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Review

Mammalian aldehyde oxidases: genetics, evolution and biochemistry

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Abstract. Mammalian aldehyde oxidases are a small group of proteins belonging to the larger family of molybdo-flavoenzymes along with xanthine oxidoreductase and other bacterial enzymes. The two general types of reactions catalyzed by aldehyde oxidases are the hydroxylation of heterocycles and the oxidation of aldehydes into the corresponding carboxylic acids. Different animal species are characterized by a different complement of aldehyde oxidase genes. Humans contain a single active gene, while marsupials and rodents are characterized by four such genes clustering at a short distance on the same chromosome. At present, little is known about the physiological relevance of aldehyde oxidases in humans and other mammals, although these enzymes are known to play a role in the metabolism of drugs and compounds of toxicological importance in the liver. The present article provides an overview of the current knowledge of genetics, evolution, structure, enzymology, tissue distribution and regulation of mammalian aldehyde oxidases.

Keywords. Aldehyde oxidase, molybdo-flavoenzyme, AOX1, AOH1, AOH2, AOH3, drug metabolism, pseudogene.

Introduction

Aldehyde oxidases are a small group of proteins belonging to the larger family of molybdo-flavoenzymes (MFEs) and are classified under a single EC number (EC 1.2.3.1). MFEs are enzymes requiring FAD and a particular form of organic molybdenum, known as the molybdenum cofactor (MoCo) [1, 2], for their catalytic activity. MoCo is a molybdopterin in eukaryotes, while it is a molybdopterin nucleotide in prokaryotes [2, 3]. The biochemical pathway leading to the synthesis of MoCo in both prokaryotic and eukaryotic organisms is complex and the details have only recently been elucidated [2].

As illustrated in Figure 1A, MFEs are structurally conserved proteins whose catalytically active form consists of two identical subunits of 140 – 150 kDa according to the enzyme considered. The MFE subunit has a typical tripartite structure in which an approximately 20-kDa N-terminal, a central 40-kDa and a 85-kDa C-terminal domains are recognized. The three domains have similar amino acid sequences in all the members of the family and are separated by two hinge regions whose primary structure is much less conserved [4]. The N-terminal domain is characterized by two non-identical iron-sulfur (2Fe/2S) centers, the central domain contains the FAD-binding site, whereas the large C-terminal domain consists of the

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substrate-binding pocket lying in close proximity to the MoCo-binding site [4]. In plants and animals, two types of MFE are known, aldehyde oxidases and xanthine oxidoreductases (XORs). The two types of enzymes are classified in the same sub-group because they have similar structures. Furthermore, unlike other molybdoproteins, such as sulfite oxidases and nitrate reductase, aldehyde oxidases and XORs require post-translational sulfuration of MoCo to become catalytically active [2, 5–9]. The reaction is carried out by a recently characterized sulfurase which substitutes one of the *oxo* groups of the MoCo with a *sulfo* double bond. Deficits in this specific sulfurase are responsible for a very rare human genetic disease known as the combined form of xanthinuria (type II xanthinuria), which is characterized by the absence of both aldehyde oxidase and XOR activities in affected patients [10, 11]. Other members of the XOR and aldehyde oxidase sub-family of MFEs are prokaryotic enzymes, such as carbon monoxide dehydrogenases [12, 13] and quinoline oxidases [14].

Unlike XOR, which has been known and studied for over 100 years, the amount of literature available on aldehyde oxidases is limited. Aldehyde oxidases catalyze the general reaction depicted in Figure 1B, acting on a large array of substrates. Typical substrates are compounds containing an aromatic heterocycle or aromatic aldehydes. N-heterocycles can be hydroxylated on the ring, while aldehydes are oxidized into the corresponding carboxylic acid. In a certain sense, the term 'aldehyde oxidase' is a misnomer, as these MFEs do not necessarily act on substrates containing an aldehyde functionality. In a typical catalytic cycle, the substrate is oxidized to the product at the molybdenum center. The reducing equivalents are passed to the flavin, which is reoxidized by molecular oxygen. The 2Fe/2S centers mediate the transfer of electrons between MoCo and the flavin cofactor and serve as electron sinks, storing reducing equivalents during catalysis [15]. The proposed reaction mechanism underlying the oxidation of an aldehyde to the corresponding carboxylic acid is summarized in Figure 1C. The scheme highlights the significance of a highly conserved Glu residue present in the substrate pocket of all aldehyde oxidases [15].

While XORs are represented throughout evolution from bacteria to humans, aldehyde oxidases are thought to be present predominantly in multicellular plants and animals [4], although reports of the existence of the latter enzymes in certain bacteria are available [16–19]. The physiological function of aldehyde oxidases in plants is beginning to be elucidated, as the enzymes are involved in the synthesis of abscisic acid, a phytohormone regulating growth under stressful conditions [5, 7, 20–24]. Clues as to the physiological significance of aldehyde oxidases in simple animal organisms like insects have been gathered. Indeed, one of the aldehyde oxidase isoforms expressed in the cabbage armyworm, *Mamestra brassicae*, is involved in the chemo-reception of pheromonal stimuli in the antennae [25]. Information on the physiological role played by aldehyde oxidases in vertebrates, mammals and humans in particular is lacking, despite the ever increasing amount of data on their primary structure and evolutionary history.

In this review article, we will provide an updated picture of current knowledge on aldehyde oxidases, focussing on the vertebrate enzymes. We will discuss the available literature and some new information recently obtained in our laboratory, concentrating on the structure, genetics, phylogeny and regulation of this group of enzymes. It must be emphasized that the current nomenclature of aldehyde oxidases found in the major public databases is confusing, often provisional and needs to be revised. The reader is referred to Table I for an updated list of the various aldehyde oxidases identified and characterized so far or predicted on the basis of the available sequenced genomes. As the various aldehyde oxidase genes and products are identified with different names and acronyms, we decided to adopt a unifying nomenclature throughout this review. The term 'aldehyde oxidase(s)' is used in a general sense, whenever no distinction between the various isoforms of the family is meant. When dealing with specific gene products, particularly in the vertebrate realm, we will use the nomenclature that we proposed originally [26]. We will refer to the various rodent proteins and their orthologues in other species as AOX1 (aldehyde oxidase 1, the first vertebrate aldehyde oxidase identified and characterized), AOH1, AOH2 and AOH3 (aldehyde oxidase homologues 1, 2 and 3).

Different organisms have a variable complement of aldehyde oxidase genes: an evolutionary perspective

Until a few years ago, it was believed that the complement of vertebrate MFEs consisted of only two members, aldehyde oxidase (AOX1) and XOR. In humans, it was known that the two proteins were the products of distinct genes residing on the p and q arms of chromosome 2 [27–30]. A similar situation was thought to be also true in the case of most other vertebrates. However, the picture turned out to be more complex, with the identification and structural characterization of novel murine genes coding for different forms of aldehyde oxidases [26, 31, 32]. In this section, we will discuss the information available

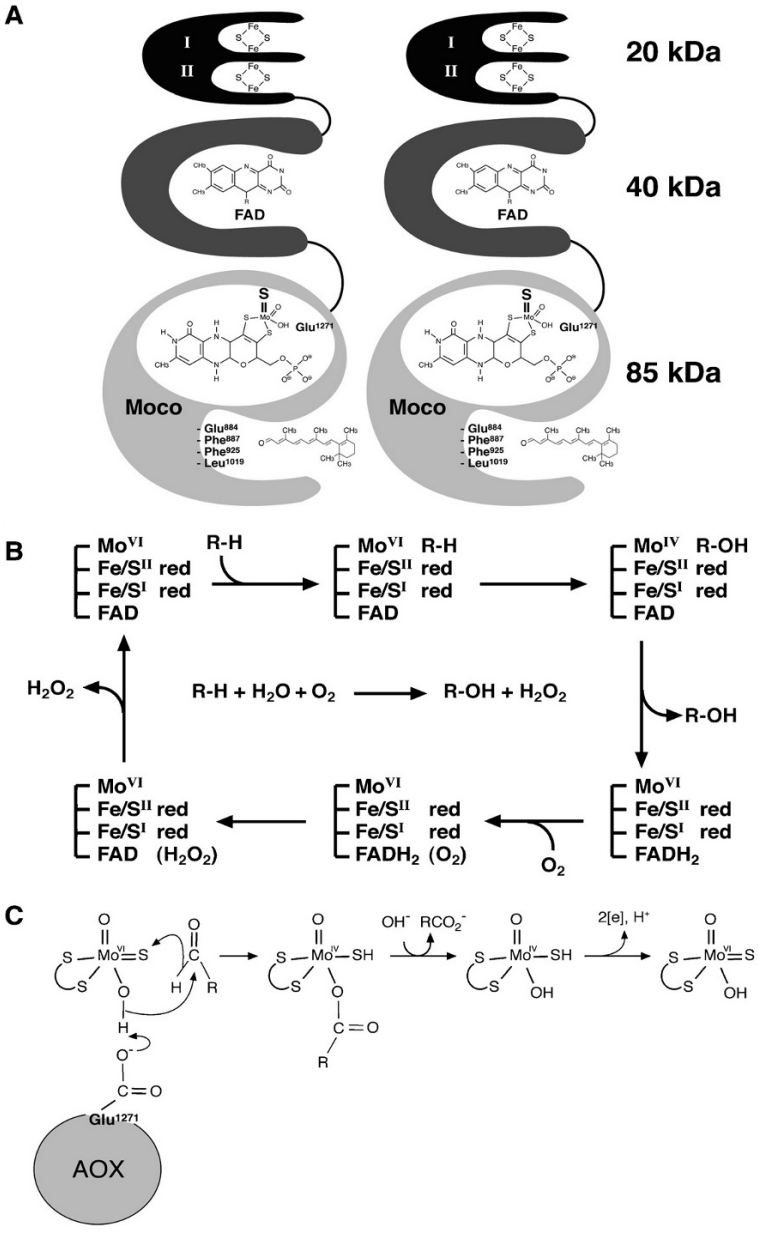

Figure 1. Domain composition of prototypical aldehyde oxidases and reaction mechanism. (*A*) The panel shows that all mammalian MFEs are homodimers consisting of two identical subunits. They comprise an N-terminal domain in which the two 2Fe/2S redox centers (I and II) are localized. The N-terminal domain is linked to the FAD-binding domain through a poorly conserved and relatively unstructured hinge region. The last two domains are linked by a second hinge region. This C-terminal domain contains the MoCo-binding site, which is located within the substrate pocket. The post-translational sulfuration of MoCo is highlighted. The highly conserved amino acid residues located in the substrate pocket along with the Glu residue important for the catalysis are also shown. Numbering of these residues refers to the bovine AOX1 sequence. In the drawing, the structure of the aldehyde oxidase substrate, retinaldehyde, is shown inside the substrate pocket. (*B*) A simplified catalytic cycle for aldehyde oxidases is shown. The various redox centers of MFEs are ordered from top to bottom according to their involvment in charge transfer. The scheme indicates that the substrate (R-H) is oxidized to the product (R-OH) at the molybdenum center. The reducing equivalents are passed to the flavin, which is reoxidized by molecular oxygen. The 2Fe/2S centers [always shown in their reduced ('red') state for the sake of simplicity] are thought to mediate the transfer of electrons between MoCo and the flavin cofactor, and to serve as electron sinks, storing reducing equivalents during catalysis. (*C*) The panel shows the proposed reaction mechanism for aldehyde oxidases (AOX). Conversion of aldehydes to the corresponding carboxylic acids proceeds via base-assisted nucleophilic attack of the Mo-OH on the substrate carbonyl, with concomitant hydride transfer to the Mo=S. The Glu residue shown is directly involved in the catalysis of the reaction as indicated.

Table 1. Aldehyde oxidase nomenclature.

Organism			Protein				
common name	specific name	abbr.	our symbol	DB name	Gene symbol	accession number	DB
Human	*Homo sapiens*	Hs	**AOX1**	aldehyde oxidase 1	AOX1	NP_001150	G
Chimpanzee	*Pan troglodytes*	Pt	**AOX1**	similar to aldeyde oxidase	nd	ENSPTRP00000021853	E
Orangoutan	*Pongo pygmaeus*	Pp	**AOX1**	hypothetical protein	nd	CAH91253	G
Rhesus monkey	*Macaca mulatta*	Mam	**AOX1**	similar to aldehyde oxidase 1	nd	XP_001089327	G
Rhesus monkey	*Macaca mulatta*	Mam	**AOH2**	similar to aldehyde oxidase 4	nd	XP_001089798	G
Rhesus monkey	*Macaca mulatta*	Mam	**AOH3**	similar to aldehyde oxidase 3-like 1	nd	XP_001089912	G
Macaque	*Macaca fascicularis*	Mf	**AOX1**	aldehyde oxidase	AOX1	AB201545	G
Rabbit	*Oryctolagus cuniculus*	Oc	**AOX1**	retinal oxidase	nd	BAA81726	G
Mouse	*Mus musculus*	Mm	**AOX1**	aldehyde oxidase 1	Aox1	NP_033806	G
Mouse	*Mus musculus*	Mm	**AOH1**	aldehyde oxidase 3	Aox3	NP_076106	G
Mouse	*Mus musculus*	Mm	**AOH2**	aldehyde oxidase 4	Aox4	NP_076120	G
Mouse	*Mus musculus*	Mm	**AOH3**	aldehyde oxidase 3-like 1	Aox3l1	NP_001008419	G
Rat	*Rattus norvegicus*	Rn	**AOX1**	aldehyde oxidase 1	Aox1	NP_062236	G
Rat	*Rattus norvegicus*	Rn	**AOH1**	aldehyde oxidase 3	Aox3	NP_001008527	G
Rat	*Rattus norvegicus*	Rn	**AOH2**	aldehyde oxidase 4	Aox4	NP_001008523	G
Rat	*Rattus norvegicus*	Rn	**AOH3**	aldehyde oxidase 2	Aox2	NP_001008522	G
Dog	*Canis lupus familiaris*	Cf	**AOH2**	aldehyde oxidase 2	AOH2	DQ150104	G
Dog	*Canis lupus familiaris*	Cf	**AOH3**	aldehyde oxidase 3	AOH3	DQ150105	G
Horse	*Equus caballus*	Ec	**AOX1**	hypothetical protein	nd	XP_001500761	G
Horse	*Equus caballus*	Ec	**AOH1**	hypothetical protein	nd	XP_001500709	G
Horse	*Equus caballus*	Ec	**AOH2**	similar to aldehyde oxidase 2	nd	XP_001503642	G
Horse	*Equus caballus*	Ec	**AOH3**	similar to aldehyde oxidase 3	nd	XP_001497159	G
Cow	*Bos taurus*	Bt	**AOX1**	aldehyde oxidase 1	AOX1	NP_788841	G
Cow	*Bos taurus*	Bt	**AOH2**	similar to aldehyde oxidase 2	nd	XP_596585	G
Cow	*Bos taurus*	Bt	**AOH3**	–	–	our reconstrucion	–
Opossum	*Monodelphis domestica*	Md	**AOX1**	hypothetical protein	nd	XP_001379598	G
Opossum	*Monodelphis domestica*	Md	**AOH1**	similar to aldehyde oxidase 1	nd	XP_001379605	G
Opossum	*Monodelphis domestica*	Md	**AOH2**	similar to aldehyde oxidase 2	nd	XP_001379630	G
Opossum	*Monodelphis domestica*	Md	**AOH3**	similar to aldehyde oxidase 3	nd	XP_001370277	G
Chicken	*Gallus gallus*	Gg	**AOX1**	aldehyde oxidase 1	AOX1	DQ150102	G
Chicken	*Gallus gallus*	Gg	**AOH**	aldehyde oxidase 2	nd	DQ150103	G
African frog	*Xenopus laevis*	Xl	**AOX1**	MGC81880	nd	AAH74143	G
Fugu	*Takifugu rubripes*	Tr	**AOX1**	aldehyde oxidase	AOX1	SINFRUP00000131133	E
Pufferfish	*Tetraodon nigroviridis*	Tn	**AOX1**	aldehyde oxidase	AOX1	GSTENP00031889001	E
Zebrafish	*Danio rerio*	Dr	**AOX1**	aldehyde oxidase 1	aox1	XP_699030	G
Fruitfly	*Drosophila melanogaster*	Dm	**AOX1**	CG18522	DmAO1	AAF55207.1	G
Fruitfly	*Drosophila melanogaster*	Dm2	**AOX2**	CG18519	DmAO2	AAF55208.2	G
Fruitfly	*Drosophila melanogaster*	Dm3	**AOX3**	CG6045	DmAO3	AAF55209.1	G
Fruitfly	*Drosophila melanogaster*	Dm4	**AOX4**	CG18516	DmAO4	AAF55210.1	G
Flatworm	*Caenorhabditis elegans*	Ce	**AOX1**	hypothetical protein B0222.9	nd	AAK71353	G

Table 1 *(Continued)*

Organism			Protein				
common name	specific name	abbr.	our symbol	DB name	Gene symbol	accession number	DB
Flatworm	*Caenorhabditis elegans*	Ce	**AOX2**	hypothetical protein F15E6.6	nd	AAB92058	G
Thale cress	*Arabidopsis thaliana*	At	**AOX1**	aldehyde oxidase	AAO1	BAA28624	G
Thale cress	*Arabidopsis thaliana*	At	**AOX2**	aldehyde oxidase	AAO2	BAA28625	G
Thale cress	*Arabidopsis thaliana*	At	**AOX3**	aldehyde oxidase	AAO3	BAA82672	G
Thale cress	*Arabidopsis thaliana*	At	**AOX4**	aldehyde oxidase	AAO4	BAA90299	G
Tomato	*Lycopersicon esculentum*	Le	**AOX1**	aldehyde oxidase	AO1	AAG22605	G
Tomato	*Lycopersicon esculentum*	Le	**AOX2**	aldehyde oxidase	AO2	AAG22606	G
Tomato	*Lycopersicon esculentum*	Le	**AOX3**	aldehyde oxidase	AO3	AAG22607	G
Corn	*Zea mays*	Zm	**AOX1**	aldehyde oxidase	zmAO-1	BAA23226	G
Corn	*Zea mays*	Zm	**AOX2**	aldehyde oxidase-2	zmAO-2	BAA23227	G

The table lists the proteins mentioned in this review. The names of the different organisms are indicated on the left. The accession numbers, the names used in the public databases (DB, GenBank, G, Ensembl, E) as well as the provisional or definitive symbols of the corresponding genes are shown on the right. The acronyms that we suggest and used in the article are highlighted in bold. nd, not determined.

on existing aldehyde oxidases with a focus on their evolution and phylogenetic relationship to XOR.

Bacteria, fungi and slime molds

XORs are very ancient and highly conserved enzymes, being represented in all organisms from prokaryotes to plants, animals and humans, while aldehyde oxidases seem to be much more recent enzymes. Indeed, the presence of functionally equivalent aldehyde oxidases in prokaryotes is not yet firmly established. Recent data reporting on the existence of such enzymes in certain bacteria [16, 18, 19, 33] need further scrutiny. Furthermore, prokaryotic MFEs, like iso-quinoline oxidases [14] are different enzymes, despite a certain degree of similarity with vertebrate aldehyde oxidases in terms of amino acid sequence and substrate specificity. Finally, *Desulfovibrio gigas* aldehyde oxidoreductase [4, 34–40], the first molybdo-enzyme ever crystallized, lacks the FAD domain and is only distantly related to vertebrate aldehyde oxidases.

The fungus *Aspergillus nidulans* is certainly devoid of aldehyde oxidase genes and is characterized by a locus (*HXA*) coding for the prototypic 150-kDa monomeric subunit of XOR [41]. The gene consists of four exons interrupted by three intervening sequences. The structure of *HXA* is substantially different from that of the prokaryotic counterparts, as exemplified by *Escherichia coli.* In the bacterium, XOR is the product of three structural genes organized in an operon, which is completed by a further gene coding for a putative chaperone. The three structural genes encode the N-terminal, intermediate and C-terminal regions of the eukaryotic MFE monomeric subunit (see Fig. 1). Thus, the transition of prokaryotes to eukaryotes is accompanied by the consolidation of the three subunits of the bacterial XOR holoenzymes in a polypeptide chain coded for by a single gene. MFE genes of the aldehyde oxidase type are also absent in the slime mold, *Dictyostelium discoideum*, which is predicted to contain a single XOR-encoding, xanthine dehydrogenase (*XDH*) gene, consisting of two exons.

Flatworms

The most primitive eukaryotic organism showing evidence of an aldehyde oxidase gene is the flat worm, *Caenorhabditis elegans.* Indeed, the genome of this worm is predicted to contain two aldehyde oxidase genes and one *XDH* characterized by exon structures that are more complex (16 exons in all cases) than those observed in the fungus and slime mold. The first aldehyde oxidase gene (*CeAOX1*) located on chromosome V is predicted to code for a protein of 1217 amino acids. The second one (*CeAOX2*) has the same number of predicted exons, though it encodes a 1228-long polypeptide and maps to chromosome IV. Finally, the *XDH* locus (Wormbase: F55B11.1) also maps to chromosome IV and is predicted to be translated into a protein of 1358 amino acids. Notably, unlike the situtation in vertebrates (see below), only a small number of exon-intron junctions are conserved in *XDH* relative to the two aldehyde oxidase genes.

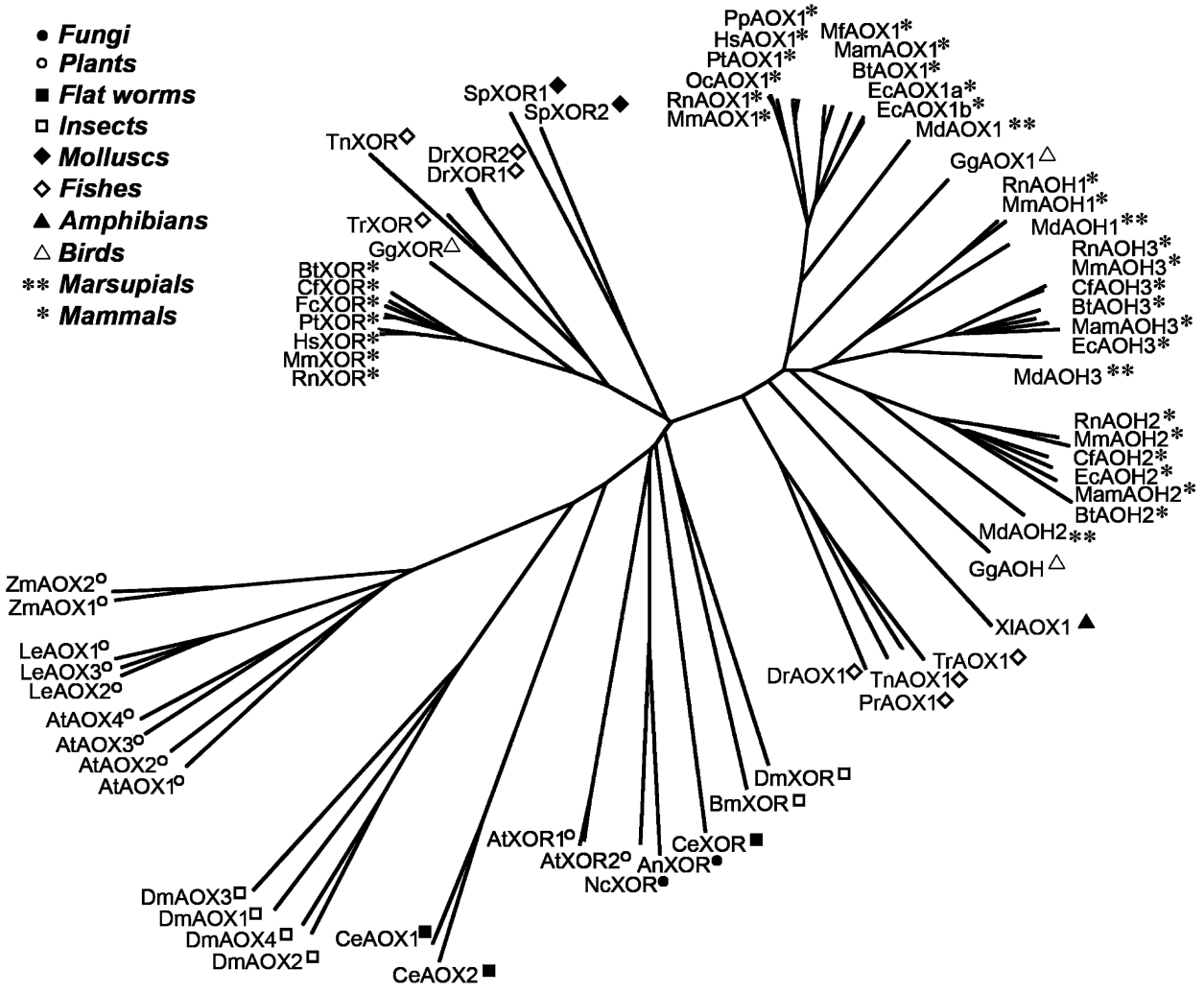

Figure 2. Phylogeny of eukaryotic MFEs. An unrooted dendrogram was obtained by the Phylip method after a CLUSTAL-W computer-aided alignment of the indicated proteins. The various organisms are indicated with the abbreviations shown in Table.

Plants

Plants are characterized by a number of aldehyde oxidase genes that vary from two in *Zea mays* to three and four in *Lycopersicon esculentum* and *Arabidopsis thaliana*, respectively. These are accompanied by the presence of two *XDH* genes in *A. thaliana.* As shown in Figure 2, the protein products of the various plant aldehyde oxidase genes are very similar and cluster together. However, the amino acid sequences of *A. thaliana* AOX1–4 are closer to one another than to any of the aldehyde oxidases present in the two other plants. This suggests a common origin of all plants aldehyde oxidase genes from the same ancestor and subsequent independent gene multiplication.

Insects

A blast search of the *Drosophila melanogaster* genome, using the sequence of mouse AOX1 as a probe, identified four uncharacterized genes clustered at a short distance from one another on chromosome 3R showing all the features of MFEs of the aldehyde oxidase type (*DmAOX1*, *DmAOX2*, *DmAOX3* and *DmAOX4*). This cluster of genes maps to the same chromosome as *XDH* (*DmXdh* or *rosy* locus), which, nevertheless, lies approximately 2.5 Mb upstream. While *DmXdh* consists of four exons, *DmAOX*s are characterized by six or seven predicted exons. Comparison of the structure of the four *DmAOX* genes after alignment of the corresponding protein products demonstrates a certain degree of conservation in some of the intron/exon junctions.

Fishes and amphibians

Vertebrates are characterized by a number of active aldehyde oxidase genes that ranges from 1 to 4 (Fig. 3). Rodents have the largest number of aldehyde oxidase genes, as described below. A striking feature of all vertebrate aldehyde oxidase genes is the

presence of 35 structurally conserved exons [4]. Perfect conservation of 35 out of the 36 exon/intron junctions between the vertebrate aldehyde oxidases and XDHs represent compelling evidence for the common origin of the two kinds of gene [4].

The fish, *Danio rerio* (zebra fish), is characterized by a single aldehyde oxidase gene located on chromosome 22. The corresponding protein shows the highest level of similarity to rodent AOX1. Stretches of DNA containing sequences related to other vertebrate aldehyde oxidase genes are also observed on chromosome 17. However, these sequences do not seem to code for complete and active MFE proteins. The presence of a single aldehyde oxidase gene in fishes is supported by the data available in *Poecilia reticulata*, *Takifugu rubripes* and *Tetraodon nigroviridis*. A similar situation seems to be true in amphibians, as exemplified by the *Xenopus laevis* genome, which is predicted to contain a single locus coding for an aldehyde oxidase with the highest similarity to AOX1 of other animal sources. However, relative to AOX1 s of different origin, the primary structure of the predicted gene product has a number of sequence anomalies that need to be supported by experimental data.

Birds

The only data on the presence and structure of aldehyde oxidase genes in birds were obtained in *Gallus gallus* [42]. In this species, we demonstrated the presence of two genes located at a short distance from each other on chromosome 7. The two genes are transcribed in the same direction and code for a protein with high similarity to mouse AOX1, and another highly related structure, which we named AOH (aldehyde oxidase homologue). The AOH designation is justified by the fact that the protein shows the same level of overall similarity to mouse AOX1, AOH1, AOH2 and AOH3.

Rodents

The mouse genome is characterized by the presence of an aldehyde oxidase gene cluster on chromosome 1 band c1. The cluster extends for approximately 350 kb and consists of four genes in a head-to-tail configuration. Determination of the primary structure of the corresponding proteins demonstrated that the four gene products are highly related, showing an overall similarity of approximately 60 % at the amino acid level. Going from the telomere to the centromere, the first locus to be encountered is *Aox1*, which is the orthologue of the bovine gene coding for the first mammalian aldehyde oxidase ever sequenced [43]. As we will see, this is also the orthologue of the sole active aldehyde oxidase gene present in humans. The second mouse gene, which we named *Aoh1*, lies approximately 5 kb from *Aox1* and is separated from *Aoh2* by approximately 20 kb. The cluster is completed by *Aoh3*, lying approximately 10 kb downstream of *Aoh2*. The presence of an aldehyde oxidase gene cluster is not a peculiarity of the mouse genome, as an identical situation is observed in the rat [31]. In rats, the gene cluster maps to chromosome 9q31 [31], which is largely syntenic to mouse chromosome 1.

Marsupials

The presence of four aldehyde oxidase genes is observed also in the more primitive mammal, opossum (*Monodelphis domestica*), a prototypical marsupial. The first draft of the genome available in the NCBI database indicates the presence of three such genes clustering on chromosome 4. The arrangement of the genes in the opossum cluster is virtually identical to that observed for the first three mouse loci. The recognizable *Aox1* orthologue is located about 10 kb upstream of the *Aoh1* counterpart, which, in turn, lies approximately 33 kb upstream of *Aoh2*. What differentiates the opossum from the rodent genomes is the presence of the *Aoh3* orthologue on a distinct chromosome. Indeed the *Aoh3* locus maps to chromosome 7. It remains to be established whether the four aldehyde oxidase genes predicted in opossum code for an equivalent number of active proteins.

Dogs and bovines

Dogs (*Canis familiaris*), are endowed with two, while bovines (*Bos taurus*) have three aldehyde oxidase genes. The two canine and the three bovine genes cluster on chromosome 37 and chromosome 2, respectively. Interestingly, dog chromosome 37 is characterized by the presence of stretches of DNA with a high level of similarity to rodent *Aox1* and *Aoh1*. However, the DNA sequences do not seem to code for active proteins [42]. The two active dog genes are the orthologues of rodent *Aoh2* and *Aoh3*, whereas the *Aox1*, *Aoh2* and *Aoh3* orthologues are represented in the bovine genome. Although sequencing of the *B. taurus* genome is still incomplete, the approximately 115-kb DNA sequence interposed between the *Aox1* and *Aoh2* orthologues does not seem to have any similarity with the exonic or intronic sequences of rodent *Aoh1*. Interestingly, the updated version of the bovine genome sequence indicates that *AOH2* precedes and is not located downstream of *AOH3*, as previously inferred [42]. Furthermore, the two genes are transcribed in the same direction and on the same strand as AOX1, which is in line with the general characteristics of the aldehyde oxidase cluster in other mammals. So far the existence and the primary structure of the putative protein products of bovine

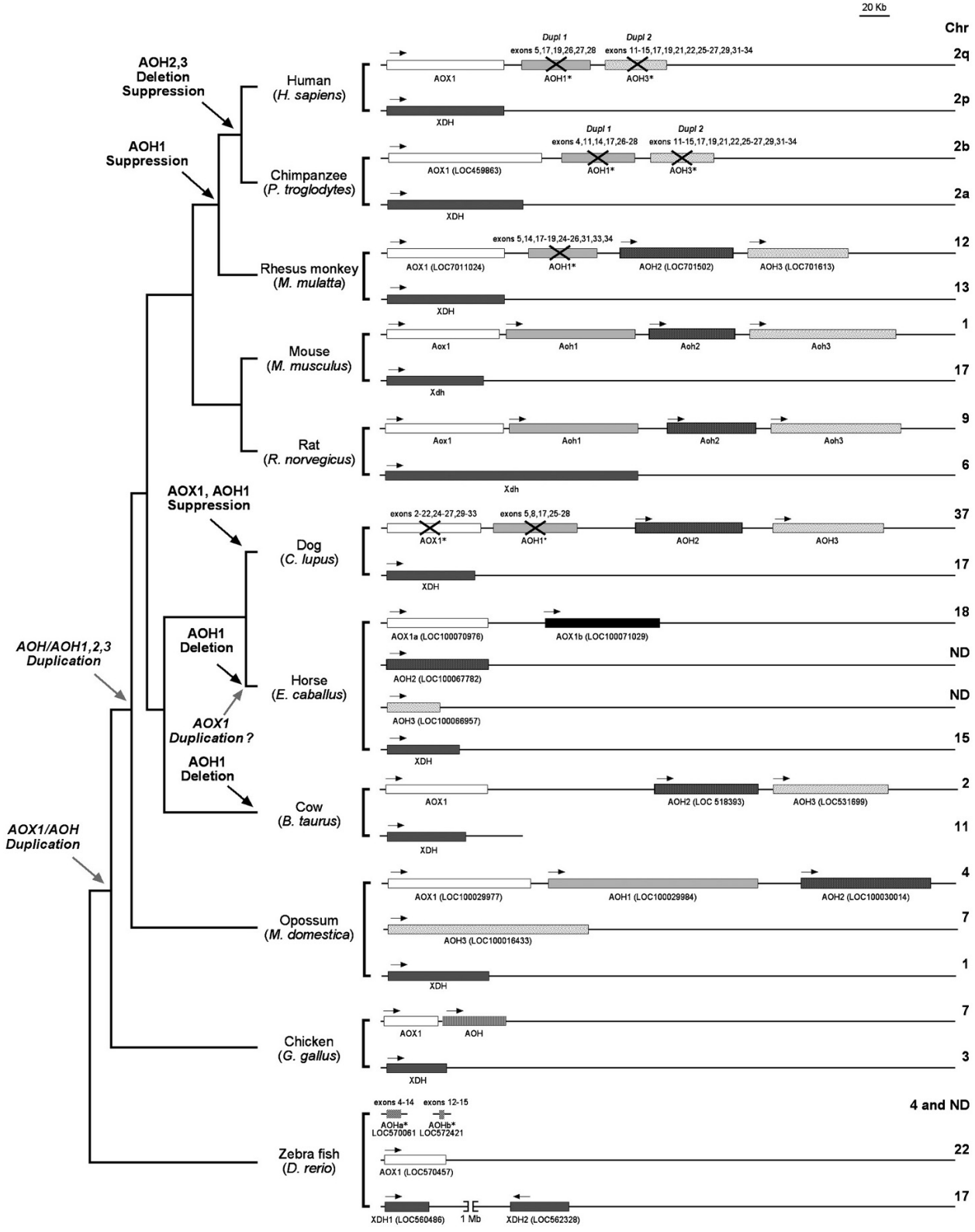

Figure 3. MFE genes in vertebrates. The figure shows a schematic representation of the MFE genes in vertebrates for which complete or almost complete genomic sequence data are available. Orthologous genes are indicated with the same shadowing. The direction of transcription is indicated by arrows. Pseudogenes are crossed through and asterisked. The exons identified in the pseudogenes are also indicated. The chromosomal location is shown on the right (ND, undetermined). Whenever the structure of the gene is predicted solely on the basis of the genomic sequence, and the corresponding cDNAs have not been isolated, the GenBank locus number (LOC) is indicated. The rooted phylogenetic diagram on the left illustrates the relative evolutionary distance between the various animal species considered. This is accompanied by an indication of the suggested timing of the duplication, suppression and deletion events.

AOH2 and *AOH3* rests on the presence of uninterrupted open reading frames (ORFs) coding for polypeptides of 1308 and 1347 amino acids, respectively. On the basis of the strict conservation in the length of all vertebrate aldehyde oxidases, these sequences seem to be slightly shorter and longer than expected, respectively. The two predicted amino acid sequences are likely to be mostly, but not completely correct, since they are not confirmed by experimental data.

Horses

Analysis of the first draft of the genome sequence suggests that the complement of aldehyde oxidase genes may be different in horses (*Equus caballus*) relative to dogs or bovines, despite the evolutionary proximity. In horses, two aldehyde oxidase genes map to chromosome 18 at a distance of approximately 42 kb from each other. The two putative genes are predicted to code for proteins of 1397 and 1467 amino acids, respectively. Comparison with the four mouse aldehyde oxidases demonstrates that the two horse proteins show the highest level of identity with AOX1 (81 % in both cases). The data suggest that one of the horse proteins may have no orthology with any of the other known mammalian aldehyde oxidases. Two further aldehyde oxidase genes of unknown chromosomal location are predicted in the horse genome. One of them codes for a protein of 1335 amino acids, which is the likely orthologue of rodent AOH2, and the other consists of 822 amino acids, representing an incomplete MFE, with substantial similarity to rodent AOH3.

Primates

Humans are endowed with a single aldehyde oxidase, namely the mouse AOX1 orthologous protein [4]. The protein is the product of a gene mapping to chromosome 2q32.3–33.1. However, two distinct DNA stretches (*Dupl1* and *Dupl*2) in which putative exons coding for protein fragments with remarkable similarity to *Aoh1* and *Aoh3* are easily recognized at a short distance from the *AOX1* locus. *Dupl1* and *Dupl*2 are transcribed into mRNAs that do not seem to code for protein products, given the presence of in-frame stop codons. *Dupl 1* and *Dupl* 2 presumably represent the vestiges of the *Aoh1* and *Aoh3* genes that underwent a process of genetic suppression. The human situation is similar to that observed in dogs and bovines, though the number and type of aldehyde oxidase genes undergoing genetic suppression in the three species is different.

The chimpanzee (*Pan troglodytes*) is the closest relative of humans and a small portion of chromosome 2b recapitulates almost exactly the situation observed on human chromosome 2q32.3–33.1. In the non-anthropomorphic primate, Rhesus monkey (*Macaca mulatta*), a relevant gene cluster is predicted to be located on chromosome 12 and consists of three loci coding for the putative protein orthologues of AOX1 (1414 amino acids), AOH2 (1322 amino acids) and AOH3 (1453 amino acids). The intergenic distances between *AOX1* and *AOH2* is approximately 85 kb, whereas a DNA fragment of 10 kb separates *AOH2* from *AOH3.* The 85-kb region contains a number of sequences bearing similarity with various dispersed exons of the mouse *AOH1* [M. Terao and E. Garattini, unpublished observations]. Assembly of these exons does not result in the prediction of an ORF corresponding to a putative aldehyde oxidase. On the basis of these, data the existence of three active aldehyde oxidase genes is predicted.

The phylogenesis of aldehyde oxidases

The data presented in the previous section indicate that the evolutionary history of aldehyde oxidase genes is characterized by a series of subsequent and separate gene duplication and suppression events that led to the present enzymes in plants, insects and vertebrates [4]. The availability of the primary structures of MFE proteins and genes allows a further reconstruction of aldehyde oxidase evolution. Figure 2 shows the phylogenetic tree of eukaryotic MFEs. It is now generally accepted that the XOR-coding gene, *XDH*, is the ancestor of all aldehyde oxidase genes. Aldehyde oxidase genes are likely to be the result of at least two independent duplication events from the ancestral *XDH* gene(s) as we originally suggested [26] and Rodriguez-Trelles *et al.* [44] discussed extensively in an elegant study. The hypothesis of two independent duplications is supported by the dendrogram shown, as the group of vertebrate aldehyde oxidases lies on the opposite side of the XOR cluster relative to the plant and lower-organism counterpart. The dendrogram also demonstrates higher similarity among vertebrate orthologous proteins in different organisms than among homologous proteins in the same organism. This further suggests that the duplication events leading to the extant complement of vertebrate aldehyde oxidase genes occurred before species divergence.

The most ancient member of the vertebrate aldehyde oxidase family is *AOX1*, whose orthologues can be traced back to marine organisms and amphibians. With the appearance of avians, *AOX1* underwent a further process of duplication giving rise to *AOH*, which is easily identified in the chicken genome [42]. That *AOX1* and *AOH* are gene duplication products is also substantiated by the conserved exon architecture [42]. The position of the AOH protein product at the

root of the dendrogram branch in which mammal AOH1, AOH2 and AOH3 cluster suggests ancestorship. At the gene level, the idea is supported by the tightly conserved exon structures of the mammalian *AOH1*, *AOH2* and *AOH3* and the *G. gallus AOH* gene. The dendrogram indicates that the order of distance from avian AOH is AOH2 < AOH1 < AOH3. However, it is currently impossible to say whether this reflects a similar series of ordered duplication events. As to this observation, the only comment possible is that the order of phylogenetic distance does not reflect the chromosomal order of the three genes in all the vertebrate species analyzed. The presence of AOX1, AOH1, AOH2 and AOH3 orthologues in *M. domestica* (opossum) demonstrates that the full process of aldehyde oxidase gene multiplication ended before the appearance of placentates.

Interestingly, rodents and the evolutionary more ancient marsupials seem to be the only extant types of animal maintaining the full complement of four aldehyde oxidase genes. Indeed, the phylogenetic history of other mammalian aldehyde oxidase genes is dominated by a progressive series of gene suppression events (see also Fig. 3). The pattern of gene suppression by deletion or generation of pseudogenes is not very clear, and different animal species have lost different numbers and types of aldehyde oxidase homologues. Bovines suppress *AOH1* by deletion, while carnivores, like dogs, show inactivation of *AOX1* and *AOH1*, with the generation of two inactive pseudogenes. Horses seem to have a rather unique composition of aldehyde oxidases, with two genes resembling *AOX1* in addition to the potential orthologues of *AOH2* and *AOH3*. Primates, such as chimpanzees, and humans are characterized by the presence of two pseudogenes, *AOH1* and *AOH3*, as well as the deletion of *AOH2*. Interestingly, gene inactivation is a sequential process in the evolution of primates, as macaques maintain three seemingly active aldehyde oxidase genes, *AOX1*, *AOH2* and *AOH3*, whereas *AOH1* is only a pseudogene [M. Terao and E. Garattini, unpublished data].

In summary, based on the data discussed, we propose that exon separation preceded gene duplication and species divergence in the evolution of vertebrate aldehyde oxidases. On the other hand, species divergence may have preceded gene duplication and exon separation in the case of aldehyde oxidases from lower organisms.

Toward the definition of the structural elements distinguishing different aldehyde oxidases and XORs

As seen in the previous section, the family of vertebrate aldehyde oxidases consists of a variable number of different gene products according to the animal species considered. The amino acid sequence of all these enzymes is highly related and shares a remarkable level of similarity with the other MFE, XOR. The similarity between aldehyde oxidases and XOR is not limited to the primary and extends to the secondary and tertiary structure of the two enzymes. Although bovine XOR is the only MFE [45] for which the crystallographic coordinates are available, the tridimensional structure of any aldehyde oxidase can be easily predicted using the XOR template and commonly available softwares and algorithms. Computer-assisted prediction of the tridimensional structures of mouse AOX1, AOH1, AOH2 and AOH3 leads to models that are almost superimposable on the experimentally determined tridimensional structure of bovine XOR.

Despite the tremendous level of similarity, aldehyde oxidases and XORs are different enzymes in terms of substrate and inhibitor specificity as well as cofactor requirement and biochemical function. XORs are relatively restricted as to substrate specificity, since they recognize rather selectively xanthine and hypoxanthine. In contrast, neither xanthine nor hypoxanthine are good substrates for any of the aldehyde oxidases purified and characterized so far, including mouse AOX1, AOH1, AOH2 and AOH3. Aldehyde oxidases are endowed with relaxed substrate specificity and can accommodate in their substrate pocket various types of compounds which are generally characterized by an aldehyde functionality, an aromatic or heterocyclic structure. Furthermore, allopurinol and oxypurinol are potent inhibitors of XOR, while they inhibit aldehyde oxidases only very modestly [46]. Conversely, a recent survey of selective inhibitors conducted on 239 drugs demonstrated that the most potent inhibitor of human AOX1 is the selective estrogen receptor modulator, raloxifene ($IC_{50} = 2.9$ nM), with tamoxifen, estradiol and ethinyl estradiol also being potent inhibitors [47]. None of these compounds inhibits XOR efficiently. Menadione, β-carboline and chlorpromazine are also specific inhibitors of aldehyde oxidase but they are totally inactive in terms of XOR inhibition [46, 48–55].

On the basis of the above discussion, it is clear that one of the priorities is to define the structural features and the amino acid residues that are at the basis of the enzymatic characteristics that distinguish aldehyde oxidases from XORs. This is of interest not only in the field of structural biology and protein engineering but

also from an evolutionary point of view, as definition of these structural details may give insights into the ways new enzymes with different functions are created by alteration of existing protein scaffolds. It is also important to establish whether there are significant structural features differentiating mammalian from insect or plant aldehyde oxidases, as the two groups of proteins are believed to have a different evolutionary history and are likely to serve different and perhaps totally unrelated functions in the organisms of origin. Finally, comparison of the fine structural characteristics of the various members of the aldehyde oxidase family may also help us to define whether different forms within the same species, such as rodent AOX1, AOH1, AOH2 and AOH3, can be predicted to metabolize the same or different substrates. All this is particularly timely, since information on the enzymatic characteristics, substrate and inhibitor specificity is starting to build up, as specific methods for the purification of different aldehyde oxidases are now available [26, 31, 56] and there has been substantial progress in the development of strategies aimed at obtaining recombinant MFEs and aldehyde oxidases in their catalytically active form [57, 58].

In the remainder of this section, we will try to summarize and discuss the available information on the structural characteristics of aldehyde oxidases. This will be done by comparing the amino acid sequences of the most important structural domains of these proteins, using Figures 4 and 5 as references. As illustrated, we aligned all the aldehyde sequences known or predicted from the corresponding genomes using the CLUSTAL-W algorithm and the CINEMA software package [59]. Bovine XOR (BtXOR) is added as a reference standard in the comparison.

N-terminal 20-kDa domain

Figure 4A shows the alignment in the region containing the domain encompassing the first 2Fe/2S redox center. The amino acid sequence in this region is very conserved in all the aldehyde oxidase proteins and does not diverge significantly from the XOR counterpart. As expected, the four Cys residues (Cys43, Cys48, Cys51 and Cys73 in BtXOR) involved in the coordination of the two iron atoms are strictly conserved. The most variable stretch of amino acids is located just upstream of the fourth Cys. However, the stretch does not show any consistent difference within the various forms of aldehyde oxidases or between aldehyde oxidases and bovine XOR. The presence of a Cys in all *D. melanogaster* aldehyde oxidases (Cys52 in DmAOXs 1–4) instead of an otherwise strikingly conserved Val residue indicates a major and specific difference in the structure of this domain between insects and mammals. Another detail of major interest is the presence of a conserved Gly residue (Gly42 in BtXOR) in all mammalian AOX1 s and AOH1 s. With the exception of the two proteins predicted in the horse genome, the residue is invariably substituted by an Ala in all AOH3 s and a Ser in all AOH2 s. This amino acid may mark subtle differences in the structural and functional characteristics of the domain occurring in different isoenzymatic forms of mammalian aldehyde oxidases. Noticeably, a Met residue is present in the homologous position of all *D. melanogaster* aldehyde oxidases.

The alignment in the region corresponding to the second non-identical 2Fe/2S redox center is shown in Figure 4B. The region is dominated by a high degree of conservation in all sequences except for a short amino acid stretch which is present only in plant aldehyde oxidases and a missing sequence in bovine AOH2. As the primary structure of the bovine AOH2 protein is only predicted from the corresponding genome, the missing sequence may represent a mistake in exon prediction and assembly. As expected and as already observed for the previous 2Fe/2S redox center, strict conservation of the four Cys (Cys113, Cys116, Cys148 and Cys150 in BtXOR) coordinating the iron atoms is observed. Altogether the data suggest that the domain does not contribute significantly to differentiate the structure of XORs and aldehyde oxidases and does not contain determinants responsible for potential enzymatic differences among the various aldehyde oxidase isoforms.

Intermediate 40-kDa domain

Mammalian XORs exist in two forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO). XDH is converted into XO irreversibly by limited proteolysis and reveribly by oxidation [4]. The dehydrogenase uses NAD instead of oxygen as the final acceptor of the electrons generated by the oxidation of hypoxanthine and xanthine [60]. As shown in Figure 5A, a Tyr residue (Tyr393 in BtXOR) placed in the context of a conserved stretch of amino acids in the 40-kDa intermediate domain of XORs is responsible for the binding of NAD [61]. As expected for enzymes using molecular oxygen, but not NAD, as the final electron acceptor, all aldehyde oxidases are devoid of this Tyr residue. In this position, all mammalian AOX1s are characterized by a Cys residue (Cys400 in HsAOX1) that is not present in other mammalian aldehyde oxidase isoforms or in AOX1s of lower vertebrates, insects and plants. This may cause a substantial distortion of the AOX1 structure in this region, as the amino acid is replaced by a Leu or a Ser in all the other types of mammalian aldehyde oxidases.

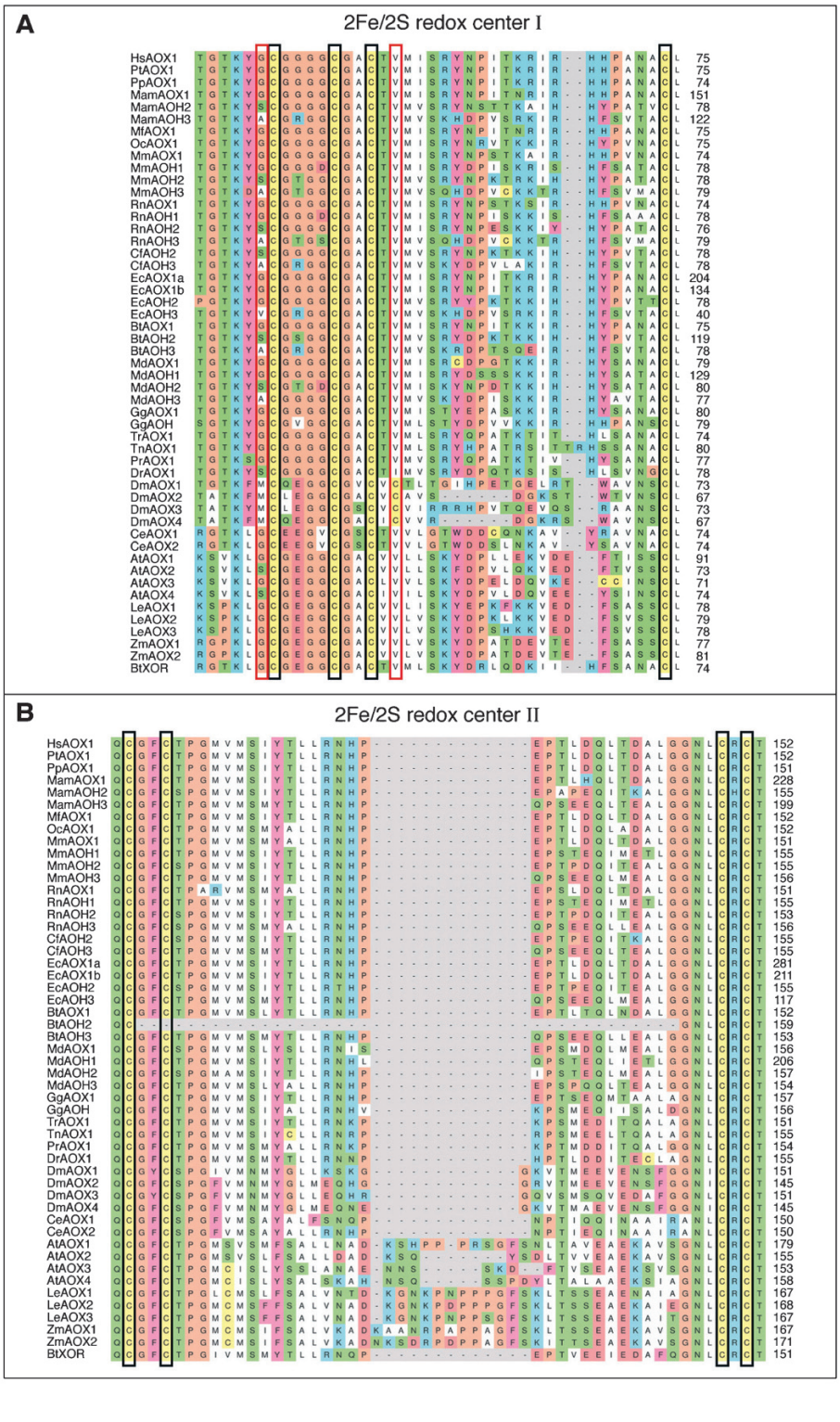

Figure 4. Alignment of aldehyde oxidases and bovine XOR: the two 2Fe/2S redox centers. The indicated amino acid sequences were aligned with the CLUSTAL-W algorithm and the regions corresponding to the first (*A*) and second (*B*) 2Fe/2S redox centers are shown. The names of the aldehyde oxidases of the various organisms, and bovine XOR (bottom; NP_776397) as a reference, are indicated on the left with the same abbreviations used in Figure 2. The number on the right side indicates the position of the last amino acid residue of each sequence. The black boxes indicate the four cysteins involved in the coordination of the two iron atoms. The red boxes indicate residues of significance for the determination of the molecular environment in different types of aldehyde oxidases (see text). Amino acid color code: white, A,I,L,M,V; yellow, C; red, D,E; pink, F,W,Y; orange, G,P; blue, H, K, R; green, N,Q,S,T.

C-terminal 85-kDa domain

The C-terminal domain of all MFEs is characterized by a fingerprint sequence, which, in its latest version, reads as follows: (Gly/Ala)XXX(Lys/Arg/Asn/Gln/His)X(11,14)(Leu/Ile/Val/Met/Phe/Trp/Tyr/Ser)XXXXXXXX(Leu/Val/Ile/Met/Phe)X(Cys/Phe)XX(Asp/Glu/Asn)ArgXX(Asp/Glu), where the amino acids in parentheses show alternative residues, while the X symbols indicate any amino acid. As evident from Figure 5B, all the aldehyde oxidase primary structures adhere to the consensus sequence strictly. It is remarkable, however, that a specific

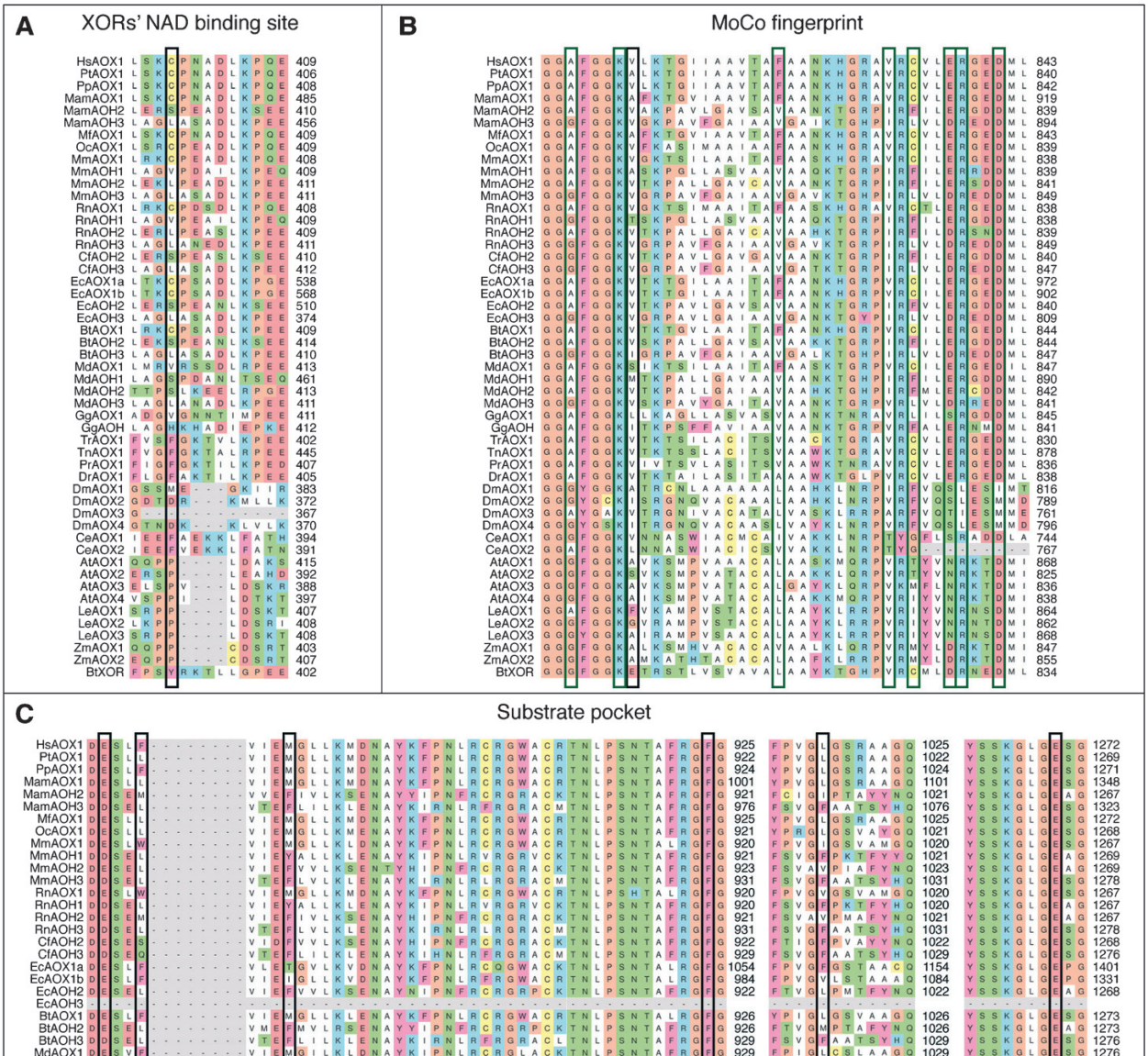

Figure 5. Alignment of aldehyde oxidases and bovine XOR: the NAD-binding site, the MoCo fingerprint region and the substrate pocket. The indicated amino acid sequences were aligned with the CLUSTAL-W algorithm, and the regions corresponding to the NAD-binding site (*A*) the MoCo fingerprint region (*B*) and part of the substrate pocket (*C*) are shown. Amino acid color code: white, A,I,L,M,V; yellow, C; red, D,E; pink, F,W,Y; orange, G,P; blue, H, K, R; green, N,Q,S,T. (*A*) The black box indicates the Tyr residue implicated in NAD binding, conserved in all XORs (not shown) and absent in all aldehyde oxidases. (*B*) Green boxes indicate the residues contained in the molybdo-flavoprotein fingerprint sequence (see text). The black box indicates the Glu802 present in bovine XOR and important for the recognition of hypoxanthine and xanthine. The residue is not conserved in aldehyde oxidases. (*C*) The residues implicated in the differential shaping of the substrate pocket of aldehyde oxidases and XORs (see text) are boxed in black. The Arg880 and Glu1261 of bovine XOR important for the recognition of the substrate and the mechanism of catalysis are also boxed in black.

residue in the sequence distinguishes vertebrate AOX1s from AOH1/AOH2 and AOH3 homologues. Indeed, with the exception of the avian protein, all vertebrate AOX1s contain a Cys in the position corresponding to Cys834 of human AOX1. Invariably, this amino acid is replaced by a Phe in vertebrate AOH1s and AOH2s, whereas a Leu is observed in AOH3s.

Both aldehyde oxidases and XOR are characterized by an active site which is buried approximately 10–15 Å away from the surface [4]. The active site can be reached through a funnel-shaped cavity dominated by the presence of hydrophobic residues able to accommodate the ring structures of the aldehyde oxidase and XOR substrates or inhibitors. As aldehyde oxidases and XORs are characterized by distinct substrates and inhibitors, it is predicted that the tunnel is different in the two enzymes. The existence of substrates, like pyridoxal, recognized by mouse AOX1 and AOH1, but not by AOH2 [M. Terao and E. Garattini, unpublished data], as well as the identification of β-carboline as a far better inhibitor of mouse AOH1 than AOX1, further suggests that the tunnel may vary even in different aldehyde oxidase isoenzymes. In bovine XOR, Leu873, Ser876, Phe914 and Phe1009 play important roles in determining the shape and characteristics of the tunnel. Furthermore, the charged amino acids Glu802 and Arg880 of BtXOR are important for the positioning of the selective XOR substrates, hypoxanthine and xanthine, into the active site. Finally, Glu1261 is instrumental in catalyzing the oxidation of hypoxanthine into xanthine and xanthine into uric acid. Figure 5C demonstrates that Leu873, conserved in all the XORs so far characterized, is consistently replaced by a negatively charged amino acid (Glu or Asp) in all vertebrate aldehyde oxidases. The conservation of Ser876 is limited to vertebrate XORs with the notable exception of the *G. gallus* orthologous protein (data not shown). However, in this case too, a replacement to a Trp, Leu or Phe is typically observed in vertebrate aldehyde oxidases. Phe914 is conserved in all vertebrate aldehyde oxidases. In contrast, Phe1009 is very ill conserved and substituted by a variety of other residues. Taken together, the results suggest that the substrate tunnel is likely to be rather different in the two types of enzyme, in line with the observed differences in substrate and inhibitor specificities. Figure 5B indicates that Glu802 is commonly replaced by a hydrophobic amino acid, most commonly Val, Ile or Ala, in aldehyde oxidases without any preference for specific isoenzymatic forms. Similarly, Arg880 is invariably substituted by a hydrophobic amino acid in all the aldehyde oxidases. The corresponding amino acid is always a Met in vertebrate AOX1s, whereas it is a Phe or Tyr in AOH1s, AOH2s or AOH3s of different origin. This suggests that AOX1 may be different from the other aldehyde oxidases in terms of preferred substrates. The central role of the Glu802 and Arg880 equivalent amino acids (Glu803 and Arg881) in determining human XOR substrate specificity was recently demonstrated experimentally by site-directed mutagenesis [58]. Human XOR and its Glu803-to-valine (E803V) and Arg881-to-methionine (R881M) mutants were expressed in *E. coli*. The E803V mutation almost completely abrogated the activity toward hypoxanthine and xanthine as substrates. On the other hand, the R881M mutant lacked activity towards xanthine, but retained slight activity towards hypoxanthine. Both mutants, however, exhibited significant aldehyde oxidase activity. Unlike wild-type XOR, the mutants were not subject to time-dependent inhibition by allopurinol.

Glu1261 of BtXOR is strictly conserved at an equivalent position in all the aldehyde oxidase sequences analyzed. The only exception is represented by one of the two aldehyde oxidases expressed in *C. elegans* (CeAOX2). However, this discrepancy is only apparent, as the relevant Glu residue is present and simply shifted by one amino acid in the alignment. Hence the Glu residue in question is fundamental for the mechanism of the reaction catalyzed by both XOR and aldehyde oxidases, justifying its insertion in the scheme illustrated in Figure 1C and just proposed by Hille [15, 62].

In summary, on the basis of the above discussion, it is clear that aldehyde oxidases are different from XORs predominantly in the intermediate and C-terminal 40- and 85-kDa domains. This indicates substantial differences around the FAD-, MoCo- and substrate-binding sites. There are specific amino acid residues that mark AOX1s selectively and differentiate them from all the other aldehyde oxidases. The same is also true in the case of mammalian AOH2 and AOH3 that show discriminatory amino acids in the domain containing the first 2Fe/2S center and the MoCo fingerprint sequence. Interestingly, the former domain also contains a Cys residue that is peculiar to insect aldehyde oxidases.

The expression of different mammalian aldehyde oxidases is tissue and cell specific

Definition of the tissue- and cell-specific expression of aldehyde oxidases in humans and other mammals may provide clues as to the function of these enzymes.

AOX1 in humans and primates

The distribution of aldehyde oxidase at the cellular level has been investigated in a variety of human tissues with the use of methods whose specificity is questionable[19, 63, 64]. In the most extensive study, the richest source of AOX1 proved to be the liver [19], although the enzyme was also found in respiratory, digestive, urogenital and endocrine tissues. In the respiratory system, it was particularly abundant in epithelial cells from the trachea and bronchium, as well as in alveolar cells. In the digestive system, AOX1 was observed in epithelia of the small and large intestines. Furthermore, the proximal, distal and collecting tubules of the kidney were immunostained with various intensities, while the glomerulus was not. Staining was observed also in the ductuli and glandular epithelia of the prostate. Moreover, the adrenal gland, cortex, and notably the zona reticularis, showed strong immunostaining. The expression of AOX1 in the human central nervous system (CNS) has been the object of a single study [65] spurred by the claim that the protein was the product of the disease gene of the recessive familial form of amyotrophic lateral sclerosis (ALS). In this study, Berger *et al.* [65] demonstrated the presence of the AOX1 transcript in the glial cell population of the spinal cord. Obviously this does not eliminate the possibility that other structures in the human CNS express the transcript too, and specific work aimed at a better definition of the anatomical localization of AOX1 in the brain is warranted.

Studies on the tissue and cell distribution of aldehyde oxidases in primates other than humans are limited to the baboon [66–67]. Isoelectric focusing and cellulose acetate electrophoresis were used to examine the multiplicity and distribution of aldehyde oxidases from tissues of baboons. In line with the existence of a single active *AOX1* gene in primates, single forms of aldehyde oxidase were found in baboon tissue extracts, with the highest activities in liver [67]. In general, the tissue distribution of aldehyde oxidase in baboons and humans is concordant. It would be interesting to establish whether similar expression patterns are observed in another primate like the Rhesus monkey, for two reasons. First, Rhesus monkeys are popular experimental models of drug metabolism and they are thought to represent one of the best proxies of humans. Second, as reported above, there is evidence that the complement of aldehyde oxidase genes is different in Rhesus monkeys and humans. Studies to define this point are the focus of current research in our laboratory.

AOX1 and AOH1 in rodents

As expected, the majority of studies on the tissue distribution of aldehyde oxidases have been conducted in rodents [68]. However, a direct comparison with the results obtained in humans is difficult, given the multiplicity of enzymatic isoforms present in mice and rats. We studied the tissue and cell distribution of aldehyde oxidases in adult mice [26, 31, 32, 69, 70] using immunological and *in situ* hybridization techniques. The data obtained by Western blot analysis are specific for each aldehyde oxidase isoenzymatic form, as validated mono-specific anti-peptide antibodies raised against non-conserved regions of AOX1, AOH1, AOH2 and AOH3 were used. Similarly, the cRNA probes utilized for the *in situ* hybridization experiments are devoid of biases due to cross-hybridization.

The pattern of tissue-specific AOX1 and AOH1 expression is largely overlapping. The richest source for the two aldehyde oxidases is the hepatocyte component of the adult liver, as demonstrated by *in situ* hybridization of the corresponding transcripts. This suggests functional redundancy of the two enzymes in liver. However, it is interesting to note that AOX1 and AOH1 differ as to the time of appearance in the liver of the developing mouse. The AOH1 transcript is already detectable in newborn mice, while the AOX1 counterpart takes time to appear and is measurable only in the fully developed animal [32]. So far, we do not know whether the same or a different population of hepatocytes is responsible for the synthesis of AOX1 and AOH1. This is an important point to be clarified as we do not know whether AOX1 and AOH1 serve the same or different functions in the liver and other target tissues. Histological localization of aldehyde oxidase in rat hepatic tissues demonstrated that the distribution of the activity is uneven, being seen mainly in the pericentral rather than the periportal area [71]. Unfortunately, we do not know whether the enzymatic method adopted in this study highlights AOX1, AOH1 or, more likely, a mixture of the two enzymes. The second richest source of both AOX1 and AOH1 is the mouse lung. The presence of aldehyde oxidase immunoreactivity was confirmed in the lung of rats at the level of the bronchial epithelium [66]. In our survey, the only other peripheral mouse tissue where significant amounts of AOH1 mRNA are detectable is the testis, where spermatogonia seem to be the predominant cell type expressing the transcript. The situation is slightly different in the case of AOX1 mRNA, which is also detectable in the testis, but is evident in the heart as well, and is particularly abundant in the epithelial layer of the esophagus [69]. However, both the esophagus and the heart seem to be sites where

AOX1 mRNA expression is not accompanied by translation into the corresponding protein [69]. Translational and post-translational regulation of mammalian MFE gene expression must always be taken into account when analyzing tissue and cell distribution data, as a dichotomy between mRNA and protein levels is not an unusual finding for this type of enzyme [24, 72]. Studies demonstrating the presence of small amounts of aldehyde oxidase activity in tissues other than those discussed above are available. Aldehyde oxidase-bearing cells are present in the esophageal, gastric and intestinal epithelium. Weak immunoreactivity was observed in the gastric glands and intestinal goblet cells [66].

Further data indicative of the tissue distribution of the four mouse aldehyde oxidases can be obtained by browsing through the OMIM section of the NCBI website (http://www.ncbi.nlm.nih.gov/). The section contains a virtual assessment of the relative abundance of known and characterized transcripts based on sequential analysis of gene expression (SAGE) experiments and expressed sequence tags (EST) representation in a collection of libraries. Mining this database with the *AOX1* mRNA (accession number NM_009676, OMIM reference: Mm.26787) resulted in the following quantitative pattern of tissue-specific expression: inner ear > vascular tissue > liver > lung ≫ female genital tract, mammary gland, spleen and brain. This is similar to what observed for *AOH1* (NM_023617, OMIM reference: Mm.20108), whose expression pattern is liver > lung > testis > female genital tract ≫ muscle, eye, pancreas, gastrointestinal tract.

Expression of mouse AOX1 in the CNS has been the object of a specific *in situ* hybridization study [70]. By far the highest levels of the AOX1 transcript were observed in the choroid plexus, the organ devoted to the production and reabsorption of the cephalorachidian fluid. Discrete expression of the mRNA was also observed in the neuronal component of the brain and spinal cord. Specific *AOX1* signals were found to be associated with the cell bodies of the cephalic nerve motor neurons and the motor neurons of the anterior horns in the spinal cord. These data have been recently confirmed and expanded with the release of the data obtained by the Allen Brain Atlas initiative. This is a collection of serial sagittal and coronal sections of the mouse brain that were hybridized with DNA probes corresponding to a large number of identified and characterized genes. The dataset is publicly available on line (http://www.brainatlas.org/aba/) and can be interrogated to define the detailed anatomical localization of cells expressing specific genes. According to a first analysis of the data present in the website, *AOX1* is the aldehyde oxidase showing the highest level of expression and the most prominent pattern of anatomical distribution. Beside the choroid plexus and the motor neuron nuclei of the cephalic nerves, expression of the *AOX1* transcript is observed also in the medulla, olfactory bulb, midbrain, pons and cerebellum. The level of *AOH1* expression in the brain is relatively low, and the localization of the transcript is predominantly if not exclusively limited to the structures of the choroid plexus.

AOH2 and AOH3 in rodents

Relative to AOX1 and AOH1, the tissue- and cell-specific expression of mouse AOH2 and AOH3 is much more restricted. Western blot and *in situ* hybridization experiments indicate that by far the richest source of AOH2 is the Harderian gland, the major exocrine gland located in the intra-orbital cavity of rodents and many other vertebrates [M. Terao and E. Garattini, unpublished data]. In this location, the protein is so abundant that it was isolated in pure form for a first enzymatic and biochemical characterization [M. Terao and E. Garattini, unpublished data]. The only other tissues containing detectable amounts of AOH2 are the keratinized epithelia lining the oral cavity, the esophagus, the most proximal portion of the stomach and the epidermis [26, 32]. In the oral cavity, AOH2 mRNA is particularly enriched in the cornified epithelium of the taste papillae. A homogeneous pattern of *AOH2* expression is observed in the basal and intermediate layers of the epidermis, though patches of high-level expression are observed around the hair folliculi [M. Terao and E. Garattini, unpublished data].

The anatomical distribution of AOH3 in the peripheral tissues is absolutely restricted, as the only organ where the protein is detected is the Bowman's gland. The Bowman's gland is the principal exocrine gland located in the sub-mucosal layer of the nasal cavities. AOH3 is very abundant and represents approximately 5% of all the cytosolic proteins. AOH3 is also present in a small cell population (sustentacular cells) located in the apical layer of the nasal neuro-epithelium. Interestingly, sustentacular cells have been reported to have the same embryonal origin as Bowman's gland cells [73–75].

With a few exceptions, the profile of tissue distribution predicted for AOH2 (NM_023631, OMIM reference: Mm.244525) and AOH3 (NM_001008419, OMIM reference: Mm.425033) is remarkably different. The richest source of *AOH2* mRNA in the adult mouse is the inner ear followed by the head and neck region and the skin. The transcript is far less abundant but represented in the liver, pancreas and eye. Surprisingly, however, very large amounts of AOH2 are predicted to be present during the early stages of

development and specifically in the zygote. Subsequently, in the embryo and fetus, expression of the transcript seems to be silenced. AOH3 expression is restricted to the head and neck.

As documented by the data available in the Allen Brain Atlas site, *AOH2* and *AOH3* mRNAs are expressed in the brain at much lower levels than *AOX1* and *AOH1*. The order of quantitative representation in the mouse brain is *AOH2* $\gg$ *AOH3*. Though a detailed description of the pattern of region-specific expression of the two transcripts is beyond the scope of this section, once again it is interesting to note that the majority of the specific signals detected is at the level of the choroid plexus structures. Whether, the observation reflects cross-hybridization with the *AOX1* mRNA or represents *bona fide* colocalization of all the aldehyde oxidases remains to be established.

Aldehyde oxidases in other vertebrates

Studies on the tissue- and cell-specific distribution of aldehyde oxidases in animal species other than mouse and rat are rare. The activity of aldehyde oxidase and XOR was compared and contrasted in crude homogenates prepared from guinea pig liver, lung, kidney, intestine, spleen and heart [76]. The activity of the latter enzyme was highest in the liver, whereas XOR was predominant in the small intestine. This is similar to the reports in mouse for both AOX1 and XOR [4]. Using cellulose acetate zymograms, single forms of aldehyde oxidase were observed in horse tissue extracts, with the highest activities in liver [77]. Given the presence of two predicted aldehyde oxidase genes in the horse genome, these data need to be confirmed with more specific and sensitive methodologies. With the help of a mono-specific antibody raised against the purified protein and the isolated cDNA, the tissue distribution of the bovine AOX1 and corresponding mRNA was determined. Once again, AOX1 is expressed at high levels in liver, lung, and spleen, and at a much lower level in many other organs [43].

As seen previously, the presence of aldehyde oxidase activity in the liver is a characteristic of many animal species. However birds and carnivores, like dogs, are an exception to this general rule [42]. Only trace amounts of aldehyde oxidase activity are measured in chicken liver and these are due to the expression of AOX1. Dogs are completely devoid of liver aldehyde oxidase activity, indicating that the enzyme is not necessary for the homeostasis of this organ. This is consistent with the absence of active *AOX1* and *AOH1* orthologues (see Fig. 3). The absence of liver aldehyde oxidase activity in dogs is important in drug metabolism studies, as this animal may not represent a good proxy of the human situation.

Physiological substrates of mammalian aldehyde oxidases: many hypotheses, little experimental evidence

Analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp) website for the presence of metabolic pathways involving aldehyde oxidase(s) (EC 1.2.3.1) suggests a number of potential physiological substrates. The enzymes have been implicated in the catabolism of the amino acids isoleucine, leucine and valine, because of their ability to carry out the oxidation of (S)-methylmalonate semi-aldehyde into methylmalonate (pathway: ko00280). Another possible substrate is a serotonin metabolite, gentisate aldehyde, which places aldehyde oxidase within the context of the tyrosine degradation pathway (pathway: ko00350). Notably, oxidation of gentisate aldehyde and (S)-methylmalonate semi-aldehyde can also be carried out by an NAD-dependent aldehyde dehydrogenase (EC 1.2.1.3) and aryl-aldehyde dehydrogenase (EC 1.2.1.29), another NAD-dependent dehydrogenase.

Endogenous indols and pyridoxal

Potentially more relevant physiological substrates of aldehyde oxidases are 5-hydroxyindoleacetaldehyde (pathway: ko00380), a serotonin oxidation metabolite, and pyridoxal, the dephosphorylated form of the active vitamin B6 metabolite (pathway: ko00750). The biotransformation of 5-hydroxyindoleacetaldehyde into the corresponding acid can also be catalyzed by an NAD-dependent dehydrogenase [78], while oxidation of pyridoxal into 4-pyridoxic acid is carried out solely by aldehyde oxidase, albeit in insects [79]. In this pathway, aldehyde oxidases may act downstream of the mitochondrial monoamine oxidase (MAO) enzyme. A role for AOX1 or AOH1 in this metabolic pathway in the CNS is possible, as the enzymes are expressed in the mouse (AOX1 and AOH1), as well as human (AOX1) brain. Interestingly, there is evidence that AOH1 is synthesized by serotonergic murine neurons (gigantocellular reticular nucleus, Allen Brain Atlas), lending further support to the involvement of aldehyde oxidases in the catabolism of the monoamine. Indoles, like the phytohormone indole-3-acetaldehyde are excellent substrates of plant aldehyde oxidases [80–86]. As indole-3-acetate is not only a phytohormone but also an intermediate metabolite of the pathway leading to the synthesis of melanin [87], it is plausible that one or more aldehyde oxidases,

such as AOX1 or skin-specific AOH2, have relevance for pigment biosynthesis in vertebrates.

Pyridoxal is an established substrate of aldehyde oxidases *in vitro* [88] and this seems to be of particular physiological relevance in the case of insects [78, 89–91]. Indeed, one of the as yet uncharacterized *D. melanogaster* aldehyde oxidases (DmAOX1, DmAOX2, DmAOX3 or DmAOX4) is likely to be the enzyme which has long been known by the name of pyridoxal oxidase [74, 85–87]. It remains to be established whether the vitamin B6 metabolite is a significant *in vivo* aldehyde oxidase substrate in mammals and humans too. In this context, it is interesting to note that we have evidence that pyridoxal can be oxidized by purified mouse AOX1 and AOH1, although it is not an efficient substrate in the case of AOH2 (M. T. and E. G., unpublished data). This is consistent with the lack of symptoms amenable to alterations in the homeostasis of pyridoxal in the AOH2 knock-out mice generated in our laboratory [M. Terao and E. Garattini, unpublished data].

Nicotinamide

The involvement of aldehyde oxidases in the degradation of another vitamin, nicotinamide, is suggested by a number of studies on the ability of purified or enriched preparations of aldehyde oxidase from human [92], monkey [92], rat [93, 94], rabbit [95] and guinea pig liver [96], and bovine eye [97] to oxidize N1-methylnicotinamide. For these reasons, animal aldehyde oxidases are included in the nicotinamide biochemical pathway (pathway: ko00760) of KEGG and are believed to catalyze the oxidation of N1-methylnicotinamide to N1-methyl-2-pyridone-5-carboxamide or N1-methyl-4-pyridone-5-carboxamide. Whether aldehyde oxidases play a significant role in nicotinamide degradation *in vivo* requires further experimental evidence. Unlike the animal counterparts, plant aldehyde oxidases do not seem to utilize this substrate efficiently [86].

Retinaldehyde

A proposed physiological substrate of aldehyde oxidases that is drawing attention is retinaldehyde, the immediate precursor of retinoic acid. Retinoic acid is the active form of vitamin A and is present *in vivo* predominantly in the form of the geometric isomer all-*trans* retinoic acid (ATRA). ATRA is the best known morphogen and controls many aspects of vertebrate embryo development [98]. In adult mammals, ATRA is believed to regulate numerous physiological processes, including the homeostasis of the immunological and hematopoietic systems, as well as the keratinization of the epidermis [99]. The metabolism of vitamin A is very complex and subject to many layers of control. Basically, retinol, the major dietary precursor of vitamin A is oxidized by retinol dehydrogenases into all-*trans* retinaldehyde (RAL), which is subsequently transformed into ATRA. At least three mouse NAD-dependent cytosolic dehydrogenases are known to be involved in this metabolic step, i.e. ALDH1A1 (formerly RALDH1, NCBI acc. no. of the corresponding mRNA: NM_013467), ALDH1A2 (formerly RALDH2, NM_009022), ALDH1A3 (formerly RALDH3, NM_053080). Degradation of ATRA into inactive hydroxylated metabolites is carried out by cytochrome P-450-dependent mono-oxygenases, like CYP26A and CYP26B.

The involvement of aldehyde oxidases in RAL oxidation to ATRA was first discovered in rabbit liver cytosol, where it was observed that a fraction of the oxidizing activity did not require addition of NAD and was due to an MFE [100–102]. Subsequently, this observation was extended to mouse liver. Direct evidence for the involvement of an aldehyde oxidase in the oxidation of RAL to ATRA came with the demonstration that recombinant mouse AOX1 expressed in *E. coli* supports the enzymatic reaction [57]. Given the existence of multiple forms of aldehyde oxidase in mice, this raises the question as to whether RAL is an efficient substrate not only of AOX1 but also of AOH1, AOH2 and AOH3. We provided evidence that this is the case, demonstrating that purified preparations of AOX1 from the liver of DBA/2 mice [56], AOH1 from the liver of CD1 mice [26], AOH2 from the mouse Harderian gland [E. Garattini and M. Terao, unpublished data] and AOH3 from the mouse Bowman's gland are all capable of oxidizing RAL into ATRA with equal efficiency.

Concluding that any of the reported mouse aldehyde oxidases are significant for the metabolism of RAL *in vivo* on the basis of the available *in vitro* data is unwarranted and requires further experimental support. In this context, the following points are worth discussing. The V_{max} of the oxidation from RAL to ATRA calculated for AOH1 (40 nmol/min per milligram protein) and AOX1 (180 nmol/min per milligram protein) do not vary significantly from those reported for ALDH1A1 (57 nmol/min per milligram protein) and ALDH1A2 (105 nmol/min per milligram protein). However, the K_m of AOH1 (70 μM) and AOX1 (31 μM) for the same reaction is more than one order of magnitude higher relative to aldehyde dehydrogenases (ALDH1A1 = 1.4 μM; ALDH1A2 = 0.7 μM). As the concentrations of free retinoids *in vivo* are in the nanomolar range, this suggests that aldehyde oxidases may acquire significance in the oxidation of RAL only in tissues where ALDH1A1, A2 and A3 are absent or in specific conditions of retinoid overload. It is unlikely that AOX1 or AOH1 play any role in the control of

endogenous RAL and ATRA in the adult mouse hepatic tissue. Indeed, *ex vivo* experiments measuring the ability of crude mouse liver cytosolic extracts to oxidize exogenously added RAL in the presence and absence of NAD indicate that NAD-dependent activity (Aldh activity) is at least threefold higher than the NAD-independent counterpart (AOX1 and AOH1 combined activities) [E. Garattini and M. Terao, unpublished data]. It is also unlikely that aldehyde oxidases are important in controlling ATRA during the development of the embryo, as aldehyde oxidases are present in target tissues predominantly after birth. Furthermore, constitutive Aldh1a2 knock-out mice are embryonic lethal as the result of a phenotype amenable to ATRA deficiency [103].

Other potential substrates

It is possible that at least certain isoforms of aldehyde oxidase act on odorants, pheromones and compounds conferring taste to food. Though largely speculative, there is evidence that this may be a viable option in the case of AOH2 and AOH3. AOH2 is concentrated in the taste papillae in the tongue, where the enzyme may serve an accessory function controlling the duration and the intensity of the stimulus on the taste receptor. This function may be important in the perception of bitter and sweet taste. Indeed, quinine, a widespread and very bitter compound, is a recognized substrate of aldehyde oxidases [104]. By the same token, the two aldehyde oxidase substrates, vanillin and benzaldehyde, are widespread, volatile and very strong sweeteners. It is conceivable that the appearance of AOH2 provided a competitive advantage to terrestrial animals in terms of appropriate food selection and protection from poisonous and often bitter substances present in the environment. In addition, suppression of the gene in primates, humans and other animal species may reflect a progressive dispensability of the enzyme. AOH3 may serve a similar accessory function in the case of odorants or pheromones, justifying its selective localization in the nasal mucosa. Thus, the function of AOH3 may be similar to that of an aldehyde oxidase isoform described in the antennae of some insects [25, 105, 106]. Once again the corresponding gene might have been suppressed in primates and humans, which are characterized by a much less sophisticated olfactory system than many other mammals.

Mammalian aldehyde oxidases represent an important drug-metabolizing system in the cytosol of the hepatic cell

As shown in Figure 6, aldehyde oxidases are potentially capable of oxidizing a relatively large array of substrates, which is not necessarily limited to compounds containing an aldehyde functionality. Interest in aldehyde oxidase as a drug-metabolizing enzyme arose long before the identification of different isoforms in various animal species. As most of the studies on the significance of aldehyde oxidase activity in drug metabolism were conducted with crude liver cytosolic extracts or, at best, semi-purified preparations, caution should be exercised in evaluating the data present in the literature. The results obtained with human liver specimens may be appropriate, as the organ seems to express the product of a single aldehyde oxidase gene (*AOX1*). In contrast, studies performed in similar preparations obtained from popular experimental models such as mouse, rat, Rhesus monkey, and possibly guinea pig, are likely to be questionable. Indeed, as we have seen in previous sections, the mouse is characterized by the presence of two liver aldehyde oxidases and the predominant form expressed in most mouse strains is AOH1 and not AOX1 [56]. Furthermore, different strains of mice seem to be characterized by variable ratios of AOH1 and AOX1 in their hepatic tissue [26, 32]. Similar observations are likely to apply to the rat [26]. While the complement of aldehyde oxidases in guinea pig is still unknown, we have seen that Rhesus monkey may contain three active aldehyde oxidase genes (see Fig. 3).

With the above-mentioned caveats in mind, there is evidence in the literature that liver aldehyde oxidase(s) can be considered the cytosolic equivalent of the more popular microsomal cytochrome P450 (CYP) drug-metabolizing system. It is possible that aldehyde oxidases act in concert with CYP, activating or inactivating various types of drug and compounds of toxicological interest [107–116]. The list of drugs metabolized by aldehyde oxidases is long and includes anti-tumor and anti-viral [115] agents, as well as compounds acting in the CNS [50, 117–119]. The reader is referred to the excellent review article by Beedham [114] for a comprehensive treatment of the topic. Our attention will be limited to a few drugs of major interest that allow us to discuss some general points.

Immunosuppressive and anti-neoplastic agents

Methotrexate is a powerful inhibitor of dihydrofolate reductase and is used for the treatment of acute lymphocytic leukemia and rheumatoid arthritis. A

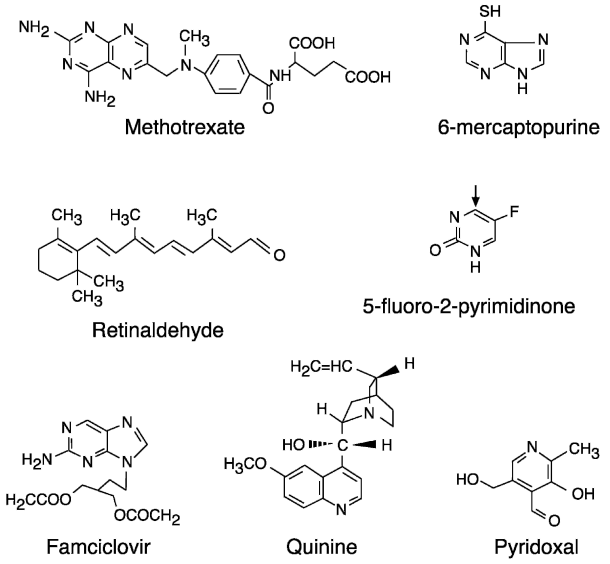

Figure 6. Chemical structure of some aldehyde oxidase substrates. The chemical structures of some of the mammalian aldehyde oxidase substrates discussed in the text are illustrated.

major metabolic step is hydroxylation to 7-hydroxy-methotrexate (7-OH-MTX) that is carried out by liver aldehyde oxidase [120]. Production of 7-OH-MTX is relevant both in terms of pharmacological activity and toxicity. Interestingly, methotrexate does not seem to be a substrate of CYPs [121]. The level of hepatic and extra-hepatic aldehyde oxidase activity has an important role in the bioavailability of the drug. This was clearly demonstrated in studies performed in rats, where marked strain and age differences in the amounts of 7-OH-MTX excreted were observed and correlated with aldehyde oxidase activity [55, 122]. A similar observation was made in human livers, where there is a high degree of inter-individual variability as to methotrexate hydroxylation and this correlates with aldehyde oxidase levels [123].

6-mercaptopurine (6-MT) is an immunosuppressant and a cytotoxic drug used for the treatment of pediatric and adult neoplastic disorders. 6-MT is also a major metabolite of azathioprine, another immunosuppressant and drug used for the treatment of autoimmune disorders [124]. Aldehyde oxidase has long been known to oxidize 6-mercaptopurine to the corresponding N-hydroxyl metabolite in humans and various animal species [124, 125]. Similar to 7-OH-MTX, the hydroxylation product of 6-MT is considered to be a major and largely inactive metabolite. Once again, differences in the oxidative metabolism of 6-MT and azathioprine in the liver and other tissues may have important implications for the clinical use of the two drugs.

Anti-malarial and anti-viral drugs

Two other drug classes of significant interest for aldehyde oxidase metabolizing activity are anti-malarial and anti-viral agents. As to the first group of drugs, quinine is oxidized by rabbit hepatic and guinea pig aldehyde oxidase [104]. Oxidation of quinine via aldehyde oxidase appears to be the predominant pathway with lower concentrations of microsomal metabolites identified. The 2′-quininone derivative of quinine seems to be the major metabolite produced by aldehyde oxidase. The high variability in the ability of different animal species to dispose of quinine is attributed to variations in the levels of liver aldehyde oxidase activity. Rat or dog liver shows low and negligible quinine-oxidizing activity, whereas baboon liver contains high levels of the drug-metabolizing activity. Marmoset and guinea pigs have the closest spectrum of activity to humans [126]. The low level of drug-metabolizing activity reported is in line with our data showing that *AOX1* and *AOH1* are inactive pseudogenes in dogs [42]. However, it is difficult to reconcile the results observed in rats with the significant amounts of both AOX1 and AOH1 present in rat liver [31]. A possible explanation is that AOH1 is the major form of aldehyde oxidase expressed in rat liver, as observed in the mouse counterpart, and the enzyme may not metabolize quinine efficiently.

The anti-viral agent famciclovir is another drug efficiently oxidized by aldehyde oxidase(s) [115, 127]. Famciclovir, a 9-substituted guanine derivative, is a new anti-viral agent which undergoes rapid hydrolysis and oxidation to yield the active anti-herpes agent, penciclovir. Studies with human liver cytosol indicate that the oxidation of the penultimate metabolite, 6-deoxypenciclovir, to penciclovir is catalyzed by AOX1. This is one of the first examples of the role exerted by aldehyde oxidases in the bioactivation of pro-drugs. Indeed, targeting of aldehyde oxidase metabolizing activity for the bioactivation of pro-drugs has been proposed for other types of agents such as 5-fluoro-2-pyrimidone, a precursor of the anti-neoplastic agent 5-fluorouracil [128, 129]. AOX1 is potentially useful in the bioactivation of pro-drugs in human liver and lung, given that the two tissues are the only ones reported to express significant amounts of this enzymatic activity.

Compounds of toxicological interest

Aldehyde oxidases are important not only for the metabolism of compounds of medicinal interest, but are also of toxicological relevance. Phthalazines are classical environmental pollutants that can be efficiently metabolized by aldehyde oxidases [130]. Aldehyde oxidases were recently identified as the nitroreductase of mammalian liver that is responsible

for the reduction of imidacloprid, a member of the newest major class of insecticides, the neonicotinoids [131–133]. Both aldehyde oxidases and XOR were shown to be involved in the nitroreduction of the environmental pollutants 2-nitrofluorene, 1-nitropyrene, and 4-nitrobiphenyl in the skin of various mammalian species. All these compounds are reduced to the corresponding amino derivatives by both types of enzyme although the relative contribution of each enzymatic activity varies in different animal species [134]. In rodents, it is possible that the reaction is catalyzed by the skin-specific aldehyde oxidase isoform, AOH2. The above-mentioned toxic compounds were considered because they exemplify the ability of aldehyde oxidases to also catalyze reductive reactions in the presence of a suitable compound acting as a donor of reducing equivalents. Another example of this type of aldehyde oxidase-catalyzed reaction is the reductive metabolism of aromatic nitro compounds. The carcinogenic 1-nitropyrene is reduced to the corresponding amine by rabbit liver cytosol in the presence of 2-hydroxypyrimidine, an electron donor of aldehyde oxidase [135]. It remains to be established whether any of these bioreductive enzymatic reactions catalyzed by aldehyde oxidases have any significance *in vivo*.

Acetaldehyde

Aldehyde oxidases have been implicated in the metabolism of acetaldehyde, the toxic metabolite of ethanol, and a role for the enzyme in ethanol-induced hepatotoxicity has been proposed. The involvement of aldehyde oxidase in acute and chronic liver toxicity has been linked to the ability of the enzyme to produce toxic reactive oxygen species (ROS) as a consequence of acetaldehyde oxidation [136]. However, we could not substantiate the relevance of AOX1 and/or AOH1 in the oxidation of acetaldehyde to acetic acid in mouse liver. For this type of experiment, we compared the DBA/2 and the CD1 strains of mice for their ability to metabolize acetaldehyde following acute and chronic administration of ethanol [56]. Relative to the control CD1 animals, DBA/2 mice are characterized by only trace amounts of liver AOH1 and a dramatic reduction of AOX1 in the same tissue. However, the levels of acetaldehyde in the livers of ethanol-administered CD-1 and DBA/2 mice were similar, indicating that neither enzyme is involved in the biotransformation of acetaldehyde *in vivo*. The observation is consistent with the fact that acetaldehyde, the toxic metabolite of ethanol, is a poor substrate of both enzymes. This does not necessarily exclude the possibility that aldehyde oxidases are somehow involved in ethanol-induced toxicity *in vivo*. A possible and interesting mechanism supporting this idea comes from a paper by Mira et al. [137]. Ethanol metabolism by alcohol dehydrogenase produces acetaldehyde and NADH, with a subsequent increase of the NADH/NAD^+ ratio. According to the authors, NADH is a substrate of aldehyde oxidase, and oxidation of the dinucleotide leads to the formation of the toxic superoxide anion radical. The apparent K_m of NADH for aldehyde oxidase is reported to be approximately 28 μM, a much smaller value than that reported for acetaldehyde (1 mM). On the basis of these data, a vicious cycle which increases oxyradical production is suggested: aldehyde dehydrogenase reduces NAD^+ to NADH, which is oxidized by aldehyde oxidase, generating reactive oxidative species plus NAD^+ available again for reduction by the former enzyme.

The levels of hepatic aldehyde oxidase activity are variable: inter-individual variability in the human population is mirrored by inter-strain variations in experimental animals

As observed in the case of many other enzymes involved in drug metabolism, genetic and epigenetic mechanisms regulate the activity of aldehyde oxidases. This is an important aspect to consider given the variety of drugs and toxicants that can be metabolized by liver aldehyde oxidases both in humans and in experimental animals. In this section, the data available on the topic in human and experimental animals are reviewed.

Human studies

Definition of AOX1 enzymatic activity in individual patients may be a useful parameter for dose adjustment and for the prevention of unnecessary side effects during drug treatment. The available data indicate that there is variability in the levels of liver aldehyde oxidase in the human population. When methotrexate 7-hydroxylase was assayed in six human liver cytosols, a 48-fold range of inter-subject variation was observed. The variation correlated with the concentrations of aldehyde oxidase activity using benzaldehyde as a substrate [123]. Development of a non-invasive method for the measurement of aldehyde oxidase *in vivo* [138] lies at the basis of a further study aimed at defining age-specific and inter-individual variations in the human population. In the study, developmental changes of aldehyde oxidase activity were investigated in 101 children. Interestingly, aldehyde oxidase activity rapidly increases with the subjects' age up to about one year. The findings suggest that activity begins to increase soon after birth. The authors conclude that dose adjustment

based on the individual level of activity should be made in children below 1 year of age [139].

At present the molecular mechanisms at the basis of the observed differences in the individual levels of liver aldehyde oxidase are unknown. However, it is likely that variability is related to the presence of nucleotide polymorphic sites (SNPs) in the regulatory or coding regions of the *AOX1* gene. A number of polymorphic sites located in the introns and 5′- or 3′-untranslated regions of the human *AOX1* gene are present in the dbSNP section of the NCBI database. However, the functional significance of these SNPs is unknown. Only four non-synonymous SNPs are reported in exons 11, 21, 31 and 34. Moreover, the frequency of these alleles is known only for the SNP located in exon 34. Four polymorphic sites resulting in missense mutations in the coding exons of *AOX1* have been reported in the population of the Churchill County of Nevada [140]. We also studied the frequency and type of allelic variants in 180 individuals representative of the Italian population. We defined three novel polymorphic sites producing missense mutations (exon 22, rs41309768 in the dbSNP database and submitted data) in addition to the same polymorphism in exon 30 found in the previous study. Although there is no experimental support for the functional significance of any of these mutations, some of them are predicted by the PolyPhen software (hhtp://genetics.bwh.harvard.edu/pph/) to affect negatively the catalytic activity of AOX1 and may be at the basis of the low liver aldehyde oxidase activity observed in certain individuals.

Animal studies

Variations in the levels of aldehyde oxidase activity in different strains of experimental animals, like rats and mice, may reflect the inter-individual variations observed in the human population. These differences have been the object of a few studies. Aldehyde oxidase-catalyzed 2-oxidation activity of RS-8359, a monoamine oxidase inhibitor, was investigated in liver cytosolic fractions from ten rat strains. More than two log differences between the highest activity in the and the lowest activity strain were observed [120]. Differences in the 7-hydroxylation of methotrexate and benzaldehyde by aldehyde oxidase were observed in liver cytosols of different rat strains [43, 123]. Among six Wistar strains, the Slc:Wistar rats showed exceptionally low oxidation activity that was comparable to that of the F344/DuCrj strain [138, 141]. As to the mechanisms underlying these differences, the information available is still incomplete. Two of the studies report a general correlation between activity and aldehyde oxidase protein levels, though the methodologies used do not permit distinguishing between AOH1 and AOX1 [141, 142].

More detailed information on the potential mechanisms responsible for inter- or intra-strain variations in the levels of liver aldehyde oxidase activity was obtained in three recent studies performed in rats and mice. The first one suggests genetic mechanisms involving a specific sequence polymorphism in the coding region of rat *AOX1* [143]. Donryu rats show a dimorphic pattern for the 2-oxidation activity of the MAO inhibitor, RS-8359, with about 20- to 40-fold variations in the V_{max}/K_m values between low- and high-activity groups. Differences are consistent with changes in the isoelectric point value of AOX1 caused by a Gly110 to Ser mutation in the protein sequence [143]. In a second paper, the expression levels of aldehyde oxidase dimeric protein were determined in different rat strains. The results suggest that rat strains with low aldehyde oxidase activity lack the ability to produce the catalytically active dimer and express only the monomeric form of the enzyme(s) [144]. In the third report, we observed that mouse strains can be divided into two major groups: a first one, exemplified by DBA/2 and CBA/2 mice, which is characterized by low levels, and a second one including CD1 and C57Bl/6J mice, showing high levels of aldehyde oxidase activity in liver. The deficiency observed in DBA/2 and CBA/2 mice is due to an almost complete lack of *AOH1* mRNA and corresponding protein expression, as well as a significant decrease in AOX1 protein and transcript. Indeed, DBA/2 mice contain approximately 0.5 % of the amounts of AOH1 observed in the liver of the CD1 strain, and the animals can be considered functional knock-outs for this gene. The phenomenon is not due to genetic alterations in the coding sequence of the *AOH1* gene, but rather to epigenetic silencing. Indeed, the 5′-flanking region of the DBA/2 *AOH1* gene is characterized by a level of DNA methylation that is much higher than that observed in the corresponding DNA region of the CD1 counterpart [56]. Recent results indicate that the deficit in DBA/2 mice may have a more complex explanation, as the animals are deficient not only in AOH1 and AOX1, but also the AOH2 protein [M. Terao and E. Garattini, ubpublished data].

Quantitative differences in the levels of liver aldehyde oxidase activity are evident also in different animal species and this is of particular importance when we want to extrapolate drug metabolism data from experimental animals to humans. Kitamura *et al.* [123] reported that liver aldehyde oxidase activity is highest in rabbits, followed by rats, hamsters and monkeys. However, the order of aldehyde oxidase activity among animal species may vary depending on

the substrate considered. In other studies, the enzyme has been reported to be high in monkey and humans but low in rats [145].

The expression of aldehyde oxidases is regulated by endogenous and exogenous factors

While a certain amount of data on the regulation of plant aldehyde oxidases is available, there are very few studies on the control of the animal counterparts by endogenous and exogenous stimuli [20, 83, 84, 146–148]. There is an equal paucity of data on the DNA regulatory elements and the transcriptional factors influencing the activity of the vertebrate aldehyde oxidase genes. Finally, studies on the post-transcriptional regulation of aldehyde oxidase expression are virtually non-existent. This gap in knowledge needs to be filled, as we are gathering evidence that the expression of mouse aldehyde oxidases is highly regulated. Suffice it to say that we do not have any clue as to the mechanisms that regulate the restricted tissue-specific expression of mouse AOX1, AOH1, AOH2 and AOH3. With respect to this last problem, we have observed that the *AOH3* mRNA is characterized by the presence of an alternative spliced form of the 3′-untranslated region that may regulate the stability of the transcript and explain the high expression levels observed in Bowman's gland [31]. A further observation that may be relevant in the context of gene regulation by anti-sense transcripts and microRNAs is the existence of an anti-sense mRNA of unknown significance originating from intron 26 of the mouse *Aoh1* gene [26].

Gender and sex hormones

In experimental animals, like mice and rats, the level of aldehyde oxidase activity in different tissues is influenced not only by the genetic background but also by the gender. The first observation on sex-dependent regulation was made by Holmes [149] in a historical paper describing the presence of two distinct liver aldehyde oxidase activities showing higher levels in male than female adult mice. Both activities were significantly reduced by castration of adult males and increased following testosterone administration to castrated males and normal female mice. These observations were subsequently confirmed by Ventura and Dachtler [150, 151]. These authors also observed that estrogen administration reduced liver aldehyde oxidase activity of male animals [151]. In a subsequent article, purification of aldehyde oxidase activities in female and male mice did not show any significant difference in the substrate preference and inhibition profile of the two preparations [152]. We further elaborated on the isoenzymatic forms of aldehyde oxidases expressed in mouse liver, as well as the molecular mechanisms and stimuli responsible for the observed sexual dimorphism [32, 69]. We demonstrated that there is a dichotomy between the levels of *AOX1* mRNA and protein, suggesting translational control. Indeed, despite similar amounts of *AOX1* mRNA, the levels of the AOX1 enzyme and corresponding polypeptide were significantly higher in male than in female animals [69]. Treatment of female mice with testosterone increased the amounts of both *AOX1* mRNA and the relative translation product to levels similar to those in male animals. All this suggests that estrogens may regulate expression of *AOX1* at the translational level directly or indirectly, while androgens exert a direct or indirect transcriptional control. Subsequently, we demonstrated that not only AOX1 but also AOH1 is regulated in a similar way [32].

It is possible that gender-specific regulation of AOX1 and AOH1 by androgens and estrogens is an indirect effect mediated, albeit incompletely, by other hormones and growth factors. The participation of circulating growth hormone (GH) as a regulator of sex differences in hepatic aldehyde oxidase activity was examined in the mouse [153]. Neonatal pretreatment with glutamate or aspartate, which are known to reduce circulating GH levels, decreased male aldehyde oxidase activity to female levels. The original situation was restored by subsequent injections of human GH. The changes in aldehyde oxidase activity in male mice caused by the excitotoxic amino acids were not observed in females. Hypophysectomy markedly decreased hepatic activity in male and to a lesser extent in female mice. The activity in hypophysectomized male mice was restored by administration of human GH. Treatment with testosterone did not increase aldehyde oxidase activity in aspartate-treated or hypophysectomized females.

It remains to be established whether regulation of AOX1 and AOH1 by sex hormones is a liver-specific effect or is observed in other organs, like lung, testis and brain, that express significant amounts of one or both isoenzymatic forms. Furthermore, it would be interesting to know whether common DNA regulatory elements control the androgen-dependent as well as the basal and tissue-specific expression of both AOX1 and AOH1. In fact, the two enzymes seem to be coregulated in many respects and the corresponding genes are located one next to the other at a very short distance in the mouse and rat aldehyde oxidase gene clusters. Unfortunately, the *cis*-regulatory elements of mammalian aldehyde oxidases have not been the object of any systematic study. So far only the minimal elements responsible for the basal level of human and

mouse *AOX1* transcription have been characterized [27, 154]. The human *AOX1* gene was found to possess a structurally complex region in the upstream DNA that contains sequences for a proximal promoter, enhancer sites and silencer elements. The transcription factors Sp1 and Sp3 seem to play a central role in the basal activity of the proximal promoter.

Other endogenous stimuli

Beside sex hormones and growth factors, the only other endogenous stimulus which has been demonstrated to influence aldehyde oxidase expression is adiponectin. Adiponectin is a protein hormone that modulates a number of processes, including glucose regulation and fatty acid catabolism. Adiponectin is produced exclusively by the adipose tissue and secreted into the bloodstream where it is very abundant. Adiponectin activates peroxisome proliferator-activated receptor-alpha (PPAR-alpha) and protects the liver from the steatosis caused by obesity or alcohol. Recombinant adiponectin downregulates AOX1 expression. Obesity is associated with low adiponectin, reduced hepatic PPAR-alpha activity and fatty liver, and AOX1 was found induced in the liver of rats on a high-fat diet when compared to controls. The current data indicate that adiponectin reduces AOX1 by activating PPAR-alpha, whereas fatty liver disease is associated with elevated hepatic AOX1 [155]. All this may have patho-physiological relevance, as AOX1 is known to produce toxic ROS as by-products of the enzymatic reactions catalyzed. High AOX1 may be associated with higher ROS, which are known to induce liver fibrogenesis.

Exogenous stimuli

A number of exogenous stimuli have been reported to modulate aldehyde oxidase expression. Oral administration of phthalazine or 1-hydroxyphthalazine to female rabbits causes an increase in the specific activity of liver aldehyde oxidase and XOR [156]. Intraperitoneal injection of the alkylating agents N-methyl-N′-nitro-N-nitrosoguanidine, N-methyl-N-nitrosourea and methyl methanesulfonate (MMS) into rats also causes an induction of liver aldehyde oxidase [157]. Similar to what observed in the case of XOR [158], dioxin (TCDD) has been demonstrated to induce both liver AOX1 and AOH1 [159]. AOX1 is induced by TCDD in mouse hepatoma cells. *AOX1* mRNA levels are not increased by the same stimulus in mutant derivatives of hepatoma cells lacking either functional aryl hydrocarbon receptor (AHR) or aryl hydrocarbon receptor nuclear translocator (ARNT) proteins. This demonstrates that transcriptional induction of AOX1 in response to TCDD occurs through the AHR pathway. TCDD induction of AOX1 mRNA is also observed in mouse liver. In this experimental condition, induction of AOX1 protein is accompanied by a similar effect on AOH1, further demonstrating coregulation of the two proteins. In line with this, consensus sequences for the binding of the AHR/ARNT dimer in the flanking regions of the *Aox1* and *Aoh1* genes are recognizable. The chemopreventive agent phenethyl isothiocyanate is also capable of inducing the *AOX1* transcript through a transcriptional mechanism. This requires the presence of the Nrf2 transcription factor, as induction is not observed in Nrf2 knock-out mice [160].

Conclusion and future perspectives

Our intention in this review article has been to provide an overall picture of the current information available on aldehyde oxidases, with particular emphasis on the mammalian enzymes, a group of MFEs which is largely neglected in the scientific literature relative to the prototypical member of the family, i.e. XOR. The limited interest raised by aldehyde oxidases so far stems from the obvious observation that this group of enzymes is still looking for physiological functions and substrates. Unlike animal and plant XORs, which play a well-established and key role in the catabolism of purines, there are only a limited number of proposed physiological substrates for aldehyde oxidases. A further problem in the field is the presence of multiple and tissue-specific forms of aldehyde oxidases that vary in different animal species. The various aldehyde oxidase isoforms may recognize distinct substrates and carry out different physiological tasks. Despite these limitations, the recent explosion of information available on the primary structure of aldehyde oxidases in many organisms is likely to boost interest in these enzymes. Indeed, there is already enough scientific evidence that the aldehyde oxidase family represents a significant paradigm from an evolutionary standpoint [4, 44].

At the practical level, it is important to reassess the current nomenclature of aldehyde oxidase genes and products, taking into account some of the problems raised in this review. A less confusing nomenclature is going to help the process of expert annotation, which is the core aspect of publicly available gene and protein databases. A sensible and informative nomenclature is particularly important for the annotation of multi-gene families, and the aldehyde oxidase family is no exception. In choosing a more appropriate nomenclature and more informative gene symbols, it is important to keep in mind that the very term ‘aldehyde oxidase’ given to the enzymes classified

under the EC number 1.2.3.1 is inappropriate. Indeed, aldehyde oxidases act on a number of substrates that do not contain aldehyde functionalities, as observed several times in this article. This type of enzyme is better described as molybdenum- and flavin-dependent oxidase and we propose that a new nomenclature should take this into account.

At the scientific level, a number of issues need to be clarified and the following type of studies are predicted in the next future. A better enzymatic characterization of the various isoforms of aldehyde oxidases in specific animal species is a priority, if we want to make progress in the functional characterization of these proteins. So far we have relied on the development of efficient purification procedures using tissues particularly enriched for the aldehyde oxidase isoform of interest as the starting source. This is an approach that we have pioneered and resulted in efficient schemes for the purification of mouse AOX1 and AOH1 from the liver [26, 56], AOH2 from the Harderian gland [M. Terao and E. Garattini, unpublished data] and AOH3 from the nasal mucosa [31]. All these methods are potentially useful for the purification of aldehyde oxidases from other animal species and sources [42]. With this type of approach, we have observed that there is a high degree of overlapping substrate specificity among AOX1, AOH1, AOH2 and AOH3, although the enzymes differ for certain characteristics. All the enzymes utilize with similar efficiency retinaldehyde, while pyridoxal does not seem to be a good substrate for AOH2 [M. Terao and E. Garattini, unpublished data]. Furthermore, there is a remarkable difference in the ability of β-carboline to inhibit AOX1 and AOH1. Further studies in this direction are clearly needed. In particular, it is important to define in more detail the structural domains and the amino acid residues that dictate specificities of the various aldehyde oxidase isoforms. This requires site-specific mutagenesis and structural studies that are possible only on recombinant proteins. To this aim, the progress made in the development of efficient methods for the expression of catalytically active recombinant MFEs in prokaryotic heterologous systems is likely to be of particular significance. Indeed, there are two general problems associated with the difficulties in obtaining large-scale quantities of MFEs. The structure of MoCo in prokaryotes and eukaryotes is different. There is an objective difficulty in assembling active MFEs in common eukaryotic expression systems due to the relative deficiency of the MoCo synthetic machinery, which is incapable of keeping up with the synthesis of the apoproteins. Purification of recombinant or native aldehyde oxidases in high yields is fundamental for the achievement of another important goal, i.e. crystallization of the first aldehyde oxidase. Indeed, only availability of the crystal structure of this type of enzyme is going to provide us with the necessary information on the molecular determinants responsible for the differences between aldehyde oxidases and XORs in terms of substrate pocket, substrate tunnel, and so on.

Direct approaches to define the biological importance of the various aldehyde oxidases are needed. The generation of knock-out animals for each of the four different mouse aldehyde oxidases is likely to provide useful information. Our laboratory has already embarked on this endeavor and has generated two lines of animals in which the *AOH2* and *AOH3* genes have been constitutively knocked out. We are currently in the process of generating similar animals for the other two MFE genes, *AOX1* and *AOH1*. So far, the *AOH2* knock-out animal is the one that has been characterized more thoroughly at the phenotypic level. Consistent with the expression of the enzyme only in adult animals, *AOH2* knock-out mice are viable and transmit the genetic deficit in a mendelian fashion, indicating that the enzyme is not important for the development of the embryo. However, we have preliminary data suggesting the relevance of the enzyme in the control of the local synthesis of ATRA in the Harderian gland [M. Terao and E. Garattini, unpublished data]. It is entirely possible that important clues on the physiological function of this class of enzymes will come only with the generation of double and triple knock-outs of the various aldehyde oxidase isoforms, since the system may be redundant, as observed for other homogeneous families of proteins (for example, retinoic acid receptors).

Given the importance of AOX1 in the metabolism of numerous types of drugs and environmental pollutants, studies in at least two directions are called for. Generation of a genetically engineered mouse with a complement of liver aldehyde oxidases similar to that observed in humans is going to be extremely useful for drug metabolism, pharmacodynamic and toxicological studies. In the same vein, we need to obtain more thorough insights into the existence and frequency of *AOX1* allelic variants in the human population and their effects on the catalytic activity of the enzyme. Correlative studies linking these allelic variants to the drug-metabolizing phenotype of individual patients is going to represent a further step in the development of the concept of personalized medicine.

Finally, an ever increasing number of genomes is going to be sequenced in the next few years. The information acquired on the number and amino acid sequence of aldehyde oxidases, as well as the structure of the

corresponding genes, in different living organisms is likely to provide us with a better and more definitive view of the interesting evolutionary history of this family of MFEs.

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