Site Directed Mutagenesis of Amino Acid Residues at the Active Site of Mouse Aldehyde Oxidase AOX1

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Abstract

Mouse aldehyde oxidase (mAOX1) forms a homodimer and belongs to the xanthine oxidase family of molybdoenzymes which are characterized by an essential equatorial sulfur ligand coordinated to the molybdenum atom. In general, mammalian AOs are characterized by broad substrate specificity and an yet obscure physiological function. To define the physiological substrates and the enzymatic characteristics of mAOX1, we established a system for the heterologous expression of the enzyme in Escherichia coli. The recombinant protein showed spectral features and a range of substrate specificity similar to the native protein purified from mouse liver. The EPR data of recombinant mAOX1 were similar to those of AO from rabbit liver, but differed from the homologous xanthine oxidoreductase enzymes. Site-directed mutagenesis of amino acids Val806, Met884 and Glu1265 at the active site resulted in a drastic decrease in the oxidation of aldehydes with no increase in the oxidation of purine substrates. The double mutant V806E/M884R and the single mutant E1265Q were catalytically inactive enzymes regardless of the aldehyde or purine substrates tested. Our results show that only Glu1265 is essential for the catalytic activity by initiating the base-catalyzed mechanism of substrate oxidation. In addition, it is concluded that the substrate specificity of molybdo-flavoenzymes is more complex and not only defined by the three characterized amino acids in the active site.

Introduction

The molybdenum cofactor (Moco) is found in a class of widely distributed proteins collectively known as molybdoenzymes. Moco containing enzymes are divided into three separate groups on the basis of structure, cofactor and spectroscopic characteristics: the dimethylsulfoxide (DMSO) reductase, the xanthine oxidase, and the sulfite oxidase family [1]. Members of the xanthine oxidase family comprise xanthine dehydrogenase (XDH, EC 1.17.1.4), xanthine oxidase (XO, EC 1.17.3.2), and aldehyde oxidase (AO, EC 1.2.3.1). All these proteins are characterized by an equatorial sulfur ligand at the Moco essential for the enzymatic activity [2], and belong to the class of complex molybdo-flavoenzymes (MFEs) containing two nonidentical [2Fe-2S] clusters and FAD as additional cofactors [3,4].

Vertebrate xanthine oxidoreductases (XOR) are the products of single orthologous genes and are the key enzymes in the catabolism of purines, oxidizing hypoxanthine to xanthine and xanthine to the terminal catabolite uric acid, with the concomitant reduction of NAD⁺ (XDH) or O₂ (XO) [5].

The gene family of vertebrate AOs is much more complex and the corresponding protein products have been the object of fewer studies. While the human genome is characterized by a single orthologue, and three highly related genes named AO homologue-1 (AOH1), -2 (AOH2), and -3 (AOH3) [4,6–8]. In other vertebrates and mammals the number of functionally active loci varies, as a consequence of gene duplication and suppression events [4]. The multiple AO gene cluster consists of the human AOX1 orthologue, and three highly related genes named AO homologue-1 (AOH1), -2 (AOH2), and -3 (AOH3) [4,6–8]. In other vertebrates and mammals the number of functionally active loci varies, as a consequence of gene duplication and suppression events [4]. The multiple AO isoforms are expressed tissue-specifically [4,7], and may recognize distinct substrates and carry out different physiological tasks. The overall level of amino acid identity between AO and XOR proteins is approximately 50%, which indicates that the two proteins originated from a common ancestral precursor [3].

While the biochemical function of XOR is well established, the biochemical and physiological functions of AO are still largely obscure [4]. Monogenic deficits of mammalian AO isoforms have not been reported to date. In humans, AOX1 and XOR do not seem to play a vital role, as genetic deficiencies in the Moco sulfurase (MCSF) gene, causing a defect of both enzymes, are associated with mild symptoms such as the formation of kidney stones [9,10]. AOs in general are characterized by a broad substrate specificity and play an important role in the metabolism
of drugs and xenobiotics [3]. In animals, AOs have a significant toxicological role, detoxifying xenobiotics of wide structural diversity. The enzymes oxidize aromatic aza-heterocycles containing a –CH=N= chemical function (e.g. phthalazine and purines), aromatic or non-aromatic charged azaheterocycles with a –CH=N= moiety (e.g. N³-methylhycinotinamide and N³-methylphthalazinum) or aldehydes, such as benzaldehyde, retinal and vanillin [3]. AO and XOR share some common substrates and the relative selectivity of the two types of enzymes has been systematically reviewed [11]. AO may have a role in the degradation of vitamins like nicotinamide and pyridoxal or in the oxidation of all-trans retinaldehyde to all-trans retinoic acid, the active metabolite of vitamin A [3]. Upon oxidation of aldehyde substrates, AO produces significant amounts of highly toxic reactive oxygen species, O₂⁻ and hydrogen peroxide [12,13].

The reaction mechanism of substrate oxidation of MFEs has been only described in detail for *Rhodobacter capsulatus* XDH [14], a bacterial XDH sharing high similarities to eukaryotic XOR both at the structural level and on the basis of amino acid sequence identity [15,16]. In the oxidized enzyme, the metal is in the Mo(VI) oxidation state, bearing an oxo (=O), an hydroxo (-OH) and an equatorial sulfido (=S) ligand. Site-directed mutagenesis showed that Glu₉⁷₃₀ is a fundamental residue for the catalytic reaction by abstracting a proton from the Mo-OH group, which then nucleophilically attacks the substrate carbon atom to be hydroxylated [14]. Two other conserved residues at the active site of XORs, Glu₉₂₉₂ and Arg₉₃₁₀, are involved in substrate binding and transition state stabilization [14,17]. In mouse AOX1 (mAOX1), the glutamate acting as an active site base is also highly conserved (E₁₂₆₅), however, the glutamate involved in substrate binding is exchanged by a valine (V₈₀₆₆), and the arginine involved in transition state stabilization is exchanged to a methionine (M₈₈₄) [3].

To study the importance of these amino acids in substrate specificity for AOX1, we established a system for heterologous expression of mAOX1 in *E. coli*. To ensure a high level of incorporation of the sulfido ligand at the Moco site, mouse MCSF (mMCSF) was cloned and coexpressed in this system. The incorporation of the sulfido ligand at the Moco site, mouse MCSF expression of mAOX1 in *E. coli* TP1000 cells.

**Table 1. Purification of recombinant mAOX1 after expression in *E. coli* TP1000 cells.**

| Step         | Volume | Protein* | Totala | Benzyaldehyde oxidizing activity | S.A. | Yield | P.F. |
|--------------|--------|----------|--------|---------------------------------|------|-------|------|
|              | ml     | mg       | units  | units/mg | %    | -fold |
| Cytosol      | 150    | 1611     | 135    | 0.1     | 100  | 1     |
| Ni-NTA       | 14     | 6.6      | 0.76   | 0.12    | 0.57 | 1.2   |
| Superoxide   | 12     | 0.84     | 0.35   | 0.43    | 0.27 | 4.2   |
| BAS          | 20     | 0.1      | 0.23   | 0.23    | 0.17 | 23    |

*Total protein was quantified with the Bradford assay.
*The activity was measured by monitoring the decrease in absorption at 600 nm in the presence of 500 μM benzyaldehyde and 100 μM DCPIP.
*Specific enzyme activity (units/mg) is defined as the oxidation of 1 μM benzyaldehyde per min and mg of enzyme under the assay conditions.

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Characterization of mAOX1

Heterologous Expression, Purification and Characterization of mAOX1

Several heterologous expression systems for mammalian AOs have been described. However a main drawback of all these systems is the low catalytic activity of the purified enzymes due to a low Moco content [18–20]. To overcome this problem, we designed an expression system that allowed the simultaneous expression of mAOX1 and mMCSF in the *E. coli* TP1000 strain, and ensured a higher level of sulfurized Moco insertion into the enzyme [21].

Recombinant mAOX1 was purified from a 12-liter *E. coli* culture using sequential Ni-NTA chromatography, size exclusion chromatography and benzamidine sepharose affinity chromatography. The purification protocol used for mAOX1 is summarized in Table 1. The overall purification from the soluble fraction was more than 23-fold with a yield of 0.17%, and the final specific activity with benzyaldehyde of the purified protein was 2.5 U/mg. This compares well to the activity reported for mAOX1 purified from mouse liver [7]. We demonstrated that the low increase in specific activity after Ni-NTA is due to the intrinsic aldehyde oxidizing activity of *E. coli* enzymes of the xanthine oxidase family present in the crude extract (data not shown). Size exclusion chromatography of the purified protein resulted in a single peak with an approximate molecular mass of 300 kDa, showing that the protein existed as a homodimer in solution (data not shown). SDS-polyacrylamide gels performed under reducing conditions demonstrated the presence of one major band with a size of 130 kDa after purification (Fig. 1). In these experimental conditions three additional bands with sizes of 120 kDa, 80 kDa, and 50 kDa were also observed (Fig. 1). Electrospray mass spectrometry analysis of the respective gel slices revealed that these bands were degradation products of mAOX1 (data not shown). These bands were also observed in AO purified from other sources, e.g. in AO purified from rat liver [12]. As observed in our data, AO purified from rat liver displayed several bands of sizes of 150, 130, 80, and 45 kDa on SDS-polyacrylamide gels, but not after native-PAGE [12]. Thus we conclude, that the degradation products occur due the reductive conditions during the SDS-PAGE.

The visible absorption spectrum (Fig. 2) of recombinant mAOX1 is similar to those of mAOH1 purified from mouse liver and shows the presence of FeS and FAD as prosthetic groups [8]. The iron content was measured by ICP-OES and showed a saturation of 90% (Table 2). Since the ratio of 450:550 in the UV-Vis spectrum was shown to be 3, this implicates that the protein is fully saturated with FAD [1]. The Moco content of mAOX1 was quantified after its conversion to Form A and related to the molybdenum content of the protein (Table 2), revealing that no demolybdo-mAOX1 was present in the purified fractions. To determine the content of the terminal sulfur ligand required for mAOX1 activity, the cyanolysable sulfur was quantified and in addition, absorption spectra of oxidized mAOX1 and benzaldehyde reduced enzyme were recorded under anaerobic conditions (data not shown). From the reduction spectra the amount of active mAOX1 was calculated to be 20%. While purified mAOX1 was 70% saturated with Moco (Table 2), the purified enzyme was only 20% saturated with the sulfido ligand required for enzyme activity.
EPR spectroscopy of the mAOX1 FeS clusters

Fig. 3 shows the EPR spectra of the FeS clusters of dithionite-reduced mAOX1 wild-type (trace a), together with the corresponding simulations (traces b–e). The spectra show signals from the reduced FAD cofactor to the flavin semiquinone and from some remaining Mo(V). Most prominent are however the characteristic EPR signals assigned to the two iron sulphur centers FeSI and FeSII, which are similar for all members of the xanthine oxidase family that have been described to date [1,22]. FeSI has EPR properties showing an almost axial g-tensor, similar to those of many other [2Fe-2S] proteins, being fully developed at relatively high temperatures (60 K), while FeSII has unusual EPR properties for [2Fe-2S] species with a strongly rhombic g-tensor, showing broad lines and being only observed at much lower temperatures (20 K). The g-values and linewidths were evaluated by simulating the superimposed spectra of FeSI, FeSII and reduced FAD [Fig. 3] using the program EasySpin [23]. The double-integrated simulated spectra for the single iron-sulfur clusters display a ratio of 1:1 indicating the presence of both clusters FeSI and FeSII in the same amount in the protein. The obtained g-values are given in Table 3. The flavin semiquinone (FAD) has been simulated by using an isotropic g-value of 2.0 and a linewidth of 1.9 mT. This linewidth is comparable with that from other flavins [1,22]. The Moco (MoV) has been neglected in the simulations.

CD-Spectroscopy

CD-spectra were measured in the visible region in both the reduced and oxidized forms (Fig. 4). The spectrum of the oxidized wild-type enzyme exhibited strong negative dichroic bands at approximately 350–400 nm and 520–580 nm, and intensive positive bands between 400 and 500 nm (Fig. 4). From the various maxima and infections, transitions can be identified at 378 (−), 434 (+), 474 (+), and 556 (−) nm. Upon reduction with dithionite, the spectrum changed markedly with less intense transitions at 370 (−), 408 (+), and 478 (−) nm. The visible CD-

Table 2. Determination of the Moco and iron content of mAOX1 and R. capsulatus XDH and variants.

| Protein | Moco content % | Fe-content % |
|---------|----------------|-------------|
| mAOX1:  |                |             |
| WT      | 70             | 90          |
| V806E   | 46             | 101         |
| M884R   | 66             | 97          |
| V806E/M884R | 44     | 108         |
| E1265Q  | 60             | 83          |
| R. capsulatus XDH: | |             |
| WT      | 75             | 99          |
| E232V   | 43             | 95          |
| R310M   | 63             | 88          |
| E232V/R310M | 49     | 97          |

Moco was quantified as described in Materials and Methods. Moco content of wild-type mAOX1 and R. capsulatus XDH was set to the calculated molybdenum content determined by ICP-OES, and Moco determined as Form A in the AOX1 variants was compared to that value.

Iron was determined by ICP-OES as described in Materials and Methods.

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spectra of reduced and oxidized mAOX1 are very similar in shape and intensity to those of XOR [24,25], showing that both FeSI and FeSII are present in the recombinant enzyme. Steady state kinetics of mAOX1 wild-type and active site variants E1265Q, V806E, M884R, and V806E/M884R

To determine the role of amino acids Glu1265, Val806, and Met884 for the oxidation of aldehydes by mAOX1, the variants E1265Q, V806E, M884R, and the double variant V806E/M884R were generated and purified. Fig. 5 shows wild-type mAOX1 with the purified variants on a native polyacrylamide gel, showing that all proteins had about the same purity. Since some of the variants were inactive with benzaldehyde, the Moco content of the variants was determined in comparison to wild-type mAOX1, and showed the following range of Moco saturation: E1265Q 60%, V806E 46%, M884R 66%, and V806E/M884R 44% (Table 2). The lower Moco content of the purified variants may be due to the modified purification protocol necessary for the purification of these mAOX1 variants, which did not bind to the benzamidine sepharose. The iron content of the variants varied between 83–108%, reflecting that the FeS clusters were not influenced by the amino acid exchanges at the active site (Table 2). In addition, the variants showed almost identical CD-spectra in comparison to wild-type mAOX (data not shown).

With the exception of retinaldehyde, steady state kinetics of wild-type mAOX1 were performed by varying the concentrations of the substrates benzaldehyde, phtalazine, acetaldehyde, xanthine and hypoxanthine, and by using DCPIP as electron acceptor (Experimental procedures). In consideration of the fact that the protein was only 20% active, the enzyme activities compared well to the values obtained for native mAOX1 [7]. In particular the $k_{cat}$ values determined with benzaldehyde were almost superimposable (Table 4). The $k_{cat}$ values determined for retinaldehyde and spectra of reduced and oxidized mAOX1 are very similar in shape and intensity to those of XOR [24,25], showing that both FeSI and FeSII are present in the recombinant enzyme.

Table 3. EPR linewidths and g-values of FeSI and FeSII from mAOX1.

| Protein   | Cluster | g-values | Linewidth [mT] |
|-----------|---------|----------|----------------|
| Rabbit liver AO | FeSI\(^{a}\) | 2.018 | 1.930 | 1.918 | 1.6 |
|           | FeSII\(^{a}\) | 2.106 | 2.003 | 1.915 | - |
| mAOX1     | FeSI\(^{b}\) | 2.019 | 1.927 | 1.912 | 2.6 |
|           | FeSII\(^{b}\) | 2.085 | 1.971 | 1.90 | 4.0 |

\(^{a}\)AOX wild-type from rabbit liver, values from [36].

\(^{b}\)g-strain was included in the simulation with 0.01 for $g_z$. Estimated error of g-values: $\pm 0.004$ for FeSI and $\pm 0.008$ for FeSII.

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not used by the recombinant mAOX1 under our assay conditions. The purine substrates xanthine and hypoxanthine were and retinaldehyde. Acetaldehyde showed the lowest catalytic efficiency were the aromatic aldehydes phthalazine, benzaldehyde and retinaldehyde. Acetaldehyde were a little higher in comparison to native mAOX1, but were in the same range. The substrates with the highest catalytic efficiency were the aromatic aldehydes phthalazine, benzaldehyde and retinaldehyde. Acetaldehyde showed the lowest catalytic efficiency. The purine substrates xanthine and hypoxanthine were not used by the recombinant mAOX1 under our assay conditions. The catalytic parameters for the mAOX1 variants E1265Q, V806E, M884R, and V806E/M884R were also determined. As shown in Table 4, variants E1265Q and V806E/M884R were unable to metabolize any of the aldehyde and purine substrates tested. While the corresponding kcat values were generally decreased, the Km value for benzaldehyde, phthalazine and acetaldehyde were drastically increased by introducing the V806E amino acid exchange in mAOX1. For the variant V806E, the catalytic efficiency decreased by a factor of 10 for benzaldehyde, retinaldehyde and acetaldehyde, while the value was only decreased 3-fold for phthalazine as substrate. Thus, the affinity for small and symmetric aromatic aldehydes are affected by this mutation, while the affinity for more hydrophobic aldehydes like retinaldehyde was increased, albeit with a concomitant decrease in kcat. In contrast, the M884R variant was inactive with phthalazine and acetaldehyde as substrates, and for both benzaldehyde and retinaldehyde kcat and Km were decreased.

Comparison of steady state parameters of the reverse amino acid exchanges (E232V, R310M, and E232V/R310M) introduced into R. capsulatus XDH

The catalytic mechanism of R. capsulatus XDH was proposed to involve three amino acids at the active site: GluB730 is thought to act as an active site base in the initial step of the reaction, while GluB232 is involved in substrate binding and transition state stabilization and R310 is involved in transition state stabilization and orientation of the substrate at the active site. Since the catalytic mechanism of R. capsulatus XDH is well characterized, we compared the steady state kinetic data derived for wild-type mAOX1 and active-site variants with amino acid exchanges introduced into the active site of R. capsulatus XDH to reverse the amino acids found at the active site of XDH to the ones conserved in mAOX1. Thus, the XDH variants E232V, R310M, and the double variant E232V/R310M were generated.

Table 4. Steady-state kinetic parameters of recombinant mAOX1 and variants with different aldehyde and purine substrates.

| substrate | Kinetic parameters | mAOX1-WT | V806E | M884R | V806E/M884R | E1265Q |
|-----------|------------------|----------|-------|-------|-------------|--------|
| benzaldehyde | Kd[kM] | 97.7 ± 21.5 | 634.5 ± 76.4 | 7130 ± 2580 | n.d. | n.d. |
| | kcat[kM]^{-1} | 317.6 ± 28.5 | 151.0 ± 20.8 | 76.4 ± 17.8 | n.d. | n.d. |
| phthalazine | Kd[kM] | 11.4 ± 4.0 | 28.55 ± 0.35 | n.d. | n.d. | n.d. |
| | kcat[kM]^{-1} | 128.1 ± 13.0 | 103.0 ± 1.0 | n.d. | n.d. | n.d. |
| retinaldehyde | Kd[kM] | 55.8 ± 8.8 | 22.2 ± 2.8 | 7.5 ± 2.0 | n.d. | n.d. |
| | kcat[kM]^{-1} | 49.5 ± 8.7 | 13.9 ± 2.4 | 13.4 ± 0.4 | n.d. | n.d. |
| acetaldehyde | Kd[kM] | 17500 ± 4900 | 52900 ± 17800 | n.d. | n.d. | n.d. |
| | kcat[kM]^{-1} | 519.9 ± 112.3 | 160.6 ± 38.1 | n.d. | n.d. | n.d. |
| xanthine | Kd[kM] | 0.030 ± 0.002 | 0.0031 ± 0.0003 | - | - | - |
| | kcat[kM]^{-1} | n.d. | n.d. | n.d. | n.d. | n.d. |
| hypoxanthine | Kd[kM] | - | - | - | - | - |
| | kcat[kM]^{-1} | n.d. | n.d. | n.d. | n.d. | n.d. |
| | kcat[kM]^{-1} | - | - | - | - | - |

*Apparent kinetic parameters were recorded in 50 mM Tris, 1 mM EDTA, pH 7.5 by varying the concentration of substrate in the presence of 100 μM DCP/IP as electron acceptor.

n.d., none was detectable.

- , not determined.

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As shown in Table 2, the Moco content of the purified XDH proteins was reduced and varied from 43%–63%, depending on the introduced amino acid exchange. Steady state kinetics of XDH were performed by varying the concentrations of the substrates benzaldehyde, phthalazine, retinaldehyde, acetaldehyde, xanthine, and hypoxanthine, by using DCPIP as electron acceptor. Compared to wild-type XDH, the activity of the variants with purine substrates was drastically decreased, while oxidation of aldehydes as substrate was increased (Table 5). We were able to successfully purify the XDH-E B232V/RB310M double variant.

**Discussion**

Here, we report a system for the heterologous expression of mAOX1 in *E. coli*. Our system tried to overcome one of the main problems associated with the expression of recombinant mammalian MFEs in bacteria, i.e. the production of large proportions of inactive demolybdob and desulfo enzymes, as observed in the case of human XOR [20]. To ensure a higher sulfuration level of AOX1, we engineered *E. coli* for the simultaneous synthesis of the mMCSF and mAOX1 proteins. After coexpression with mMCSF, mAOX1 contained a 50% higher level of the terminal sulfido ligand of Moco (data not shown), although only 20% of the purified enzyme existed in the catalytically active form. This suggested two possibilities: either the conditions for the expression of both proteins have to be further optimized, or the sulfuration of Moco in bacteria and eukaryotes is different. Thus in prokaryotes, sulfuration of Moco precedes insertion into MFEs [26].

The recombinant mAOX1 displayed similar catalytic properties in comparison to the enzyme purified from mouse liver [7]. This demonstrates that the protein was correctly folded in mMCSF and mAOX1 proteins. After coexpression with mMCSF, mAOX1 contained a 50% higher level of the terminal sulfido ligand of Moco (data not shown), although only 20% of the purified enzyme existed in the catalytically active form. This suggested two possibilities: either the conditions for the expression of both proteins have to be further optimized, or the sulfuration of Moco in bacteria and eukaryotes is different. Thus in prokaryotes, sulfuration of Moco precedes insertion into MFEs [26]. Thus it remains possible that due to this difference, heterologous expression of mammalian MFE’s in *E. coli* will not give rise to a complete sulfurated enzyme. However, we also tried expression of AOX1 in *Pichia pastoris*, and this expression system also did not give rise to a higher sulfuration level of AOX1. Thus, it also remains possible, that part of the sulfido ligand of mAOX1 is exchanged to an oxo-ligand during purification of the enzyme.

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**Table 5.** Steady-state kinetic parameters of recombinant *R. capsulatus* XDH and variants with different aldehyde and purine substrates.

| substrate | Kinetic parameters* | XDH-WT | E232V | R310M | E232V/R310M |
|-----------|---------------------|--------|-------|-------|-------------|
| benzaldehyde | $k_{cat}/K_m$ | 540 ± 146 | 39.9 ± 7.0 | 552 ± 188 | 25.9 ± 15.5 |
| phthalazine | $k_{cat}/K_m$ | 1240 ± 210 | 310 ± 80 | n.d. | 420 ± 73 |
| retinaldehyde | $k_{cat}/K_m$ | 6.3 ± 1.3 | 38.2 ± 10.5 | 154.8 ± 7.1 | 36.6 ± 1.1 |
| acetaldehyde | $k_{cat}/K_m$ | 0.39 ± 0.06 | 3.3 ± 0.9 | 10.9 ± 0.2 | 3.0 ± 0.2 |
| xanthine | $k_{cat}/K_m$ | 59 ± 10 | 175 ± 7 | n.d. | n.d. |
| hypoxanthine | $k_{cat}/K_m$ | 3300 ± 460 | 85.6 ± 15.3 | n.d. | n.d. |

*Apparent kinetic parameters were recorded in 50 mM Tris, 1 mM EDTA, pH 7.5 by varying the concentration of substrate in the presence of 100 µM DCPIP as electron acceptor. n.d., none was detectable.

- not determined.

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liver AO were also similar [29] and are consistent with an axial signal for FeSI.

Eukaryotic XOR and AO are similar in protein structure and prothestic group composition, but have different characteristics of substrate specificity both at the molybdenum center and at the FAD center [3,11]. While mAOX1 is a true oxidase using molecular oxygen as electron acceptor at the FAD site, XOR exists in two interconvertible forms: NAD⁺ is the substrate for the XDH form of XOR, while after proteolytic cleavage or intramolecular disulfide formation, the XO form is generated, which uses O₂ instead of NAD⁺. The hydroxylation of aldehyde and purine substrates is catalyzed at the molybdenum center in both AO and XOR. However, the former catalyzes the hydroxylation of aldehydes more efficiently than the latter [11]. On the other hand, purines such as hypoxanthine and xanthine are good substrates of XOR, but poor substrates of AO. The crystal structure of R. capsulatus XDH with alloxan bound to the active site [15], in addition to site-directed mutagenesis studies [14], proposed that the purine substrates are bound and oxidized as follows: Catalysis is initiated by abstraction of a proton from the Mo-OH group by the conserved active site GluB730, followed by the nucleophilic attack on the carbon of the substrate and the concomitant hydride transfer to the Mo = S of the molybdenum center [14]. This reaction yields an intermediate with the hydroxylated product coordinated to the molybdenum via the newly introduced hydroxyl group. The catalytic sequence is completed by displacement of the bound product from the molybdenum coordination sphere by hydroxide from solvent. This is followed by intramolecular electron transfer to the FeS and FAD cofactors, as well as deprotonation of the Mo-SH to give the oxidized form of the molybdenum center. In addition, Glu4232 and Argg310 of R. capsulatus XDH were shown to be involved in the binding, orientation and transition state stabilization of the substrate [14,17].

A similar reaction mechanism as the one described for XOR have been proposed for AOs [4], although, this proposal has never been supported by experimental data based on site-directed mutagenesis. Heterologous expression of mAOX1 in E. coli enabled us to study the involvement of specific residues at the active site in more detail for the first time. We exchanged residues Glu1265, Val806, and Met884 to the ones identified in the active site of XDH. For direct comparison reasons, the reverse amino acid exchanges were introduced to the active site of R. capsulatus XDH. Our results showed that the amino acid exchanges in XDH resulted in the complete loss of activity towards purine substrates for the E₉₂₃₂V/R₉₃₁₀M double variant, and a higher activity with aldehyde substrates. In contrast to Yamaguchi et al. [20], we were able to purify the E₉₂₃₂V/R₉₃₁₀M double XDH variant. However, the reverse was not the case for amino acid exchanges in mAOX1. The amino acid exchanges V₈₀₆₃E/M₈₈₄₃R in mAOX1 resulted in a complete loss of enzyme activity with both purine and aldehyde substrates. Both residues at the active site of mAOX1 seem to be important for the binding of substrates, since the M₈₈₄₃R variant either completely lost the activity with phthalazine or acetaldelyde as substrates, or the activity with retinaldehyde and benzaldehyde was drastically reduced, with a concomitant increase of Kₘₚ for benzaldehyde. In general, the V₈₀₆₃E exchange resulted in an increase in Kₘₚ for most substrates (except retinaldehyde) and in a decrease in kₐₚ. Thus, while the ability to bind and utilize aldehyde substrates was decreased by converting the two residues to the ones found in XDH, purines were still not converted by the mAOX1 variants. While in XDH the two residues seem to determine which substrates are efficiently bound at the active site, in mAOX1 more factors determine the binding and conversion of substrates. mAOX1 seems to be more adapted to aldehyde substrates and the substrate specificity can not be converted back to purine substrates by two amino acid exchanges at the active site. The active site of XDH is deeply buried in the enzyme, but reachable through a funnel-shaped cavity that is wider on the surface. Hydrophobic residues, able to accommodate the ring structures of the aromatic substrates dominate the channel in XOR (e.g. Leu₉₃₀₃, Pro₉₃₀₆, Phe₉₃₄₄, Phe₉₅₄₉ in R. capsulatus XDH) [15]. These residues are exchanged to more bulky, charged or hydrophobic amino acids in mAOX1: Glu₈₇₇, Thr₉₈₀, Phe₉₁₈, and Ile₁₀₁₃ [3]. It is possible that the aromatic purine residues analyzed in this study, are unable to enter the active site of mAOX1.

Finally, the mAOX1-E1265Q variant was catalytically inactive. This result is consistent with an essential catalytic role for this residue [4]. Like in XOR, Glu1265 of mAOX1 may function as an active-site base to abstract a proton from the Mo-OH leading to the nucleophilic attack on the aldehyde substrate molecule, which is followed by a hydride transfer to give the transition state intermediate (Fig. 6). The roles of residues Met884 and Val806 are proposed to be the stabilization of substrate binding. Since the aromatic aldehydes tested in this study are symmetric, the orientation of the substrate at the active site is not as important as in XOR [17].

In total, the established expression system of mAOX1 in E. coli can further be used for detailed site-directed mutagenesis and structure-function studies of mammalian MFEs. In future studies it is planned to characterize the homologues of mAOX1 in rodents in more detail. Analysis of substrate specificities will give insights into the potential physiological roles of these enzymes.

Materials and Methods

Bacterial Strains, Plasmids, Media and Growth Conditions

E.coli TP1000 (ΔmobAB) cells [30] were used for the coexpression of mAOX1 wild-type and variants with mMCSF. E. coli expression cultures were grown in LB medium under aerobic conditions at 30°C for 24 h. Ampicillin (50 μg/ml) chloramphenicol (150 μg/ml), sodium molybdate (1 mM) and isopropyl-β-D-thiogalactoside (IPTG) (20 μM) were used when required.

Cloning Expression and Purification of mouse AOX1 and variants

The cDNA of mAOX1 was cloned from mouse CD1 liver [31], using primers designed to allow cloning into the NdeI-SalI sites of the expression vector pTrcHis [32]. The resulting plasmid was designated pSL205, and expresses mAOX1 as a N-terminal fusion protein with a His₆-tag. By using PCR mutagenesis the amino acid exchanges V₈₀₆₃E, M₈₈₄₃R, V₈₀₆₃E/M₈₈₄₃R and E1265Q were introduced into mAOX1. The mMCSF cDNA fragment was cloned from mouse liver using primers for the cloning into the Ndel-XhoI sites of the expression vector pACYC184 (Novagen), and the resulting plasmid was designated pSS110.

For heterologous expression in E. coli, pSL205 and pSS110 were transformed into TP1000 cells [30]. To express recombinant proteins, cells were grown at 30°C in LB medium supplemented with 150 μg/mL ampicillin, 50 μg/ml chloramphenicol, 1 mM molybdate, and 20 μM IPTG. After 24 h, cells were harvested by centrifugation, resuspended in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and disrupted by several passages through a French Press cell. The supernatant was mixed with 3.5 mM of Ni-nitrilotriacetic (NTA) resin per 12 liters of cell growth. The slurry was transferred to a column and washed with 10 column volumes of 50 mM sodium phosphate, 300 mM NaCl,
Enzyme assays

Enzyme assays were carried out at 30 °C in Tris buffer (50 mM, 1 mM EDTA, pH 7.5) in a final volume of 500 μL. Total enzyme concentration was 30 nM for wild-type mAOX1 and 50–200 nM for the variants. Enzyme activity was monitored spectrophotometrically at 600 nm with 100 μM 2,6-dichlorophenolindophenol (DCPIP) as electron acceptor and calculated using the extinction coefficient of 16.1 mM−1 cm−1 for DCPIP [34].

To determine retinaldehyde oxidase activity, the assay described by Vila et al. [7] was used with some modifications. Purified mAOX1 (2 μg) was incubated in the dark for 10 min at 30°C in 100 μL of 10 mM potassium phosphate buffer, pH 7.4 and the reaction was started by the addition of 0.01–10 mM all-trans-retinaldehyde. The reaction was stopped with 100 μL of butanol/methanol (95:5 v/v) containing 0.005% w/v of butylated hydroxytoluene (0.005% w/v) (Sigma-Aldrich). The organic phase was separated and 20 μL were subjected to high performance liquid chromatography on a C18 reverse phase column (4.6×250-mm ODS Hypersil, 5 μm). The production of the oxidation product all-trans-retinoic acid was determined at 340 nm and quantified by using a calibration curves.

Absorption Spectra during Anaerobic Reduction

Purified mAOX1 in 50 μM of 50 mM Tris, 1 mM EDTA, pH 7.5, was incubated in an anaerobic chamber (Coy Lab Systems) for 2 h at 4°C before benzaldehyde was added to a final concentration of 300 μM. Complete reduction was achieved by the addition of 20 mM sodium dithionite. Spectra were recorded in 0.15 mL cuvettes using a Shimadzu UV-2401 PC spectrophotometer.

Metal and Moco/MPT analysis

The molybdenum and iron contents of the purified proteins were quantified by ICP-OES analysis with a Perkin-Elmer Optima 2100 DV. The samples were wet-ashed at a concentration of 4 μM in a volume of 500 μL by the addition of 500 μM 65% nitric acid and incubated over night at 100°C. The samples were further diluted by the addition of 4 mL of water. As reference, the multi-element standard solution XVI (Merck) was used. The complete Moco content of mAOX1 in comparison to the variants was quantified after conversion to its fluorescent degradation product Form A as described earlier [35].

CD-spectroscopy

UV/visible CD spectra of 2.1 mg/mL enzyme samples were recorded in 50 mM Tris, 1 mM EDTA, pH 7.5 using a Jasco J-715 CD-spectrophotometer. The scanning mode was set step-wise, each nm a data pitch was recorded, the response time was 2 seconds and each measurement was repeated 4 times.
Electron Paramagnetic Resonance Spectroscopy

9.5 GHz X-band EPR spectra were recorded on a Bruker ESP300E spectrometer equipped with a TE102 microwave cavity. For temperature control between 5 K and 300 K an Oxford ESR 900 helium flow cryostat with an Oxford ITG4 temperature controller was used. The microwave frequency was detected with an EIP frequency counter (Microwave Inc.). Magnetic field was calibrated using a LiLiF standard with a known g-value of 2.002293 ± 0.000002 [36]. Samples (typically 0.1 mM enzyme) were prepared in quartz tubes with 4 mm outer diameter. Chemical reduction, in order to generate the reduced Fe(II)-Fe(III) in the FeS clusters, was performed by adding a small volume of anaerobic sodium dithionite solution to the protein solution under a weak stream of argon gas (20-fold excess dithionite with respect to the protein). The sample tubes were frozen, after a change of colour was observed (typically 30 s reaction time) in liquid nitrogen. Baseline corrections, when required, were performed by subtracting a background spectrum, obtained under the same experimental conditions from a sample containing only a buffer solution. Simulations of the experimental EPR spectra, based on a spin Hamilton operator approach, were performed with the program EasySpin [23]. Second integrals from the simulated spectra of reduced FeS and FeSII were used to estimate the relative amount of both clusters in the respective samples.

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Author Contributions

Conceived and designed the experiments: SL. Performed the experiments: SS. Analyzed the data: MS. Contributed reagents/materials/analysis tools: MT EG FL. PH. Wrote the paper: SL.

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