REVIEW ARTICLE

Mammalian molybdo–flavoenzymes, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology

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The molybdo–flavoenzymes are structurally related proteins that require a molybdopterin cofactor and FAD for their catalytic activity. In mammals, four enzymes are known: xanthine oxidoreductase, aldehyde oxidase and two recently described mouse proteins known as aldehyde oxidase homologue 1 and aldehyde oxidase homologue 2. The present review article summarizes current knowledge on the structure, enzymology, genetics, regulation and pathophysiology of mammalian molybdo–flavoenzymes. Molybdo–flavoenzymes are structurally complex oxidoreductases with an equally complex mechanism of catalysis. Our knowledge has greatly increased due to the recent crystallization of two xanthine oxidoreductases and the determination of the amino acid sequences of many members of the family. The evolution of molybdo–flavoenzymes can now be traced, given the availability of the structures of the corresponding genes in many organisms. The genes coding for molybdo–flavoenzymes are expressed in a cell-specific fashion and are controlled by endogenous and exogenous stimuli. The recent cloning of the genes involved in the biosynthesis of the molybdenum cofactor has increased our knowledge on the assembly of the apo-forms of molybdo–flavoproteins into the corresponding holo-forms. Xanthine oxidoreductase is the key enzyme in the catabolism of purines, although recent data suggest that the physiological function of this enzyme is more complex than previously assumed. The enzyme has been implicated in such diverse pathological situations as organ ischaemia, inflammation and infection. At present, very little is known about the pathophysiological relevance of aldehyde oxidase, aldehyde oxidase homologue 1 and aldehyde oxidase homologue 2, which do not as yet have an accepted endogenous substrate.

Key words: aldehyde oxidase, molybdenum, molybdenum cofactor, molybdo–flavoenzymes, xanthine dehydrogenase, xanthine oxidase.

INTRODUCTION

Molybdenum is a transition metal that is incorporated, as a biologically active cofactor (molybdenum cofactor; MoCo), into a class of widely distributed proteins known collectively as molybdo-enzymes or molybdo-proteins. Nitrate reductase (EC 1.6.6.1) and sulphite oxidase (SO; EC 1.8.3.1) are among the most prominent members of the family. The molybdo–flavoenzymes (MFEs) are a homogeneous subgroup of molybdo-proteins characterized by the fact that they need not only the MoCo, but also a flavin cofactor, for their catalytic activity [1–3]. MFEs are present in the bacterial [4], fungal [5], plant [6,7] and animal [8–10] kingdoms, and represent a group of structurally and biochemically related proteins. In humans and other mammals, until a few years ago it was believed that the subfamily of MFEs consisted of only two members, i.e. xanthine oxidoreductase [XOR; xanthine dehydrogenase (XD) form, EC 1.1.1.204; xanthine oxidase (XO) form, EC 1.1.3.22] and aldehyde oxidase (AOX1; EC 1.2.3.1). (Throughout the text, we will refer to human aldehyde oxidase and the corresponding orthologous proteins as AOX1. The relative coding genes will be referred to as *AOX1*.) XOR has been the object of many reports and has long been recognized as the key enzyme in the catabolism of purines, oxidizing hypoxanthine into xanthine and xanthine into the terminal catabolite, uric acid. The biochemical and physiological functions of AOX1 are still largely obscure.

In the last few years, novel members of the MFE family have been identified in prokaryotic and eukaryotic organisms [11–14]. In particular, the identification of at least three novel mouse MFEs with structural similarity to both AOX1 and XOR ([10,14]; M. Terao, unpublished work) has opened up new scenarios. This has dramatically improved our understanding of the evolution of MFEs. In addition, the availability of the crystal structures of a few members of the protein family [15–17] has greatly increased our insight into the molecular details regulating the mechanisms of catalysis of MFEs. The current availability of the necessary molecular tools is promising important advances in the elucidation of the role that MFEs play in mammalian pathophysiology. In addition, further insight into the regulation of these enzymes will come from the recent cloning of the genes involved in the complex biochemical pathway leading to the synthesis of MoCo.

For all of these reasons, we feel that a survey of mammalian MFEs is particularly timely. The decision to focus on mammalian enzymes is dictated by the established or potential relevance that these proteins have in the biomedical realm. Although this review is meant to be comprehensive and to summarize our current knowledge on the structure, genetics, regulation, function and pathophysiology of MFEs, it does not

Abbreviations used: AOH, aldehyde oxidase homologue; AOX1, aldehyde oxidase; CODH, carbon monoxide dehydrogenase; DgAOR, *Desulfovibrio gigas* aldehyde oxidoreductase; MCSU, MoCo sulphurase; MFE, molybdo–flavoenzyme; MoCo, molybdenum cofactor; MPT, molybdopterin; SO, sulphite oxidase; TCDD, tetrachlorodibenzo-*p*-dioxin; XD, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase.

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cover all topics in the same depth. Much more information is available on XOR relative to what is known for AOX1 and the most recently discovered mammalian MFEs. Similarly, more emphasis is given to the structural and genetic results, as they currently outweigh the functional data. Furthermore, a significant proportion of the review is dedicated to the phylogenesis of MFE proteins and related genes. Finally, a section summarizing the data available on the genes and proteins involved in the biosynthesis of MoCo is included. This is justified by the importance of the post-translational events that regulate the insertion of the cofactor into the holo-enzymic forms of MFEs.

GENERAL CHARACTERISTICS AND STRUCTURE OF MFEs

Currently, the family of mammalian MFEs consists of XOR, AOX1 and two recently identified mouse proteins, AOH1 (aldehyde oxidase homologue 1) and AOH2, which are characterized by remarkable structural similarity to AOX1 [8,10,14,18,19]. We have also some evidence for a fifth and related mouse enzyme, temporarily denoted AOH3 (E. Garattini and M. Terao, unpublished work). The primary structures of XOR and AOX1 were determined with a classical approach involving protein purification, cDNA cloning and indirect determination of the complete sequence of the predicted polypeptide. By contrast, the identification and characterization of AOH1 and AOH2 are the result of a typical reverse genetics approach. The cDNAs coding for AOH1 and AOH2 were identified by interrogating the GenBank Nucleotide Sequence Database for sequences showing significant similarity to those of both AOX1 and XOR [10,14]. Subsequently, confirmation of the existence of the AOH1 and AOH2 proteins was obtained by immunological methods and, in the case of AOH1 only, by protein purification [10].

Most of the available data on the metal ion content, substrate specificity, and kinetic, spectroscopic and tertiary structure characteristics of mammalian MFEs are the result of purification studies conducted on native proteins extracted from relevant sources. This is due to the fact that efficient expression of catalytically active recombinant MFEs in heterologous systems is still a problem, although expression of *Drosophila melanogaster* XOR in *Aspergillus nidulans* [20] and of mouse AOX1 in *Escherichia coli* have been reported [21]. In fact, popular host organisms either do not contain the appropriate MoCo biosynthetic machinery (*E. coli*) or cannot provide enough MoCo to keep up with MFE apoprotein synthesis (*Pichia pastoris* or baculovirus).

As illustrated schematically in Figure 1, mammalian MFEs are cytosolic proteins characterized by a similar general structure. The enzymes are homodimers consisting of identical subunits of approx. 150 kDa [9,10,14,22]. The subunits have a typical and easily recognized tripartite structure. An N-terminal domain of approx. 20 kDa containing two 2Fe/2S redox centres is followed by a 40 kDa flavin-containing region and a 85 kDa C-terminal domain comprising the MoCo and the substrate-binding sites.

XOR is the prototypical member of the MFE family

The enzyme for which we have the largest amount of information is XOR, as this protein has been the object of intense study at the enzymological and spectroscopic levels for many years [23–25]. XOR has been purified from bovine [26] and human [27] milk, as well as from rat [28,29], mouse [9] and rabbit [30] liver. Although XOR oxidizes a variety of compounds, including purines, pteridines and aldehydes, hypoxanthine and xanthine are thought to be the physiological substrates of the enzyme. Regardless of the species and the source used for isolation, there is relatively little variability in terms of K_m, V_{max}, catalytic-centre activity (turnover number) and final specific activity when these parameters are measured using hypoxanthine or xanthine as a substrate. Moreover, all mammalian XORs are inhibited by allopurinol, one of the oldest known and most selective inhibitors of the enzyme [31]. XOR can be extracted from mammalian sources in the form of a dehydrogenase (XD) or an oxidase (XO). In the first case, the electrons deriving from the oxidation of hypoxanthine or xanthine reduce NAD^+ to NADH. In the second case, electrons are transferred directly to molecular oxygen with the production of superoxide and, secondarily, of hydrogen peroxide [2]. XD can be converted reversibly or irreversibly into XO by oxidation [33] or by specific proteolytic cleavage [34]. Irreversible conversion of purified rat XD into XO is achieved *in vitro* by controlled trypsin cleavage [35]. Under these experimental conditions, the homodimeric form of XOR is cut at two points at the level of each monomeric subunit; however, the cleaved fragments remain complexed [35]. The cleavage sites are located within the two hinge regions connecting the 2Fe/2S- to the flavin-containing domain, and this to the MoCo-containing region [35]. Conversion of XD into XO is not a general characteristic of all eukaryotic XORs. Indeed, chicken liver XOR is present only in the XD form and is resistant to conversion [2,33–36].

The complete primary structures of XORs from various mammalian organisms are known, as the cDNAs coding for the human [19,37,38], bovine [28,39], mouse [40], rat [35] and cat [41] orthologous proteins have been cloned. Mammalian XORs have similar length and conserved amino acid sequences (80 % or more overall identity). Given their sequence similarity, it is not surprising that all mammalian XORs are easily aligned along their entire length (see Figure 2A as an example). The subdivision into the three basic structural domains, typical of the MFEs, is easily recognized in all XOR sequences. In fact, the three domains have relatively conserved sequences, and are separated by less conserved amino acid stretches that act as hinge regions. The highest degree of similarity among XORs is at the level of the 2Fe/2S N-terminal domain, where the eight cysteine residues involved in the co-ordination with the four iron atoms are strictly conserved.

XOR, AOX1 and related enzymes have different enzymic characteristics, but similar primary structure

AOX1 has been purified in its catalytically active form from bovine and rabbit liver [21,22], and the recombinant form of the mouse enzyme has been expressed in *E. coli* and purified from the engineered micro-organism [21]. In addition, a method for the purification of AOH1 free from contamination by AOX1 has been described [10]. At present the low amounts of AOH2 expressed in skin, which is the richest source of the enzyme, have prevented purification of the protein. AOX1, the oldest and most thoroughly studied enzyme, has broad substrate specificity. The enzyme oxidizes aromatic azaheterocycles containing a –CH=N– chemical function (e.g. phthalazine and purines), aromatic or non-aromatic charged azaheterocycles with a $-CH=N^+-$ moiety (e.g. N^1-methylnicotinamide and *N*-methylphthalazinium) or aldehydes, such as benzaldehyde, retinal and vanillin [42]. Several substrates of AOX1 are of toxicological or pharmacological interest, and they include the toxic metabolite of ethanol, acetaldehyde [43,44], as well as anti-neoplastic and

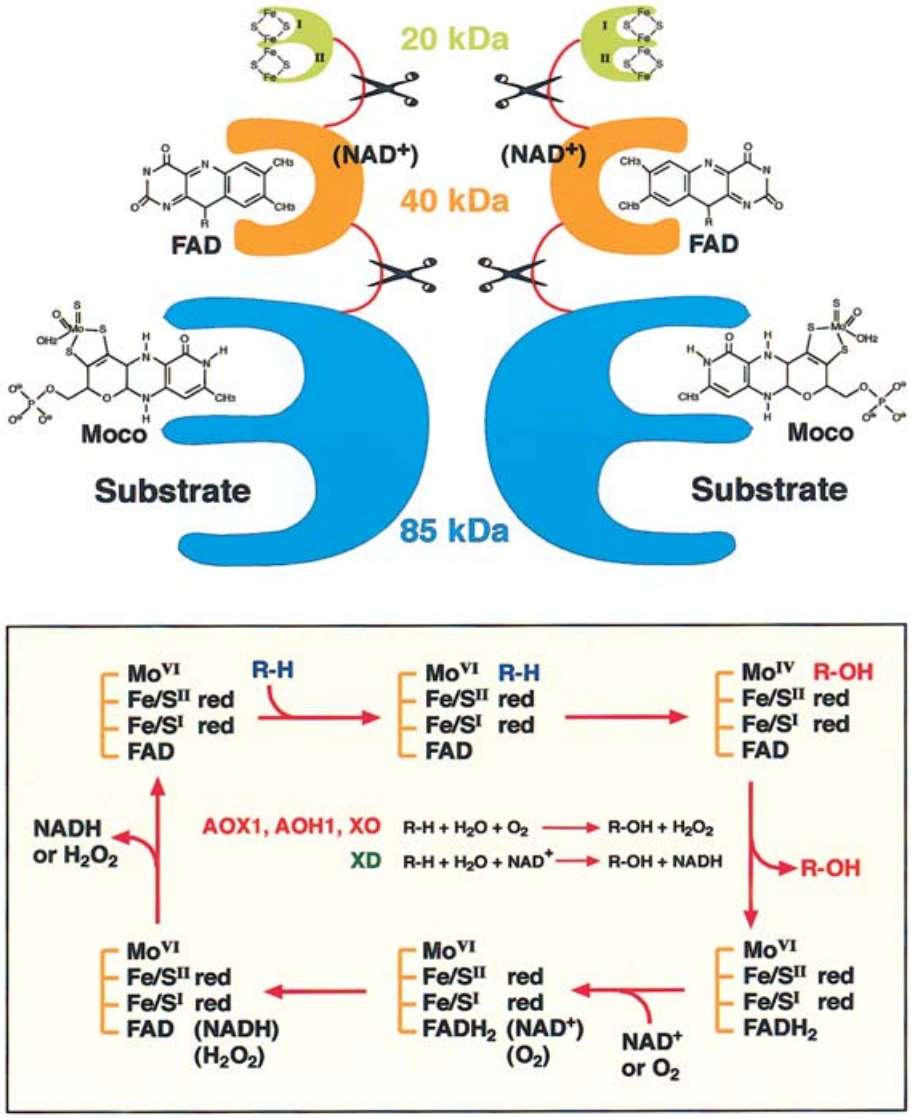

Figure 1 Domain composition of a prototypical MFE

All mammalian MFEs are homodimers consisting of two identical subunits that form a complex in the cellular cytosol. They consist of a 20 kDa N-terminal domain (light green) in which the two spectroscopically distinct 2Fe/2S redox centres (I and II) are localized. This is linked to the 40 kDa FAD-binding domain (orange) through a poorly conserved and relatively unstructured hinge region. In the XD form of XOR, the FAD-containing domain binds NAD (NAD^+). The sequences necessary for this binding are not conserved in AOX1 and related enzymes. The 85 kDa domain contains the MoCo-binding site, which is located within the substrate pocket. The 45 kDa and 85 kDa domains are linked by a second relatively unstructured hinge region. In rat XOR the two hinge regions can be cleaved (scissors) at identified amino acid residues by trypsin or trypsin-like proteolytic enzymes. This results in the conversion of the XD form of XOR into the XO form. Whereas XO, AOX1, AOH1, and possibly AOH2 and AOH3, use oxygen as the final acceptor of the electrons resulting from the oxidation of the substrate, producing superoxide oxygen radicals or hydrogen peroxide, XD uses NAD^+, reducing it to NADH. In the simplified reaction mechanism shown at the bottom of the figure, the various redox centres 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 centre. The reducing equivalents are passed to the flavin, which is re-oxidized by molecular oxygen (NAD^+ in the case of the XD form of XOR). The Fe/S centres [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.

anti-viral agents, such as methotrexate [45], 6-mercaptopurine [46] and famcyclovir [47]. Some of the AOX1 substrates are common to XOR, and the relative selectivity of the two enzymes has been systematically reviewed in a relatively old but comprehensive study [48]. Very little is known about the substrate specificity of AOH1 and AOH2, which, nevertheless, are likely to be isoenzymic forms of AOX1. We know that benzaldehyde is a substrate for both AOX1 and AOH1, and this substrate forms the basis of an electrophoretic assay which is used to measure the two enzymes [10,14,49]. The oxidase activity of AOX1 is inhibited by compounds such as dicoumarol [50] or methadone [51], although the specificity of the two inhibitors for the enzyme is questionable.

The cDNAs coding for human [8], bovine [22], rat [52] and mouse [18] AOX1, as well as those for mouse AOH1 and AOH2 [14], are available. When the complete sequence of the first AOX1 was determined, it was immediately evident that the primary structure of the protein was extremely similar to those of the various mammalian XORs available at the time [22]. This was the first demonstration that AOX1 and XOR are two related enzymes, not only in terms of general structure and biochemical characteristics but also in terms of amino acid sequence. The

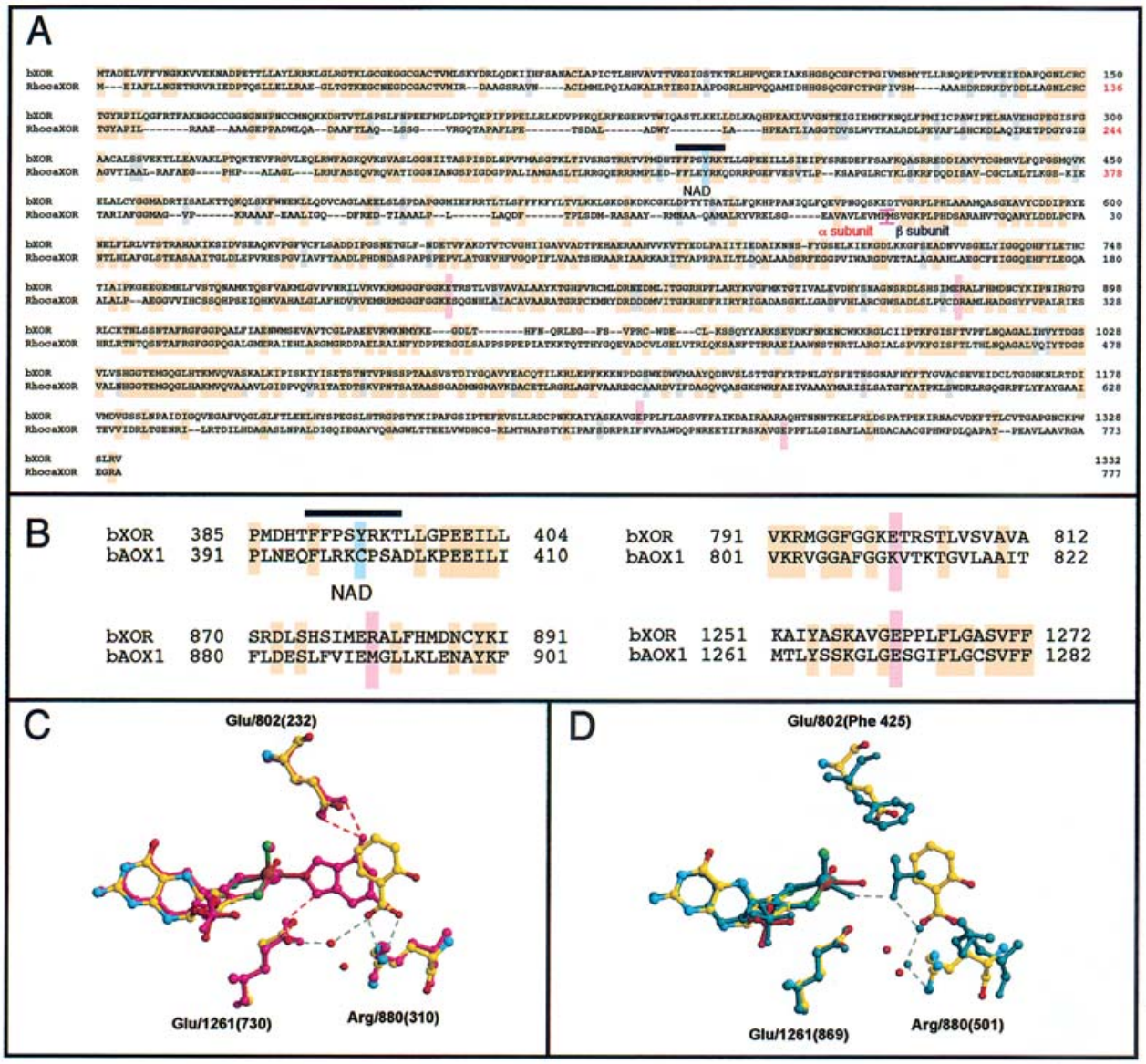

Figure 2 Structural details of selected MFE proteins

(**A**) Alignment of the *R. capsulatus* (RhocaXOR) and bovine XOR (bXOR) protein sequences. The panel demonstrates that a prototypical prokaryotic XOR protein dimer, consisting of two chains coding for the two Fe/S redox centres and FAD-binding site (α subunit) as well as the MoCo-binding domain (β subunit), can be aligned with a prototypical mammalian XOR single-chain monomer. Identical and similar amino acids in the two sequences are boxed in yellow and grey respectively. The position of the sequence corresponding to the conserved NAD^+-binding site identified in chicken XOR is indicated by a thick solid line above the two sequences. The relevant tyrosine residue is boxed in light blue. The position of the C-terminus of the *R. capsulatus* XOR α chain and the N-terminus of the corresponding β chain is indicated by two square brackets in magenta. Amino acid residues involved in the binding of the substrate at the molybdenum centre are boxed in pink. (**B**) Comparison of bovine XOR and AOX1. The panel shows an alignment of the XOR and AOX1 sequences surrounding the NAD^+-binding site and the three amino acid residues known to be involved in the binding of the substrate at the molybdenum centre. It is evident that the Tyr residue necessary for the binding to NAD^+ in XORs is replaced by a different residue in bovine AOX1. This is typical of all AOX1s, AOH1, AOH2 and AOH3, and is consistent with the fact that these proteins do not bind NAD^+ and are pure oxidases. In a similar fashion, Arg^{880} and Glu^{802}, two residues known to be important for the positioning of the substrate in the active site of bovine XOR, are not conserved in bovine AOX1, species orthologues or related enzymes. This suggests that different MFEs act on different types of substrates, and is in line with the fact that hypoxanthine and xanthine are not utilized by AOX1s, AOH1 or AOH2 as efficiently as by XOR. (**C**) Comparison of the active-site structure of bovine XD complexed with salicylate (shown in colour code) and *R. capsulatus* XD complexed with alloxanthine (represented in magenta and with residues labels in parentheses). In *R. capsulatus* XD, alloxanthine is bound directly to the Mo (Mo–N-8 distance = 2.1 Å) and replaces the OH/OH_2 ligand. The alloxanthine molecule is stabilized in the active site by interactions with Glu^{232}, Glu^{730} and Arg^{310}. In bovine XOR, salycilate binding is stabilized by hydrogen-bonding interactions with Glu^{1261} via a water molecule and to the conserved Arg^{880}. (**D**) Comparison of the active-site structure of bovine XD–salicylate (shown in colour code) and DgAOR–propan-2-ol (represented in green and with residue labels in parentheses) complexes. Whereas Glu^{869} and Arg^{501} are conserved in DgAOR, Phe^{425} is replaced by a Glu residue in XORs (Glu^{802} in bovine XOR) and is involved in the orientation of the purine molecules.

amino acid sequences of AOX1 from different animal species can be easily aligned along their entire lengths with practically all types of XORs. The overall level of similarity between AOX1 and XOR proteins is approx. 50 %, which clearly indicates that the two MFEs originated from a common ancestral precursor [10,14,22]. The subsequent cloning of the AOH1 and AOH2 cDNAs from mouse liver and skin respectively [14] demonstrated that the mammalian MFE family is larger than originally thought. The two

Table 1 DgAOR and structurally characterized MFEs

MCD, MPT cytosine dinucleotide.

Enzyme	Molecular mass (kDa)	Cofactors	Subunit composition	Refs	Pdb code
DgAOR	2 × 100	2 × (2 [2Fe/2S]) 2 × (Mo-MCD)	α_2	[62,174]	1ALO, 1HLR
Oligotropha carboxydivorans CODH	2 × 137	2 × (2 [2Fe/2S]) 2 × (Mo-MCD) 2 × FAD	$(\alpha\beta\gamma)_2$	[66]	1QJ2
Bovine milk XD/XO	2 × 145	2 × (2 [2Fe/2S]) 2 × (Mo-MPT) 2 × FAD	α_2	[17,68]	1FO4/ 1FIQ
Rhodobacter capsulatus XD	2 × 135	2 × (2 [2Fe/2S]) 2 × (Mo-MPT) 2 × FAD	$(\alpha\beta)_2$	[16]	1JRO

novel mouse MFEs are very similar to each other (63 % amino acid identity) and show a remarkable level of residue conservation with both AOX1 and XOR proteins from various sources [10,14]. However, AOH1 and AOH2 are more closely related to mouse AOX1 (approx. 60 % identity) than they are to the corresponding XOR (approx. 50 % identity). This justifies the acronym adopted to designate the two novel proteins. As exemplified in Figure 2(B), there are some notable amino acid differences that distinguish the AOX1, AOH1 and AOH2 sequences from those of XORs. The FAD-binding domain of XORs contains a short, conserved sequence that has been demonstrated to represent the NAD^+-binding site of chicken XD [53,54]. The corresponding sequence in AOH1, AOH2 and AOX1 from various animal and plant sources is completely divergent. In addition, a strictly conserved Arg residue in the MoCo domain of XORs is substituted by different amino acids in AOX1, AOH1 and AOH2 [10,14,18,22]. Interestingly, the Arg residue is important for the positioning of hypoxanthine and xanthine in the substrate pocket of these enzymes [17,55]. Taken together, these sequence details suggest that not only AOX1, but also AOH1 and AOH2, are likely to be pure oxidases. In addition, they are consistent with the fact that hypoxanthine and xanthine are substrates for all XORs, but not for AOX1, AOH1 or AOH2 [10].

Comparison of the crystal structures of XORs gives insight into the enzymology of mammalian MFEs

MoCo-containing enzymes are divided into four separate groups on the basis of structure, cofactor and spectroscopic characteristics: the DMSO reductase, the XO (including mammalian MFEs), the SO and the aldehyde ferredoxin oxidoreductase families. As the following discussion is limited to MFEs, the reader is referred to a relatively recent and comprehensive review article [56] for a more general treatment of the structural characteristics of molybdenum-containing enzymes. However, as SO is the only mammalian MoCo-containing protein besides MFEs, and given its critical requirement for human health [57–60], it is worthwhile mentioning that the structural details of the protein are known. In fact, this enzyme has been crystallized from chicken liver [61]. The catalytically active SO protein does not contain Fe/S centres, is homodimeric, and its monomer consists of two domains. The MoCo domain of approx. 42 kDa is much smaller than that of MFEs and is characterized by a dioxo Mo centre. The second domain consists of a haem iron bound to a haem domain of approx. 10 kDa. In spite of its classification in the molybdo-protein family, crystallization of the protein [61] confirms that SO has a structure completely different from that of MFEs, which are a distinct set of enzymes.

Much of the information available on the secondary/tertiary structures of MFEs derives from the crystallization of bovine and *Rhodobacter capsulatus* XORs, and of the structurally related proteins *Desulfovibrio gigas* aldehyde oxidoreductase (DgAOR; note that this protein is not a real MFE, as it lacks the FAD cofactor and the corresponding domain) and *Oligotropha carboxydivorans* CO dehydrogenase (CODH) [15–17,62–64].

As mentioned above, MFEs are organized as dimers, and the monomers act as independent subunits. Each subunit contains a Mo atom co-ordinated to a tricyclic pyranopterin, two spectroscopically distinguishable 2Fe/2S centres (classified as type I and II) and an FAD cofactor. The cofactors are embodied within the protein polypeptide, which has a remarkably similar fold in all of the structurally characterized enzymes (Table 1).

There are differences in terms of subunit composition among the various crystallized structures. All the cofactors of the bovine milk enzyme (XO/XD) are bound within a single, large polypeptide (α_2 structure). By contrast, in *R. capsulatus* XOR ($\alpha_2\beta_2$ structure), subunit α binds the two 2Fe/2S centres and the FAD cofactor, while subunit β incorporates MoCo. In the case of CODH, each of the three independent subunits harbours distinct cofactors: the two 2Fe/2S centres in subunit α, FAD in subunit β and MoCo in subunit γ, such that the overall structure corresponds to a dimer of trimers. This is similar to what is predicted for *E. coli* XOR [65]. The nature of the nucleotide bound to the pyranopterin moiety is dictated by the species of origin rather than the enzymic function of the protein considered. In eukaryotic enzymes, MoCo is always in the monophosphate form (molybdopterin; MPT), while both the MPT (*R. capsulatus* XOR) and the MPT cytosine dinucleotide (DgAOR and CODH) forms are observed in prokaryotes.

With the exception of CODH, in which a Mo/S/Cu substructure has been identified [66], all the other enzymes contain a similar molybdenum active site. The metal site adopts a 5-fold co-ordination state, with the dithiolene moiety, one oxo group and one hydroxy/water ligand defining the equatorial plane, and a Mo–S ligand at the apical co-ordination site. The sulphido ligand is known to be essential for catalytic activity [67], and its position in DgAOR has been determined on the basis of resulphuration experiments carried out on single crystals [63]. In resulphurated crystals (1.8 Å electron density maps) the sulphido ligand is identified unambiguously [63]. In the case of the *R. capsulatus* or bovine XD structures, solved and refined to lower resolutions of 3.0 Å and 2.1/2.5 Å (XD/XO) respectively, the authors reported

Table 2 Significant amino acid residues in the substrate- and Moco-containing domains of MFEs and DgAOR

The amino acid residues that have been shown to participate in substrate recognition (**A**) and in the formation of the tunnel leading to Moco (**B**) in bovine and *R. capsulatus* XORs are listed. The residues identified in the corresponding positions of the bovine AOX1 protein sequence and the DgAOR crystal structure are shown for comparison. Note that amino acid correspondence in bovine AOX1 is based solely on a CLUSTAL-W alignment of the protein sequence with bovine and *R. capsulatus* XORs.

Bovine XO/XD	*R. capsulatus* XD	Bovine AOX1	DgAOR
(A)			
Glu^{802}	Glu^{232}	Lys^{812}	Phe^{425}
Arg^{880}	Arg^{310}	Met^{890}	Arg^{501}
Glu^{1261}	Glu^{730}	Glu^{1271}	Glu^{869}
(B)			
Leu^{873}	Leu^{303}	Glu^{883}	Phe^{494}
Ser^{876}	Pro^{306}	Glu^{886}	Leu^{497}
Phe^{914}	Phe^{344}	Phe^{924}	Tyr^{535}
Phe^{1009}	Phe^{459}	Leu^{1019}	Leu^{626}

uncertainty as to the chemical identification of the Mo ligands. Hence, in these proteins, the Mo ligands can be inferred only by analogy with DgAOR and from mechanistic studies [12,67].

A typical feature of the enzymes listed in Table 1 (and of MPT-containing enzymes in general) is the active site, which is buried (approx. 10–15 Å away from the surface), but reachable through a funnel-shaped cavity that is wider on the surface (15–20 Å diameter) and narrower at mid-height in close proximity to the Mo atom (6 Å diameter). Hydrophobic residues, able to accommodate the ring structures of the aromatic substrates or inhibitors of XOR, dominate the channel. Most of these residues are conserved in *R. capsulatus* XOR, bovine XO/XD and DgAOR (Table 2) or are substituted by amino acids that preserve hydrophobicity. In the case of bovine XD complexed with the tightly bound inhibitor TEI-6720 [2-(3-cyano-4-isobutoxyphenyl)-4-methyl-5-thiazolecarboxylic acid], the inhibitor molecule fills the channel that leads into the buried MPT active site. The inhibitory action of TEI-6720 is due to it blocking the access of the substrate to the enzyme. The location of the inhibitor in the channel is stabilized by several hydrogen bonds and hydrophobic interactions with the generally conserved neutral and aromatic residues Phe^{649}, Leu^{648}, Leu^{873}, Phe^{914}, Phe^{1009}, Val^{1011}, Phe^{1013} and Leu^{1014}.

In bovine XOR, Glu^{1261}, Arg^{880} and Glu^{802} (see Figure 2C and Table 2) reside close to the Mo active site and are important in substrate recognition and enzymic catalysis. Glu^{1261} is highly conserved not only in XORs of prokaryotic and eukaryotic origin, but also in AOX1, AOH1, AOH2 and AOH3. In all of the structures analysed, the two charged oxygens of the carboxylic group of Glu^{1261} are close to the Mo atom, at a distance that varies between 2.7 Å (for *R. capsulatus* XOR) and 3.0 Å. As proposed for DgAOR [63], this Glu residue has a role in catalysis, since it promotes the nucleophilicity of the water ligand and is likely to bind directly to Mo at intermediate steps of the reaction mechanism. Arg^{880} is conserved in all XORs and in DgAOR (Arg^{501}), but not in AOX1, AOH1 or AOH2. The residue may be important for the positioning of purine substrates in the Mo active site. This is supported by the observation that point mutations of the corresponding Arg residue (Arg^{911} to Gln or Gly) in *Aspergillus nidulans* XOR cause changes in the hydroxylation position of the 2-hydroxypurine ring from C-8 to C-6 [55]. Finally, Glu^{802}, which lies on the other side of the purine ring, is conserved only in XORs.

The structures of bovine XO and XD were determined from crystals of the proteins complexed with the inhibitor salicylate [17]. Interestingly, salicylate is placed in a position in the active site similar to that of the inhibitor molecule propan-2-ol in DgAOR (Figure 2D). However, while propan-2-ol establishes a hydrogen bond with the water/hydroxy ligand of MoCo, the carboxylic group of salicylate is hydrogen-bonded to the guanidinium group of the conserved residue Arg^{880}. The crystal structure of *R. capsulatus* XOR has been solved in both its native and alloxanthine-inhibited forms (crystallized in the presence of allopurinol) [16]. In this last situation, alloxanthine binds directly to the Mo atom (Mo–N-8, 2.1 Å), replacing the OH/OH_2 ligand [16], similar to what has been reported for allopurinol-inhibited bovine XOR [68].

The crystallization of MFEs has contributed greatly to the elucidation of the molecular mechanisms underlying the interconversion of XD and XO. In this process, the electron acceptor changes from NAD^+ to dioxygen. Most of the structural differences between XD and XO are believed to be due to conformational changes in the NAD^+-binding site and the redox potentials of the FAD cofactor, such that the flavin semiquinone is more stable in XD than in XO. Consistent with this view, the main structural differences between XD and XO are due to changes in the conformation of the FAD domain [17]. Although they maintain similar folds, the two forms of XOR show the largest conformational differences around the FAD active site. When the transition from XD to XO occurs, a change in conformation of the loop Gln^{423}–Lys^{433} is observed. This produces a structural rearrangement that results in a different electrostatic potential surrounding the FAD-binding pocket and reduced accessibility to the FAD isoalloxazine ring.

GENETICS AND EVOLUTION

The structures of the human [69–71] and mouse [72,73] *XOR* and *AOX1* genes are completely defined. At present, the structures of the mouse *AOH1* and *AOH2* loci are available [10], and we have recently identified *AOH1* and *AOH2* orthologues in the rat genome and cloned the relevant cDNAs (M. Terao and E. Garattini, unpublished work). However, the situation is different in the case of the human genome, as described below.

Mammalian *XOR* genes: an example of exon multiplication during phylogenesis

The human *XOR* locus maps to chromosome 2p22 [74], whereas the mouse counterpart is located on chromosome 17 [72]. Human and mouse *XOR* genes consist of 36 relatively short exons, and span approx. 80 and 85 kb respectively. Not surprisingly for two orthologues, all of the exon/intron junctions of the human and mouse *XOR* genes are perfectly conserved in terms of both position and type. The number of exons in mammalian *XOR* genes is larger than that observed in the orthologous genes of *Aspergillus nidulans* (four exons) [5], *Caenorhabditis elegans* (16 exons) [75], and insects such as *Drosophila melanogaster* (four exons) [76], *Bombix mori* (six to eight exons) [77] and *Calliphora vicina* (four exons) [78]. In spite of the remarkable disparity in the number of exons, the positions of most of the exon/intron junctions are concordant in the various species homologues. As to definition of the regulatory elements of the human and mouse *XOR* genes, few functional studies are available [71,79–82]. The 5′-flanking regions of the two genes are devoid of a canonical TATA box, which is substituted by an initiator element. These DNA regions contain most of the elements

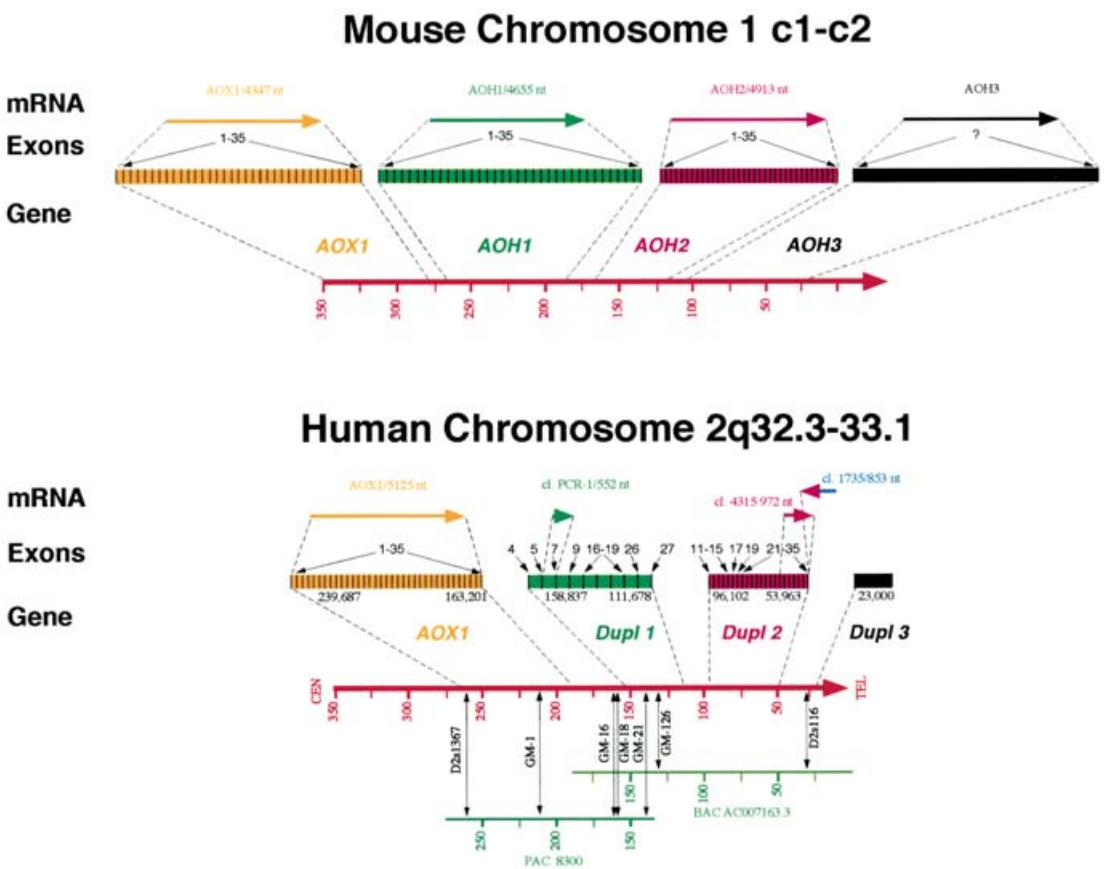

Figure 3 Mouse and human MFE gene clusters

Shown is a schematic representation of the MFE gene clusters on mouse chromosome 1 and human chromosome 2. In the mouse, the cluster consists of four genes, *AOX1*, *AOH1*, *AOH2* and *AOH3*. Except for *AOH3*, the structure of which is not yet completely known, each gene comprises 35 exons and codes for a transcript (coloured arrows) as well as a corresponding functional MFE. The length of each transcript is indicated in nucleotides (nt) above each arrow. In humans, one functional gene (*AOX1*) is followed by three DNA regions (*Dupl. 1*, *Dupl. 2* and *Dupl. 3*) characterized by the presence of exon sequences showing high nucleotide similarity to exons contained in human *AOX1* and mouse *AOX1*, *AOH1*, *AOH2* and *AOH3*. In *Dupl. 1*, *Dupl. 2* and *Dupl. 3*, the exons are numbered according to their similarity to the corresponding exons of human *AOX1*. The names and lengths of the clones isolated by hybridization screening of human liver cDNA libraries or PCR amplification of human liver mRNA are indicated above the arrows. Clone 1735 is a cDNA deriving from a polyadenylated transcript running in the opposite direction relative to clone 4315 and complementary to part of the 4315 mRNA. For this reason the corresponding arrow is shown in two colours (purple and red). CEN, centromere; TEL, telomere. The red scale underneath the gene loci is in kb. The green lines represent relevant human BAC (bacterial artificial chromosome) or PAC (P1 artificial chromosome) clones with their relative size in kb. The recent availability of the complete nucleotide sequence of the human genome confirmed the presence of *AOX1*, *Dupl. 1* and *Dupl. 2* in a contiguous fragment (gi 15987253). The positions of some important microsatellite markers are indicated by vertical black arrows. The numbers underneath human *AOX1*, *Dupl. 1*, *Dupl. 2* and *Dupl. 3* indicate the numbers of the first and last nucleotides of the *AOX1* gene or of the regions of similarity within each duplication.

responsible for the constitutive expression of XOR, but do not seem to comprise sequences dictating tissue and cell specificity or responses to endogenous and exogenous stimuli.

AOX1, *AOH1* and *AOH2* cluster on mouse chromosome 1

As illustrated schematically in Figure 3, the mouse *AOX1*, *AOH1* and *AOH2* genes are clustered close together on chromosome 1 band c1 [10]. *AOX1* is the most 5′ gene and is separated from *AOH1* by approx. 5 kb. In turn, *AOH1* is approx. 15 kb away from *AOH2*. Very recent data obtained in our laboratory demonstrate that the cluster consists of a fourth locus represented by the gene encoding AOH3, which is located approx. 9 kb downstream of *AOH2* (M. Terao and E. Garattini, unpublished work). *AOX1*, *AOH1* and *AOH2* have very similar structures, consist of 35 exons and range from 98 to 60 kb in length. The positions and types of intron/exon junctions are perfectly conserved among the three genes, and we have evidence that the same is true for *AOH3* as well. All this indicates that the gene cluster must have been generated through one or more events of tandem duplication. Interestingly, 34 out of 36 junctions are strictly conserved in mouse and human *XOR* genes relative to *AOX1*, *AOH1* or *AOH2*. The two notable exceptions are represented by exon/intron junctions 7 and 26. The latter is of particular significance, as exon 26 in *AOX1*, *AOH1* and *AOH2* is split by an extra junction present only in the two *XOR* genes. This accounts for the fact that *XOR* genes consist of 36 exons, whereas the genes coding for other MFEs consist of 35 exons. The protein alignment and gene structure data clearly indicate that all known mammalian MFEs have a common origin and have evolved from an ancestral precursor.

AOH1 is characterized by a curious anomaly. The gene has the potential to synthesize two mRNAs differing only in the presence or absence of an extra portion of 5′-untranslated region transcribed from an unusual leader exon [10]. This leader exon is located within intron 26 of the *AOH1* gene and is transcribed in the opposite direction relative to all of the other exons. For this reason, we proposed that one of the AOH1 transcripts is the result of an unusual type of *trans*-splicing event [10].

At present, very little is known about the regulatory elements of the *AOX1*, *AOH1* or *AOH2* genes. The 5′-flanking regions of *AOX1* and *AOH2* contain functional promoters, which are active in many cell types. Similarly, the two putative promoter regions located upstream of the two leader exons have been shown to activate the transcription of exogenous reporter genes [10].

The human *AOX1* gene cluster: simplification in complexity

The human *AOX1* gene maps to chromosome 2q32.3-2q33.1 [83]. As shown in Figure 3, this genomic region contains a complete *AOX1* gene [71]. Sequences homologous to mouse *AOH1* (duplication 1; *Dupl. 1*), *AOH2* (*Dupl. 2*) and *AOH3* (*Dupl. 3*) have also been identified in the same genomic region. The *AOX1* gene and the three boxes of homology are separated by approx. 4–5 kb, and are ordered on chromosome 2 with the *AOX1* centromere proximal, followed by *Dupl. 1*, *Dupl. 2* and *Dupl. 3*. Several microsatellites have been ordered within this human DNA region, including GM-1, GM-16 and D2S116, a marker for amyotrophic lateral sclerosis.

The human *AOX1* gene spans approx. 80 kb of DNA and contains 35 exons, whose boundaries are perfectly coincident with those of the mouse orthologue. Importantly, exon 14 contains the microsatellite locus marker GM-1. The DNA regions corresponding to *Dupl. 1*, *Dupl. 2* and *Dupl. 3* do not seem to represent genes coding for functional MFEs. *Dupl. 1* and *Dupl. 2* preserve only exons corresponding to nt 543–3616 and nt 1105–4058 respectively of AOX1 cDNA. While some exons coding for the FAD (exons 7–18 of *AOX1*) and the MoCo (exons 19–34 of *AOX1*) domains are conserved exactly in *Dupl. 1* and *Dupl. 2*, some are completely unrecognizable. In particular, most of the exons corresponding to the FeS-binding domain and the FeS signature sequences (exons 1–6 of *AOX1*), which are strictly conserved in all eukaryotic and prokaryotic MFEs, are not present in *Dupl. 1* or *Dupl. 2*. Significantly, many of the *AOX1* exons identified in *Dupl. 1* and *Dupl. 2* contain translational stop codons in all three reading frames. Relevant examples of this phenomenon are represented by exon 4 of *Dupl. 1* and exon 15 of *Dupl. 2*. Evidence for a third pseudogene (*Dupl. 3*) is presently incomplete, although a poorly conserved region downstream of *Dupl. 2*, which represents approx. 30 % of the 5′ region of mouse *AOH3*, has been identified.

DNA regions contained in *Dupl. 1* and *Dupl. 2* are transcribed, as screening of human cDNA libraries resulted in the identification of two clones. A 992 nt liver cDNA (clone *4315* in Figure 3) shows 80 % conservation with human *AOX1* and mouse *AOH1* exons 30–34, matches AOX1 mRNA from nt 3600 to 4159 and is derived from *Dupl. 2*. A second, 899 nt cDNA (clone *1735*), obtained from a human testes cDNA library, represents an RNA transcribed in the opposite direction relative to *AOX1* and clone *4315*, and it partially overlaps (with 100 % nucleotide identity) the *4315* cDNA. We have an additional cDNA clone, derived from the 5′-terminus of *Dupl. 1*, which contains sequences corresponding to *AOX1* exons 5–7. However, all efforts to link exons 5–7 to other exons by reverse transcription–PCR have failed.

Taken together, these data indicate that the human genome is likely to retain a single functional *AOX1* gene and three tandem gene duplications with similarity to mouse *AOH1*, *AOH2* and *AOH3*. Although, at present, the potential for long-range RNA splicing or other complex processing has not been rigorously excluded, the simplest and most tenable interpretation of available data indicates that these duplications are pseudo-genes replacing the mouse *AOH1*, *AOH2* and *AOH3* loci. Overall, the human *AOX* cluster on chromosome 2 greatly resembles the mouse counterpart on chromosome 1 in terms of number, order and polarity of the duplications. This strongly indicates that the most proximal genes of the human and mouse clusters are orthologues, and justifies the use of the same symbol, *AOX1*, to define them.

Towards the definition of MFE phylogenesis

Figure 4 shows an updated phylogenetic tree for MFEs. The ancestral precursor of all MFEs is likely to be an XOR of prokaryotic origin, since oxidases with biochemical and structural characteristics similar to those of AOX1, AOH1 or AOH2 are not known in this type of organism. The prokaryotic XOR precursor holo-enzyme must have been the product of three distinct genes, coding for the 2Fe/2S-, the FAD- and the MoCo-containing domains, as observed in the case of the XOR protein synthesized in the extant bacterial species *E. coli*. In a subsequent evolutionary step, the three coding genes were consolidated in a single open reading frame coding for the entire 150 kDa XOR subunit typical of eukaryotic organisms. An *XOR* gene of this type is evident in the fungus *Aspergillus nidulans* [5]. The appearance and consolidation of *XOR* must have been followed by one or more duplication events leading to the generation of other MFE genes of the *AOX1* and *AOH* types (referred to collectively as *AOX*s from now on). One such duplication is evident in the flat nematode *Caenorhabditis elegans*, whose genome contains a canonical *XOR* gene as well as a second, structurally related locus with at least two features typical of *AOX* genes (absence of the conserved NAD^+-binding sequence and presence of the Arg residue important for positioning of the hypoxanthine in the substrate-binding pocket) [75]. A situation very similar to that in the mouse genome is observed in the plant *Arabidopsis thaliana* and in the insect *Drosophila melanogaster*, which are characterized by the presence of one *XOR* and four other structurally related MFE genes similar to *AOX*s [84–86]. This raises the question as to whether the MFEs, other than XOR, present in *C. elegans*, *D. melanogaster* and plants have any phylogenetic relationship with mammalian AOX1 and related enzymes. A number of considerations makes this possibility rather unlikely. First, mammalian AOX1, AOH1 and AOH2 proteins show a higher degree of identity with all XORs than with the AOXs identified in *C. elegans*, *D. melanogaster* and *A. thaliana*. Secondly, the degree of similarity between mammalian AOX1, AOH1 or AOH2 and the respective XOR homologues is greater than that observed between AOXs and XORs from the same species in *C. elegans* and *D. melanogaster*. Thirdly, while the number, position and type of exon/intron junctions of all mammalian MFE genes are almost completely concordant, the same parameters are much more relaxed in the nematode and the fly. Taken together, the available data support the view that the process of duplication of MFE genes from *XOR*s took place independently in mammals and other animal species. Furthermore, they are consistent with the fact that the duplication of the *AOX1*, *AOH1* and *AOH2* genes is a very recent event. Finally, it is unlikely that the *AOX*s observed in *C. elegans*, *D. melanogaster* and *A. thaliana* are orthologues of and evolved into mammalian AOX1, *AOH1*, *AOH2* or even *AOH3*. For reasons that are as yet unclear, the process of mammalian evolution appears to have led to the disappearance of three functional *AOX* genes in humans.

As a concluding observation, it is interesting to note that the genomes of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* do not contain *XOR* or related genes encoding MFEs. In addition, while the genome of the fishes *Fugu*

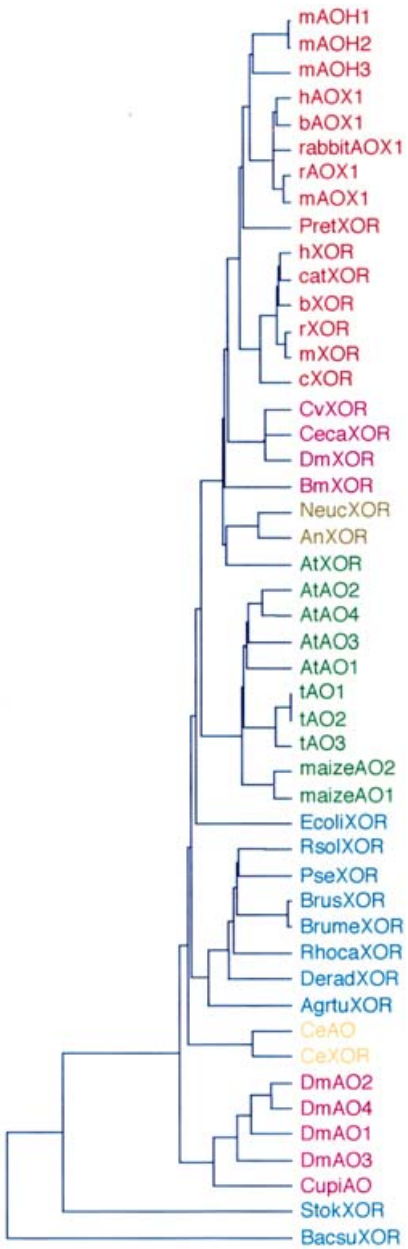

Figure 4 Phylogenesis of MFEs

The figure shows a phylogenetic tree obtained by comparison of the amino acid sequences of many different MFEs with the CLUSTAL-W program. Proteins of animal, insect, fungal plant, bacteria and nematode origin are indicated in red, magenta, brown, green, blue and yellow respectively. GenBank accession nos. are as follows: mAOX1 (mouse AOX1), NM009676; mAOH1, NP_ 076120; mAOH2, AAD51028; mXOR, X62932; mAOH3, M. Terao and E. Garattini unpublished work; hAOX1 (human AOX1), XM_ 002522; hXOR, NM_ 000379; bAOX1 (bovine AOX1), X87251; bXOR, X83508 or X98491; rabbit AOX1, AB009345; PretXOR XOR [*Poecilia reticulata* (fish)], AAK59699; cXOR (chicken XOR), D13221; cat XOR, AF286379; rAOX1 (rat AOX1), Q9Z0U5; rXOR, NM_ 017154; CvXOR (*Calliphora vicina* XOR), X07323; CecaXOR (*Ceratitis capitata* XOR), AAG47345; DmAO1 (*Drosophila melanogaster* AOX1), AE003709 (protein I.D. AAF55207.1); DmAO2, protein I.D. AAF55208.2, DmAO3, protein I.D. AAF55209.1; DmAO4, protein I.D. AAF55210.1; DmX ^[ÔR, Y00308; BmXOR (*Bombyx mori* XOR), D38159; NeucXOR (*Neurospora crassa* XOR), CAD37030; AnXOR (*Aspergillus nidulans* XOR), X82827; AtAO1 (*Arabidopsis thaliana* AOX1), AB005804; AtAO2, AB005805; AtAO3, AB016622; AtAO4, AB037271; AtXOR, AL161586; tAO1 (tomato AOX1), AAG22606; tAO2, AAK52409; tAO3, AAK52410; maizeAO1 (maize AOX1), D88451; maizeAO2, D88452; EcoliXOR (*Escherichia coli* XOR), Q46801, Q8X6C5 and Q46799; RsolXOR (*Ralstonia solanacearum* XOR), CAD15802 and CAD15803; PseXOR (*Pseudomonas aeruginosa* XOR), NP_ 250215 and NP_ 250214; BrusXOR (*Brucella suis* XOR), AAN29297 and AAN29296; BrumeXOR (*Brucella melitensis* XOR), NP_540493 and NP_540492; RhocaXOR (*Rhodobacter capsulatus* XOR), CAA04470 and CAA04469; DeradXOR (*Deinococcus radiodurans* XOR), NP_296359 and NP_285502; AgrtuXOR (*Agribacteriun tumefaciens* XOR), NP_532984 and NP_532982; CeAO (*Caenorhabditis elegans* AOX), Z83318; CeXOR, protein I.D. CAB05902.1; CupiAO (*Culex pipiens quinquefasciatus* AOX), AF202953; StokXOR (*Sulfolobus tokodaii* XOR), BAB65050 and BAB65049; BacsuXOR (*Bacillus subtilis* XOR), O32145, O32144 and O32143.

rubripes and *Poecilia reticulata* have at least an *XOR* gene, that of the zebra fish *Danio rario* does not contain a similar sequence. A computer search of the whole genomes of *Saccharomyces cerevisiae*, *S. pombe* and *D. rario* revealed the absence not only of MFEs but also of sequences similar to those of other molybdo-proteins, such as nitrate reductase and SO. Incidentally, the lack of SO is a characteristic of the zebra fish, as the protein is synthesized by other types of fishes, such as *F. rubripes* and *Merluccius productus* [87]. The genomes of *Saccharomyces cerevisiae*, *S. pombe* and *D. rario* also seem to be devoid of coding sequences with similarity to most of the proteins involved in the synthesis of MoCo. Thus the absence of molybdo-proteins and MFEs, along with absence of the complete MoCo synthetic machinery, suggest that molybdenum is not an essential element for the life of these three organisms.

TISSUE DISTRIBUTION AND REGULATION

The expression of mammalian MFE genes has been studied using a number of techniques, including cytochemistry, immunohistochemistry and Western blot analysis at the protein level, as well as Northern blot, reverse transcription–PCR and *in situ* hybridization at the mRNA level [10,14,88–91]. When analysing the available results, the following points should be considered. First, in the case of data obtained with immunological techniques, the specificity of the antibodies has not always been assessed. This may be a significant pitfall, as all mammalian MFEs are structurally very similar. Secondly, mRNA expression does not necessarily correlate with synthesis of the corresponding catalytically active protein. This is particularly true in the case of the holo-form of MFEs, whose assembly is complex and controlled by many distinct gene products. Thirdly, cytochemistry relies on the specificity of the substrates and/or the inhibitors used, and absolutely selective reagents for XOR, AOX1 and related enzymes are not available. Finally, XOR and other MFEs show significant species-specific variations in their levels of expression.

XOR is expressed in a tissue-specific fashion

Most of the data relating to the tissue and cell distributions of XOR have been obtained in experimental animals, such as mice [40,91] and rats [88]. In these animals, the highest amounts of XOR enzymic activity are measurable in the first part of the intestinal tract. A decreasing gradient of XOR expression is observed as we proceed from the proximal to the distal portion of the small intestine. The epithelial lining of the duodenum and the jejunum are particularly rich in XOR mRNA and protein [40,91]. A similar gradient is also observed in the case of adenosine deaminase, the enzyme that precedes XOR in the metabolic pathway leading to the production of uric acid from purines [92]. This has led to the suggestion that expression of the two enzymes is coupled. A similar distribution of XOR in the human small intestine is supported by data reporting the presence of large amounts of the corresponding activity in the epithelial and goblet cells of the proximal intestine [93].

In humans and rodents, liver and lung are also rich sources of XOR activity, protein and mRNA [40,90,91]. Both *in situ*

hybridization [91] and cytochemical [94] studies demonstrate that the enzyme is expressed in the hepatocytic component of the organ. In human liver, only the periportal subpopulation synthesizes the enzyme [90]. Significant amounts of XOR mRNA and enzymic activity are also expressed in the cellular component of the alveolar septi of the mouse lung [90]. In contrast, the human lung expresses barely detectable amounts of XOR [95]. In humans, the presence of detectable quantities of XOR activity in other organs and cells, such as the heart and the brain, has also been challenged [96–99]. This is of particular importance, as many studies have suggested a role for XOR in the pathogenesis of the cell damage observed following ischaemia/reperfusion of cardiac and nervous tissue [100,101]. Based on data obtained in the bovine [102], it has long been thought that XOR is present in the capillary endothelium of many organs and tissues. However, this idea has been challenged by the finding that circulating XOR adheres to the inner aspect of the blood vessels through interaction with as yet uncharacterized proteoglycan structures [103,104]. With regard to this, it is interesting to note that, unlike other MFEs, XOR (predominantly in the XO form) is present in human and mouse plasma [105,106].

The mammary gland presents a good example of tissue- and stage-dependent expression of XOR. Under normal conditions, low levels of XOR mRNA, protein and enzymic activity are associated with the mammary myoepithelium [107–109]. During the final phase of pregnancy and the whole period of lactation, a striking induction of XOR activity, which is the result of an increase in levels of the corresponding transcript, is observed. XOR induction parallels the growth and development of the alveoli and the beginning of the secretory phase. XOR activity reverts to background levels following involution of the gland at the end of lactation. This phenomenon has been carefully studied in mice and rats, and it is likely to proceed in a very similar fashion in humans.

XOR, like all other mammalian MFEs, is considered to be localized primarily in the cellular cytoplasm. However, in the myoepithelial cell of the mammary gland, the intracellular compartmentalization of XOR is not entirely cytoplasmic. In cultures of human mammary epithelial cells, the enzyme has been demonstrated to have both an intracellular and a surface localization [110]. Importantly, during lactation, XOR is an essential protein component of the secretory fat droplet and is physically associated with other major milk proteins. The thiol-bond-dependent association of XOR with butyrophilin and adipophilin is of particular significance and indicates a vital role in milk secretion [107]. This idea has been supported by some of the data obtained in XOR knockout animals as detailed below. XOR activity has been determined not only in milk but also in other body fluids, such as blood. This is different from what has been observed for mouse AOX1, AOH1 and AOH2, which cannot be detected in plasma by either immunological or enzymic methods (M. Terao and E. Garattini, unpublished work). The presence of XOR in milk and blood raises the question of whether the enzyme is the only MFE that can be actively secreted outside the cell. No information is presently available with respect to this specific point, although lack of a typical N-terminal secretory sequence in the primary structure of XOR suggests that, if active protein secretion is indeed taking place, the process does not involve the typical secretory pathway.

XOR expression is induced by various pathophysiological stimuli

The degree of *XOR* gene expression and the level of activity of the corresponding enzyme is regulated by various types of stimuli and through different molecular mechanisms. Cytokines are known regulators of the XOR protein and, among these, interferons stand out [112–115]. Treatment of mice with type I interferon and interferon inducers, such as polyinosine/cytidine and bacterial lipopolysaccharide [40,91], results in the induction of *XOR* gene expression in various tissues, with the notable exception of the duodenum [40,91]. Stimulation of XOR synthesis can be replicated *in vitro* in different cell types, including epithelial cells [112] and fibroblasts [114]. The phenomenon is the consequence of an increase in the transcriptional activity of the *XOR* gene [114]. This suggests that XOR mediates some aspects of the biological activity of interferons. Particular attention has been paid to the anti-proliferative and anti-viral activities of this cytokine [116] and to the ability of interferon to depress the levels of cytochrome P450-dependent mono-oxygenase activity in the liver [117–119]. However, experiments involving XOR inhibition do not support an involvement of this enzyme in the therapeutic or toxic effects of interferons [116].

A second series of cytokines that have been shown to induce the expression of XOR are tumour necrosis factor α, interleukin-1 and interleukin-6 [112,113,120]. Again, these agents act by inducing the transcription of the *XOR* gene into the corresponding mRNA [112,113]. At present it is not known whether stimulation of XOR by tumour necrosis factor α, interleukin-1 or interleukin-6 is cell- or tissue-specific, as the phenomenon has been reported in human kidney epithelial cells [113], but not in rat pulmonary microvascular cells [115]. Induction of XOR by these primary or secondary pro-inflammatory cytokines suggests a potential role for the enzyme in inflammation.

Corticosteroids, such as dexamethasone or cortisone, increase the expression of the *XOR* gene in human kidney epithelial cells [113], the rat mammary gland [110] and the mouse HC11 cell line, an experimental model of mammary epithelial cell differentiation [107]. Cortisone is a hormone associated with lactation in the mouse and may be the primary stimulus responsible for the induction of XOR during the lactation period. In this respect, prolactin, another important lactogen, may play an accessory role. In fact, combinations of prolactin and corticosteroids are more effective than the single components in inducing XOR and other milk-secretory markers in HC11 cells [107]. Although corticosteroids are lactating hormones, they are also anti-inflammatory agents. Hence it is curious that anti-inflammatory compounds and pro-inflammatory cytokines share the ability of inducing XOR.

Other exogenous stimuli capable of inducing XOR are PMA [121–128] and tetrachlorodibenzo-*p*-dioxin (TCDD) [129,130]. Keratinocytes are believed to be the cell type responsible for the synthesis of XOR in inflamed skin following application of PMA [126]. XOR induction by PMA is also observed in cells of different origin [121,123], suggesting that the effect is not cell-specific. The modality by which the phorbol ester increases XOR activity is currently unknown, although increased activity seems to be accompanied by conversion of XD into XO. In addition, it is not clear if PMA is a direct inducer or stimulates the production of pro-inflammatory cytokines, which represent the ultimate effectors. Topical administration of PMA is instrumental in inducing the promotion phase of experimental skin carcinogenesis. However, suppression of XOR activity by allopurinol does not affect PMA-dependent inflammation, skin hyperplasia or tumour progression in this experimental model [127]. TCDD is a widely distributed environmental pollutant with serious toxic effects on the liver and skin. This compound induces XOR activity in mouse liver through activation of the TCDD receptor, Ahr [129]. Increased production of superoxides by TCDD-induced XOR may be responsible for the liver damage caused by this toxic agent.

Oxygen tension is another critical determinant of XOR intracellular activity. In general, hyperoxic conditions tend to depress XOR enzymic activity, while hypoxia enhances XOR expression and is responsible for the rapid conversion of XD into XO [131–133]. These phenomena are observed in various cell types, including endothelial and lung cells [90]. Conversion of XD into XO during ischaemia/reperfusion of various organs forms the basis of the hypothesis that XOR plays a role in the tissue damage observed in this pathological situation [90].

In conclusion, despite its established biochemical function in the catabolism of purines, a basic and general cellular pathway, *XOR* does not show the characteristics of a typical housekeeping gene, not only because of its tissue- and cell-specific expression, but also because it can be regulated by a variety of different stimuli.

The pattern of expression of AOX1 and related enzymes is different from that of XOR

The information available on the tissue distribution, as well as the regulation, of AOX1 and related enzymes is limited. In the mouse [9,10,18], the organ that expresses the largest amounts of AOX1 is the liver. It is likely that a similar situation applies in humans [8,134], rats [52] and cattle [22]. For this reason, it has generally been accepted that AOX1 is a liver-specific enzyme; however, this is not accurate, at least in the mouse. Low but significant amounts of the protein and corresponding mRNA are present in the lung and testis [10,14]. The tissue distribution of AOX1 entirely overlaps that of AOH1. However, comparison of *in situ* hybridization results suggests that different, although partially superimposable, subpopulations of hepatocytes are responsible for the expression of the *AOX1* and *AOH1* genes [10]. Furthermore, AOH1 is expressed in the hepatocyte during the final phases of fetal development, whereas AOX1 expression is evident only in the liver of the mature animal [10]. This suggests a function for AOH1 during liver development. The expression profile of AOH2 is entirely different from that of AOX1 or AOH1. Significant amounts of AOH2 mRNA and protein are observed only in keratinized epithelia, such as the epidermis and the mucosa of the oral cavity, the oesophagus and the first part of the stomach [10]. In liver, lung and testes, whenever AOX1 or AOH1 apoproteins are present, the corresponding benzaldehyde-oxidizing activities are measurable ([10,14]; M. Terao, unpublished work), which demonstrates that the two enzymes are catalytically active. At present it is not known whether the AOH2 protein synthesized in relevant tissues is also in its catalytically active state. In fact, determination of AOH2 activity is not possible, as a specific substrate has not yet been found and the enzyme does not metabolize any of the substrates utilized by AOX1 and AOH1, including benzaldehyde, phenanthridine and retinaldehyde (E. Garattini and M. Terao, unpublished work).

Trace amounts of AOX1 transcript and protein are detectable in the mouse brain and spinal cord. This is due to the fact that the *AOX1* gene is active only in a minor population of cells. The AOX1 transcript is selectively expressed in the epithelial cells of the choroid plexus, the organ involved in the secretion and reabsorption of the cephalo-rachidian fluid, as well as in the motor neurons of the brain and spinal cord [135]. At present, it is not known whether the human central nervous system has a similar cell distribution of AOX1, although one study suggested the presence of the corresponding RNA only in the glial and not in the neuronal component of the anterior horns of the spinal cord [83].

Both liver AOX1 and AOH1 are expressed in a gender-specific fashion. The hepatocytes of male mice synthesize much larger amounts of the two enzymes than those of female animals [14]. AOX1 and AOH1 proteins can be induced by chronic administration of testosterone to female animals ([14,18]; M. Terao and E. Garattini, unpublished work). This phenomenon is mediated by an increase in the levels of AOX1 and AOH1 mRNAs, suggesting that the transcriptional rate of the two corresponding genes is modulated by the male sex hormone [14]. Modulation is likely to be indirect, and growth hormone and/or somatomedins have been proposed to mediate the action of testosterone [136]. Interestingly, regulation of AOX1 and AOH1 by testosterone seems to be tissue-specific, as no sex-related differences in the levels of AOX1 and AOH1 have been observed in the lung [10].

Assembly of the holoenzymic form of MFEs is controlled by the complex machinery regulating MoCo biosynthesis

Further control of the expression and biosynthesis of MFEs is exerted by the availability of MoCo, which needs to be assembled into the apoprotein. The majority of our present knowledge about MoCo biosynthesis stems from studies of MoCo mutants in *E. coli*, where five MoCo-specific operons comprising more than 15 genes are known. The MPT structure of MoCo is conserved in all organisms; hence it is tempting to speculate that (part of) the biosynthetic pathway for MoCo may also be similar in all organisms [137]. Indeed, nearly all *E. coli* MoCo proteins have counterparts in eukaryotes. Six proteins are involved in MoCo biosynthesis in humans, plants and fungi [138]. In humans, MoCo biosynthesis proceeds in three stages (Figure 5).

In stage 1, starting from a guanosine derivative (most probably GTP), a unique and complex reaction sequence [139] results in the formation of precursor Z as the first stable intermediate of MoCo biosynthesis [140]. This reaction is catalysed by the proteins MOCS1A and MOCS1B. Unlike in other eukaryotes, these two proteins are encoded by a single gene (*mocs1*) in humans [141]. The corresponding transcript is bicistronic, with two consecutive reading frames separated by a stop codon. The first reading frame encodes MOCS1A, and the second one MOCS1B. Further transcripts of the *mocs1* gene have been found [142] that are spliced in order to bybass the normal termination codon of *mocs1A*. MOCS1A is a FeS-cluster binding protein that probably belongs to the newly described class of 'radical SAM-proteins' [143], generating a radical species during catalysis [144]. The function of MOCS1B is unknown.

In the second stage of MoCo biosynthesis, two sulphur atoms are incorporated into precursor Z. This reaction is catalysed by the enzyme MPT synthase, a heterotetrameric complex of two small (MOCS2A) and two large (MOCS2B) subunits that stoichiometrically converts precursor Z into MPT. The sulphur is bound to the C-terminus of MOCS2A as thiocarboxylate. In a separate reaction, sulphur is transferred to the small subunit of MPT synthase to re-activate the enzyme for the next reaction cycle of precursor Z conversion [138]. MOCS3 is involved in this process of re-activation and sulphur transfer, and cysteine is the likely donor of the reactive mobile sulphur [138]. As with *mocs1*, the two subunits of human MPT synthase are encoded by one gene, named *mocs2* [145]. On the bicistronic mRNA, the first reading frame codes for the small subunit MOCS2A and the second one for the large subunit MOCS2B. The two reading frames overlap, and exhibit a frameshift of +1 for *mocs2B*. In both cases of bicistronic expression, the first of the two encoded proteins always shows a typical double-glycine motif at its

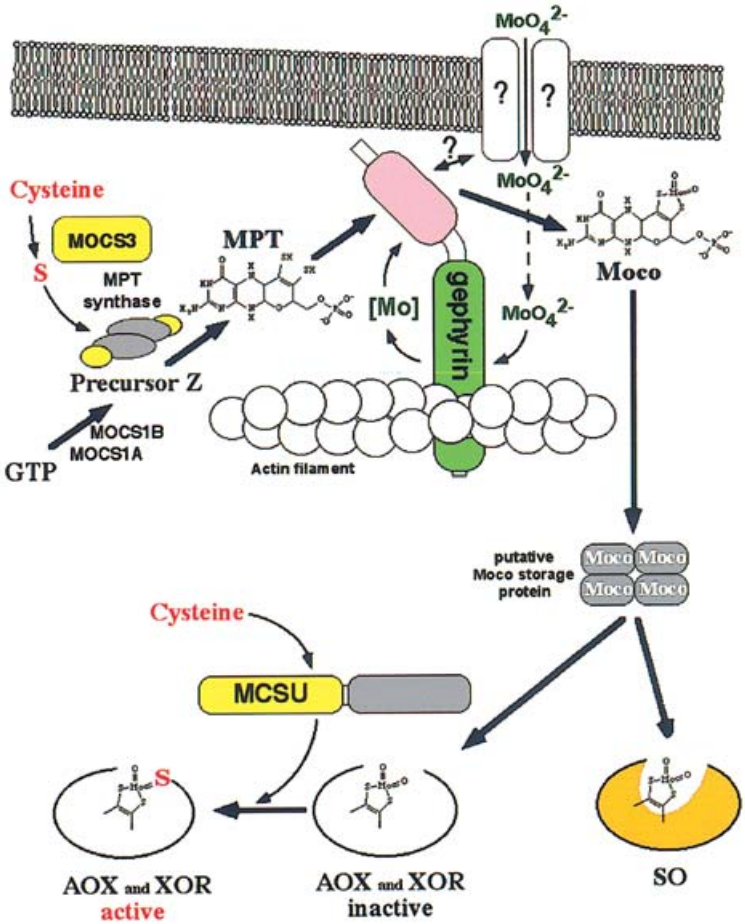

Figure 5 Model for MoCo biosynthesis in human cells

MOCS1A and MOCS1B convert GTP into precursor Z. MPT synthase, consisting of its subunits MOCS2A and MOCS2B, inserts sulphur into precursor Z and converts the precursor into MPT. MPT synthase is sulphated by MOCS3. Subsequently, MPT is bound to gephyrin, which is located under the plasmalemma and bound to an actin filament. A putative molybdate-anion channel is proposed that interacts with gephyrin to facilitate molybdate channelling to the N-terminal domain of gephyrin. This domain generates an activated form of Mo that is incorporated by the C-terminal domain into the bound MPT. MPT is highly sensitive to oxidation; therefore we suggest that the rapid conversion of precursor Z via MPT into MoCo occurs in a multienzyme complex anchored by gephyrin on the cytoskeleton. Finally, MoCo is bound by a putative MoCo storage protein that supplies the cofactor to the Mo-enzymes. SO needs no further modification of MoCo, while AOX1 and XO require a final maturation step, in which the sulphurase MCSU replaces an oxygen atom with a sulphur, thereby activating the enzymes.

C-terminus. For the small subunit of MPT synthase (MOCS2A), we know that the C-terminus is functionally essential. Therefore the observed bicistronic expression of the two MOCS2 proteins is a further indication of strong functional pressure for maintaining the free C-terminus in MOCS2A, and possibly also in MOCS1A. Bicistronicity would ensure co-linear expression and implies that the newly synthesized and interacting proteins are close together. Such micro-compartmentalization is certainly advantageous in view of the low substrate concentrations that occur during MoCo biosynthesis. The human *mocs* genes are expressed at a very low level, and their mRNAs can be detected in all organs, albeit with varying abundance [141,145]. In particular, muscle tissue and liver are rich in *mocs* expression.

In stage 3, Mo has to be transferred to MPT, and this requires the uptake of molybdate. While a high-affinity transport system has been described in *E. coli* [146], in humans nothing is known about the way in which Mo is taken up into the cell. Having crossed the plasma membrane, Mo has to be inserted into MPT. Mutants defective in this step produce MPT, and can be partially restored by growing them on high-molybdate medium, as was shown for the murine cell line L929 [147]. Insertion of molybdenum into MPT is catalysed by the protein gephyrin, which was initially described as a neuroreceptor anchor protein linking glycine receptors in the postsynaptic membrane to the subcellular cytoskeleton [148]. Based on the identity of gephyrin with its plant homologue Cnx1, its additional function in MoCo biosynthesis was proven [149]. Gephyrin knockout mice not only show the expected absence of synaptic glycine receptor clustering, but also develop symptoms identical with those of MoCo deficiency [150]. The identification of a *gephyrin* gene deletion in a patient with symptoms typical of MoCo deficiency has been described [151]. It is clear that gephyrin combines two different functions: (1) biosynthetic activity in MoCo formation, and (2) a structural role in receptor clustering. What could be the functional significance of the cytoskeletal binding of gephyrin in terms of MoCo biosynthesis? As in the case of the bicistronic expression of the *mocs1* and *mocs2* genes, one can conclude that, for higher eukaryotic cells, it becomes important to facilitate substrate–product flow, which could result in microcompartmentalization of a hypothetical MoCo-biosynthetic multienzyme complex. Therefore anchoring to submembranous cellular structures such as the cytoskeleton (1) might help in organizing such a biosynthetic machinery, (2) would

ensure the rapid and protected transfer of labile intermediates within the reaction sequence from GTP to MoCo, and (3) would bring biosynthetic multienzyme complex close to an as yet unknown molybdate-anion channel providing the metal for MoCo synthesis.

Following its biosynthesis, MoCo has to be assembled into Mo-containing enzymes, and the various steps involved in this process are ill-defined. The availability of sufficient amounts of MoCo is essential for the cell to meet its changing demands for synthesizing Mo-enzymes. The existence of MoCo-storage proteins would be a good way to buffer the supply and demand of MoCo. In fact, MoCo-binding proteins have been described for algae and higher plants (e.g. [152]), but their detailed function and reaction within the cell are still unknown. The mechanisn of insertion of MoCo into Mo-enzymes is not understood. For insertion of MoCo into the target apoenzymes in the living cell, either (as yet unknown) chaperones would be needed or the MoCo-storage proteins may become involved at this stage. For some bacterial Mo-enzymes, system-specific chaperones are required for MoCo insertion and protein folding, e.g. XDHC for *Rhodobacter capsulatus* XOR [153].

A final maturation step is specific to MFEs. In fact, XOR and AOX, and presumably also AOH1, AOH2 and AOH3, are enzymes with a mono-oxo Mo centre, in contrast with SO, which is a dioxo Mo-enzyme. This peculiar feature requires the addition of a terminal inorganic sulphide to the Mo site, and this is catalysed by MoCo sulphurase (MCSU) (Figure 5). The human *MCSU* gene has been recently cloned, but most of our information on the protein is from plant studies. Plant MCSU is a two-domain protein whose N-terminus shares significant sequence identity with the bacterial sulphurase NifS. A pyridoxal phosphate-dependent mechanism of (trans)sulphuration has been proposed [154], in which a MCSU-bound persulphide, resulting from the desulphuration of free L-cysteine to L-alanine, is likely to be transferred to Mo. The C-terminal domain, which is not present in NifS proteins but is common to all MCSU proteins, is probably responsible for mediating the contact between XOR/AOX and the trans-sulphurase domain of MCSU proteins [155]. From the regulatory point of view, the activity of MCSU could be an important switch for controlling the amount of functional AOX1/XDH molecules in the cell.

Given the complexity of MoCo biosynthesis, some observations on the pathophysiological relevance of the process are warranted. MoCo is essential in most, if not all, organisms, and defective MoCo has detrimental or lethal consequences due to the pleiotropic loss of all Mo-enzymes. In humans, a combined deficiency of SO and XOR was described [156] which is now named 'human MoCo deficiency'. This pathological entity is very rare, although more than 80 cases have been studied worldwide [157]. The disease is autosomal recessive and occurs in all racial groups. Patients affected show neonatal seizures, severe neurological abnormalities, dislocated ocular lenses, feeding difficulties and dysmorphic features of the brain and head, and die in early childhood [158]. No therapy is available to cure the symptoms of this disease. Other, exceedingly rare monogenic defects involving one of the steps of MoCo biosynthesis have been described. Human patients showing deficiency in MOCS1 or MOCS2 proteins [159] are known, while no patients defective in *mocs3* have been described. A final and interesting class of patients is represented by individuals deficient in MCSU. These patients suffer from xanthinuria type II [160] and have symptoms very similar to those observed in those with a hereditary deficit of XOR.

As a concluding point, it should be underscored that the overall picture of human MoCo deficiency is very similar to that of the monogenic hereditary disease known as 'isolated SO deficiency' [161]. In fact, individuals suffering from either genetic defect exhibit identical symptoms. This observation led to the conclusion that the pathophysiology of MoCo deficiency is due mainly to the absence of SO activity. At present, it is not clear whether the neurological symptoms observed in human MoCo deficiency and isolated SO deficiency occur as a result of increased levels of toxic sulphite or of a shortage of sulphate, which is necessary for the formation of sulpholipids present in the myelin sheaths of nerve axons, or a combination of the two effects. Clearly, in humans, loss of SO activity is a much more serious problem than loss of XOR and/or AOX1.

FUNCTION AND PATHOPHYSIOLOGY

The biochemical function of XOR is well established: the enzyme is involved in the catabolism of purines, oxidizing hypoxanthine into xanthine and xanthine into uric acid [90]. Uric acid is a terminal catabolite in humans, whereas in other mammals, including rodents, the compound is metabolized further into allantoin by uricase. In contrast, recognized physiological substrates for AOX1, AOH1 and AOH2 have not yet been described.

XOR is a double-faced protein with pro- and anti-oxidant potential

Although a physiological substrate and a biochemical function of XOR are available, this does not necessarily translate into a recognized homoeostatic role for the enzyme. In humans, the only described genetic deficit of XOR (human xanthinuria type I) is a rare, albeit benign, pathological condition. The only symptoms of xanthinuric patients are colics, resulting from hypoxanthine stones in the liver and kidney [162,163]. This suggests a largely dispensable role for the enzyme in the homoeostasis of the human organism. However, caution should be exercised in drawing any conclusion, given the extreme rarity of xanthinuria and the possibility of functional compensation in xanthinuric patients. Indeed, the tissue- and cell-specific expression of XOR suggests a local and specialized physiological function, which may be related to the ability of the enzyme to produce the superoxide anion (a strong oxidizing agent) or uric acid (a potent antioxidant).

Toxic oxygen radicals have long been recognized as microbicidal agents; therefore XOR has been postulated to serve a defensive role against micro-organisms in organs and body fluids [90]. Interest in this concept has been renewed by the observation that, in certain conditions, XOR-derived superoxide anions interact with nitrous oxide producing peroxynitrite, a very strong oxidant [164]. At present, the microbicidal role of XOR is consistent with the protective action exerted by the protein in *Salmonella typhimurium* infection [165], and with the fact that mediators of infection and inflammation, such as cytokines and interferons [40,91,114,115,120], induce the enzyme. However, this contrasts with experimental evidence implicating XOR and derived oxygen radicals as pathogenic mediators of infection. In this regard, it has been shown that administration of XOR inhibitors protects mice from pneumonia [166].

A role for XOR in mammogenesis and lactogenesis has also been suggested [108,109,112], in accordance with the high levels of the enzyme observed in the mammary gland of pregnant and lactating animals. With respect to this, the binding of XOR to butyrophilin, one of the main components of the milk fat globule, is particularly intriguing [106].

A wealth of data implicate the XO form of XOR in the tissue damage observed following ischaemia/reperfusion. This

is the consequence of an attractive and debated theory first proposed by Granger and McCord [167,168]. Ischaemia causes the accumulation of hypoxanthine through catabolism of ATP. In parallel, hypoxia-induced activation of proteases leads to the conversion of XD into XO. The oxygen reperfusion of the ischaemic organ, the availability of significant amounts of hypoxanthine and the presence of XOR predominantly in the XO form together cause increased production of toxic oxygen radicals, which exacerbate the cellular damage set in motion by hypoxia. This scheme is applicable to any organ containing significant amounts of XOR, including heart, liver, intestine and kidney. In a subsequent elaboration, it has been proposed that circulating XO is responsible for the tissue damage observed during the process of multiple organ failure that often follows local ischaemia. The theory in its two forms has been validated in the experimental animal, whereby inhibition of XOR decreases the production of oxygen radicals and protects from ischaemic damage (e.g. [168]). Nevertheless, a number of points need to be considered. First, all of the available data were obtained using XOR inhibitors which are not entirely specific. Secondly, there is no convincing demonstration that XD is indeed converted into XO during ischaemia. Thirdly, and most importantly, the levels of catalytically active XOR in rodent tissues are much higher than those observed in human tissues. The phenomenon of ischaemia/reperfusion injury is particularly evident in the case of the human heart, in which the amounts of XOR are barely detectable [89].

Very recently, the generation of XOR knockout mice has been reported [169]. Surprisingly, homozygous deletion of the *XOR* gene is incompatible with life, as $XOR^{-/-}$ mice are runted and die within 6 weeks of birth. Although the reason for this is unknown, the phenomenon is in apparent contrast with what is observed in xanthinuric patients. The results obtained in mice suggest either that humans have a greater potential to compensate for the XOR deficit or that there is a substantial difference in the metabolic pathway(s) controlled by the enzyme in rodents and humans. The observation that $XOR^{+/-}$ female animals have a deficit in lactogenesis is of particular interest [169]. In fact, it confirms the importance of XOR in the homoeostasis of the mammary gland. Furthermore, it suggests that XOR has a functional role in lactogenesis that is independent of its enzymic activity [169]. This adds unexpected complexity to the problem of XOR pathophysiology.

AOX1, AOH1 and AOH2: three enzymes looking for a substrate and a function

The pathophysiological significance of AOX1 and the related mouse enzymes AOH1 and AOH2 is even more obscure than for XOR. Single monogenic deficits have not been described for any of the three proteins. AOX1 is characterized by broad substrate specificity, and this makes it an important enzyme in the metabolism of drugs and xenobiotica [43–48]. However, the enzyme also potentially catalyses the oxidation of endogenous products involved in various metabolic pathways. AOX1 may participate in the metabolism of neurotransmitters, oxidizing the 5-hydroxytryptamine (serotonin) metabolite 5-hydroxyindoleacetaldehyde and the monoamine metabolite dihydroxymandelaldehyde into the corresponding acids (for metabolic pathways, see www.genome.ad.jp/kegg). In the tyrosine pathway, the enzyme is believed to compete with aryl aldehyde dehydrogenase (EC 1.2.1.29) for the oxidation of gentisate aldehyde into gentisate, the precursor of hydroquinone. In the degradation pathway of valine, leucine or isoleucine, it is possible that AOX1 biotransforms (*S*)-methylmalonate semialdehyde into methylmalonate, a precursor of succinyl-CoA. Finally, AOX1 may have a role in the degradation of vitamins such as nicotinamide and pyridoxal. In the first case, it is proposed to oxidizeN^1-methylnicotinamide into N^1-methyl-2-pyridone-5-carboxamide or N^1-methyl-4-pyridone-5-carboxamide; in the second case it may transform pyridoxal into 4-pyridoxate [170].

Another potential substrate of AOX1 of physiological importance is all-*trans*-retinaldehyde, the precursor of all-*trans*-retinoic acid, the active metabolite of vitamin A. AOX1 oxidizes retinal into retinoic acid [21], and we recently observed that the same is true for AOH1 (M. Terao and E. Garattini, unpublished work). Retinoic acid is a key regulator of the homoeostasis of keratinized epithelia and a recognized morphogen of the vertebrate organism [171]. In this context, it would be important to establish if not only AOX1 and AOH1, but also AOH2, is capable of oxidizing retinaldehyde, as this latter enzyme is expressed in keratinized epithelia, classic target tissues of vitamin A (M. Terao and E. Garattini, unpublished work).

Currently there is no solid evidence that AOX1 or any of its related enzymes plays a role in any pathological situation. AOX1 may play an indirect and potentially protective role in the hepatic damage caused by ethanol in alcoholics. In fact, the enzyme may detoxify the liver through the biotransformation of acetaldehyde into the corresponding acid. However, with regard to the significance of AOX1 in the metabolism of acetaldehyde, it should be emphasized that various aldehyde dehydrogenase isoforms have the potential to compete with hepatic AOX1 for this substrate. Finally, the location of AOX1 very close to a number of genetic markers co-segregating with the juvenile form of amyotrophic lateral sclerosis (ALS), a very rare form of motor neuron degeneration, originally suggested that the enzyme represents a biologically plausible candidate disease gene [83]. However, the real disease gene was recently isolated by positional cloning [172,173] and was shown to lie in close proximity to, but to be different from, *AOX1*.

CONCLUSION AND FUTURE PERSPECTIVES

As illustrated in the previous sections, significant progress has been made in the identification and structural characterization of the various members of the MFE family. However, information on the regulation and function of MFEs is still largely unsatisfactory. We know very little about the endogenous and exogenous stimuli regulating the activity of the various members of the MFE family. This is accompanied by an even greater lack of knowledge on the molecular determinants, such as transcription factors, that modulate the rate of expression of the MFE genes in the relevant tissues and cell types. A significant effort is needed to define the *cis*-regulatory elements of the various mammalian MFE genes. More importantly, we still have few clues about the physiological substrates of MFEs, and only a limited understanding of the function that these enzymes have in the mammalian organism. It is expected that progress in determination of the physiological relevance of MFEs will come from the generation of knock-out mice for members of the MFE family other than XOR. We are currently pursuing this avenue and have already generated homozygous knock-out mice for the AOH2 enzyme. In addition, we have identified two laboratory strains of mice with a relatively specific deficit in the expression of AOH1. Both lines of animals are viable and we are in the process of analysing any alterations in the phenotype (M. Terao and E. Garattini, unpublished work).

A final comment on the current nomenclature of MFEs is warranted. The recent identification of at least five distinct, but structurally very similar, members of the MFE family

calls for a revision of the nomenclature used in the scientific literature, as a certain confusion in the field is evident. The available biochemical, structural and genetic evidence indicates that human and mouse AOX1, AOH1, AOH2 and AOH3 are likely to represent tissue- and/or developmental-stage-specific variants of an enzymic activity traditionally referred to as AOX. The internationally accepted symbol for the genes encoding this type of enzyme is *AOX*, and the only functional human *AOX* gene is currently referred to as *AOX1* in the NCBI data bank. For this reason, we have chosen to refer to the orthologous mouse gene as *AOX1* throughout this review. Although the acronyms AOH1, AOH2 and AOH3, which we proposed and utilized for the three proteins in this and previous publications [10–14], are justified, they may be confusing. Here we propose that the symbols AOH1, AOH2 and AOH3 be substituted by AOX2, AOX3 and AOX4 respectively to define the different proteins, and that the notations in italics be used to define the corresponding genes. This type of nomenclature is likely to simplify the annotation process of the various mammalian genomes and lead to an unequivocal definition of the various genes and expressed sequence tags (ESTs) present in the public data banks.

This work was supported financially by grants from the Consiglio Nazionale delle Ricerche (Progetto Finalizzato Biotecnologie), Ministero Italiano dell'Università e della Ricerca (MIUR), the Associazione Italiana per la Ricerca contro il Cancro and the EEC Training Network Program (XONET). M.J.R. acknowledges financial support from FCT, project POCTI/35078/BME/2000. E.G. and M.T. are grateful to Dr Mami Kurosaki, Dr Monica Barzago and Dr Ruth Vila-Pont for their skilful help in collecting some of the data described.

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Received 15 January 2003/4 February 2003; accepted 10 February 2003
Published as BJ Immediate Publication 10 February 2003, DOI 10.1042/BJ20030121