations of this newly identified AOX and explorative studies of the enzyme as industrial biocatalyst. Second, we determined the X-ray structure of PcAOX, which will represent the second known AOX structure, adding insights into the structural features and catalytic mechanism of this class of enzymes. The structural information in turn can guide the rational design of PcAOX into tailor-made biocatalysts.
3.2. Experimental section

3.2.1. Protein expression and purification

The open reading frame for AOX from *P. chrysosporium* (Accession number HG425201, UniProtKB/TrEMBL: T2M2J4) was purchased from GeneScript (Piscataway, NJ, USA) with optimized codons for protein expression in *E. coli*. The *aox1* gene was amplified from the delivered plasmid using Phusion High-Fidelity DNA polymerase (Thermo Scientific) and the corresponding pairs of primers: forwards 5′– ATGGGTCATCCGGAAGAAGTTG–3′ and reverse 5′– TTAGCGGACTTGTTGCGTAGCC–3′. The purified PCR products (100–200 ng) were incubated with 0.5 U Taq polymerase (Roche) and 0.75 mM dATP at 72 °C for 15 min to introduce the 3′-A overhangs. The resulting insert DNA fragments were ligated into the pET-SUMO vector according to the instruction manual of the Champion pET SUMO expression system (Invitrogen). AOX was expressed in *E. coli* BL21(DE3), grown in Terrific Broth containing 50 μg/mL kanamycin and 1% (w/v) glucose at 37 °C. Protein expression was induced when the cells reached OD600 ∼0.7–0.8 by adding 1 mM isopropyl β-D-1-thiogalactopyranoside. The cells were grown at 24 °C until late stationary phase and harvested by centrifugation at 4600 × g for 10 min (Beckman–Coulter JA-10 rotor, 4 °C) and the cell pellet was stored at −20 °C.

For protein purification, cells were resuspended in lysis buffer (50 mM KPi pH 7.8, 400 mM NaCl, 100 mM KCl, 20 mM imidazole, 10 μM FAD) and mechanically disrupted by sonication using a VCX130 Vibra-Cell (Sonics & Materials, Inc., Newtown, USA) at 4 °C (5 sec on, 10 sec off, 70% amplitude, total of 5 min). After removal of unbroken bacteria and cellular debris by centrifugation (20000 × g, Beckman–Coulter JA-25.5 rotor, 4 °C, 45 min), the supernatant was loaded onto a 5 mL HisTrap HP column (GE Healthcare) pre-equilibrated in the same buffer. The elution of the recombinant enzyme with the His-SUMO tag was facilitated by a 20–500 mM imidazole gradient. Fractions containing the pure enzyme as indicated by SDS-PAGE were pooled, desalted, and concentrated into buffer containing 50 mM KPi pH 7.5 using a 30-kDa MWCO Amicon (Millipore) centrifugal filter unit. To obtain the native enzyme, the His-SUMO tag was cleaved by incubating with 10% (mol/mol) SUMO protease (Invitrogen) overnight at 4 °C in lysis buffer supplemented with 1 M urea. The native enzyme was purified from the cleavage mixture by gel-permeation using a Superdex 200 10/300 GL (GE Healthcare) column in 10 mM Tris/HCl pH 7.5, 100 mM NaCl prior to crystallization experiments.

3.2.2. Biochemical characterization

The UV–Vis spectrum of His-SUMO-AOX was recorded from 250 to 650 nm in 50 mM Tris/HCl pH 7.0 before and after addition of 0.1% (w/v) sodium dodecyl sulfate (SDS). The extinction coefficient of PcAOX was determined based on that of free FAD as described before (Macheroux 1999).

The pH optimum for enzyme activity was determined based on oxygen consumption rates using methanol as substrate. The reaction contains 40 mM Britton–Robinson buffer at various pH values (Britton and Robinson 1931), 100 nM enzyme, 25 mM methanol in a final volume of 1 mL, and activity was mon-
Alcohol oxidase from *P. chrysosporium* was monitored for 5 min at 23 °C using a Hansatech Oxygraph instrument (Hansatech Instruments Ltd., Norfolk, UK). Prior to the measurements, the zero oxygen level was calibrated by complete reduction using sodium dithionite.

AOX thermostability was determined based on the apparent melting temperature $T_m$ of the enzyme using the ThermoFAD protocol as previously described (Forneris et al. 2009) with a MiniOpticon real-time PCR detection system and 48-well RT-PCR plates (Biorad Laboratories, Hercules, CA, USA). Each well has a final volume of 20 μL containing 2.0 μL of 100 μM AOX stock (in 50 mM KPi pH 7.5) diluted in the tested buffers (in duplicate).

To determine the steady-state kinetics parameters, AOX activity was measured at 25 °C in 50 mM KPi pH 7.5 using a Cary 100 Bio UV–Visible spectrophotometer (Varian Inc., USA). H$_2$O$_2$ generated by the oxidase was converted into a visible product by including in the reaction horseradish peroxidase (HRP, 20 U/mL, Sigma), 4-aminoantipyrine (0.1 mM) and 3,5-dichloro-2-hydroxybenzene-sulfonic acid (1 mM). This assay results in the formation of a pink to purple quinoid product that can be monitored at 515 nm ($\varepsilon_{515} = 26$ mM$^{-1}$ cm$^{-1}$) (Federico et al. 1997).

### 3.2.3. Protein crystallization, X-ray data collection, and structure determination

The native AOX from *P. chrysosporium* was crystallized using the sitting-drop vapor diffusion technique at 20 °C by mixing equal volumes of 9.3 mg/ml enzyme (in 10 mM Tris/HCl pH 7.5, 100 mM NaCl, 66 μM FAD) and mother liquor containing 14% (w/v) PEG3350, 0.2 M potassium acetate. Prior to X-ray data collection, crystals were cryoprotected in solution containing 18% (w/v) PEG3350, 0.2 M potassium acetate, 20% (v/v) glycerol and flash-cooled by plunging into liquid nitrogen. X-ray diffraction data to 2.4 Å were collected at the ID23-EH2 and ID29 beamlines of the European Synchrotron Radiation Facility in Grenoble, France (ESRF). Image indexing, integration, and data scaling were processed with Xds package (Kabsch 2010b; Kabsch 2010a) and programs of the CCP4 suite (Winn et al. 2011). The diffraction images appeared strongly anisotropic, extending to 3.1, 2.6, and 2.4 Å resolution in the direction of a*, b*, and c*, respectively, which resulted in a high $R_{sym}$ value (Table 3.1). The high-resolution shell cutoff was determined based on the correlation coefficient of half-datasets (CC$_{1/2}$) and visual inspection of the quality of the electron density map as previously described (Lang et al. 2014; Karplus and Diederichs 2015).

The AOX structure was solved by molecular replacement using Phaser (McCoy et al. 2007) and the coordinates of *P. pastoris* AOX1 [PDB ID code 5HSA (Koch et al. 2016)] which shares 52% sequence identity with PcAOX as the search model devoid of all ligand and water molecules. Manual model rebuilding and structure inspection was carried out with Coot (Emsley and Cowtan 2004), whereas alternating cycles of refinement were performed with Refmac5 (Murshudov et al. 1997). PISA server was used to analyze the oligomeric organization of the protein and the molecular interface area (Krissinel and Henrick 2007). Figures were drawn with UCSF Chimera (Pettersen et al. 2004). The detailed data processing statistics of the collected dataset are summarized in Table 3.1.
Table 3.1: Data collection and refinement statistics

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>Space group</td>
<td>P2₁</td>
</tr>
<tr>
<td>Resolution (Å)</td>
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</tr>
<tr>
<td>a, b, c (Å)</td>
<td>112.7, 204.0, 116.5</td>
</tr>
<tr>
<td>$R_{\text{sym}}$ a,b (%)</td>
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<td>Completeness b (%)</td>
<td>98.1 (99.5)</td>
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<td>Unique reflections</td>
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<td>Multiplicity b</td>
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<td>$I/\sigma$ b</td>
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<tr>
<td>$CC_{1/2}$</td>
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<tr>
<td>Number of atoms:</td>
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<td>- protein</td>
<td>40862</td>
</tr>
<tr>
<td>- FAD, glycerol, water</td>
<td>8 x 53, 5 x 6, 700</td>
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<td>Average B value for all atoms (Å²)</td>
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<td>$R_{\text{free}}$ b,c (%)</td>
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<td>Rms bond angles (°)</td>
<td>1.55</td>
</tr>
<tr>
<td>Ramachandran outliers (%)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*a $R_{\text{sym}} = \sum |I_i - \langle I \rangle|/\sum I_i$, where $I_i$ is the intensity of $i^{th}$ observation and $\langle I \rangle$ is the mean intensity of the reflection.

*b Values in parentheses are for reflections in the highest resolution shell.

$c R_{\text{cryst}} = |F_{\text{obs}} - F_{\text{calc}}|/|F_{\text{obs}}|$ where $F_{\text{obs}}$ and $F_{\text{calc}}$ are the observed and calculated structure factor amplitudes, respectively. $R_{\text{cryst}}$ and $R_{\text{free}}$ were calculated using the working and test sets, respectively.

3.3. Results and discussion

3.3.1. Expression and purification of PcAOX

The production of soluble PcAOX, a fungal protein, was facilitated by codon optimization for *E. coli* expression and presumably also by the fusion to a small ubiquitin-like modifier (SUMO) at the N-terminus of the protein. The SUMO fusion is known to enhance the expression level and solubility of partially insoluble proteins, which were often encountered with enzymes of eukaryotic origin that are expressed in *E. coli*. From one liter of culture, around 600 mg of purified yellow-colored His-SUMO-PcAOX could be obtained after one step of purification. The purified enzyme displayed a typical flavoprotein UV-Vis absorbance spectrum, with two absorbance maxima at 385 and 455 nm (Figure 3.1). Upon unfolding the enzyme with 0.1% (w/v) SDS, the absorbance spectrum of the released flavin was recorded and used to determine the extinction coefficient of the enzyme ($\varepsilon_{455} = 10.3 \text{mM}^{-1} \text{cm}^{-1}$).

The native PcAOX was obtained by cleaving the N-terminal His-SUMO tag from the fusion enzyme by proteolytic treatment with SUMO protease. The UV-Vis absorbance spectrum of native PcAOX is essentially identical to that of the fusion enzyme. The apparent catalytic rates measured with ethanol were also
comparable for both forms of PcAOX. These results indicated that the SUMO fusion at the N-terminus has no significant effects either on the microenvironment around the flavin cofactor or the enzyme activity. Nevertheless, the native PcAOX precipitated shortly upon storage at 4 °C, implicating that the His-SUMO fusion is crucial for maintaining the protein stability. For this reason, except for the crystallization experiments, we decided to use His-SUMO-PcAOX for the rest of our study.

3.3.2. pH optimum and thermostability of PcAOX
To evaluate the optimal pH for PcAOX activity, methanol oxidation rates were measured between pH 3.0–10 in Britton–Robinson buffer. The enzyme displayed a clear preference for neutral to basic conditions (Figure 3.2), with an optimal pH value of 9.0. The oxidase retained >75% of its activity between pH 7 and pH 10. Beyond pH 6.0 and pH 11, the activity dropped sharply below 50% of that at pH 9.0. This is in good agreement with the pH profile reported for native AOX isolated from P. chrysosporium (Linke et al. 2014).

The melting temperatures of PcAOX determined by the ThermoFAD method at various buffers indicated that the enzyme is highly thermostable (Table 3.2).

**Figure 3.1:** Absorption spectra of His-SUMO-PcAOX in 50 mM Tris/HCl pH 7.0 before (solid line) and after addition of 1% (w/v) SDS (broken line)

**Figure 3.2:** Effect of pH on methanol oxidation. The reaction contains 40 mm Britton–Robinson buffer, 100 nm AOX, 25 mM methanol, and activity was monitored by following oxygen consumption for 5 min at 23 °C using a Hansatech Oxygraph instrument.
Table 3.2: Unfolding temperatures of PcAOX in different buffers determined by the ThermoFAD method. B–R: 40 mM Britton–Robinson buffer

<table>
<thead>
<tr>
<th>Condition</th>
<th>( T_m [^\circ C] )</th>
<th>Condition</th>
<th>( T_m [^\circ C] )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM KPi pH 7.5</td>
<td>58</td>
<td>B–R buffer pH 9.0</td>
<td>40</td>
</tr>
<tr>
<td>50 mM KPi pH 7.5</td>
<td>57</td>
<td>B–R buffer pH 10</td>
<td>35</td>
</tr>
<tr>
<td>50 mM Tris/HCl pH 7.5</td>
<td>58</td>
<td>B–R buffer pH 11</td>
<td>31</td>
</tr>
<tr>
<td>50 mM HEPES pH 7.5</td>
<td>61</td>
<td>100 mM NaCl, 50 mM KPi pH 7.5</td>
<td>56</td>
</tr>
<tr>
<td>B–R buffer pH 3.0</td>
<td>31</td>
<td>500 mM NaCl, 50 mM KPi pH 7.5</td>
<td>55</td>
</tr>
<tr>
<td>B–R buffer pH 4.0</td>
<td>47</td>
<td>1 M glycerol, 50 mM KPi pH 7.5</td>
<td>58</td>
</tr>
<tr>
<td>B–R buffer pH 5.0</td>
<td>62</td>
<td>5 M glycerol, 50 mM KPi pH 7.5</td>
<td>58</td>
</tr>
<tr>
<td>B–R buffer pH 6.0</td>
<td>62</td>
<td>20 mM MeOH, 50 mM KPi pH 7.5</td>
<td>55</td>
</tr>
<tr>
<td>B–R buffer pH 7.0</td>
<td>57</td>
<td>500 mM MeOH, 50 mM KPi pH 7.5</td>
<td>57</td>
</tr>
<tr>
<td>B–R buffer pH 8.0</td>
<td>53</td>
<td>100 mM EtOH, 50 mM KPi pH 7.5</td>
<td>58</td>
</tr>
</tbody>
</table>

Over a wide range of pH values from pH 5.0 to pH 8.0, the \( T_m \) is >53 °C. The enzyme is less thermostolerant at pH 5.0 and pH 9.0. Addition of NaCl has virtually no effect on the enzyme thermostability. However, we noticed that in the absence of salt, concentrated enzyme tended to precipitate at room temperature. The ThermoFAD experiments suggest that PcAOX tolerates various water-miscible solvents such as methanol (0.5 M), ethanol (0.1 M), and glycerol (5 M), as judged by their minor influence on the \( T_m \) values.

### 3.3.3. Steady-state kinetics

To determine the steady-state kinetics of PcAOX, an HRP-coupled assay was used to probe the \( \text{H}_2\text{O}_2 \) production rates upon oxidation of the model substrates methanol and ethanol. The initial reaction rates were recorded and could be fitted well using the Michaelis–Menten kinetic model (Table 3.3). The determined \( K_m \) values for methanol and ethanol of PcAOX (2.4 and 15 mM, respectively) differ somewhat from previously reported values [36.6 and 22.5 mM, respectively (Linke et al. 2014)], most probably due to the assay conditions such as temperature and buffers used. The \( k_{cat} \) values for both substrates were found to be similar, around 18 s\(^{-1}\). The \( k_{cat} \) and \( K_m \) values of PcAOX are in close agreement with the kinetic parameters of AOX1 from \( P. \text{pastoris} \) \([k_{cat} \text{ and } K_m \text{ for methanol are } 0.6 \text{ mM and } 5.7 \text{ s}^{-1}, \text{respectively (Koch et al. 2016)}]\). As observed for other AOXs, methanol also represents the best substrate for PcAOX with a specificity constant of 7.2 \( \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \), which is very similar to PpAOX1 (9.5 \( \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \)).

Regrettably, and in contrast to the results from Linke et al., we invariably observed only a very low activity when using glycerol as a substrate. At extremely high substrate concentrations (1.0–4.0 M), the observed rate varied between 0.1–0.2 s\(^{-1}\), which is too low to be catalytically relevant for physiological conditions or industrial applications.
Table 3.3: Steady-state kinetic parameters for AOX measured with the HRP-coupled assay

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ [mM]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>$k_{cat}/k_m$ [M$^{-1}$ s$^{-1}$]</th>
</tr>
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<tbody>
<tr>
<td>methanol</td>
<td>2.4 ± 0.1</td>
<td>18 ± 0.1</td>
<td>7.2 × 10$^3$</td>
</tr>
<tr>
<td>ethanol</td>
<td>15 ± 0.9</td>
<td>19 ± 0.4</td>
<td>1.3 × 10$^3$</td>
</tr>
</tbody>
</table>

3.3.4. AOX overall structure

PcAOX crystallized as a homo-octamer that, similarly to PpAOX1, can be interpreted as either a tetramer of dimers or a dimer of two tetramers facing each other (Figure 3.3). Each monomer comprises residues 2 to 645 and a non-covalently bound FAD molecule. Within each monomer, two domains can be recognized which is the typical topology found in the members of the glucose–methanol–choline (GMC) oxidoreductase superfamily (Fraaije and Mattevi 2000): a substrate binding domain and an FAD-binding domain. The latter is characterized by the typical Rossmann fold featuring a sandwich of a 5-stranded parallel sheet and a 3-stranded antiparallel sheet, whereas the substrate binding domain comprises a 6-stranded antiparallel sheet (Figure 3.4). Relatively to the octameric oligomer, the substrate binding domain of each monomer is buried inside the octamer and involved exclusively in the interactions between the two tetramers, whereas the FAD-domain is located closer to the periphery of the oligomer (Figure 3.3b). Within a tetramer, one subunit interacts with each of the two adjacent subunits by 16 hydrogen bonds and 6 salt bridges, which involves an area of approximately 1525 Å$^2$, accounting for about 6% of its accessible surface. The inter-tetramer interface, however, is far more extensive as indicated by the burial of 11% (approximately 3000 Å$^2$) of the monomer surface, including 38 inter-subunit hydrogen bonds and 3 salt bridges. Therefore, upon octamer formation, each monomer buries about 25% of its solvent-accessible surface area, which strongly stabilizes the oligomer.

The monomers from PcAOX and PpAOX1 are closely related, as indicated by a root mean square deviation of 0.92 Å for 642 common pairs of Cα atoms upon superposition of the monomer in the two enzyme structures (Figure 3.4). A few major differences between the two structures entail a shorter loop (573–574 in PcAOX, corresponding to 579–586 in PpAOX1) in the FAD-binding domain and a short insert in the vicinity of the substrate binding domain (525–538 in PcAOX, corresponding to 521–544 in PpAOX1). The latter, so-called an enabling loop, facilitates formation of both dimeric and tetrameric sub-assemblies in PpAOX1 (Koch et al. 2016). In addition, PpAOX1 carries a peroxisomal targeting signal (PTS) at the C-terminal extension that was believed to be crucial for functional octamer assembly maturation (Koch et al. 2016). Interestingly, despite a high structural similarity, PcAOX does not contain a signal sequence, thus localizes instead in the cytosol (Linke et al. 2014).
3.3.5. The active site of PcAOX

As the majority of the GMC oxidases, eight PcAOX subunits contain a dissociable, tightly bound FAD cofactor buried deep inside the FAD-binding domain in an elongated shape, thus preventing the release of the cofactor during catalysis (Figures 3.3 and 3.4) (Vonck et al. 2016). Similarly to PpAOX1, the isoalloxazine ring in PcAOX is found in a bent conformation. A distinctive feature of AOX1 from *P. pastoris* involves a modified FAD cofactor where the isoalloxazine ring is attached to an unusual arabinyl instead of a ribityl chain (the sugar C2′ carbon’s configuration is changed from *R* to *S*) (Koch et al. 2016). In contrast, PcAOX does not harbor such modified flavin, probably due to interspecies variations.
Figure 3.4: Superposition of P. chrysosporium AOX monomer A (in deep sky blue) with that of the homologous P. pastoris AOX1 [in light gray, 52% sequence identity, PDB ID 5HSA (Koch et al. 2016)]. The overall topology of the two structures and the active site are highly conserved. In both structures, the flavin cofactor adopts essentially the same position in the active site with a similar bent conformation of the isoalloxazine ring. Besides a longer loop of 12 amino acids at the N-terminus in PcAOX1 (which is not visible in the figure due to the orientation), the major differences between the two proteins lie in the length of some loops that are labelled with the corresponding residues. FAD is drawn as sticks with carbon atoms in yellow (for PcAOX) or in light gray (for PpAOX1), oxygen atoms in red, nitrogen atoms in blue, and phosphorous atoms in orange.

and/or growth conditions.

The substrate binding site in PcAOX is completely solvent-inaccessible, a remarkable feature that has been observed for PpAOX1 and also members of the vanillyl alcohol oxidase family (Mattevi et al. 1997; Nguyen et al. 2016). The two recurrent elements of the GMC-type oxidase, i.e., His561 and Asn604 positioned close to the FAD ring, are strictly conserved in PcAOX (corresponding to His567 and Asn616 in PpAOX1) (Figure 3.5). The active site histidine residue serves as a general base catalyst, abstracting protons from the substrate alcohol, whereas the asparagine residue acts as a hydrogen donor (Dijkman et al. 2013; Romero and Gadda 2014). Careful inspection of the active site of PcAOX revealed a weak but consistent $F_o - F_c$ peak contoured at 3.0σ level in front of the N5 atom at the si side of the flavin from several subunits (data not shown). Superposition with PpAOX1 indicated the presence of a water molecule occupying exactly the same position in PcAOX. This peak locates at a distance suitable for hydride transfer to the N5 atom of the flavin and is in hydrogen bonding distance with the side
Figure 3.5: The active site architecture of *P. chrysosporium* AOX (carbon atoms in deep sky blue, with FAD in color code as in Figure 3.4). The refined $2F_o - F_c$ electron density map (contoured at 1σ) is shown for FAD as dark gray chicken-wire. The structure is superposed and comparatively analyzed with that of *P. pastoris* AOX1 (carbon atoms in light gray). Residues forming the catalytic pocket in both structures are displayed, featuring a tight and highly solvent-inaccessible substrate binding cavity. The orientation and color coding is the same as in Figure 3.4.

chains of His561 and Asn604 (corresponding to His567 and Asn616 in PpAOX1). This water molecule was believed to occupy the position of oxygen atom of the aldehyde product in PpAOX1 (Koch et al. 2016). In PcAOX the electron density peak in the same position was nonetheless too weak to model a water molecule.

Comparative analyses showed that other active site residues, most of which are either aromatic and/or hydrophobic, are largely conserved in PcAOX (Figure 3.5). Different amino acids between the two AOXs include Cys311, Phe313, His394, Phe402, and Phe419 in PpAOX1, which are replaced with Thre315, Leu317, Phe399, Tyr407, and Tyr419 in PcAOX, respectively. Despite the seemingly considerable changes, the bulkiness and the hydrophobicity of the residues remains equivalent, hence the size and the shape of the substrate cavity are essentially the same. These active site residues are less conserved when compared with more distantly related members of the GMC oxidase family, which confirms their role in tuning substrate specificity. Within the two AOX structures it is noteworthy the replacement of Phe313 in PpAOX1 by Leu317 in PcAOX, which in the latter generates extra space for substrate binding (Figure 3.5). This may partly explain the observation that while glycerol is not a substrate for PpAOX1, it can be converted by PcAOX, although with very poor efficiency.
3.4. Discussion

With the rapid increase in biodiesel production and the massive availability of its by-product glycerol, effective (bio)catalysts that can convert glycerol into value-added products are in demand (Johnson and Taconi 2007; Pagliaro et al. 2007; Zhou et al. 2008). Among these, oxidases that can oxidize glycerol are highly desirable as such biocatalysts have the potential to produce enantiomerically pure glyceraldehyde or glyceric acid. Thus, an efficient glycerol oxidase would allow the conversion of glycerol into valuable building blocks while the concomitantly produced \( \text{H}_2\text{O}_2 \) may also be of value. Attempts to engineer the available alcohol/polyol oxidases, e.g., alditol oxidase, into a “glycerol oxidase” by directed evolution and rational design have resulted in ineffective glycerol oxidases with poor catalytic efficiencies. The best alditol oxidase mutant for oxidation of glycerol was found to display only a \( k_{\text{cat}} \) of 0.06 s\(^{-1}\) (Gerstenbruch et al. 2012).

The recent identification of a novel AOX from the white-rot basidiomycete \( P. \ chrysosporium \) that was suggested to be responsible for the glycerol oxidase activity has prompted us to further investigate the reported enzyme. Whereas most of AOXs are of eukaryotic origin, hence rendering efficient overexpression of AOX-encoding genes a challenge in prokaryotic hosts, PcAOX was found to be well expressed in \( E. \ coli \). In fact, AOX from \( P. \ chrysosporium \) represents the first fungal methanol oxidase within the GMC superfamily that can be heterologously expressed in \( E. \ coli \) with a rather impressive high yield: >600 mg of pure protein from 1 L of culture can be obtained by merely one purification step. The enzyme was found to be active on methanol and ethanol with high and similar catalytic efficiencies, displaying similar kinetic properties when compared with a prototypical AOX (e.g., AOX from \( P. \ pastoris \)—PpAOX1). The steady-state kinetic parameters deviate somewhat from those reported by Linke et al., which may be due to differences in experimental conditions (Linke et al. 2014). Unexpectedly, activity towards glycerol was found to be very low (\( k_{\text{obs}} = 0.2 \text{ s}^{-1} \) at 4 M glycerol). The reason for such discrepancy with reported kinetic data by Linke et al. is currently unknown.

For a better insight into the molecular basis of the rather restricted substrate scope of PcAOX and other enzyme properties, we set out to elucidate the structure of this fungal AOX. We succeeded in determining the crystal structure of native PcAOX at 2.40 Å. Inspection of the active site revealed that the substrate binding pocket is an exceptionally small and remarkably solvent-inaccessible cavity (Figure 3.5). This is in good agreement with a clear preference for methanol as the best substrate, similar to the \( P. \ pastoris \) AOX1 (Koch et al. 2016). Modelling a glycerol molecule into the active site (data not shown) invariably showed that the cavity has just about enough space to accommodate this polyol. Moreover, the high hydrophobicity of the substrate binding pocket allows limited hydrogen bonding interactions between the modeled glycerol and PcAOX. It is gratifying that these features highly correlate with the observed low affinity and activity of the enzyme towards glycerol in our study. Based on the available structural analysis, rational design attempts are currently being made in our laboratories.
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