Evolution, expression, and substrate specificities of aldehyde oxidase enzymes in eukaryotes

Mineko Terao[‡], Enrico Garattini[‡], Maria João Romão[§], and ⓘ Silke Leimkühler[¶][1]

From the [‡]Laboratory of Molecular Biology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, via La Masa 19, 20156 Milano, Italy, the [§]UCIBIO–Applied Biomolecular Sciences Unit, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal, and the [¶]Department of Molecular Enzymology, Institute of Biochemistry and Biology, University of Potsdam, Karl-Liebknecht-Str. 24–25, 14476 Potsdam, Germany

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Aldehyde oxidases (AOXs) are a small group of enzymes belonging to the larger family of molybdo-flavoenzymes, along with the well-characterized xanthine oxidoreductase. The two major types of reactions that are catalyzed by AOXs are the hydroxylation of heterocycles and the oxidation of aldehydes to their corresponding carboxylic acids. Different animal species have different complements of *AOX* genes. The two extremes are represented in humans and rodents; whereas the human genome contains a single active gene (*AOX1*), those of rodents, such as mice, are endowed with four genes (*Aox1-4*), clustering on the same chromosome, each encoding a functionally distinct AOX enzyme. It still remains enigmatic why some species have numerous AOX enzymes, whereas others harbor only one functional enzyme. At present, little is known about the physiological relevance of AOX enzymes in humans and their additional forms in other mammals. These enzymes are expressed in the liver and play an important role in the metabolisms of drugs and other xenobiotics. In this review, we discuss the expression, tissue-specific roles, and substrate specificities of the different mammalian AOX enzymes and highlight insights into their physiological roles.

Mammalian aldehyde oxidases (AOXs)[2] are metal-containing enzymes that require the molybdenum cofactor (Moco), two [2Fe-2S] clusters, and FAD for their catalytic activity (1, 2). AOXs belong to the xanthine oxidase family of Moco-containing enzymes along with xanthine oxidoreductase (XOR), bacterial carbon monoxide dehydrogenases, periplasmic aldehyde oxidoreductases, and other molybdoenzymes from prokaryotes. Humans contain a total of four molybdoenzymes, namely aldehyde oxidase 1 (AOX1) (3), xanthine oxidoreductase (4), sulfite oxidase (5), and the mitochondrial amidoxime-reducing component (6). In humans, the only essential molybdoenzyme is sulfite oxidase (7–9).

Eukaryotic AOXs are cytosolic proteins that are expressed in many tissues of various organisms, including insects, rodents, and humans (10, 11). AOXs oxidize purines, pyrimidines, and pteridines and are involved in nicotinic acid metabolism (12). In addition, AOXs bio-transform organic aldehydes into the corresponding carboxylic acids, hydroxylate heteroaromatic rings, and catalyze the reduction of nitro- and sulfo-groups (13–18). Given this broad substrate specificity, human AOX1 is an emerging enzyme in the context of drug metabolism (19, 20). Indeed, the number of drug molecules recognized and metabolized by AOXs is increasing, and this has raised the interest of pharmaceutical companies (21, 22).

Despite these many characterized activities, the physiological role of mammalian AOXs, is still a matter of debate (23). The genomes of many prokaryotic and eukaryotic organisms contain genes coding for AOX enzymes (11). In mammals, the complement of *AOX* genes varies according to the animal species considered (10, 11, 24). Mice and rats are characterized by the largest number of active *AOX* genes (*Aox1*, *Aox2*, *Aox3*, and *Aox4*),[3] each coding for a different AOX enzyme (Fig. 1) (11). In contrast, humans and the majority of primates contain a single active *AOX* gene, which is the m*Aox1* orthologue (10). The multiplicity of *AOX* genes observed in many vertebrates is the result of a series of duplication events from a common ancestor gene. In the mammalian species characterized by multiple *AOX* genes, the loci cluster on a short region of the same chromosome.
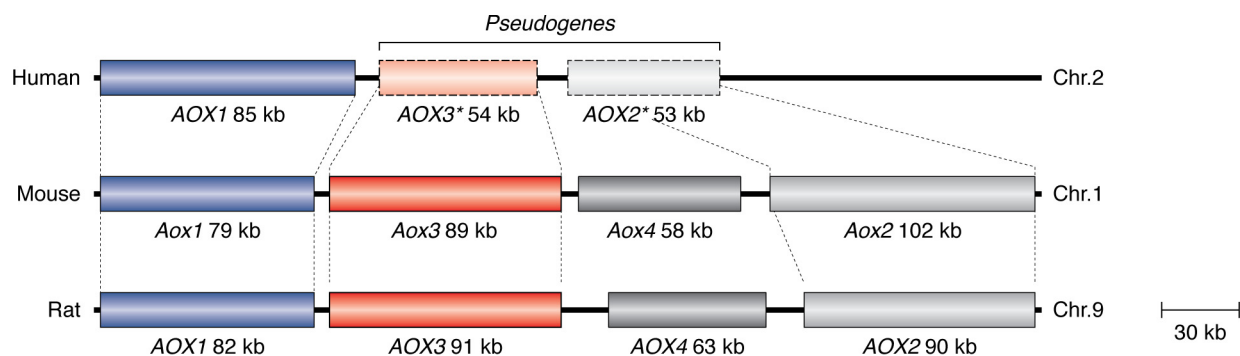
The reason why mammals other than humans express multiple AOX enzymes is unknown (25). Nevertheless, the decrease in the number of active AOX isoenzymes from rodents to humans is the result of progressive deletion/inactivation of the corresponding genes (24). It can be speculated that AOX2, AOX3, and AOX4 exert tissue-specific functions in rodents, which are dispensable in humans. The physiological role and demand for several AOX enzymes in certain organisms may find an answer following definition of the physiological

Figure 1. Organization of the human, mouse, and rat *AOX* genes. Shown is a *schematic representation* of the *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus AOX* genes and pseudogenes. Pseudogenes are marked by an *asterisk*.

function and substrate specificity of the various mammalian AOXs.

In this review, we discuss the evolution, expression, substrate specificities, and structures of the different mammalian AOX enzymes. The major aim is to give an overview on what is known about the potential roles of these different enzyme forms with a focus on the four enzymes present in mice. First insights into the physiological role of one specific enzyme form could be drawn from the physiological studies of m$Aox4^{-/-}$ knockout mice. Further, the role of reactive oxygen species (ROS) production by AOX enzymes is discussed, because they were produced in much higher proportions than previously appreciated. For the role of human AOX1 in drug metabolism, the reader is referred to numerous specialized reviews in the literature covering this topic (12, 20, 25–28).

Domain structure and catalytic mechanism of the AOX enzymes

AOX enzymes are present in both eukaryotes and prokaryotes. A common catalytic mechanism has been proposed for all AOXs. Before we focus on the different AOX enzymes present in mammals, we introduce the differences and common principles in the primary structures of AOX enzymes in different organisms. AOXs in general are Moco-containing enzymes that, with some exceptions in prokaryotes, contain two [2Fe-2S] clusters and FAD as additional prosthetic groups. First, it is important to give a brief overview on the structural characteristics of prokaryotic AOX enzymes to highlight the differences from and similarities with the eukaryotic counterparts. In bacteria and archaea, the xanthine oxidase family of molybdoenzymes is complex, as it includes CO dehydrogenases (29), aldehyde oxidoreductases (30–32), and quinoline-metabolizing (31) and nicotinic acid–metabolizing (33) enzymes. In particular, the domain structure, the subunit composition, and the cofactor complement of prokaryotic AOXs is variable (Fig. 2) (30–32). *Rhodobacter capsulatus/Veillonella atypica* xanthine dehydrogenase (XDH) (34), *Desulfovibrio gigas* aldehyde oxidoreductase (MOP) (30), and *Escherichia coli* periplasmic aldehyde oxidoreductase (PaoABC) (35) are the enzymes showing the highest structural similarities with eukaryotic xanthine oxidoreductases and AOXs. The redox-active centers of the bacterial and archaeal members of the xanthine oxidase family are contained in separate subunits, and exceptions to the general cofactor composition have also been identified (Fig. 2).

R. capsulatus XDH is organized as an $(\alpha\beta)_2$ heterodimer, and it contains the eukaryotic form of Moco (assigned as Mo-molybdopterin (Mo-MPT), Fig. 3). In contrast, *V. atypica* XDH consists of an $\alpha\beta\gamma$-heterotrimer, and it contains the MPT cytosine dinucleotide (MCD) form of Moco (Fig. 3). In addition, MOP lacks the FAD domain, and it consists of two identical subunits organized as an α_2 dimer (30). Each subunit contains the two [2Fe-2S] clusters and the MCD cofactor. Finally, the periplasmic aldehyde oxidoreductase from *E. coli* is characterized by a structure consisting of an $\alpha\beta\gamma$-heterotrimer, which exceptionally includes an additional [4Fe-4S] cluster proximal to the FAD in the β-subunit (35).

In comparison, the xanthine oxidase family of eukaryotic molybdoenzymes consists solely of AOXs (EC 1.2.3.1) and XORs. Two interconvertible forms of the mammalian XOR enzyme are known, xanthine oxidase (XO; EC 1.17.3.2) and XDH (EC 1.17.1.4). The domain structure of eukaryotic AOXs is highly conserved, and the catalytically active enzymes are organized as α_2 homodimers (Fig. 2) (36, 37). Each subunit of the catalytically active AOX and XOR homodimer contains three cofactor-binding domains. The N-terminal domain binds two [2Fe-2S] clusters known as FeSI and FeSII. The central domain contains the FAD binding site, whereas the C-terminal domain (4, 38–41) harbors the Moco in the Mo-MPT form (Fig. 3). The three domains are connected by flexible linker regions. The characteristic of the molybdoenzymes belonging to the xanthine oxidase family is the presence of an essential sulfur atom acting as an equatorial sulfido ligand to the molybdenum atom (Mo=S, Fig. 3) (42–45). The presence of this Mo=S ligand is at the basis of the cyanide-dependent inactivation of these molybdoenzymes (46). Cyanide treatment results in the formation of an inactive desulfo form, and these enzymes can be reversibly reactivated by treatment with sulfide and dithionite (42, 43).

The major structural differences among the members of the eukaryotic xanthine oxidase family are observed at the FAD site (3, 38). These differences are particularly evident between the FAD sites of AOXs and XORs, which react with the terminal electron acceptor. The final electron acceptor is molecular oxygen in the case of AOXs and XO, whereas it is NAD$^+$ in the case of XDH (47). The enzymes can be reversibly converted from the XDH form to the XO form, which has been shown to involve the formation of a new disulfide bond, between two cysteine
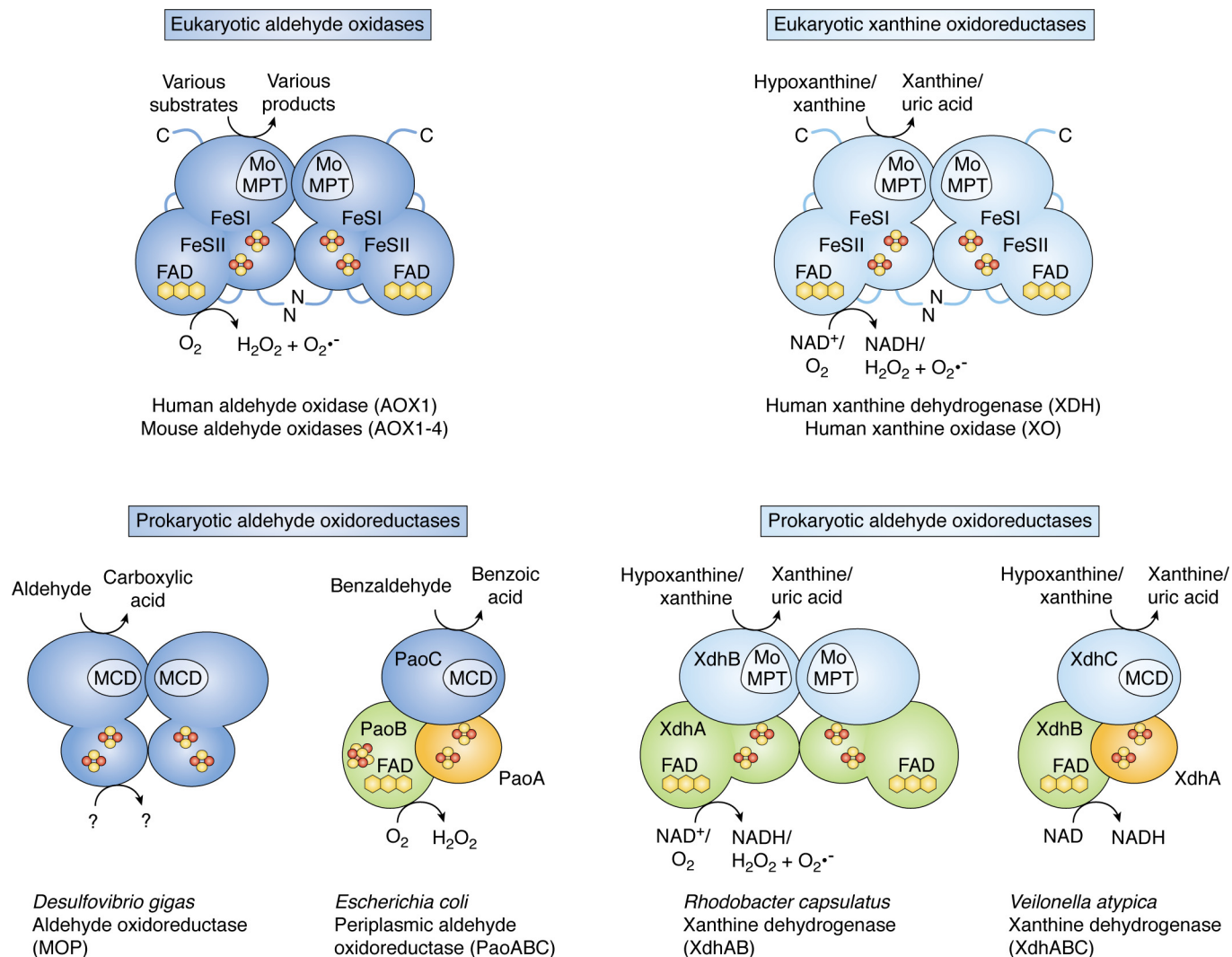
Figure 2. Overview of the subunit composition and cofactor organization of aldehyde oxidases and xanthine oxidoreductases. In eukaryotes, the catalytically active forms of AOXs and XORs consist of α_2 homodimers. Each subunit of the dimer consists of separate domains containing the Moco catalytic site, two distinct [2Fe-2S] redox centers, and the FAD-binding site. In the prokaryotic enzymes, the [2Fe-2S] cluster-binding subunits are shown in *orange*, the subunits binding the FAD cofactor are *colored* in *green*, and the subunits containing the Moco are *colored* in *blue*. The FAD domain is absent in the *D. gigas* aldehyde oxidoreductase. In the *R. capsulatus* xanthine dehydrogenase, the iron-sulfur and flavin-binding domains of the protein constitute one subunit (XdhA), and the Mo-MPT–binding domain constitutes a second (XdhB) subunit. In the *V. atypica* xanthine dehydrogenase, the iron-sulfur centers are located in one subunit (XdhA), the flavin in a second (XdhB), and the Moco as MCD in a third (XdhC) subunit. In the PaoABC from *E. coli*, a [4Fe-4S] cluster is present in proximity to the FAD cofactor on the PaoB subunit. Potential substrates and electron acceptors are indicated.
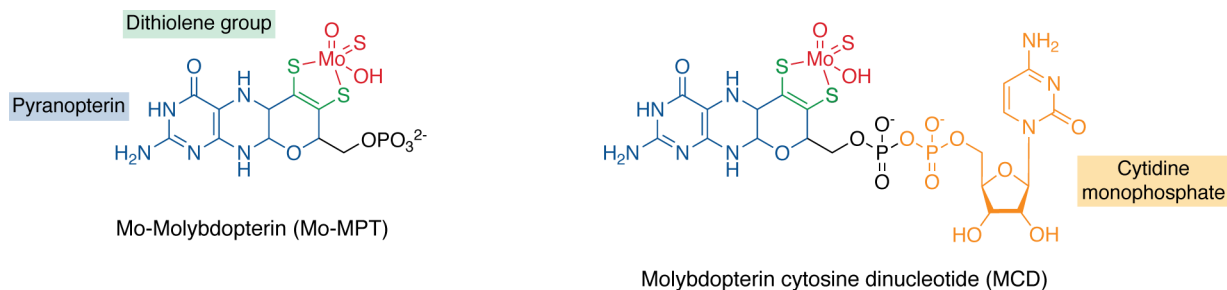
Figure 3. Forms of the molybdenum cofactor present in enzymes of the xanthine oxidase family. Characteristic to enzymes of the xanthine oxidase family is a sulfido ligand at the equatorial position of the molybdenum atom. In eukaryotic enzymes of this family, the basic form of Moco is a pyranopterin, named Mo-MPT, which coordinates the molybdenum atom (*colored* in *red*) by the characteristic dithiolene group (*colored* in *green*) at the C1′ and C2′ positions of the pyranopterin ring (*colored* in *blue*). In bacteria, the MPT core can be modified by an additional CMP nucleotide (*colored* in *orange*) at the phosphate group (*colored* in *black*), forming MCD.

residues at the FAD site (Cys^{535} and Cys^{992} in bovine XOR). In addition, the conversion of XDH into XO requires a structural rearrangement of the 11-residue loop (Gln^{423}–Lys^{433} in bovine

XOR) located in close proximity to the FAD cofactor (4). In addition, the movement of this loop leads to a change in the electrostatic environment around the FAD cofactor (48) and
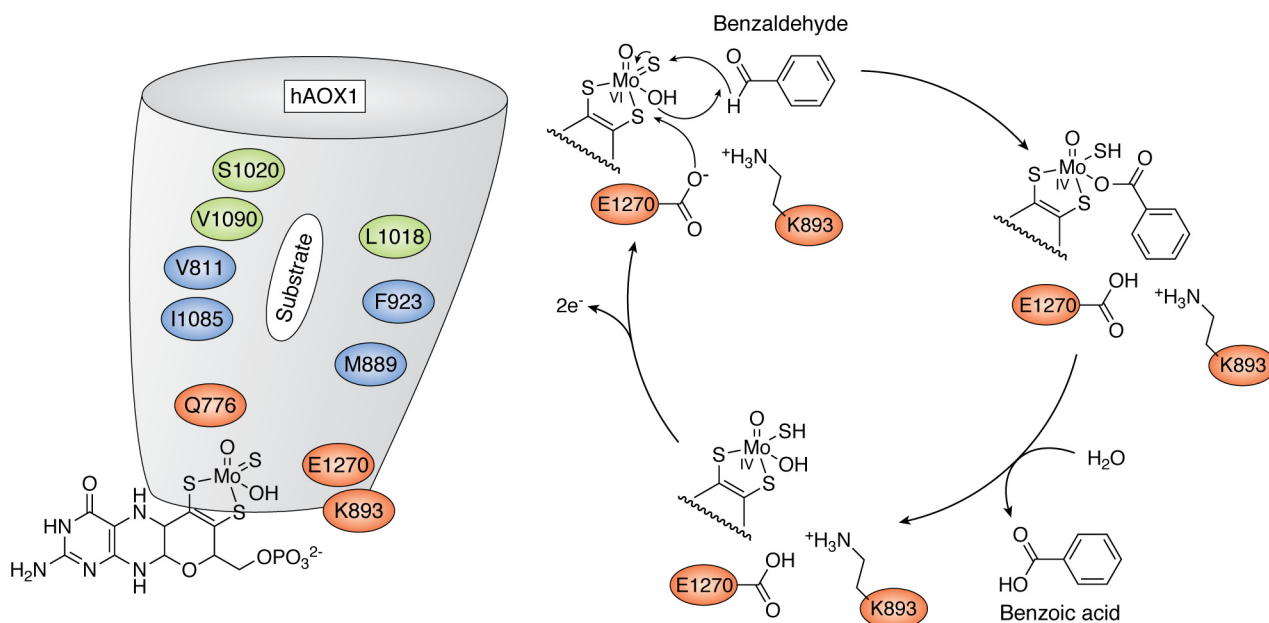
Figure 4. The catalytic mechanism of human AOX1. Shown is a representation of the proposed reaction mechanism for human AOX1, as exemplified for the substrate benzaldehyde. For details, see "Domain structure and catalytic mechanism of the AOX enzymes."

blocks the access of NAD^+ to its binding site (4). All of these structural elements involved in XDH/XO conversion are absent in mouse and human AOXs. This might explain the preference of AOX toward oxygen as the final electron acceptor (2).

The C-terminal 85-kDa domains of both AOXs and XORs contain the Mo-MPT cofactor, which is the site of substrate conversion. The substrate binds to the active site, and it is converted into its product by an oxygen atom derived from water. There are structural features along the substrate access funnel that might account for the observed differences in substrate specificities between AOX and XOR enzymes. In AOXs, the funnel is wider and more anionic than in XORs. This implies that AOXs are characterized by a better access of larger and bulkier substrates as well as a broader substrate specificity. The role exerted by specific amino acid residues of the active site in the catalytic activity of mammalian AOXs has been elucidated by site-directed mutagenesis studies performed with heterologous expression systems developed in *E. coli* (49–53). The active site of AOXs contains only a few highly conserved amino residues (Glu[1270], Phe[923], Lys[893], and Gln[776]; human AOX1 numbering), as many other residues are species-specific and differ in the human and mouse enzymes (Fig. 4) (54). Glu[1270] is the essential residue for the catalytic activity of all the enzymes belonging to the xanthine oxidase family (38).

The general reaction mechanism proposed for all AOXs is a base-catalyzed mechanism starting with an initial attack of the deprotonated Glu[1270] to the -OH ligand of the Mo atom, resulting in the generation of a nucleophilic $-O^-$ group (Fig. 4). Subsequently, the activated $Mo-O^-$ ligand attacks the substrate, followed by a hydride transfer to the sulfido ligand, leading to the generation of an intermediate species. In human AOX1, this intermediate is stabilized by hydrogen-bonding interactions with the Val[811], Met[889], and Lys[893] residues of the active site. This mechanism is supported by experimental evidence with

substituted *N*-heterocycles and by DFT calculations (55). Whereas the lysine is also conserved in mammalian AOX enzymes, the valine and methionine differ among AOX enzymes, depending on their substrate specificity. The next reaction step involves the release of the product from the reduced Mo site and the binding of a water molecule. The reaction cycle is completed once Mo is reoxidized and the two reducing equivalents are transferred to molecular oxygen via the two [2Fe-2S] clusters and FAD.

The evolution of AOX genes from bacteria to humans

The evolutionary history of *AOX* and *XDH* genes has been reconstructed from the DNA-sequencing data available for many organisms from bacteria to humans (11). A phylogenetic analysis of the deduced protein sequences indicates that the extant complement of mammalian *AOX* genes is likely to originate from two distinct and primordial gene duplication events involving an ancestral *XDH* gene (24). The first duplication involves a bacterial *XDH* gene and led to the development of the current set of *AOX* genes observed in bacteria, protists, algae, and plants (Fig. 5). The second duplication is likely to have originated from a fish *XDH* gene, and it is at the basis of the present complement of vertebrate *AOX* genes (Fig. 5).

Vertebrate AOX and XDH proteins are characterized not only by high levels of similarity in terms of their amino acid sequences, but also by a similar exon-intron organization of the corresponding genes. As already mentioned, the mouse and rat genomes contain four distinct loci, *Aox1*, *Aox2*, *Aox3*, and *Aox4*, while a single active *AOX1* gene is present in humans and higher primates (Fig. 1). All mammalian *AOX* and *XDH* genes consist of 35 and 36 exons, respectively. In mammals, the exon-intron junctions of all of the *AOX* and *XDH* genes are strictly conserved (10, 24). The *Aox1*, *Aox2*, *Aox3*, and *Aox4* genes cluster on a short region of mouse chromosome 1 and rat chromosome 9 in a head-to-tail configuration (Fig. 1). The presence of
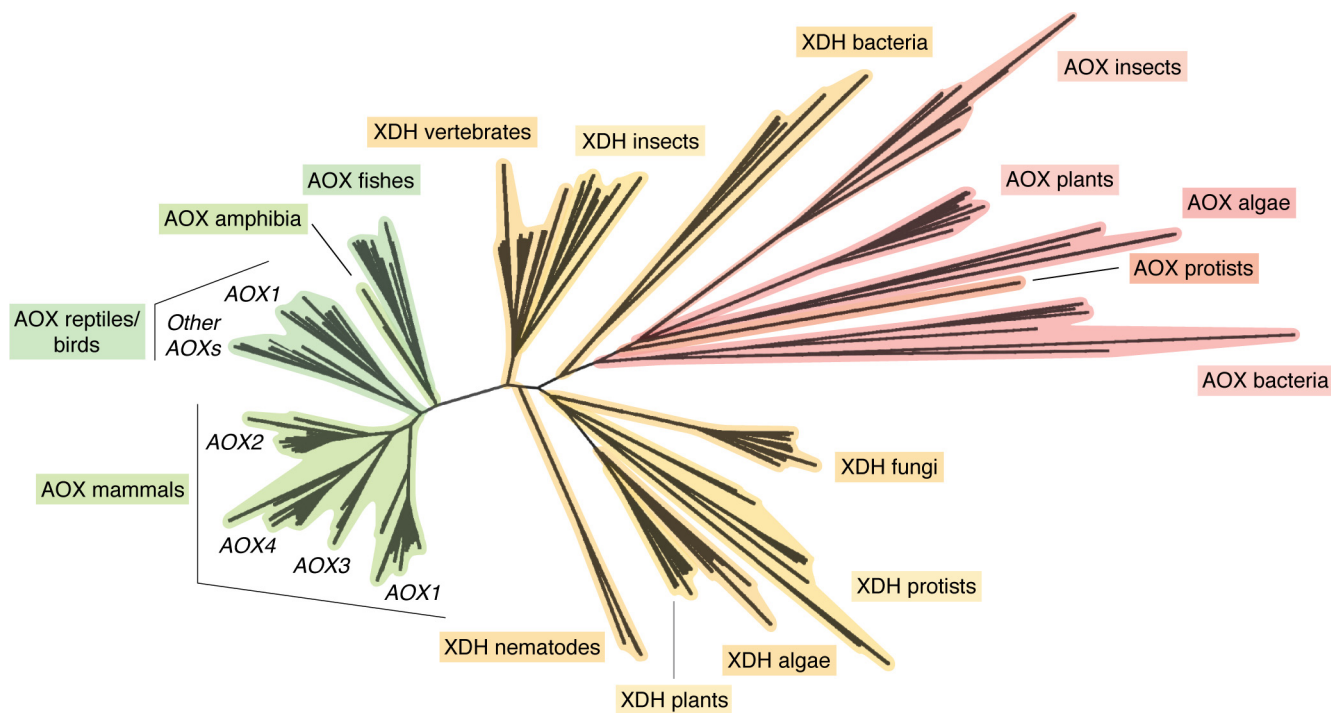
Figure 5. Phylogenetic tree of AOX and XDH proteins in prokaryotes and eukaryotes. The unrooted phylogenetic tree was generated from the available prokaryotic as well as eukaryotic AOX and XDH protein sequences. The phylogenetic tree consists of all of the AOX and XDH proteins whose structure could be predicted from the cloning of the corresponding cDNAs or deduced from genome-sequencing data.

one or more *AOX* genes resulted from a process of asynchronous *AOX* gene duplication events that occurred during the evolution of vertebrates, starting from fishes (24). *AOX1* is likely to be the most ancient gene, whereas the order of appearance of AOX3, AOX4, and AOX2 during vertebrate evolution is still unknown. However, a number of considerations led to the proposal that AOX4 is more ancient than AOX3, which, in turn, appeared earlier than AOX2. It is concluded that the presence of only a single AOX enzyme in humans is due to gene deletion and pseudogenization events. In humans, downstream of the *AOX1* gene, the *AOX4* gene has been completely deleted, whereas the genes for *AOX3* and *AOX2* were transformed into inactive pseudogenes. Cows seem to have maintained three active aldehyde oxidase genes (*AOX1*, *AOX4* and *AOX2*) on chromosome 2. The absence of nucleotide sequences with similarity to AOX3 strongly suggests that this gene has been deleted. Deletion of the *AOX3* gene seems to be a conserved feature in horses; however, our present view of the aldehyde oxidase cluster in this animal species is still incomplete. Functional inactivation of *AOX3* seems to be a common theme. The genome of dogs is characterized by two seemingly active *AOX4* and *AOX2* loci and two inactive *AOX1* and *AOX3* pseudogenes clustering on chromosome 37. The vestiges of numerous exons with nucleotide similarity to the rodent *Aox1* and *Aox3* genes are easily identified on two separate regions slightly upstream of the dog *AOX4* and *AOX2* loci. It is interesting to note that the dog is currently the only mammalian species that seems to be lacking AOX1, in addition to AOX3. Consequently, this mammal is devoid of aldehyde oxidase activity in the liver.

Tissue-specific expression of different AOX enzymes

The only mammalian species extensively characterized for the profiles of tissue- and cell-specific AOX expression are humans and mice (2). The tissue-specific pattern of human *AOX1* mRNA expression, which is based on EST (expressed sequence tag) data, is available in the UNIGENE section of the NCBI site (UniGene Hs.406238). These data indicate that detectable levels of the *AOX1* transcript are observed in many tissues. Adrenal glands, adipose tissue, and liver are the richest sources of human AOX1, followed by the trachea, the glandular epithelium of the prostate, bone, kidney, and connective tissue. The presence of human AOX1 in the adrenal gland and glandular epithelium of the prostate is consistent with a potential role of the enzyme in steroid hormone metabolism.

In mice, the expression of the human *AOX1* orthologous gene (*Aox1*, UNIGENE Mm.26787) is more restricted, and it is limited to a selected number of tissues (2). The largest amounts of *mAOX1* mRNA are present in the inner ear and the seminal vesicles, although measurable amounts of the transcript are observed also in the liver, the lung, and the central nervous system (56). Expression of the *mAox3* gene (UNIGENE Mm.20108) is also relatively restricted, as significant amounts of the corresponding transcript are found only in the liver, lung, oviduct, and testis. Interestingly, *mAOX3* and *AOX1* are co-expressed in lung and liver. In liver, AOX3 is by far the most abundant enzyme. Significant amounts of the *mAOX4* transcript (UNIGENE Mm.244525) are evident in the fertilized ovum, the inner ear, the tongue (2), and the epidermal layer of the skin (57). However, the richest source of mAOX4 is the Harderian gland, where the molybdo-enzyme accounts for

~2% of all of the cytosolic proteins. The Harderian gland is a large exocrine gland located behind the eye bulb and characterized by the secretion of a lipid-rich fluid, which lubricates the eye surface and the fur (56). The most restricted profile of expression is observed in the case of the mAOX2 enzyme (2), as the corresponding transcript (UNIGENE Mm.414292) is detectable only in the nasal cavity (58). In this location, high levels of *AOX2* mRNA are expressed in the Bowman's gland of the submucosal layer, which is responsible for the secretion of the mucous fluid. Nevertheless, AOX2 is also detectable in the sustentacular cells located in the apical layer of the nasal neuroepithelium. Given this localization, we speculate that AOX2 may play a role in the olfactory process.

Aox4 knockout mice give insights into the physiological role of the enzyme

Further insights into the tissue-specific function of the various mammalian AOXs have been and will be provided by the generation of knockout animals. Currently, $Aox4^{-/-}$ mice are the only knockout animals available, whereas the construction of knockouts in the other genes is in progress (59). $Aox4^{-/-}$ mice are viable and fertile and generally do not show any major morphological or behavioral abnormalities. Despite this, $Aox4^{-/-}$ mice are characterized by perturbations in the expression of clock genes, which are accompanied by reduced locomotor activity as well as resistance to diet-induced obesity and hepatic steatosis. All of these effects are observed in both female and male animals. Resistance to obesity is due to diminished fat accumulation resulting from increased energy dissipation. The phenomenon results from the white adipocytes of $Aox4^{-/-}$ mice undergoing trans-differentiation toward thermogenic brown adipocytes. The data obtained in this knockout model indicate that AOX4 contributes to the local synthesis and biodisposition of endogenous retinoids in the Harderian gland and skin, the principal sources of the AOX isoenzyme. The Harderian gland's transcriptome of $Aox4^{-/-}$ mice shows an overall down-regulation of direct retinoid-dependent genes as well as perturbations in the pathways controlling lipid homeostasis and cellular secretion, particularly in sexually immature animals. The skin of knockout mice is characterized by thickening of the epidermis in basal conditions and after UV light exposure. This is confirmed by analysis of the transcriptome, which shows an enrichment and overall up-regulation of genes involved in the process of epidermal thickening.

Metabolomic analyses performed in the Harderian glands of $Aox4^{-/-}$ and WT animals demonstrate the presence of 25 metabolites whose levels are significantly different in the two types of mice. Among the differentially produced metabolites, tryptophan and 5-hydroxyindolacetic acid stand out, because they are part of the serotonin/melatonin biosynthetic pathway, which controls circadian rhythms. Tryptophan is more abundant in $Aox4^{-/-}$ mice than WT mice, whereas 5-hydroxyindolacetic acid is measurable only in $Aox4^{-/-}$ mice. In addition, the levels of serotonin are significantly lower in the Harderian gland of $Aox4^{-/-}$ than WT mice. A similar trend is observed in the serum. Consistent with this, purified Harderian gland AOX4 recognizes tryptophan and 5-hydroxyindolacetic acid, but not serotonin, as substrates. Given the relevance of the tryp-

tophan/serotonin pathway for circadian rhythms and fat deposition, the data support the idea that AOX4-dependent alterations in the levels of tryptophan and 5-hydroxyindolacetic may explain the perturbations in the two processes observed in $Aox4^{-/-}$ mice.

In conclusion, the phenotype of $Aox4^{-/-}$ mouse provides insights into the physiological function of AOX4, demonstrating that the enzyme plays a role in the control of diurnal rhythms, adipogenesis, and locomotor activity, supporting a link between these three processes. Interestingly, the reported effects have no impact on life expectancy, which is the same in $Aox4^{-/-}$ and WT mice. It is possible that human AOX1 plays a similar role as mAOX4 in adipogenesis, as there are data indicating that the enzyme controls fat deposition in adipocyte cultures (60). This point requires further investigations, as human AOX1 may represent a useful molecular target for the development of new anti-obesity agents.

## Crystal structures of human AOX1 and mouse AOX3

To obtain further insights into differences of the four AOX enzymes, and additionally to reveal substrate- and inhibitor-binding sites, attempts have been made to solve the crystal structures of the four enzymes from mice and AOX1 from humans. So far, only the crystal structures of human AOX1 and mAOX3 are available. However, our knowledge on the molecular details of AOXs has greatly increased based on the 2.5 Å (PDB: 4UHW) structure of human AOX1 in addition to a 2.6 Å structure in complex with the substrate phthalazine and the inhibitor thioridazine (PDB: 4UHX) (Fig. 6) (3). These enzymes were purified after heterologous expression in *E. coli*. The crystal structure of mAOX3 has been determined at 2.8 Å resolution (38, 50) (PDB: 3ZYV) from an enzyme that was directly purified from mouse livers. Overall, human AOX1 and mAOX3 are characterized by a 65% amino acid sequence identity and are very similar in terms of overall fold.

Details on substrate binding at the Mo active site were obtained from the crystal structure of the phthalazine-thioridazine complex cocrystallized with human AOX1 (Fig. 6) (3). The structure is deemed to contain two putative gates controlling access of the substrate and release of the product from the Moco active site. Both gates correspond to mobile, unordered regions, with Gate 1 consisting of the [652]SFCFFTEAEK[661] and Gate 2 consisting of the [880]LDESL[884] amino acid sequences (Fig. 7). By comparison, Gate 2 in mAOX3 and also in the more distinctly related bovine XOR is remarkably ordered. In human AOX1, Gate 1 is predominantly composed of bulky hydrophobic residues. With the exception of Glu[660] (Asp in mAOX2), Gate 1 amino acid residues are not conserved in other members of the XOR family. In addition, Gate 1 is shorter in mouse AOX2, AOX3, and AOX4. In all AOX enzymes, Asp[881] of Gate 2 is strictly conserved, and it is always followed by another acidic residue (Glu or Asp). The side chains of these amino acids point toward the substrate channel and are likely to be involved in binding or orienting polar substrates toward the catalytic center. The active site of human AOX1 contains a few residues (Glu[1270], Phe[923], Lys[893], and Gln[776]) that are conserved in all mammalian AOXs (Fig. 7). Glu[1270] is a key residue present
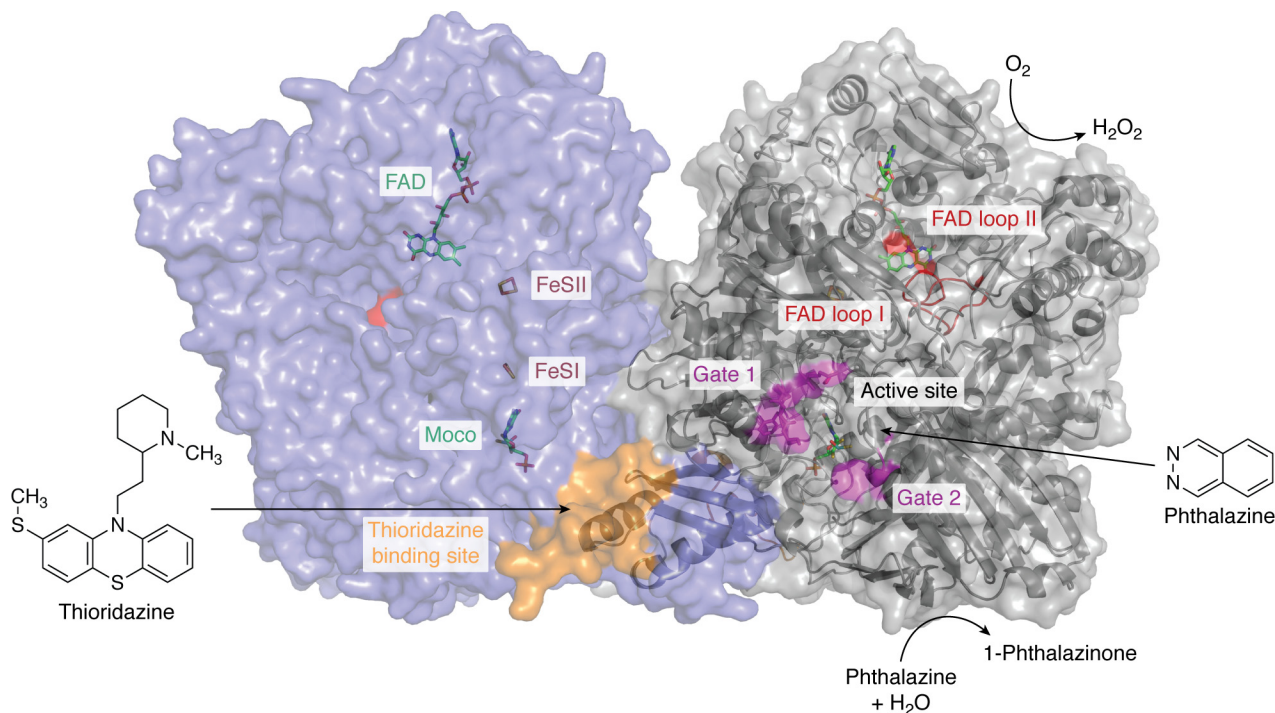
Figure 6. The crystal structure of the human AOX1 homodimer in complex with phthalazine (substrate) and thioridazine (inhibitor). The different protein cofactors (FAD, FeSII, FeSI, and Moco) are indicated and are shown in a *color-coded stick representation* on the *left monomer*. The phthalazine substrate as well as the substrate-binding site, including the flexible Gates 1 and 2, are marked in *pink* on the *right monomer*. The thioridazine-binding site is marked in *orange*. The flexible loops at the FAD site (loop I and loop II) are marked in *red* (*surface representation* on the *left monomer*, *cartoon representation* on the *right monomer*). The figure was generated using PDB entry 1UHW published by Coelho *et al.* (3).
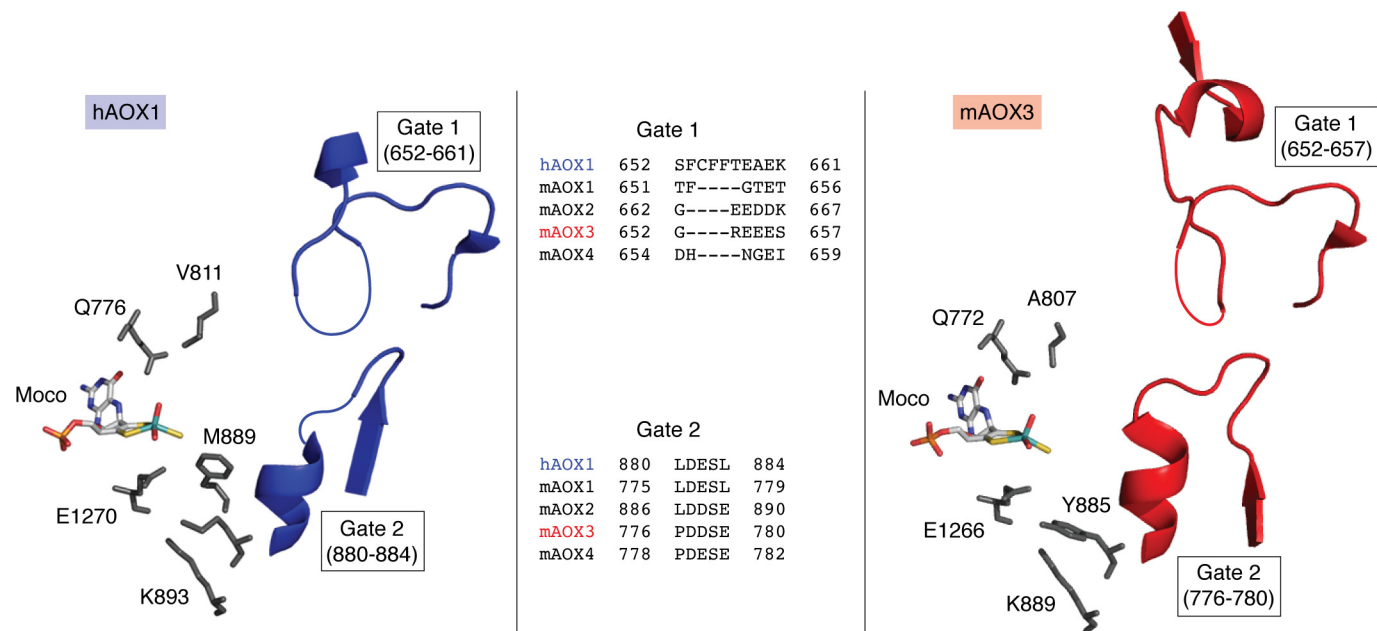
Figure 7. The active site of hAOX1 and mAOX3. Shown are the residues of hAOX1 (*left*) and mAOX3 (*right*) surrounding the Moco and the location of Gate 1 and Gate 2 at the entrance of the substrate funnel. Missing residues in the electron density (and therefore not present in the coordinate files) are indicated by *thin lines*. An amino acid sequence alignment of Gate 1 and Gate 2 of the human and mouse enzymes is given. The figure was created using PyMOL version 2.1.1.

in all members of the xanthine oxidase family, whereas Lys[893] is specific to AOXs.

Substrate specificities of the mammalian AOX enzymes

Mouse AOX1, AOX3, AOX4, and AOX2 proteins are characterized by ~60% sequence similarity and show the same domain and cofactor organization (2). To provide information

on differences in the substrate-binding regions and the funnels leading to the active site, homology modeling based on the available mAOX3 crystal structure (PDB: 3ZYV) has been performed on mouse AOX1, AOX2, and AOX4 (54). The results point to major differences among mouse AOX1, AOX2, AOX3, and AOX4 in the substrate-binding region. On the basis of these data, it has been proposed that the substrate-binding site
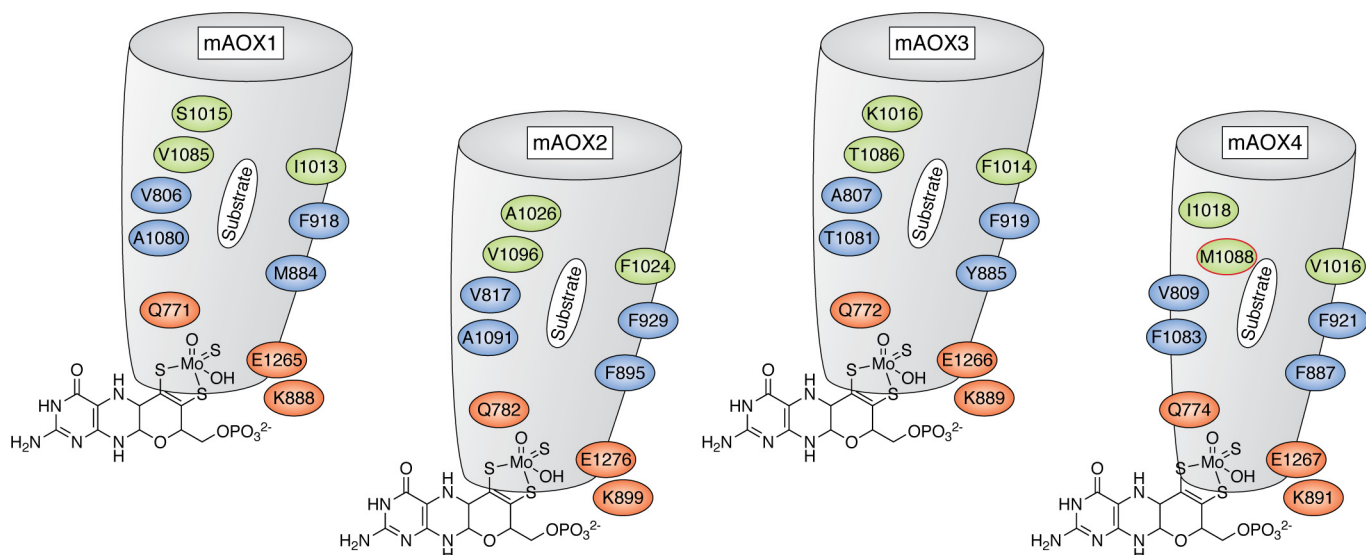
Figure 8. Active site and substrate binding funnel in mouse AOX enzymes. Representation of the substrate binding sites in mouse AOX1, AOX2, AOX3, and AOX4 in addition to residues in the conserved and nonconserved substrate-binding funnel, which selects the substrate specificity. The representation of mAOX3 is taken from the crystal structure (PDB: 3ZYV) (38), whereas those of mAOX1, mAOX2, and mAOX4 are taken from the modeled structures (54). Residues in *red* indicate the amino acids whose nature is conserved in all mouse AOX enzymes; residues in *blue* are hydrophobic residues, partially conserved and involved in substrate orientation; and residues in *green* are those specific for AOX4, with the AOX4-Met[1088] residue *highlighted* in *red*. The funnel for mAOX4 is predicted to be smaller compared with the other enzymes.

consists of a region that builds the inner active site consisting of the amino acids Gln[772], Ala[807], Phe[919], Phe[1014], Lys[889], and Glu[1266] (mAOX3 numbering) (Fig. 8). The more distal amino acids in the substrate-binding tunnel are predicted to form an enzyme-specific region, which shows remarkable differences in the four mAOX enzymes. From these comparisons, mAOX1 is proposed to have the widest substrate-binding funnel. Conversely, mAOX4 is proposed to have the narrowest funnel, which is shaped by several bulky amino acid side chains. Mouse AOX2 and AOX3 are proposed to contain funnels of similar width, but with the mAOX2 funnel likely to be more hydrophobic (54).

To test whether the predicted substrate selectivity of the four mouse AOX enzymes can be explained by the shape of the entrance pocket, the kinetic parameters of the four mAOX enzymes have been compared in a study using 30 different structurally related aromatic and aliphatic substrates (61). In this study, the 30 substrates considered were divided into azo-heterocyclic compounds and molecules containing an aldehyde group (benzaldehydes, alkyl aldehydes, and structurally related cinnamaldehydes). As a general outcome, this study showed that the three mouse AOX1, AOX2, and AOX3 enzymes have no significant differences in substrate selectivity (61). Mouse AOX1 and AOX3 are characterized by the highest catalytic activities against the majority of the common substrates considered (a general summary of selected substrates is given in Table 1) (61). Both mouse AOX1 and mouse AOX3 are mainly expressed in the liver and have evolved overlapping substrate specificities with high substrate turnover rates. It has to be highlighted that AOX1 does not use N^1-methylnicotinamide as substrate. The mAOX3 enzyme thereby often is more effective than mAOX1 under lower substrate concentrations (related to a lower K_m) (61). This role might be beneficial in the liver, where most substrate degradation pathways occur. Thus,

mAOX1 and mAOX3 might cover different niches at different substrate concentrations. The mAOX2 enzyme is also active with all substrates tested; however, the enzyme is often more effective under lower substrate concentrations (61). The role of mAOX2 in rodents still needs to be elucidated in more detail in the future. Expression of mAOX2 is highly restricted to the Bowman's gland in the nasal cavity, but no specific substrate for mAOX2 has been identified. Overall, the substrate specificities of mAOX2 are overlapping with those of mAOX1 and mAOX3. Lower substrate concentrations might be present in the Bowman's gland as compared with the liver, explaining the higher catalytic efficiencies of the enzyme, and likely, high turnover rates are also not mandatory in this tissue.

Conversely, mAOX4 is generally the least active enzyme with the highest selectivity toward the substrates considered. In particular, mAOX4 is unable to use phenanthridine, N^1-methylnicotinamide, ethylvanillin, and methoxybenzaldehyde as substrates and is characterized by a poor reactivity toward purine, whereas these compounds are generally good substrates for most of the other three mouse AOX enzymes (Table 1). Conclusively, mAOX4 is unlikely to play an important role in the oxidation of aromatic heterocyclic compounds. In addition and consistent with the predicted hydrophobicity of the substrate-binding funnel, mAOX4 does not recognize hydrophilic aromatic substrates and some N-heterocyclic compounds (54).

In conclusion, from the overlapping substrate specificities of mouse AOX1, AOX2, and AOX3, similar substrate-binding cavities were predicted according to modeling studies (54) (Fig. 8). The kinetic data show that only mAOX4 has a predicted narrower substrate-binding funnel, suggesting a higher selectivity, lower turnover numbers, and catalytic efficiencies for most substrates tested (Table 1 and Fig. 8). In general, the substrate selectivities predicted by only computationally based analyses were not confirmed by kinetic data (54).

Table 1

Substrate specificities of the mouse AOX enzymes

ND, not determined; $++++$, very good substrate ($k_{cat} > 300$ min^{-1}); $+++$, good substrate ($k_{cat} = 120-300$ min^{-1}); $++$, moderate substrate ($k_{cat} = 50-120$ min^{-1}); $+$, poor substrate ($k_{cat} = 15-50$ min^{-1}); $-$, substrate not converted.

	AOX1	AOX2	AOX3	AOX4
Former names	AOX1	AOH3, AOX3L1	AOH1	AOH2
Potential functions	ND	ND	ND	Adipogenesis, circadian clock, locomotor activity
Predominant expression in tissue	Liver	Bowman's gland	Liver	Harderian gland
Rate of O_2^- production in moles per mole of substrate	30%	40%	20%	20%
Preferred substrates/products: Aromatic aldehydes				
Benzaldehyde $\rightarrow$ benzoic acid	$+++$	$+++$	$+++$	$++$
Vanillin $\rightarrow$ vanillic acid	$++++$	$++$	$+++$	$+$
2-Methoxybenzaldehyde $\rightarrow$ 2-methoxybenzoic acid	$++++$	$+$	$++++$	$-$
***N*-Heterocyclic compounds**				
Phthalazine $\rightarrow$ phthalazinone	$++$	$+++$	$+++$	$++++$
Phenanthridine $\rightarrow$ phenanthridinone	$+++$	$+++$	$+++$	$-$
Purine $\rightarrow$ hydroxypurine	$++++$	$+++$	$++++$	$+$
N^1-Methylnicotinamide $\rightarrow$ 6-pyridone	$-$	$++$	$+$	$-$
Cinnamaldehyde-related compounds				
Cinnamaldehyde $\rightarrow$ cinnamic acid	$+++$	$++$	$+++$	$-$
4-Dimethylamino cinnamaldehyde $\rightarrow$ 4-dimethylamino cinnamic acid	$+++$	$++$	$++$	$+$
Aliphatic aldehyde				
Hexanal $\rightarrow$ hexanoic acid	$+++$	$+$	$++$	$+$

To identify the amino acids that are specific for the substrate selectivity of mAOX4, site-directed mutagenesis was performed. To this purpose, residues Val[1016], Ile[1018], and Met[1088] in AOX4 (Fig. 8) were exchanged into the corresponding amino acids present in the other mAOX enzymes. These residues are not conserved and were identified to be specific for mAOX4 (61). The M1088T exchange had a major effect on the kinetic constants of the AOX4 enzyme. The k_{cat} of this variant was increased with benzaldehyde, salicylaldehyde, phthalazine, and pentanal compared with the corresponding value of WT counterpart (61). Thus, this amino acid largely contributes to substrate binding and turnover. Conclusively, whereas the role of mAOX4 is specific to the Harderian gland, the enzyme apparently has not evolved for high substrate turnover in this tissue, because one selected amino acid exchange in an amino acid specific for mAOX4 led to an increase in activity, whereas the substrate selectivity was unaltered in this variant.

In general, the data discussed above indicate that there is an urgent need to obtain the crystal structures of the three mouse AOX enzymes (AOX1, AOX2, and AOX4), as this is the only way to identify the structural differences at the basis of the experimentally determined profiles of substrate selectivity. In fact, computational modeling data seem to provide pieces of information that are not sufficient to predict the substrate specificity of mouse AOX1, AOX2, AOX3, and AOX4.

The role of mammalian AOXs in the production of reactive oxygen species

AOXs are cytosolic proteins and use molecular oxygen as the sole electron acceptor. During substrate oxidation and molecular oxygen reduction, AOXs generate mainly H_2O_2 in addition to a proportion of O_2^- radicals. So far, XOR in its XO form has been considered to be the only molybdo-flavoenzyme involved in the generation of O_2^- (62–64). The fact that also AOXs produce a significant amount of O_2^- radicals has been largely overlooked in the past and might be of patho-physiological interest (65). Particularly, it is noticeable that hepatic AOX1 is calculated to generate 24-fold larger amounts of O_2^- than XOR. These

calculations are based on the relative levels of XO and AOX enzymatic activity in human liver (17, 65). Thus, AOX1 and other mammalian AOXs may represent significant sources of ROS in the cytosol of liver and other tissues and may play a critical role in ROS-mediated tissue injury under specific conditions (66, 67). Given this background, the abilities of the purified mouse AOX1, AOX2, AOX3, and AOX4 enzymes to produce O_2^- have been compared (53). The four mAOX enzymes generally show a different rate of O_2^- production in relation to the amount of substrate converted. In particular, mAOX2 is the most efficient producer of superoxide anions, with a rate of 40% (Table 1). As the enzyme is located in the nasal mucosa, which is one of the main sites of entrance for infective agents, it might be feasible to speculate that mAOX2 produces ROS from endogenous substrates to protect mice from viral and bacterial infections. A similar role might be played by human and mAOX1 in the liver. Also, mAOX1 was shown to produce a ratio of 30% superoxide radicals, which is higher than the reported value of 15–20% for XO (67). In contrast, mAOX3 and mAOX4 produce a rate of 20% O_2^- radicals per mole of substrate converted, a ratio that is significantly lower as compared with mAOX1 and mAOX2. In this and other tissues, it is also possible that AOX-dependent production of O_2^- might be involved in some of the toxic effects triggered by drugs and xenobiotics.

Potential physiological functions of mammalian AOXs in endogenous pathways

The functional role of AOXs in the vertebrate organisms is still largely unknown, although the broad substrate specificity supports the idea that these enzymes are involved in numerous metabolic pathways. In addition to the substrates shown in Table 1, it has to be noted that under hypoxic conditions, AOXs can also catalyze the reduction of *N*-oxides, sulfoxides, nitrocompounds, and heterocycles (12, 68–71). More recently, it was also demonstrated that AOXs hydrolyze amides (12, 72, 73). Despite this, still very little information is available on the endogenous substrates recognized by AOXs and the metabolic pathways these enzymes are involved in.

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As mentioned above, AOXs may play a role in tryptophan metabolism, as metabolomic analyses conducted in *Aox4* knockout mice demonstrated that tryptophan and 5-hydroxy-indolacetic acid levels are higher in the Harderian gland of genetically engineered than in that of control animals (62).

Nicotinamide catabolism is another metabolic pathway that may be partially controlled by AOXs. In fact, AOXs oxidize N^1-methylnicotinamide into N^1-methyl-2-pyridone-5-carbox-amide and N^1-methyl-4-pyridone-5-carboxamide. N^1-Methyl-nicotinamide is metabolized by AOX-enriched extracts obtained from human (74), monkey (74), rat (75), rabbit (76), and guinea pig liver (77), suggesting that the compound may be recognized by human and other mammalian AOX1 isoenzymes or mammalian AOX3. In this case, only purified mouse AOX3 and AOX2 proteins have been shown to recognize N^1-methyl-nicotinamide as a substrate (44).

AOXs are also suggested to be involved in vitamin A metabolism. In fact, AOXs are implicated in the oxidation of 9-*cis*-and all-*trans*-retinal into the corresponding retinoic acids. All-*trans*-retinoic acid is the active metabolite of vitamin A, and it controls many aspects of vertebrate homeostasis in both the developing and adult organisms. In vertebrate embryos, all-*trans*-retinoic acid is a well-known morphogen, and it regulates the development of numerous tissues and organs, including the central nervous system. In adult organisms, the molecule influences numerous biological processes, including vision as well as growth/differentiation of skin epithelial and hematopoietic cells. At present, all-*trans*-retinal is the candidate endogenous substrate of AOXs for which the greatest amount of supporting evidence is available. Purified mouse liver AOX1 (78, 79) and AOX3 (80), Harderian gland AOX4 (57), and nasal AOX2 (81) proteins recognize retinaldehyde as a substrate. The data were confirmed by using purified recombinant enzymes, which demonstrates that mAOX1 is endowed with the highest retinalde-hyde-metabolizing activity. In line with this, a marked decrease in the levels of all-*trans*-retinoic acid is evident in the Harderian gland and skin of *Aox4* knockout mice relative to what is observed in WT animals (57).

Roles in drug and xenobiotic metabolism

The presence of AOX1 and AOX1/AOX3 in the hepatic tissue is the basis for the role played by this class of enzymes in the metabolism of exogenous compounds, such as drugs and environmental pollutants humans and other mammals are exposed to (25, 38, 82). AOXs metabolize a variety of anti-tumor, immunosuppressive, anti-malarial, and anti-viral drugs as well as molecules acting in the central nervous system. As for the anti-tumor and immunosuppressive agents, methotrexate and 6-mercaptopurine are two prominent examples of AOX substrates. In the context of antimalarial and anti-viral drugs, liver AOXs have been shown to oxidize cryptolepine into cryp-tolepine-11-one (83) and quinine into 2-quinone (84).

AOXs metabolize not only drugs, but also molecules of toxicological interest, such as the environmental pollutants, phthalazines, which are classical AOX substrates. Different AOXs oxidize phthalazine into 1-hydroxyphthalazine, which undergoes irreversible isomerization into 1-phthalazinone (3, 16, 85). Phthalazine administration increases rabbit liver AOX activity, demonstrating that the compound is not only a substrate, but it is also an AOX inducer (86). Two other examples of relevant AOX substrates are caffeine (87) and the aromatic aldehyde, vanillin. Vanillin is a sweetener, and it is oxidized into the corresponding carboxylic acid by AOXs. Nitro-compounds are further prototypes of toxic agents with the potential to be metabolized by mammalian AOXs. Indeed, molecules containing a nitro functionality exemplify the ability of AOXs to act not only as oxidases, but also as reductases (13, 71, 88–90). For instance, various AOX isoenzymes have the potential to reduce 2-nitrofluorene, 1-nitropyrene, and 4-nitrobiphenyl, three widespread pollutants, into the corresponding amines. Interestingly, AOX-dependent reduction of these substrates is observed not only in the liver (13), but also in the skin of mice (88, 89). This has important ramifications for the human organism, as our skin contains AOX1 enzymatic activity (91), and human AOX1-dependent reduction of nitro-compounds may represent an important defense reaction against environmental pollutants.

Conclusions

The role of human AOX1 in the metabolism of xenobiotics is of general significance in drug development programs (20, 26, 27). However, the animal models used during preclinical studies represent a major problem, because unlike humans, some of the most popular animal models, such as rodents, are characterized by expression of other AOXs besides AOX1. Further, dogs and cats are not good experimental tools for metabolic studies involving human AOX1 substrates (27) because of inactivation of the *AOX1* and *AOX3* genes by pseudogenization (24, 92). Thus, it is not surprising that canine studies have failed to detect metabolites of AOX substrates either *in vitro* or *in vivo* (93, 94).

The decrease in the number of active AOX isoenzymes from rodents to humans is the result of progressive deletion/inactivation of the corresponding genes. Combining the studies summarized above, it is possible that, at least in part, the functions carried out by rodent AOX1, AOX2, AOX3, and AOX4 are combined in human AOX1, making it a multipurpose enzyme. This may have resulted from evolutionary changes in the substrate-binding funnel of human AOX1, which is wider and consequently has resulted in an increase in the ability of the enzyme to recognize physiological substrates originally specific to AOX2, AOX3, and AOX4. However, a summary of the data present in the literature mainly on the mouse enzymes implies that AOX2 and AOX4 exert specialized functions in organs and tissues that are no longer active or have become dispensable in humans. The presence of two enzymes, mAOX1 and mAOX3, in the liver might be beneficial to mice to cover a larger range of substrate concentrations to be converted by the two enzymes. More detailed future studies in addition to knockout mice in each individual AOX enzyme are expected to provide more information on the patho-physiological and tissue-specific roles of these enzymes. In particular, the role of AOX4 in adipogenesis, as revealed by the *Aox4* knockout mouse, provides important information that might be beneficial for the treatment of obesity in humans.

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