Molecular cloning of the cDNA coding for mouse aldehyde oxidase: tissue distribution and regulation *in vivo* by testosterone

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The cDNA coding for mouse aldehyde oxidase (AO), a molybdoflavoprotein, has been isolated and characterized. The cDNA is 4347 nt long and consists of an open reading frame predicting a polypeptide of 1333 amino acid residues, with 5′ and 3′ untranslated regions of 13 and 335 nt respectively. The apparent molecular mass of the translation product *in vitro* derived from the corresponding cRNA is consistent with that of the monomeric subunit of the AO holoenzyme. The cDNA codes for a catalytically active form of AO, as demonstrated by transient transfection experiments conducted in the HC11 mouse mammary epithelial cell line. The deduced primary structure of the AO protein contains consensus sequences for two distinct 2Fe–2S redox centres and a molybdopterin-binding site. The amino acid sequence of the mouse AO has a high degree of similarity with the human and bovine counterparts, and a significant degree of relatedness to AO proteins of plant origin. Northern blot and *in situ* hybridization analyses demonstrate that hepatocytes, cardiocytes, lung endothelial or epithelial cells and oesophagus epithelial cells express high levels of AO mRNA. In the various tissues and organs considered, the level of AO mRNA expression is not strictly correlated with the amount of the corresponding protein, suggesting that the synthesis of the AO enzyme is under translational or post-translational control. In addition, we observed sex-related regulation of AO protein synthesis. In the liver of male animals, despite similar amounts of AO mRNA, the levels of the AO enzyme and corresponding polypeptide are significantly higher than those in female animals. Treatment of female mice with testosterone increases the amounts of AO mRNA and of the relative translation product to levels similar to those in male animals.

Key words: aldehyde oxidase, cDNA, mouse, xanthine oxidase.

INTRODUCTION

Aldehyde oxidase (AO; aldehyde oxygen oxidoreductase, EC 1.2.3.1) belongs to the small family of molybdoflavoproteins that includes xanthine oxidoreductase (XOR) and sulphite oxidase [1]. In its catalytically active form the protein has a molecular mass of approx. 300 kDa and consists of two identical monomeric subunits of 150 kDa each [2]. The enzyme is characterized by the presence of two 2Fe–2S redox centres, one flavin and one molybdopterin-binding site in each subunit [2]. This general structure is very similar to that of XOR, a protein with which AO shows a striking level of amino acid similarity [2,3] and a common genetic origin [4,5].

AO is a toxicologically important enzymic system metabolizing various classes of xenobiotics. Along with the microsomal cytochrome P450-dependent mono-oxygenases, AO is considered to be one of the principal drug-metabolizing enzymes in the liver. The protein oxidizes and inactivates cancer chemotherapeutic agents such as methotrexate and 6-methylthiopurine [6,7] and reduces N-oxides, nitrosamines, hydroxamic acids, azo dyes and nitropolycyclic aromatic hydrocarbons [8]. In addition, AO is implicated in the metabolism of ethanol and is purported to be responsible for alcohol-generated hepatotoxicity [9].

Despite the toxicological importance of the enzyme, the physiological function of AO has not yet been elucidated. In particular it is not clear whether the oxidase acts on any specific substrate of physiological importance, although AO has been shown to catalyse the bio-transformation of retinaldehyde into retinoic acid *in vitro* [10] and has been suggested to have a role in the catabolism of monoamines [11]. In the central nervous system it is possible that the protein has a specific role in the homoeostasis of motorneurons. In fact, in humans, the AO gene has been recently implicated in the aetio-pathogenesis of the recessive form of familial amyotrophic lateral sclerosis, a serious motorneuron disease [12], and, in mice, the AO transcript selectively localizes to this type of cell [13].

Definition of the tissues and cell types expressing AO, determination of the molecular mechanisms underlying the expression of the related gene and identification of the endogenous and exogenous factors controlling the levels of the enzyme *in vivo* will be helpful in clarifying the physiological function of this molybdoflavoprotein. Physiological, pharmacological and toxicological studies on AO are easier to perform in the experimental animal. As a first step in this direction, here we describe the molecular cloning of the mouse AO cDNA and the determination of its primary structure. With this cDNA as a probe, we determined the tissue and cell types expressing the gene. Furthermore, we observed sex-specific and androgenic hormone-dependent differences in the level of expression of the apoenzyme and the holoenzyme. Finally, we demonstrated that, under basal and testosterone-induced conditions, synthesis of the catalytically active form of AO is a complex phenomenon controlled predominantly at the translational level.

MATERIALS AND METHODS

Animals

Male and female CD1 mice weighing 18–20 g were obtained from Charles River Italia (Calco, Como, Italy) and maintained in the central animal house facilities of the Istituto 'Mario Negri'. Testosterone propionate (Sigma, St. Louis, MO, U.S.A.) was dissolved in sesame oil and administered subcutaneously to

Abbreviations used: AO, aldehyde oxidase; CMV, cytomegalovirus; XOR; xanthine oxidoreductase.

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6-week-old mice once a day for 11 days at a dosage of 50 mg/kg. At the end of the experiment, animals were killed and tissues were explanted. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 February 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 12 December 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Molecular cloning and sequencing of the cDNA encoding AO

On the basis of the nucleotide sequence data from human [14] and bovine [2] AO cDNA species, pairs of degenerate, normal or inosine-containing oligonucleotides [5′-CTG(G/C)AGTACAT-TAAAGTACCAG-3′ and 5′-TA(A/G)AA(A/G)TG(C/T)TC-(C/T)TGICCICC-3′, 5′-AGTTTCTGCCATTGGATCCAAC-CC-3′ and 5′-TGTCTTCTCCTCGTTCCAGAA-3′, 5′-TCTG-GAACGAGGAGAAGACA-3′ and 5′-GTGAACTTGTCTTC-ACAGGC-3′] were synthesized and used to amplify partial AO cDNA fragments from mouse liver poly(A)$^+$ RNA by reverse-transcriptase-mediated PCR. This resulted in the molecular cloning of mAO1 (13), mAO-PCR2,6 and mAO-PCR3,3, respectively. Amplifications by PCR were performed after reverse transcription with the gene AMP kit (Cetus Perkin Elmer, Norwalk, CT, U.S.A.) in accordance with the recommended protocol. The samples were subjected to 30–35 cycles of amplification (94 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min). The cDNA bands obtained after amplification by PCR were subcloned in pBluescript (Stratagene, La Jolla, CA, U.S.A.) by using the T/A subcloning method [15]. The clones mAO-PCR2,6 and mAO-PCR3,3 were labelled with [^{32}P]dCTP [specific radioactivity $(1–2)\times10^9$ c.p.m./μg; Amersham, Little Chalfont, Bucks., U.K.] and used to screen a random-primed mouse liver cDNA library (Clontech, Palo Alto, CA, U.S.A.). Screening of the library resulted in the isolation of two additional cDNA fragments (mAO-5P,3 and mAO-1A,8), which were subcloned into the *Eco*RI site of pBluescript. Hybridization and washing of the plaque lifts were performed in stringent conditions in accordance with standard protocols [16]. The 5′-most region of the cDNA was isolated by rapid amplification of cDNA ends (RACE) with a commercially available kit (Marathon kit; Clontech) with two oligonucleotides (first primer, 5′-CCACA-GCCATACTTAGTTCCTGTG-3′, complementary to nt 121–144 of the mouse AO cDNA; nested primer, 5′-CGGAGAT-TCTTCCTCAGGTATGGT-3′, complementary to nt 94–117 of the mouse AO cDNA) synthesized on the basis of the nucleotide sequence of clone mAO-5P,3. The PCR fragment was subcloned into the *Eco*RV site of pBluescript, resulting in the clone mAO-RACE6.

Mouse AO cDNA species were sequenced in both directions by the dideoxy-nucleotide chain-termination method [17] with double-stranded DNA as templates and T7 DNA polymerase (Pharmacia, Uppsala, Sweden) in accordance with the manufacturer's instructions, using either vector primers or specific oligonucleotides (17–22 nt long). Oligonucleotides were custom-synthesized by Gibco BRL (Grand Island, NY, U.S.A.). Computer analysis of the DNA sequences was performed with the GeneWorks software package (Intelligenetics, Mountain View, CA, U.S.A.).

Construction of the full-length mouse AO cDNA and translation *in vitro*

The full-length mouse AO cDNA was reconstructed in pBluescript with the following strategy. The 5′ and 3′ fragments were amplified from mouse liver poly(A)$^+$ RNA by reverse-transcriptase-mediated PCR, under the conditions described above, with the following pairs of oligonucleotides derived from the mouse AO cDNA sequence: 5′-ggcggccgCGGAGTCATGGAC-CCCATTCAGCT-3′, corresponding to nt 7–30 (the lower-case letters correspond to a *Not*I site added to the oligonucleotide to facilitate subcloning) and 5′-GCCATGTTCCTGATCTGGGA-3′, complementary to nt 1049–1068; as well as 5′-TGGGCCAT-CTTGTCTGTGCTGTGA-3′, corresponding to nt 2010–2033, and 5′-ggcggccgGCCAATTCCTCCAGAGGTTCATGT-3′, complementary to nt 4021–4044. The PCR-amplified 5′-fragment was cleaved with *Not*I and *Hin*dIII; the PCR-amplified 3′-fragment was cut with *Eco*RI and *Not*I. The two cleaved PCR products were inserted, in the presence of a *Hin*dIII–*Eco*RI fragment obtained from clone mAO-5P,3, into the *Not*I site of pBluescript. The sequence of the reconstructed full-length cDNA was confirmed in at least one direction. Translation of the mouse AO cDNA was performed *in vitro* with a commercially available kit (TnT Reticulocyte Lysate System; Promega, Madison, WI, U.S.A.) after transcription *in vitro* with T7 RNA polymerase and translation with reticulocyte lysates in the presence of [^{35}S]methionine in accordance with the manufacturer's instructions. Products translated *in vitro* were subjected to PAGE on 6.0 % (w/v) gels under denaturing conditions; radiolabelled bands were detected by autoradiography after fluorography with En^{3}Hance (New England Nuclear, Boston, MA, U.S.A.).

Transfection of the mouse AO cDNA

The full-length mouse AO cDNA was cloned into the *Not*I site of the eukaryotic expression vector pCMVβ (Clontech) after elimination of the β-galactosidase gene by cleavage with the same restriction enzyme. The plasmid was transfected by lipofection into the HC11 mouse mammary epithelial cell line by using Lipofectamine (Gibco BRL), in accordance with the manufacturer's instructions. At 48 h after transfection, cell extracts were analysed for the presence of AO protein and enzymic activity by Western blotting and by a spectrophotometric assay respectively, as described below. The HC11 cell line, a gift from Dr. Nancy Hynes (FMI, Basel, Switzerland), was routinely passaged in RPMI 1640 medium containing 10 % (v/v) fetal calf serum, 5 μg/ml insulin (Sigma) and 10 ng/ml epidermal growth factor (Sigma).

Preparation of the subcellular fractions

The cytosolic and mitochondrial fractions of mouse liver were obtained as described by Pastorino et al. [18]. In brief, liver homogenates were prepared by resuspending the freshly isolated tissue in homogenization buffer (50 mM potassium phosphate, pH 7.3 containing 250 mM sucrose, 0.1 mM EDTA, 0.1 mM PMSF, 2 μM leupeptin and 0.15 μM aprotinin) with a Potter–Elvehjem homogenizer. Homogenates were centrifuged at 2500 ***g*** for 10 min to eliminate nuclei and cell debris; the supernatant was centrifuged at 12000 ***g*** for 30 min to pellet the mitochondrial fraction. The cytosol was separated from the microsomal fraction by ultracentrifugation at 105000 ***g*** for 60 min. All operations were performed at 4 °C.

Northern blot analysis

Northern blot analysis was performed as described previously [2], with total or poly(A)$^+$ RNA and ^{32}P-labelled mAO-PCR2,6 cDNA as a probe. To normalize for the quantity of RNA loaded on the gel, blots were sequentially hybridized either with a synthetic oligonucleotide, 5′-ACGGTATCTGATCGTCTTCG-

AACC-3′, that recognizes 18 S ribosomal RNA [19], or with a cDNA fragment coding for glyceraldehyde-3-phosphate dehydrogenase [20].

Hybridization *in situ*

The mouse AO cDNA *Pst*I–*Hin*cII fragment (nt 1943–2182) was subcloned in pBluescript and used as a template for the synthesis of sense and anti-sense riboprobes, employing T3 and T7 RNA polymerases (Stratagene) in the presence of [α-^{35}S]thio-UTP (specific radioactivity 1200 Ci/mmol; Amersham). Template DNA species were degraded by DNase I (Pharmacia); the average length of the riboprobes was adjusted to approx. 150 nt by treatment with alkali [21]. Mouse tissues were fixed overnight in 4 % (w/v) paraformaldehyde, embedded in paraffin, sectioned to 5 μm thickness and mounted on chromalum-containing gelatin-coated slides.

The conditions for the pretreatment of slides, hybridization, washing and detection by the nuclear-track emulsion technique were precisely as described previously [22,23]. At the end of the *in situ* hybridization, tissue sections were stained with haematoxylin–eosin and photographed under the microscope.

Gel electrophoresis and Western blot analysis

SDS/PAGE was performed under reducing conditions. The anti-AO polyclonal antiserum was obtained from rabbits immunized with a highly purified preparation of bovine AO; the antiserum's characteristics and specificity were described previously [2]. The antibodies cross-react with mouse AO but not with mouse XOR. For Western blot analysis, freshly isolated mouse organs were homogenized in 10 vol. of 50 mM potassium phosphate buffer, pH 7.3, containing 0.1 mM EDTA, 0.1 mM PMSF, 2 μM leupeptin and 0.15 μM aprotinin. The homogenates were ultracentrifuged at 105000 ***g*** for 1 h The cytosolic supernatants were heated at 55 °C for 10 min and centrifuged briefly to eliminate the protein precipitate. The AO protein contained in the supernatant was precipitated by the addition of an equal volume of a saturated solution of $(NH_4)_2SO_4$, resuspended in SDS/PAGE buffer and subjected to Western blot analysis. After electrotransfer of proteins separated by SDS/PAGE [6.0 % (w/v) gel] on nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), membranes were incubated sequentially with a 1:300 dilution of the rabbit anti-AO antiserum and a 1:2500 dilution of goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Sigma) for 2 h at room temperature under standard conditions [2,22,23]. Specific AO bands were detected by autoradiography on X-Omat films (Kodak, Rochester, NY, U.S.A.), with a commercially available enhanced chemiluminescence kit (ECL®; Amersham) based on a peroxidase-specific chemiluminescent substrate.

Determination of AO enzymic activity

AO enzymic activity was determined by the spectrophotometric method of Taylor et al. [24] with the AO-specific substrate phenanthridine. The assay was performed under linear reaction conditions relative to substrate and protein contents. One unit of enzyme activity is defined as that producing 1 nmol of 6-phenanthridone/min. Proteins were measured by the Bradford method with a commercially available kit (Bio-Rad, Richmond, VI, U.S.A.).

RESULTS AND DISCUSSION

Molecular cloning and structural characterization of the mouse AO cDNA

AO is known to possess a primary structure very similar to that of XOR, another molybdoflavoprotein with which it shares substrate specificity [2]. To clone the mouse AO homologue, we designed degenerate oligonucleotides corresponding to a conserved region of the human [14] and bovine [2] cDNA species, showing a high level of divergence from the corresponding sequences in XOR cDNA species. The use of these oligonucleotides resulted in the amplification of a primary PCR product from mouse liver RNA [13] that was instrumental in the isolation of other overlapping cDNA species, representing incomplete fragments of a larger mRNA. The sequence of the full-length cDNA coding for mouse AO is illustrated in Figure 1.

Mouse AO is 4347 nt long and consists of a 13 nt 5′ untranslated region, a 3999 nt open reading frame coding for a 1333-residue polypeptide and a 3′ untranslated region of 335 nt. The assignment of the first coding amino acid to the first in-frame methionine residue is currently just presumptive, owing to the lack of N-terminal sequence data for the corresponding protein. However, the putative first ATG lies within a nucleotide context similar to that observed around the first methionine codon in many eukaryotic transcripts [25]. In addition, isolation of the relative mouse AO gene, determination of the transcription start site and sequencing of the genomic region corresponding to the entire 5′ untranslated portion of the transcript demonstrate the absence of any other upstream and in-frame ATG codons (M. T., unpublished work). The predicted translation product of the mouse AO cDNA presents all the features characteristically observed in AO and XOR proteins of various origins [2,14,26–37]. The coding region of the cDNA predicts a consensus sequence (residues 43–74) typical of 2Fe–2S redox centres of the ferredoxin type [38], which is contained in AO and XOR proteins of animal and plant origin. This is followed, at a short distance, by a sequence (residues 112–155) identical with that present in the second 2Fe–2S centre of bovine and human AO proteins [2,14] as well as in all the XOR proteins so far characterized [26–34]. Another salient feature of the mouse AO cDNA-encoded polypeptide is a fingerprint sequence typical of all molybdopterin-containing polypeptides (residues 801–835). Although mouse AO is a flavoprotein, a FAD-binding consensus sequence of the type described by Correll et al. [39] is not evident, similarly to what has already been observed in all AO and XOR proteins for which sequence data are available [2,14,26–37]. Unlike XOR proteins, for their catalytic activity AO proteins do not require NAD^+ as a cofactor [2]. Consistent with that and as already observed in the bovine AO cDNA [2], the translation product of mouse AO is devoid of an amino acid sequence like that reported to be responsible for the binding of NAD^+ in chicken XOR (FFTGYRKTIVKPE; single-letter amino acid codes) [40]. Because this sequence is well conserved in all XOR proteins, its absence from our mouse clone suggests that the encoded protein does not require NAD^+ for its catalytic activity and further indicates that the cDNA codes for a genuine AO protein.

The 3′ untranslated region of the mouse AO cDNA is relatively short and does not contain a canonical polyadenylation sequence (AATAAA). However, 20 and 32 nt upstream of the poly(A) tail there are two sequences (ATTAAA and TATAAA) that might be used as polyadenylation signals. Nevertheless, at present we cannot rule out the presence of other polyadenylation consensus sequences giving rise to AO transcripts with longer 3′ ends. A comparison of the 3′ untranslated region of mouse AO with the corresponding regions of human and bovine AO cDNA species

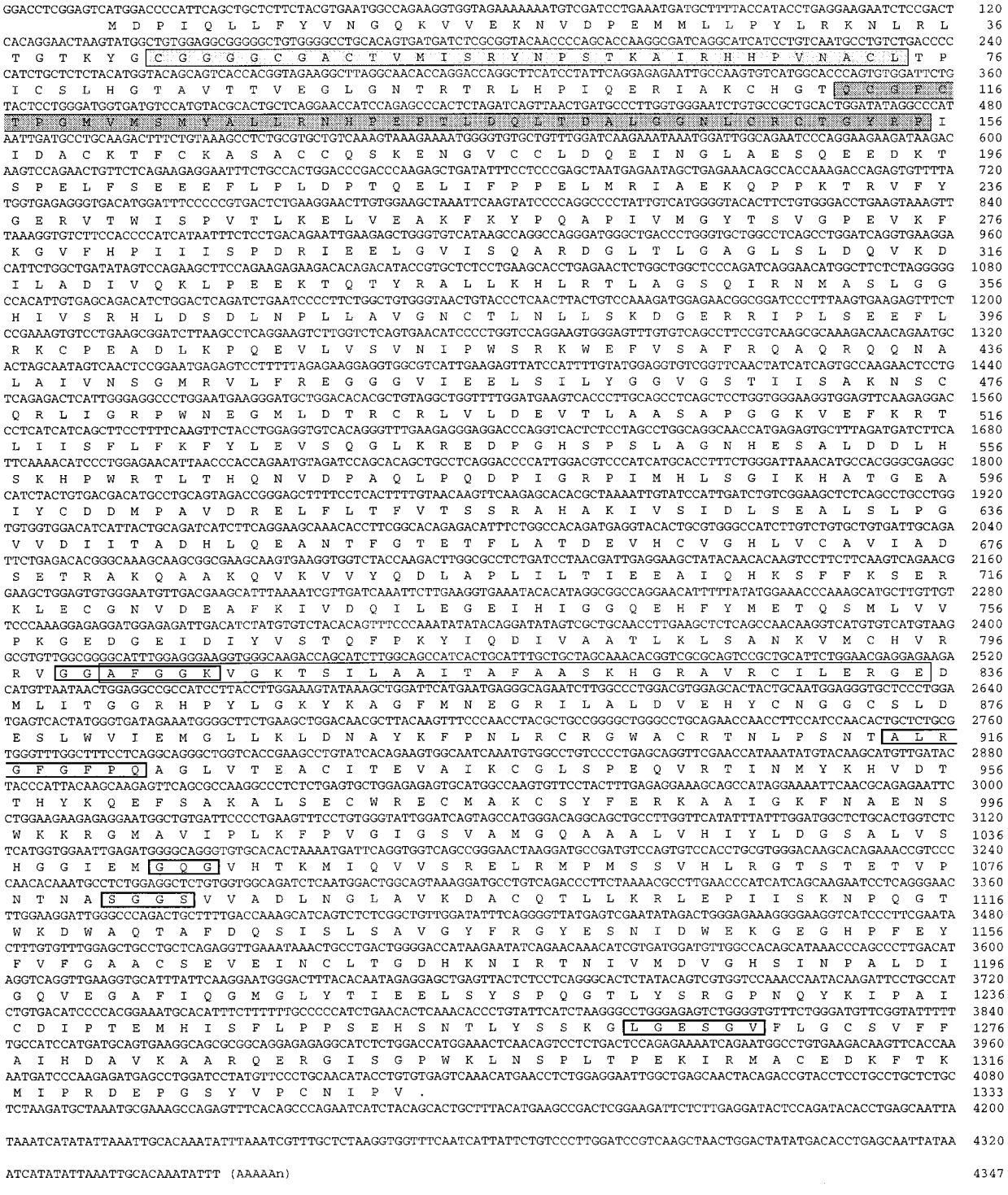

Figure 1 Nucleotide sequence and deduced amino acid sequence of mouse liver AO

The nucleotide sequence of mouse liver AO cDNA (upper line) was obtained from the overlapping cDNA fragments and is presented with its deduced amino acid sequence (lower line). Nucleotide residues are numbered in the 5′ to 3′ direction; amino acid residues are numbered from the N-terminus to the C-terminus starting from the putative first methionine residue. The sequences proposed to participate in the formation of the two non-identical iron–sulphur centres of other AO proteins are boxed (light grey, first 2Fe–2S redox centre; dark grey, second 2Fe–2S redox centre). Similarly, the molybdenum protein fingerprint sequence is indicated by an open box. The MoCoI, MocoII, MoCoIII, MoCoIV and MoCoV sequences (numbered from the N-terminus to the C-terminus), which correspond to the sequences involved in the binding of the molybdopterin cofactor and substrate in *Desulfovibrio gigas* aldehyde oxidoreductase [43] are marked by boxes with a thick line. The symbol (AAAAAn) indicates the polyadenylated tail of the transcript.

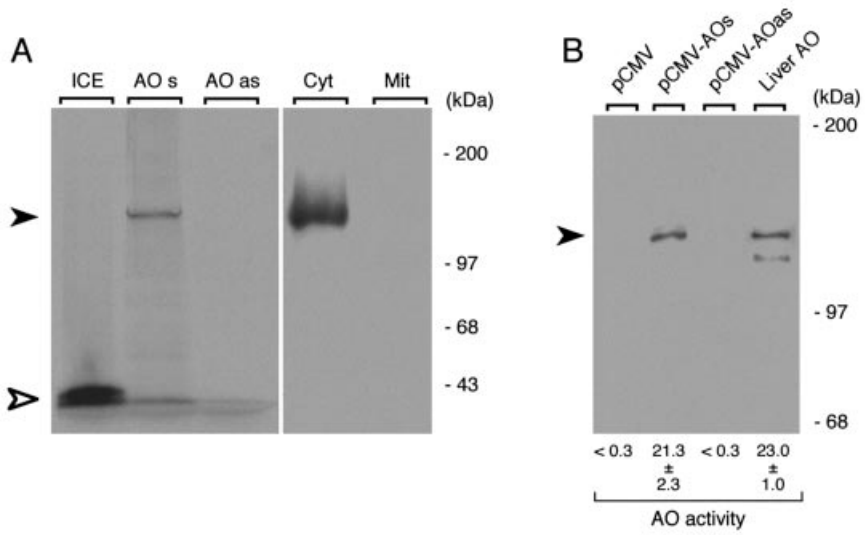

Figure 2 Translation of the mouse AO cDNA *in vitro* and subcellular distribution of the mouse liver AO protein

(**A**) The full-length mouse AO cDNA was transcribed *in vitro* in the sense (AOs) or the anti-sense (AOas) orientation with T3 or T7 RNA polymerase. The transcripts were subsequently translated with reticulocyte lysates in the presence of [^{35}S]methionine. As a positive control for the experiment, a full-length caspase-1 cDNA (ICE) (a gift from Dr. Marta Muzio, Istituto 'Mario Negri') was translated *in vitro* under the same experimental conditions. The radioactive protein products were analysed by fluorography and autoradiography after electrophoresis on 6% (w/v) polyacrylamide gels under denaturing and reducing conditions (left panel). A portion of the same gel used for the analysis of the products translated *in vitro* was loaded with 100 μg of proteins from mouse liver cytosolic (Cyt) and mitochondrial (Mit) extracts and subjected to Western blot analysis with a specific anti-AO polyclonal antibody (right panel). (**B**) HC11 mouse mammary epithelial cells were transiently transfected with the indicated plasmids (pCMV, plasmid vector; pCMV-AOs, plasmid vector containing the AO cDNA in the sense orientation relative to the CMV enhancer promoter; pCMV-AOas, plasmid vector containing the AO cDNA in the anti-sense orientation relative to the CMV enhancer promoter). At 48 h after transfection, cell extracts from three culture dishes were pooled and subjected to Western blot analysis as in (**A**). A portion of each cell extract was used for the measurement of AO enzymic activity (results are indicated at the bottom in units/mg of protein and are means $\pm$ S.D. for three culture dishes). A liver cytosolic extract was used as a positive control for the experiment (mean $\pm$ S.D. for three replicate determinations). The position of the AO protein is indicated by a solid arrow; that of the caspase-1 protein (43 kDa) is indicated by an open arrow. The positions of molecular mass markers are indicated at the right of each panel (myosin, 200 kDa; phosphorylase *b*, 97 kDa; BSA, 68 kDa; ovalbumin, 43 kDa).

demonstrates a significant degree of identity (64% and 62% respectively), suggesting a possible regulatory function conserved throughout the evolution of mammals.

Consistent with the size of the predicted polypeptide, translation *in vitro* of the transcript corresponding to mouse AO resulted in the synthesis of a polypeptide migrating with an apparent molecular mass of approx. 150 kDa on SDS/PAGE (Figure 2A). In contrast, incubation of the anti-sense transcript with reticulocyte lysates did not give rise to any detectable protein band. The apparent molecular mass of the mouse AO translation product is in line with the molecular mass of the monomeric subunit of other mammalian AO proteins [2,14]. In addition, the length of the translated polypeptide is the same as that of the protein band specifically highlighted in cytosolic extracts obtained from liver (Figure 2A) and other tissues by a polyclonal antibody recognizing murine AO on Western blot analysis (see Figure 6). In spite of the reported presence of AO enzymic activity in guinea-pig liver mitochondria [41], we did not observe significant amounts of AO protein (Figure 2A) or enzymic activity (results not shown) in this subcellular fraction. With respect to this, in mouse, the AO enzyme is selectively localized in the cytosol, because no other intracellular organelles (nuclei, microsomes and Golgi apparatus) besides mitochondria contained detectable levels of the protein on Western blot analysis (results not shown).

To test for the ability of the mouse AO cDNA to programme the synthesis of a catalytically active form of the AO enzyme *in vivo*, we constructed plasmids containing the cDNA in the sense (pCMV-AOs) and anti-sense (pCMV-AOas) orientations relative to the cytomegalovirus (CMV) enhancer–promoter. As expected from the translation experiments *in vitro*, transfection of pCMV-AOs into the HC11 mouse mammary epithelial cell line (Figure 2B) resulted in the synthesis of a polypeptide whose apparent molecular mass was the same as that of the AO monomeric subunit in liver extracts (the approx. 130 kDa band observed in liver extracts corresponds to an AO-derived degradation product frequently observed in liver extracts; see also Figure 6). In contrast, transfection of the cell line with pCMV or pCMV-AOas was not associated with detectable levels of the AO polypeptide on Western blot analysis. Consistent with these data, measurable amounts of AO enzymic activity were observed in the cytosolic extracts of pCMV-AOs-transfected HC11 cells, whereas the levels of the enzyme were below the limits of detection in cells transfected with pCMV or pCMV-AOas (Figure 2B). These results demonstrate that the mouse AO cDNA codes for a catalytically active form of the AO enzyme.

Mouse AO can easily be aligned with other AO proteins of animal and plant origin as well as with all the XOR proteins so far sequenced (results not shown). The mouse AO translation product is most similar to human and bovine AO proteins (83% and 81% identity respectively), further indicating that the protein is the murine AO homologue. Interestingly, this analysis indicates that the mouse and the two other mammalian AO proteins are more closely related to XOR proteins (51% identity with mouse XOR) of different origins than to plant AO proteins (28% identity with *Zea mais* AO1 and tomato AO). Thus, although mammalian and plant AO proteins are structurally related and catalyse similar biochemical reactions, they might have evolved independently and are likely to have different physiological significances. The mouse AO polypeptide is five and six residues

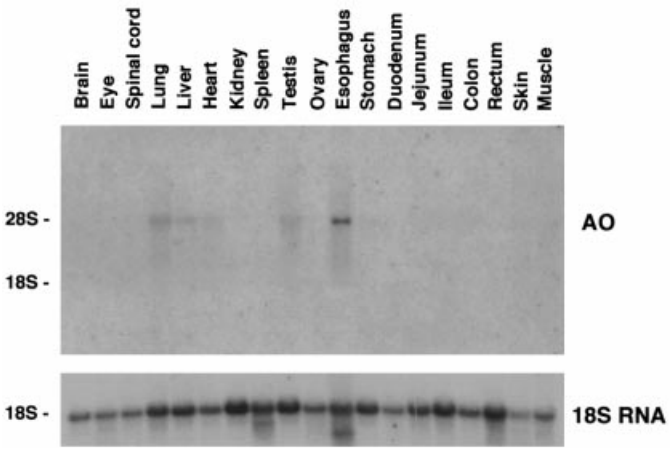

Figure 3 Tissue distribution of AO mRNA

Total RNA was obtained from the indicated tissues. RNA (30 μg per lane) was loaded on a formaldehyde/1% (w/v) agarose gel and subjected to Northern blot analysis. After transfer, nylon membranes were hybridized with the mouse AO cDNA fragment mAO-PCR2,6. The positions of 28 S and 18 S rRNA species are shown at the left. The same blot was hybridized sequentially with an oligonucleotide (5′-ACGGTATCTGATCGTCTTCGAACC-3′) recognizing 18 S rRNA (bottom panel) to illustrate the amount of RNA loaded in each lane.

shorter than human and bovine AO respectively. This difference in length is due to a short deletion from a region (residues 651–652 of mouse AO) where the three proteins show a low degree of conservation. Alignment of mouse AO with the human and bovine counterparts demonstrates the presence of three relatively conserved regions separated by two sequence blocks of much lower similarity. The limits of the three regions roughly correspond to the boundaries of the 20 kDa N-terminal domain, the 40 kDa central domain and the 85 kDa C-terminal domain, which can be obtained by trypsin cleavage of the catalytically active XOR [42] and AO (M. Terao, unpublished work) proteins. The similarity of mouse AO to all the other AO proteins is very marked towards the N-terminus. In the domain corresponding to the first 2Fe–2S centre, the four cysteine residues involved in the co-ordination of the metal ion (Cys^{43}, Cys^{48}, Cys^{51} and Cys^{73}) are strictly conserved in all the AO proteins with the notable exception of the enzyme from tomato [33]. The sequence of the second 2Fe–2S centre in mouse AO is almost completely identical with that of the human and bovine counterparts, whereas in its central portion it differs markedly from that of plant AO proteins. In this region, the protein alignment indicates the presence of two putative subdomains consisting of highly conserved sequences (QCGFCTPG and GNLCRCTGYRPI), which contain the four iron-co-ordinating cysteine residues (Cys^{113}, Cys^{116}, Cys^{148} and Cys^{150}) and are separated by a hinge region of variable length and amino acid composition. In spite of the fact that most of the amino acid residues in the two 2Fe–2S redox centres that are identical in AO proteins are also identical in XOR proteins, Tyr^{58}, Pro^{60}, Leu^{74} and Thr^{150} are typical of AO. This demonstrates that the general structures of the two centres in AO and XOR are similar, although there are subtle structural differences that might have relevance for the different catalytic activities of the two types of protein. A high level of amino acid identity between mouse and other AO proteins is also observed in the C-terminal moiety (approx. 85 kDa), where the molybdenum cofactor and the substrate binding sites are located. Complete adherence to the consensus sequences corresponding to the five domains (MoCoI–MoCoV) involved in the binding of the molybdenum cofactor and substrate identified in the crystals of *Desulfovibrio gigas* aldehyde oxidoreductase [43] is observed in all the AO proteins. Most of the identical residues observed in AO proteins are shared by XOR proteins, with the notable exceptions of Gln^{1044} in the MoCoIII, and Gly^{1083} and Ser^{1084} in the MoCoIV, of mouse AO. The flavin-containing 40 kDa central region of mouse AO is the least conserved portion of the protein. In this region, the longest stretch of amino acids that is identical in all AO polypeptides is between residues 592 and 603, in which 8 out of 12 residues are strictly conserved. All these amino acid residues are also identical in XOR proteins, which suggests the possibility that the residues have a role in binding the flavin cofactor.

Tissue and cell distribution of mouse AO mRNA and protein

As shown in Figure 3, in the male animal, the transcript coding for AO is expressed in only a few mouse tissues, as assessed by Northern blot analysis with total RNA. A single mRNA band with an apparent size consistent with that of the corresponding cDNA is expressed at detectable levels in the lung, liver, heart and testis. Lower levels of the transcript (visible only after longer exposure of the autoradiogram) are detectable in the brain, spinal cord, spleen and eye. The transcript is not measurable by this technique in the stomach, small or large intestine, skin or striated muscle. The highest amounts of AO mRNA are evident in the oesophagus. The results demonstrate that the tissue distribution of AO in mouse is slightly different from that observed in bovine species [2]. Furthermore, the tissue-specific expression and the abundance of mouse AO is markedly different from that of the closely related molybdoflavoprotein XOR [22,28]. In fact, the mouse XOR mRNA is expressed at very high levels in the first tract of the small intestine (duodenum and jejunum) and in the liver, and at lower levels in many other tissues [22]. This suggests that, despite overlapping substrate specificity [1], similar structure [3] and common genetic origin [4,5,44], AO and XOR have substantially different tissue- or cell-specific physiological functions.

To gain insight into the types of cell expressing AO mRNA in the mouse, we performed *in situ* hybridization experiments on tissue sections, with a ^{35}S-labelled mouse anti-sense AO cRNA as a probe. As shown in Figure 4(b), a uniform accumulation of grains is observed along the septa of the lung alveoli. Owing to the scattering of the radioactive signal, it is at present difficult to establish whether the endothelial or the epithelial component of the septa is responsible for the synthesis of AO mRNA. The AO transcript is also observed in the epithelial cells lining the bronchi and bronchioli (results not shown). Similarly, most hepatocytes are uniformly capable of expressing the AO transcript, as assessed by the quantity of silver grains accumulated in tissue sections at the level of this cell type (Figure 4d). This is different from what was observed for XOR, where only a small subset of hepatocytes are capable of expressing the enzyme [22]. In the heart ventricles, accumulation of silver grains is observed mainly on cardiocytes (Figure 4f). In the oesophagus, the hybridization signal is predominantly at the level of the multilayered epithelial component of the mucosa (Figures 5c and 5d). The *in situ* hybridization signal is specific, because hybridization of adjacent sections with a sense AO cRNA probe does not result in a significant accumulation of silver grains in any of the cell types contained in the organs and tissues considered (Figures 4a, 4c, 4e, 5a and 5b). The specificity of the observed *in situ* hybridization signals is further supported by the lack of silver grain accumulation, after challenge with the anti-sense AO cRNA probe, in sections

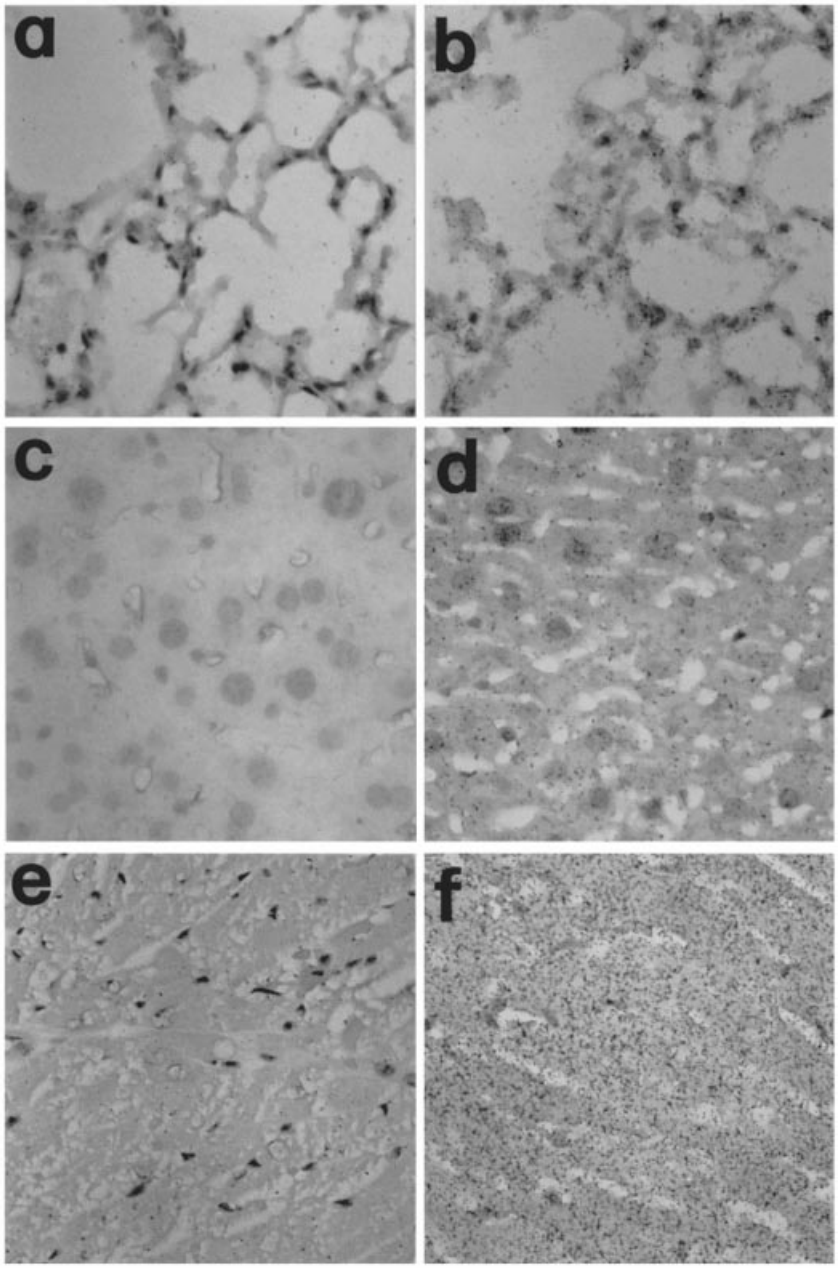

Figure 4 Localization of the mouse AO mRNA transcript in lung, liver and heart by *in situ* hybridization

Mouse tissue sections were prepared from lung (**a**, **b**), liver (**c**, **d**) and heart (**e**, **f**), and hybridized with sense (**a**, **c**, **e**) or anti-sense (**b**, **d**, **f**) mouse AO cRNA. Magnification $\times 400$.

obtained from two tissues that do not express the AO transcript, such as the stomach and the rectum (results not shown). The cell localization results for liver, heart and oesophagus were reproduced in separate *in situ* hybridization experiments with the use of a different AO cRNA probe.

The measurement of AO enzymic activity in all the tissues studied by Northern blotting experiments demonstrated that liver, lung, brain and spinal cord are the only organs in which detectable amounts of the enzyme are present (Figure 6, upper panel, and results not shown). This might be due partly to the relatively low sensitivity of the enzymic assay used, and we cannot rule out the presence of trace amounts of AO activity in other organs and tissues. However, a comparison of Figures 3 and 6 (upper panel; see columns corresponding to male animals) indicates that there is no close correlation between the levels of AO mRNA and enzymic activity in the tissues considered. In fact, the oesophagus, which contains the highest levels of the mouse AO transcript, does not show detectable amounts of the corresponding enzymic activity. Furthermore, liver and lung, which express approximately similar quantities of the mRNA, show significant differences in the levels of the catalytically active AO enzyme (the approx. 120 kDa band observed in the Western blot lanes corresponding to lung is due to a non-specific interaction between the anti-AO antibody and a protein of

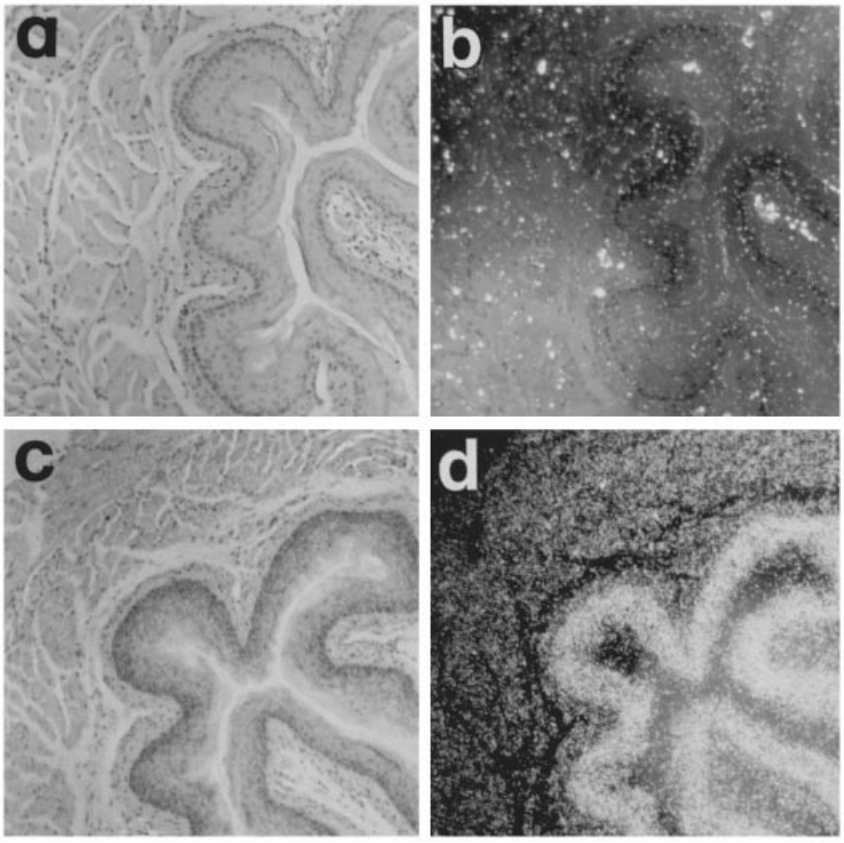

Figure 5 Localization of the mouse AO mRNA transcript in the oesophagus by *in situ* hybridization

Oesophagus was sectioned transversely and the tissue slices were hybridized with sense (**a**, **b**) or anti-sense (**c**, **d**) mouse AO cRNA. Light-field (**a**, **c**) and dark-field (**b**, **d**) micrographs are presented. Magnification $\times 100$.

unknown nature present in lung extracts at high levels). Finally, the amounts of AO activity in brain are much higher than would be expected on the basis of the level of AO mRNA accumulation. Because similar results were obtained in separate experiments on different batches of animals, our results indicate that the synthesis of the holoenzymic and catalytically active form of mouse AO in various tissues is not simply regulated by the steady-state levels of the corresponding transcript. This would not be unprecedented for molybdoflavoproteins, because the tissue-specific expression of mouse XOR is also a complex process under the influence of translational and post-translational events [22]. To gain an insight into the molecular mechanisms responsible for the discrepancies between the levels of AO mRNA and AO enzymic activity, we determined the amounts of AO protein in various tissues by Western blot analysis (Figure 6, lower panel; see columns corresponding to male animals). In all the tissues tested, the levels of AO immunoreactive protein are well correlated with AO enzymic activity. This indicates that the translation of the AO mRNA into the apoprotein in lung, liver, brain and spinal cord is followed by the assembly of the holoenzyme and that this last process is not rate-limiting. Thus, in organs such as the heart and the oesophagus, which contain significant amounts of AO mRNA, a lack of detectable enzymic activity is likely to be the result of a translational repression effect, although we cannot formally exclude the possibility that the observed effect might also be due to rapid and tissue-specific degradation of the AO protein.

Sex-regulated synthesis of the mouse AO in liver: effects of testosterone

All the experiments on the tissue-specific distribution of mouse AO mRNA and proteins described above were performed on male animals. However, Yoshihara and Tatsumi [8] reported gender-specific differences in the levels of hepatic AO enzymic activity. As shown in Figure 6 (upper panel), we confirmed this observation in our outbred mouse strain, demonstrating that the male liver has a content of AO enzyme that is approx. 7-fold higher than that in the female. This prompted us to evaluate other tissues for this phenomenon. The lung, brain and spinal cord of male and female animals show approximately the same levels of AO enzymic activity, which demonstrates that the gender-specific regulation of the enzyme might be a characteristic of the hepatic tissue. As shown in Figure 6 (lower panel), the amounts of AO enzymic activity are correlated with the levels of the AO immunoreactive protein. This indicates that, in the hepatic tissue, there are no sex-specific differences in the assembly of the AO holoenzyme from the apoprotein.

To get a further insight into the molecular mechanisms and the endogenous factors regulating the synthesis of the catalytically active hepatic enzyme in male and female mice, we measured the amounts of AO mRNA, immunoreactive protein and enzymic activity in animals treated with testosterone propionate or vehicle alone. The results of this experiment are summarized quantitatively in Table 1. Surprisingly, a Northern blot analysis

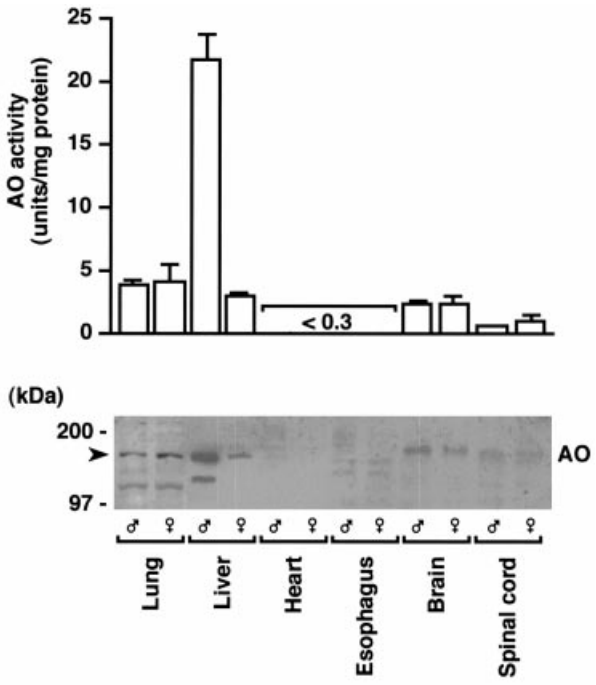

Figure 6 Tissue distribution of mouse AO enzymic activity and protein

Upper panel: AO enzymic activity was measured in portions of protein extracts obtained from the indicated tissues derived from three animals. Results are means ± S.D. The notation < 0.3 indicates that activity was below the limit of detection. Lower panel: the rest of the protein extracts were pooled and used (100 μg for each tissue sample) in a Western blot analysis. Immunoreactive bands were revealed with the use of a chemiluminescent substrate after incubation with a secondary antibody linked to horseradish peroxidase. The positions of the molecular mass markers are indicated at the left (myosin, 200 kDa; phosphorylase *b*, 97 kDa).

performed on the poly(A)$^+$ fraction of liver RNA demonstrated that the basal levels of the mouse AO transcript are similar in male and female animals after the administration of vehicle alone. Whereas the chronic administration of testosterone in male mice does not significantly affect steady-state levels of AO mRNA, treatment of female animals with the steroid results in an approx. 3-fold increase in the amounts of the transcript. As expected on the basis of the results shown in Figure 6, under basal conditions, female mice synthesize much smaller amounts of AO protein than male animals, confirming that translation of the corresponding transcript is inhibited or degradation of the polypeptide is enhanced. Treatment of male animals with testosterone has only marginal effects on the synthesis of the AO polypeptide. In contrast, the hormone causes a 9-fold induction of the protein in female livers. Measurement of AO enzymic activity, with phenanthridine as a specific substrate, shows a tight correlation with basal and testosterone-induced synthesis of the corresponding protein. In fact, male mice demonstrate approx. 8-fold the AO enzymic activity of female animals. Furthermore, testosterone selectively induces the AO enzyme in female mice approx. 9-fold.

Because AO mRNA steady-state levels are similar in the livers of male and female animals, whereas the amounts of the corresponding protein and enzymic activity are different, the sex-specific regulation of AO must be a complex and multifactorial process. In particular, the basal level of AO gene expression in both male and female mice is either independent of the sex-hormone environment or is controlled by distinct factors or hormones in the two genders. We favour the second possibility and suggest that, whereas testosterone controls the basal levels of the AO mRNA in males, females must synthesize one or more regulatory factors that compensate for the absence of the androgenic hormone and maintain high levels of the AO transcript in basal conditions. This is supported by the observation that female animals, but not males, maintain the potential to respond to testosterone with an increase in the amounts of AO mRNA. In addition, it is likely that testosterone (or other derived androgenic hormones) has an important role in controlling the amounts of the catalytically active form of AO by a translational or post-translational mechanism. In male animals the androgenic complement of hormones maintains high basal levels of the AO holoenzyme by facilitating the translation of the AO mRNA into the corresponding apoprotein or by stabilizing the synthesized polypeptide. The addition of testosterone to a female hormone environment is sufficient to activate translation (or inhibit degradation) and bring AO enzymic activity to the levels observed in males. It remains to be established whether the effect of testosterone on the expression of the AO gene and on the synthesis of the corresponding protein is a direct or rather an indirect effect, as suggested recently by Yoshihara and Tatsumi [45]. With this aim, we are currently performing experiments in castrated mice, which show a female AO expression pattern, with low levels of protein and enzymic activity (M. T., unpublished work).

As regards the biological relevance of the sex-dependent differences in AO enzymic activity, it has yet to be demonstrated

Table 1 Levels of AO mRNA, immunoreactive protein and enzymic activity in livers after treatment with testosterone

Male and female mice (three animals per experimental group) were treated with testosterone propionate (50 mg/kg) for 11 days by subcutaneous injection. Liver was isolated from a single animal and divided into halves. Poly(A)$^+$ RNA from the the first half of the organ (20 μg of RNA per lane) was used for Northern blot analysis. The filter was sequentially hybridized with a ^{32}P-labelled mouse AO cDNA probe and a glyceraldehyde-3-phosphate cDNA. Protein extracts from the second half of the liver were used for the AO enzymic assay or loaded (100 μg per lane) on an SDS/6% (w/v) polyacrylamide gel and subjected to Western blot analysis with a specific anti-AO polyclonal antibody. Immunoreactive bands were revealed with the use of a chemiluminescent substrate after incubation with a secondary antibody linked to horseradish peroxidase. AO enzymic activity was determined on an aliquot of the protein extract used for the Western blot. AO mRNA levels were calculated from the densitometric analysis of the Northern blot and normalized for the intensity of the glyceraldehyde-3-phosphate signal in each sample. AO protein levels were calculated from the densitometric analysis of the Western blotting experiment. Each experimental value is the mean ± S.D. for three separate animals. For RNA and protein levels, results are expressed relative to the value observed in vehicle-treated male animals, which is taken as 1. *Significantly different ($P < 0.01$) from the corresponding value in vehicle, measured by Student's *t* test.

Sex	Addition to vehicle	Relative mRNA content	Relative protein content	Enzymic activity (units/mg of protein)
Male	None	1.0 ± 0.2	1.0 ± 0.3	23.4 ± 1.4
	Testosterone propionate	0.7 ± 0.1	1.4 ± 0.3	26.0 ± 0.4
Female	None	0.8 ± 0.2	0.1 ± 0.06*	2.8 ± 1.6*
	Testosterone propionate	2.9 ± 0.5*	0.9 ± 0.2*	26.4 ± 0.9*

whether the phenomenon is a peculiarity of the mouse or is also observed in humans and other animal species. Nevertheless, this gender-dependent difference might have important implications for metabolism of xenobiotics and might explain sex-related differences in the therapeutic or toxic effects of certain types of drug [46]. In addition, the androgen-dependent regulation of the enzyme suggests the possibility that AO is involved in the metabolism of steroidogenic hormones.

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