The First Mammalian Aldehyde Oxidase Crystal Structure: Insights into Substrate Specificity

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Running title: The first crystal structure of mouse liver aldehyde oxidase

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Background: Aldehyde oxidases have pharmacological relevance and AOX3 is the major drug-metabolizing enzyme in rodents.

Results: The crystal structure of mouse AOX3 with kinetics and molecular docking studies provides insights into its enzymatic characteristics.

Conclusion: Differences in substrate and inhibitor specificities can be rationalized by comparing the AOX3 and xanthine oxidase structures.

Significance: The first aldehyde oxidase structure represents a major advance for drug design and mechanistic studies.

Aldehyde oxidases (AOXs) are homodimeric proteins belonging to the xanthine oxidase (XO) family of molybdenum containing enzymes. Each 150 kDa monomer contains a FAD redox cofactor, two spectroscopically distinct [2Fe-2S] clusters and a molybdenum cofactor (Moco) located within the protein active site. AOXs are characterized by broad range substrate specificity, oxidizing different aldehydes and aromatic N-heterocycles. Despite increasing recognition of its role in the metabolism of drugs and xenobiotics, the physiological function of the protein is still largely unknown. We have crystallized and solved the crystal structure of mouse liver aldehyde oxidase 3 (mAOX3) to 2.9Å. This is the first mammalian AOX whose structure has been solved. The structure provides important insights into the protein active center, and further evidence on the catalytic differences characterizing AOX and xanthine oxidoreductase (XOR). The mAOX3 3D structure combined with kinetic, mutagenesis data, molecular docking and molecular dynamics studies make a decisive contribution to understand the molecular basis of its rather broad substrate specificity.

The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org, under accession code 3zyv and r3zyvsf respectively.

Abbreviations: AOX, aldehyde oxidase; Moco, molybdenum cofactor; XOR, xanthine oxidoreductase; XDH, xanthine dehydrogenase; XO, xanthine oxidase; FAD, flavin adenine dinucleotide; MOP, D. gigas aldehyde oxidoreductase.

Aldehyde oxidases (AOXs, EC 1.2.3.1) are a small group of evolutionarily conserved proteins belonging to the family of molybdo-flavoenzymes along with xanthine oxidoreductase (XOR), the key enzyme in the catabolism of purines (1-3). In their catalytically active form, molybdo-flavoenzymes function as homodimers and require a molybdenum cofactor (Moco) as well as flavin adenine dinucleotide (FAD) to oxidize their substrates. AOXs and XORs are characterized by similar primary structures (approximately 50% amino acid identity) and are evolutionarily related enzymes (4-8). In fact, the extant complement of AOX genes evolved from an XOR ancestral precursor via a series of gene duplication and suppression/deletion events (3,9). In the animal kingdom, AOXs are present in virtually all
species from insects to humans. Different animal species contain a different complement of AOX genes encoding an equivalent number of AOX isoenzymes. In mammals, the two extremes are represented by certain rodents, such as mice and rats, which are endowed with four AOX genes, and by humans, whose genome is characterized by a single active gene. In mice, the four AOX loci (Aox1, Aox3, Aox4 and Aox3l1) form a cluster and map to a small region of chromosome 1 band c1. The products of the four mouse Aox genes are expressed in a tissue- and organ-specific fashion (1). The mouse AOX1 and AOX3 protein products are synthesized mainly in the liver (5). The richest source of AOX4 is the Harderian gland, a major structure located in the orbital cavity (4,10). Finally, AOX3l1 expression is restricted to the Bowman’s gland of the nasal mucosa (11). In these sites, the four AOXs are hypothesized to perform undefined tissue and organ specific functions, possibly acting on a different or overlapping set of substrates (1). The only functional gene present in humans is the ortholog of mouse Aox1 (6). The human AOX1 gene is located on chromosome 2p near two inactive pseudogenes, representing the vestiges of the mouse Aox3 and Aox3l1 orthologs (6). Large amounts of AOX1 are present in the cytosol of human hepatocytes, although detectable levels of the protein are also found in many other tissues (12).

Mammalian AOXs are characterized by broad substrate specificity and metabolize a wide range of endogenous and exogenous compounds (12). They oxidize organic molecules containing an aldehyde functionality into the corresponding carboxylic acid and hydroxylate numerous types of aza- and oxo-heterocycles. Given their broad substrate specificity, human AOX1 is considered to be the major cytosolic enzyme involved in phase I metabolism and inactivation of drugs as well as other xenobiotics (13). In other model organisms used for drug metabolism studies, the same role is played by other AOX isoenzymes. In mice and rats, the main drug-metabolizing enzyme is AOX3, as this is the prevalent form of AOX present in the liver. The significance of AOXs for the clearance of xenobiotics is predicted to increase in the near future (13).

Here, we report the structure of mouse AOX3, the first mammalian AOX to be crystallized. The crystallographic structure demonstrates that the catalytically active forms of mAOX3 and bXOR (14-16) have similar overall conformations, although significant structural differences are found in many functional domains, with particular reference to the substrate binding pocket and the Moco domain. Comparing the crystal structures of AOX and the related bXOR protein provided invaluable information as to the structural determinants responsible for the difference in substrate and inhibitor specificity. In addition, it shed light on possible differences in the catalytic mechanisms of the two types of enzymes. From an applied perspective, the structure of AOX1 or AOX3 will be instrumental in the generation of in silico methods for the prediction or validation of chemical structures acting as AOX substrates.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**

mAOX3 was purified from CD1 mouse liver as well as from a heterologous expression system from *E. coli*, as reported previously (17). The recombinant mAOX3 was expressed as an N-terminal fusion protein with a His6-tag. Both proteins were stored in 50 mM sodium phosphate pH 8.0, 300 mM NaCl at -80ºC until usage, without loss of activity. Final protein concentration was 10 mg/ml and 17.8 mg/ml, for the native and recombinant samples, respectively, determined using the BCA protein quantification assay (Sigma-Aldrich).

**Enzyme Assays**

Steady state kinetics were performed at 37ºC in 50 mM Tris-HCl pH 8.0 and 1 mM EDTA, using variable substrate (0–250 µM benzaldehyde, 0–50 µM phthalazine, 0–5 mM N1-methylnicotinamide and 0–600 µM phenanthridine) and purified mAOX3 (50–200 nM) concentrations. As electron acceptors, 100 µM of 2,6-dichlorophenolindophenol (DCPIP) or molecular oxygen (in the case of phenanthridine) were used in a final reaction volume of 500 µl. Enzyme activity was monitored at 600 nm for DCPIP and at 321 nm for phenanthridine. Inhibition studies of
mAOX3 were performed using 0–2 µM menadione, 0–2 µM norharmane, 0–50 µM raloxifene, (solubilized in dimethyl sulfoxide, with final solvent concentration of 1% v/v) and 0-750 µM benzamidine (solubilized in assay buffer) under standard assay conditions with phthalazine. Specific activity was calculated using molecular extinction coefficients of 21,400 M⁻¹ cm⁻¹ for mAOX3, 4775 M⁻¹ cm⁻¹ for phenanthridine and 16,100 M⁻¹ cm⁻¹ for DCPIP (18). Kinetic parameters were obtained by nonlinear fitting of the Michaelis-Menten equation or the appropriate inhibition equation [1] using R.build 2.12.00 (19).

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v = \frac{v_{\text{max}} [S]}{K_M + \frac{[I]}{K_{iC}} + [S] \left(1 + \frac{[I]}{K_{iu}}\right)}
\]

[1] Michaelis-Menten Equation with competitive (Kci) and uncompetitive (Kiu) inhibition term

Crystallization

The mAOX3 protein was pre-incubated with 10 mM dithiothreitol, for 1h at 4°C, and crystals with approximate dimensions 0.40x0.15x0.05 mm³ were reproducibly obtained at 20°C within three days, using 12-16% polyethylene glycol 8000, 0.1 M potassium phosphate pH 6.5, and 2 mM EDTA. Further details on protein crystallization were previously reported (17). The crystals were flash cooled in liquid nitrogen, using paratone oil as cryoprotectant, before transfer to a nitrogen stream. The majority of the crystals diffracted to poor resolution (~6Å) at beamline ID14-1 of the European Synchrotron Radiation Facility (ESRF, Grenoble – France) but for some of the crystals, diffraction could be improved to a resolution beyond 3Å using the beamline automatic annealing procedure.

Data Collection and Processing

An 180° data set of a native mAOX3 crystal was collected at ID 14-1 (ESRF), at 0.934Å wavelength. The crystal belongs to space group P1, with unit cell dimensions a = 90.9 Å, b = 135.3 Å, c = 147.4 Å, and α = 78.2°, β = 77.7°, γ = 89.9°, and two dimers in the unit cell (Table 1). All data were processed using iMosflm 1.0.4 (20) or XDS (21), to 2.9Å.

When processing the data with iMosflm the mosaicity value became too unstable, probably due to crystal anisotropy, until the program crashed. The data were then processed with fixed values of mosaicity ranging from 0.3 to 1.5, in 0.1 increments. The dataset with the best overall statistics was the one with a mosaicity value of 1, which was the one used for refinement. We also processed the data with XDS that gave a mosaicity value of 0.349. However, the electron density maps were clearly better when using data processed with iMosflm.

Because the data were very anisotropic some data were not usable due to spot overlapping, and some images had to be discarded. This explains why the final data set is only 89.8% complete (77.3% in the highest shell).

Structure Solution and Refinement

The structure was solved by molecular replacement with Phaser 2.1.4 (22), using the structure of bovine xanthine dehydrogenase (PDB ID: 3bdj) as a search model, after omitting all the cofactors and solvent molecules. Although the initial density was very poor, clear positive density could be observed at the expected position of the Moco, the FAD and the two FeS cofactors, indicating the correctness of the solution. Four monomers were found in the unit cell. Density modification with four-fold non-crystallographic symmetry averaging was performed using DM (CCP4) (23). The resulting electron density improved dramatically, allowing rebuilding one chain and applying NCS operators to transform it to the three other chains. Because the data was of relatively low quality, NCS restraints were maintained throughout the refinement. The reported resolution of the data is 2.9Å. The correlation coefficient between half data sets (24) for 2.9Å resolution is CC1/2 = 0.911 (number of pairs = 1214). Initial rounds of refinement were carried out in Refmac 5.5 (22), omitting all the cofactors. After several rounds of rebuilding and refining in Coot (25), the R/Rfree leveled off at about 26/33%. Switching to Phenix.refine (26) lowered the Rfree to ~30%. All the following steps were carried out in Phenix, with TLS...
refinement (8 TLS groups per monomer) and NCS restraints with automatic weighting. In the final stages of refinement, over 250 residues (~5%) would keep moving out of the Ramachandran most favored conformations. Ramachandran restraints were also added to the refinement protocol. Waters were automatically added by Phenix.refine and manually inspected in Coot. The final model contains four chains (A-D) and residues S7 to V1334, with all the cofactors present. Some loops located at the surface, linker I (P169 - T199; E227 – N231); FAD domain (G399 – I404); linker II (D538 – I545; L558 – G563); and Moco domain (R1290 – W1296; Q1321 – P1329) had no visible electron density and were omitted from the model. The deposited model contains 5018 protein residues, 654 water molecules and one sodium ion per monomer (see Table 1 for structure refinement and model statistics). The residues with disordered side chains were stubbed at Cβ.

RESULTS AND DISCUSSION

Overall Structure

The overall topology of mouse AOX3 and mammalian XORs are similar. In the homodimer, each monomer comprises 1335 residues, and can be divided into three major domains involved in binding to the cofactors (Fig.1A). The small N-terminal domain (domain I; 20 kDa, Met1-Pro169) harbors the two FeS clusters, and connects to the FAD-binding domain II (40 kDa, Thr232-Leu534), through a poorly conserved linker I region (Ser170-Asn231). The C-terminal and largest domain III (90 kDa, Leu576-Ala1335) contains the Moco binding site, with the Mo catalytic center located at the bottom of a ~15Å deep and wide pocket. All these sub-domains share a similar fold and while sub-domains III.1 and III.4 are involved in dimerization contacts. The residues with disordered side chains were stubbed at Cβ.

Iron/Sulfur Domain

The two iron-sulfur clusters are located in domain I and are designated FeSI and FeSII, according to their distinct EPR signals. Domain I can be further divided into two sub-domains: the N-terminal sub-domain with [2Fe-2S] II (Met1-Val192) and the second sub-domain (Glu93-Pro169), which binds [2Fe-2S] I. The iron saturation of the purified protein from mouse liver was determined to be 53% (17). However, the two [2Fe-2S] clusters were clearly visible in the initial electron density maps, and were refined with full occupancy (Fig.2). All the atoms in the two clusters present B factor values within the range of their surrounding atoms. This shows that only the active portion of the protein with a full occupancy of the FeS clusters has crystallized.

FAD Domain

The FAD domain can be divided into two sub-domains, comprising residues Thr232-Ser419 and Ser420-Leu534. The FAD cofactor is mainly coordinated by the first sub-domain with residues Pro263, Leu264, Asn268, Thr269, Tyr270, Ser354 and Leu411 establishing hydrogen bonds with the dinucleotide, and residues His358 and the Asp367 contacting the riboflavin (Fig.3). The isoalloxazine ring is stacked between two hydrophobic side chains: Leu344 (Phe in XOR) and Phe438 (Ile in XOR), the latter laying approximately parallel.

Moco Domain

The C-terminal domain III contains the Moco binding site, which is located at the bottom of a wide groove, leading to the catalytic center. As in related enzymes, domain III can be structurally divided into four sub-domains that include non-continuous stretches of the polypeptide chain: sub domain III.1 (Leu576-Pro698 and Glu745-Gly843), sub domain III.2 (Met699-Gln744 and Arg844-Phe964), sub domain III.3, (Asp965-Lys1009 and Phe1160-Val1334) and sub domain III.4 (Phe1010-Val1159). All these sub-domains share a similar fold and while sub-domains III.2 and III.3 are exposed to the solvent, sub-domains III.1 and III.4 are involved in dimerization contacts.

Comparison With Structurally Related Proteins
Crystal structures from several members of the molybdo-flavoenzyme family have been reported in the past few years. The first structure reported was the *D. gigas* aldehyde oxidoreductase (MOP) at a resolution of 2.25Å (later refined to 1.28Å) (27,28). Although mAOX3 and MOP share low sequence identity (23%), their overall fold is very similar. MOP lacks the FAD domain but its global structure superimposes to mAOX3 domains I and III (rmsd of 1.83Å for 614 Cα atoms). The crystal structure of xanthine dehydrogenase (XDH) isolated from the purple bacterium *R. capsulatus* (*RcXDH*) was published later at 2.7Å resolution (29). Unlike mAOX3 which is a homodimer, *RcXDH* is a homotetramer (αβ)2. The two FeS clusters and the FAD are bound to the XdhA subunit, and Moco is in the XdhB subunit. In spite of a different subunit composition, the overall fold and the arrangement of the cofactors are similar in mAOX3 and *RcXDH* and the two structures superimpose with an rms deviation of 1.09Å for 913 Cα atoms. The main difference between the two proteins is the absence of the linker II region (Lys535 -Pro575 in mAOX3) in the bacterial enzyme. The crystal structures of the bovine milk dehydrogenase (XDH) and oxidase (XO) forms of XOR were initially reported at a resolution of 2.1Å and 2.5Å, respectively (15), and later at higher resolution (1.65Å, (30)). The protein is synthesized in the dehydrogenase form, but can be readily converted into the oxidase form by oxidation of sulfhydryl residues (a reversible process), or by proteolysis (irreversible) (15,31,32). The main difference between the two protein forms is the binding of NAD+, which occurs only in the XDH form. mAOX3 and bXOR are characterized by a similar overall fold and cofactor arrangement, with an rms deviation of 1.26Å for 2358 Cα atoms. The major differences between the two enzymes concern residues involved in the mechanism of XDH to XO inter-conversion and crucial residues located within the protein active site. On one hand, lack of residues leading to a flexible loop around the FAD site might explain the fact that AOXs are pure oxidases and do not use NAD+ as the final acceptor of reducing equivalents. On the other hand, conserved amino acid residues in each enzyme at the active site are different and may explain the large differences in substrate and inhibitor specificity between mAOX3 and bXOR. In fact, similar to all the other mammalian AOXs, mAOX3 oxidizes a different set of substrates in comparison to XORs.

**XDH/XO Inter-conversion Domain**

The reversible XDH to XO inter-conversion involves the formation of a new disulfide bond, between two cysteine residues (Cys535 and Cys992), which are not conserved in mAOX3 being substituted by Tyr542 and Phe997, respectively. Both reversible and irreversible inter-conversion of XDH into XO requires structural rearrangement of an eleven-residue loop, referred to as the “variable loop” (Gln423-Lys433 in bovine XOR), which is in close proximity to the FAD cofactor. The amino acid sequences of the loop are rather divergent in mAOX3 and bXOR (Fig.4). The loop movement leads to a change in the electrostatic environment around the FAD cofactor influencing its redox potential (which changes from -410 mV in XDH to -234 in XO (30)). In XO, the loop position blocks access of NAD+ to its binding site near FAD. As a consequence, the XO form does not bind NAD+ and uses oxygen as the final electron acceptor instead. In mAOX3 the final electron acceptor is always oxygen, and we anticipated that the structure of the corresponding “variable loop” would be similar to the one observed in the XO form of XOR. In fact, the FAD cofactor exhibits similar redox potentials in XO (-234 mV) and rabbit AOX (212 mV) (30,33).

Surprisingly, the mAOX3 loop turned out to be totally superimposable with the one present in the XDH form (Fig.5). The 11 residues that comprise the loop are conserved in bovine and human XOR but not in AOXs. Of the 11 residues in the highly charged XOR loop (Q423ASRREDDIAK433) only 4 are conserved in mAOX3 (Q430APRQQNAFAT440) or human AOX1 (Fig.5). Interestingly, while six out of the eleven residues are charged in XOR, only the conserved Arg433 is charged in AOXs. In addition, XOR Arg427, which is substituted by Gln434 in mAOX3, belongs to the cluster of conserved amino acid residues (Arg335, Trp336, Arg427, Phe549, XOR numbering) responsible for the switching between XO and XDH(31,34). These residues are also not conserved in...
Arg427 corresponds to a Gln in AOXs and to an Asp in RcXDH. Trp336 (bXOR numbering) plays a major role in the inter-conversion mechanism and is substituted by a Thr in AOXs and by an Arg in RcXDH. All these different structural elements responsible for the XDH to XO conversion, are absent in the mouse and human AOXs, and are probably related to the inability of an inter-conversion mechanism by AOX.

Active Site and Substrate Access Funnel

All residues in the active site of mAOX3 have well defined electron density, but the limited resolution of the data (2.9Å) did not allow to unambiguously identify all Mo coordinating ligands. Since these are known to be the same as in XOR they were defined according to the crystal structure of homologous bXDH (2.0Å resolution) (35). As in all members of the molybdo-flavoenzyme XOR family, Mo adopts a distorted square pyramidal coordination geometry, with the two sulfur atoms from the dithiolene in the equatorial plane (Mo-S distances: 2.4 to 2.7Å - values for the 4 molecules in the a.u.). The two other equatorial positions include the catalytically essential sulfur atom (=S) (not clear in the structure due to insufficient resolution) and the equatorial OH group (Fig.2) that is the source of the transferred oxygen. An oxygen atom (=O) occupies the apical position. When comparing the active site structures of mAOX3 and bXDH (Fig.6, Table 2), important conserved residues are Gln772, Glu1266, Phe919 and Phe1014 (mAOX3 numbering). The two conserved Phe residues (Phe919 and Phe1014) are responsible for the orientation of substrate and inhibitor molecules in XORs through stacking interactions (36). As in bXOR, Gln772 makes a hydrogen bond with the apical oxygen ligand and Glu1266 is in contact with the hydroxyl ligand. This strictly conserved glutamate has been shown to be crucial for catalysis in XOR enzymes, playing a fundamental and direct role in the reaction mechanism initiating it as an active site base (28). In our studies Glu1266 was exchanged by a glutamine, and variation of this residue resulted in a complete loss of activity with different N-heterocyclic compounds as substrates (Table 3). However, a residual activity with benzaldehyde was obtained (60% reduction of activity in comparison to the wild type enzyme), which might be explained by the higher electrophilicity of the carbonyl carbon atom in aldehydes, as compared to the N-heterocyclic compounds which are substrates of XOR. Other key residues located in the catalytic core are not conserved in AOX3 and XOR, and were expected to be responsible for most of the differences in substrate binding, specificity, and catalysis. Of particular relevance are the charged residues Glu802 and Arg880 in bXDH that correspond to Ala807 and Tyr885 in AOX3, respectively (Fig.6, Table 2). It was suggested from the XDH crystal structure that Glu802 and Arg880 are essential for the correct positioning/orienting and/or activation of the substrate, through the establishment of hydrogen bonds and electrostatic interactions (36), as found in all structures of XOR complexed to inhibitors or substrates (29,37-40). Glu802 in bXDH is replaced by a valine in all AOX1 orthologs and by an alanine (Ala807) in mAOX3. This might allow the accommodation of bulkier substrates at the protein active site. In addition, an uncharged residue contributes to the necessary environment for the binding of differently charged compounds. The conserved Arg880 of bXDH is replaced by methionine in all AOX1 orthologs, a phenylalanine in all mAOX4 and all mAOX3l1 orthologs and by a Tyr885 in mAOX3. As shown in the crystal structure, the aromatic ring of this amino acid points outwards from the substrate binding cavity, possibly leading to a further increase in the ability of the mAOX3 active site to accommodate bulkier substrates (Fig.6). Mutagenesis studies of the two XDH residues, (Glu803 and Arg881 in human XOR numbering), were performed by Yamaguchi et al(41) in an attempt to alter the substrate specificity from the XOR to the AOX type. Two mutants (E803V, R881M) acquired the ability to oxidize some recognized AOX substrates, while their capacity to oxidize hypoxanthine or xanthine was impaired. The authors predicted that the substrate specificity of XOR should change completely to AO by the double mutant E803V/R881M but the latter could not be purified. In contrast, the exchange of these residues in mAOX1 (V804E, M884R and
V804E/M884R) to the ones present in XOR gave no rise in activity with xanthine or hypoxanthine as substrates (42). This finding implicated that more residues in the active center are involved in substrate binding and conversion than the two residues directly involved in substrate binding. To study the influence of these residues in mAOX3 activity, Ala807 was exchanged to a valine and Tyr885 to a methionine, the conserved residues in all AOX enzymes. The A807V variant of mAOX3 did not affect the kinetic constants with smaller substrates like benzaldehyde or phthalazine (Table 3). However, the affinity for bulkier substrates like phenanthridine was decreased, while the catalytic efficiency was slightly raised. Upon conversion of Tyr885 to methionine the kinetic constants also remained mainly the same, with small hydrophobic substrates like benzaldehyde and phthalazine (Table 3). These substrates were converted with unchanged rate constants and only a minor increase in $K_M$ was observed, possibly due to the smaller hydrophobic pocket in the Y885M variant. On the other hand, bulkier substrates like phenanthridine or more charged substrates like N1-methylnicotinamide were converted with higher efficiency. This may be due to the higher flexibility of the methionine side chain in comparison to tyrosine that may facilitate the binding of these substrates. The effects were much more pronounced in the double variants (Table 3).

Another important charged residue close to the active site of mAOX3 is Lys889, which is conserved in all AOX enzymes and replaced by His in XORs (His884). Lys889 lies ~10Å away from the Mo center and ~6Å away from Glu1266. This lysine adopts a position similar to the conserved Arg880 of XDH (Fig.6). Molecular dynamics simulations demonstrated that substrate docking into the active site causes Lys889 to move from its original position, establishing new interactions with Glu1266 and/or the substrate itself (Fig.2 SI). Lys889 is located close to the funnel pathway and limits the access of larger substrates to the protein active site. Our results show that changing Lys889 into a histidine lead to a 2–3 fold decrease in the catalytic efficiency using benzaldehyde and phthalazine as substrates, while the $K_M$ values remained the same (Table 3). This can be explained by the impaired interaction of His889 with the substrate (Fig.2 SI). Consequently, the nucleophilic attack of the Mo-OH moiety or the stabilization of the transition state is affected. This explanation is in agreement with the finding that the conversion rate constant of phenanthridine is not affected since it does not interact with Lys889 via an oxygen or nitrogen. Additionally, it is concluded that bulkier substrates or substrates unable to interact with Lys889 via an oxygen or nitrogen (such as phenanthridine or N1-methylnicotinamide, Table 3) also show a lower $K_M$ value in the histidine variant based on its slightly smaller side chain. Structural features along the substrate funnel may also account for the differences in substrate specificities between mAOX3 and XOR. In AOX3 the funnel is ~20Å wide at the surface, and becomes tighter towards the Mo catalytic center, where it is approximately 8Å wide (Fig.7). Several residues along the funnel are divergent in the two proteins, particularly: Arg717, Asp878, Glu880, Leu881 and Thr1081 of AOX3 are replaced by Leu712, Leu873, His875, Ser876 and Pro1076 in XOR (Table 2). Comparison of mAOX3 and bXDH molecular surfaces indicates that the shape and width of the substrate access funnel is more anionic and wider in the case of mAOX3 (Fig.7). Taken together our results are consistent with the entrance of larger and bulkier substrates for AOXs enzymes in contrast to XORs.

Inhibition by Benzamidine, Menadione, Norharmane and Raloxifene

Inhibition studies of mAOX3 with raloxifene revealed a mixed type inhibition with calculated inhibition parameters of $K_{ic} = 12.2 \pm 1.8 \ \mu M$ and $K_{iu} = 59.1 \pm 5.4 \ \mu M$ (Table 4) (43). The inhibition mechanism is in agreement with the two binding modes found in molecular docking studies (see below and SI). The higher rigidity of the complex in binding mode (A) can be explained by a stronger interaction, giving rise to a 5-fold lower competitive constant $K_a$ in comparison to $K_{ic}$. The inhibition constants obtained with purified mAOX3 are 5 orders of magnitude higher than the value previously reported with human liver cytosol (44). The strong inhibition in liver extracts might imply that a more effective raloxifene metabolite is
generated in the liver. In contrast, inhibition studies of purified mAOX3 with menadione showed an uncompetitive inhibition with a $K_{ic} = 1.25 \pm 0.04 \, \mu M$ (Table 4). This value is comparable to studies by Barr and Jones (45) using human liver lysates, who determined a mixed-type inhibition with $K_{ic} = 0.75 \pm 0.18 \, \mu M$ and $K_{iu} = 0.12 \pm 0.0 \, \mu M$. The difference in the inhibition mechanism might be explained by the fact that we used DCPIP instead of oxygen as terminal electron acceptor and DCPIP was shown to act as an inhibitor itself. Thus, the enzyme-DCPIP complex might prevent menadione binding in a competitive manner. Benzamidine was shown to be a competitive inhibitor of rat and bovine XO (46) with $K_{ic}$ values of 30 mM and 3 mM, respectively. Benzamidine inhibited native rat AOX and mAOX3 at similar millimolar concentrations (47,48). An estimated $K_{ic}$ of 25 $\mu M$ can be calculated from the data of Vila et al., using a competitive Michaelis-Menten model. Our data agree with these results and shows a competitive inhibition with a $K_{ic} = 22.5 \pm 0.8 \, \mu M$ (Table 4). Norharmane was shown to inhibit the 2-OH-pyrimidine:ferricyanide reaction of native mAOX3 in a non-competitive manner with $K_{ic} = 0.68 \, \mu M$ (47). Our data show a competitive inhibition with a $K_{ic} = 0.18 \pm 0.01 \, \mu M$ (Table 4). The differences between the two inhibition modes are explained by the use of ferricyanide as final electron acceptor in the former study, which does not interfere with the Mo-site, whereas DCPIP interacts with the Mo-site (49). In conclusion, raloxifene shows a different behavior in human liver lysate and purified mAOX3, showing a possible metabolism of raloxifene in the liver, which results in a more.

Molecular docking studies using raloxifene identified two different binding modes. In mode (A) (Fig.8A) is one of the phenol moieties that enters into the pocket while in mode (B), is the piperidyl group that takes its place (Fig.8B). For this reason, we performed molecular dynamics studies on the two raloxifene binding modes. As expected, in mode (A) when the inhibitor enters the active site via the phenol there is less variation in its position. Raloxifene interacts with the pocket via a hydrogen bond with Glu1266 and aromatic stacking interaction with Phe919. In mode (B) there is only a van der Waals interaction between the piperidyl group of the inhibitor and Phe919 in the Moco pocket.

**Reaction Mechanism**

The presence of some highly conserved residues at the catalytic center (Glu1266, Phe919, Gln 772, in mAOX3 numbering), as well as the type of reaction catalyzed, suggests that the reaction mechanism for both AOXs and XORs is similar, though not identical, since AOXs exhibit much broader substrate specificity. The reaction mechanism that we propose for AOXs with the substrate phthalazine is illustrated in Fig.9. The reaction starts by the nucleophilic attack of the activated Mo-OH ligand (activated by Glu1266) on the carbon atom of the substrate (carbon atom adjacent to an aromatic nitrogen atom) (i). Concomitantly, hydride transfer occurs to the sulfido ligand and an intermediate species is formed. This intermediate is stabilized by hydrogen bonding interactions with residues of the active site, namely Glu1266 and Lys889. This concerted mechanism is favored by experimental evidence with substituted N-heterocycles as well as by recent DFT calculations (50). In step (ii), the product is released from the reduced Mo site and a water molecule replenishes the vacant coordination position. The reaction cycle is closed (iii) once Mo is re-oxidized and the two reducing equivalents are transferred to molecular oxygen via the two [2Fe-2S] and the FAD cofactor. It is expected that the proton transfer from the hydroxyl ligand of Mo to Glu1266 disrupts the interaction of Glu1266 with Lys889. This forces Lys889 to move away from Glu1266 and the Mo center where it can interact with the product before the latter is released from the active site.

**Conclusions**

The present crystal structure constitutes the first of an aldehyde oxidase protein, having major importance to clarify its substrate specificity. When compared to XORs, the broader range of substrates as well as the kinetic behavior of AOXs can now be explained. The mAOX3 overall structure is similar to XORs but shows marked differences around the FAD.
Detailed analysis of the protein active center in addition to docking and simulation studies have shown that the different substrate specificities between AOXs and XORs are related to the non-conservation of several protein residues, not only in the active site as expected, but also in the active site funnel pathway. Docking studies on the mAOX3 structure enabled to reveal specific inhibitor interactions. For the highly potent human AOX1 inhibitor raloxifene, two binding modes were predicted by the computational studies, in agreement with our kinetic data. However, a different inhibition mechanism was found in purified mAOX3 in comparison to previous studies in whole-cell lysates. Site-directed mutagenesis of mAOX3 combined with molecular docking studies for several types of substrates, revealed new protein-substrate interactions, highlighting the role of the highly conserved Lys889 in substrate binding and its putative involvement in the reaction mechanism of aldehyde oxidases.

Acknowledgements. This work was financially supported by: the Portuguese Science and Technology Foundation (FCT-MCTES) through project PTDC/BIA-PRO/118377/2010, and grant SFRH/BD/37948/2007 (CC); and by the Cluster of Excellence ‘Unifying Concepts in Catalysis’ (SL and MM) coordinated by the Technische Universität Berlin and funded by the Deutsche Forschungsgemeinschaft. The exchange of researchers among laboratories was funded by the DAAD-GRICES program (MJR and SL). Grants from the Associazione Italiana per la Ricerca contro il Cancro (AIRC), the Fondazione Italo Monzino and the Negri-Weizmann Foundation to EG are also acknowledged. The authors would like to thank the ID14-1 and ID23-2 staff of the ESRF (Grenoble, France) for assistance during data collection.

REFERENCES

52. DeLano. *Schrödinger, LLC*
Table 1 - Data collection, structure refinement and model building statistics. Values in parenthesis correspond to the highest resolution shell. $R_{\text{work}} = \frac{\sum ||F_{\text{calc}}|| - ||F_{\text{obs}}||}{\sum ||F_{\text{obs}}||} \times 100$, where $F_{\text{calc}}$ and $F_{\text{obs}}$ are the calculated and observed structure factor amplitudes, respectively. ($R_{\text{free}}$ is calculated for 5% of the reflections randomly chosen for each data set).

<table>
<thead>
<tr>
<th>Crystal sample</th>
<th>mAOX3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beamline (ESRF)</td>
<td>ID14-1</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.934</td>
</tr>
</tbody>
</table>
| Unit cell parameters | $a = 90.9$, $b = 135.3$, $c = 147.4$
| | $\alpha = 78.2$, $\beta = 77.7$, $\gamma = 89.9$
| Space group | P1 |
| Number of molecules in the AU | 4 |
| Matthews coefficient (Å³/Da) | 3.23 |
| Solvent content (%) | 61.5 |
| Resolution limits (Å) | 49.9 - 2.9 |
| No. of observations | 219268 (20892) |
| No. of unique observations | 133319 (16784) |
| Multiplicity | 1.6 (1.2) |
| Completeness (%) | 89.8 (77.3) |
| $R_{\text{pim}}$ (%) | 5.6 (13.9) |
| $R_{\text{syn}}$ (%) | 5.7 (33.8) |
| $I/\sigma (I)$ | 9.6 (3.6) |
| CC₁/₂ outer shell (# of pairs) ((24)) | 0.911 (n=1214) |
| $R_{\text{free}}$ (%) | 27.02 |
| $R_{\text{factor}}$ (%) | 24.37 |
| Number of water molecules | 654 |
| Average B factor for all atoms (Å²) | 42.7 |
| RMSD from ideal geometry | |
| Bond lengths (Å) | 0.013 |
| Bond angles (°) | 1.21 |
Table 2 - Comparison of relevant residues (conserved and non-conserved) of mAOX3 (and hAOX1) (blue) and bovine XDH (green), present in the substrate-binding pocket and in the substrate access channel.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Location</th>
<th>mAOX3 (hAOX1)</th>
<th>bXDH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conserved</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Active site</td>
<td>Gln772 (Gln)</td>
<td>Gln767</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phe919 (Phe)</td>
<td>Phe914</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phe1014 (Leu)</td>
<td>Phe1009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glu1266 (Glu)</td>
<td>Glu1261</td>
</tr>
<tr>
<td><strong>Non-conserved</strong></td>
<td></td>
<td>Ala807* (Val)</td>
<td>Glu802</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyr885* (Met)</td>
<td>Arg880</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lys889* (Lys)</td>
<td>His884</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pro1015 (Gly)</td>
<td>Thr1010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyr1019 (Ala)</td>
<td>Leu1014</td>
</tr>
<tr>
<td></td>
<td>Substrate Access Channel</td>
<td>Arg717 (Arg)</td>
<td>Leu712</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asp878 (Glu)</td>
<td>Leu873</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glu880 (Leu)</td>
<td>His875</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leu881 (Phe)</td>
<td>Ser876</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thr1081 (Ile)</td>
<td>Pro1076</td>
</tr>
</tbody>
</table>
Table 3 - Kinetic parameters of mAOX3 variants obtained at 37 °C pH = 8.00 using 100 µM DCPIP (or oxygen in case of phenanthridine) as electron acceptor.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Benzaldehyde</th>
<th>N1-methylnicotinamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k_{cat} [min^{-1}]</td>
<td>K_{m} [µM]</td>
</tr>
<tr>
<td>mAOX3-WT</td>
<td>41.9 ± 0.8</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>E1266Q</td>
<td>10.2 ± 0.5</td>
<td>86.3 ± 11.3</td>
</tr>
<tr>
<td>A807V</td>
<td>41.0 ± 0.3</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Y885M</td>
<td>57.4 ± 0.8</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>A807V/ Y885M</td>
<td>43.6 ± 1.1</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>K889H</td>
<td>19.2 ± 0.5</td>
<td>2.3 ± 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Phenanthridine</th>
<th>Phthalazine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k_{cat} [min^{-1}]</td>
<td>K_{m} [µM]</td>
</tr>
<tr>
<td>mAOX3-WT</td>
<td>51.7 ± 0.6</td>
<td>32.3 ± 1.4</td>
</tr>
<tr>
<td>A807V</td>
<td>315.0 ± 5.2</td>
<td>149.5 ± 5.8</td>
</tr>
<tr>
<td>Y885M</td>
<td>266.9 ± 66.5</td>
<td>33.4 ± 11.6</td>
</tr>
<tr>
<td>A807V/ Y885M</td>
<td>218.0 ± 17.1</td>
<td>11.8 ± 1.7</td>
</tr>
<tr>
<td>K889H</td>
<td>79.9 ± 0.7</td>
<td>16.1 ± 0.6</td>
</tr>
</tbody>
</table>

1 terminal electron receptor was 100 µM DCPIP
2 terminal electron receptor was molecular oxygen in air saturated buffer
n.d. none detectable
Table 4 - Kinetic parameters of phthalazine:DCPIP reaction in the absence or presence of benzamidine, menadione, norharmane and raloxifene obtained at 37 °C pH = 8.00 using 100 µM DCPIP as electron acceptor. Only the appropriate inhibition parameters ($K_{ic}$ represents the competitive component and $K_{iu}$ the uncompetitive component) were included in the fit to Equation [1].

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$k_{cat} \text{ [min}^{-1}\text{]}$</th>
<th>$K_i \text{ [µM]}$</th>
<th>$K_{ic} \text{ [µM]}$</th>
<th>$K_{iu} \text{ [µM]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzamidine</td>
<td>40.7 ± 0.3</td>
<td>1.3 ± 0.0</td>
<td>22.5 ± 0.8</td>
<td>–</td>
</tr>
<tr>
<td>menadione</td>
<td>38.6 ± 0.4</td>
<td>1.6 ± 0.1</td>
<td>–</td>
<td>1.25 ± 0.04</td>
</tr>
<tr>
<td>norharmane</td>
<td>49.0 ± 0.6</td>
<td>2.0 ± 0.1</td>
<td>0.18 ± 0.01</td>
<td>–</td>
</tr>
<tr>
<td>raloxifene</td>
<td>43.7 ± 0.8</td>
<td>1.8 ± 0.1</td>
<td>59.1 ± 5.4</td>
<td>12.2 ± 1.8</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

FIGURE 1 - A) Ribbon representation of the mAOX3 crystal structure: left monomer in grey, and right monomer with the three different domains colored as: domain I in red (residues Met1-Pro169); domain II in green (residues Thr232-Leu534) and domain III in blue (residues Leu576-Val1335). Domain III is separated from the FAD domain by a linker II region (Lys535-Pro575). The linker regions are represented in dark grey (linker I Ser170-Asn231). The two mouse AOX3 monomers are tightly bound, with the majority of contacts established by residues present in the domain containing the Moco binding site. The molybdenum atoms from the two monomers are more than 50Å apart and most likely, the two subunits work independently as shown previously for the *Rhodobacter capsulatus* xanthine dehydrogenase (51). Homodimer approximate dimensions are 150Åx90Åx70Å. B) Arrangement and distances between the different protein cofactors: MPT, the two distinct [2Fe-2S] centers and FAD.

FIGURE 2 - Simulated annealing omit electron-density map contoured at 1.0 σ, and superimposed with the final refined model for [2Fe-2S] I, [2Fe-2S] II and molybdopterin monophosphate cofactor (MPT) (monomer A).

FIGURE 3 – LIGPLOT representation of mAOX3 environment in the FAD binding site. N and O atoms are represented as blue and red balls, respectively. Ligand bonds are in purple, non-ligand bonds are in light brown and hydrogen bonds are green dashed lines. Non-ligand residues in hydrophobic contacts with the ligand, are presented by red semi-circles with radiating spokes.

FIGURE 4 - Comparison of the amino acid sequences of mouse AOX3, human AOX1, bovine XDH, human XOR, and *R. capsulatus* XDH. The mAOX3 [2Fe-2S] I and II binding Cys residues are marked as (+) and (*) respectively. Four conserved active site residues are marked in blue, and non-conserved active site residues are marked in green. Residues in the substrate access channel are marked in orange. The “variable loop” of the FAD domain is marked in red. Black boxes indicate identical residues, and grey boxes similar residues. (Alignment was created with CLC Sequence viewer Rev 6.5.4.).

FIGURE 5 - Superposition of mAOX3 (yellow), bXDH (grey) and bXO (blue) at the FAD binding site, viewed from the solvent. The FAD and the [2Fe-2S] II are represented color coded, and correspond to the mAOX3 structure. Note the change in the “variable loop” (residues Gln423-Lys433, in bovine XDH numbering) between XO and XDH forms. The FAD molecule occupies a vast area within the protein, with the isoalloxazine ring in close proximity to the solvent accessible area, and pointing towards the FeSII center. The FAD cofactor is 9.0Å away from the FeSII, while the distance from the exocyclic NH2 of the pterin to the nearest FeSI is 5.1Å.

FIGURE 6 – Active site comparison between mAOX3 (yellow) and bovine XDH (grey). Outlined are the most important non-conserved residues (mAOX3 numbering). Also present is the XDH inhibitor oxipurinol (OXI), and the conserved XDH and mAOX3 residues Phe919, Phe1014 and Glu1266.

FIGURE 7 – Surface representation of the funnel leading to the active site in mAOX3 (A) and bovine XDH (B). The Mo atom is seen at the end of the funnel as a green sphere. The entrance is much wider in the case of mAOX3, with a narrow constriction closer to the Mo active site. Electrostatic surface potentials calculated using the Delphi program, and represented in PyMol (52) (surface potential color from -0.10 V, negatively charged in red, to +0.10 V, positively charged in blue).

FIGURE 8 – Molecular docking studies with the inhibitor raloxifene; binding modes (A) and (B).
FIGURE 9 – General reaction mechanism for aldehyde oxidases exemplified for the substrate phthalazine (mAOX3 residue number).

FIGURES

FIGURE 1
FIGURE 3
FIGURE 4
FIGURE 5

XDH/XO: 423QASRREDDIAK433
mAOX3: 430QAPRQQNAFT440
FIGURE 6
FIGURE 9
The First Mammalian Aldehyde Oxidase Crystal Structure: Insights Into Substrate specificity

Catarina Coelho, Martin Mahro, José Trincão, Alexandra T. P. Carvalho, Maria João Ramos, Mineko Terao, Enrico Garattini, Silke Leimkühler and Maria João Romão

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