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Purification, cDNA Cloning, and Tissue Distribution of Bovine Liver Aldehyde Oxidase*

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Aldehyde oxidase was purified to homogeneity from bovine liver and primary structural information obtained by sequencing a series of cleavage peptides permitted the cloning of the corresponding cDNA. The cDNA is 4,630 base pairs long, and it consists of a 102-base pair 5′-untranslated region followed by a 4017-base pair coding region and a 511-base pair 3′-untranslated region. The open reading frame predicts a 1339-amino acid polypeptide with a calculated molecular weight of 147,441, which is consistent with the size of the aldehyde oxidase monomeric subunit. The aldehyde oxidase polypeptide contains consensus sequences for iron-sulfur centers and a molybdopterin binding site. The amino acid sequence deduced from the cDNA shows significant similarity with that of xanthine dehydrogenases from various sources. The primary structure of bovine aldehyde oxidase is remarkably similar (approximately 86%) to that of the translation product of a cDNA recently isolated by Wright *et al.* (Wright, R. M., Vaitaitis, G. M., Wilson, C. M., Repine, T. B., Terada, L. S., and Repine, J. E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 10690–10694) and reported to represent human xanthine dehydrogenase. With the help of a monospecific antibody raised against the purified protein and the isolated cDNA, the tissue distribution of the bovine aldehyde oxidase protein and corresponding mRNA was determined. Aldehyde oxidase is expressed at high levels in liver, lung, and spleen, and, at a much lower level, in many other organs.

Aldehyde oxidase (aldehyde-oxygen oxidoreductase; EC 1.2.3.1; AO)[1] catalyzes the oxidation of endogenous and exogenous *N*-heterocyclic compounds in the presence of O_2 (1). The enzyme belongs to the family of molybdenum-containing protein like xanthine oxidoreductase (XD) and sulfite oxidase (2), and it represents a short oxidoreductive chain characterized by four oxidation centers, *i.e.* two iron-sulfur clusters, a flavin cofactor, and a molybdopterin cofactor (3). The structure of the molybdopterin cofactor in AO is the same as that of XD and sulfite oxidase, and it is known to be different from that present in other bacterial molybdoproteins (4, 5). Although the primary structure of AO has not yet been elucidated, it is known that AO is a homodimer, consisting of two identical subunits of 150 kDa each (6). AO is expressed in the liver of various animal species (6, 7), although the tissue and cell distribution of the protein has not been studied in detail. The oxidase is considered to be a cytosolic protein; however, in guinea pig, a recent report demonstrates the presence of small amounts of AO in mitochondria (8).

AO plays an important role in the metabolism of xenobiotics, since it is involved in the reduction of *N*-oxides, nitrosamines, hydroxamic acids, azo dyes, nitropolycyclic aromatic hydrocarbons (6), and sulfoxides (9). Furthermore, the enzyme mediates the oxidative metabolism of certain cancer chemotherapeutic agents such as methotrexate (10) and 6-methylthiopurine (11). In spite of its toxicological and pharmacological relevance, it is not known whether AO acts on any endogenous substrate, and the physiological function of the enzyme is still obscure. Recently, AO has been suggested to play an important role in the visual processes, since the enzyme catalyzes the biotransformation of the principal component of the visual pigments, retinaldehyde, to retinoic acid, the active form of vitamin A (12). Since retinoic acid has pleiotropic effects on various cell types (13), it is possible that AO participates in many other physiologically significant processes. Although a monogenic deficiency in human AO is not known, the enzymatic activity is undetectable in the very rare hereditary disease known as combined deficiency of molybdoproteins (14). The pathological condition is characterized by a deficit in the synthesis of the molybdenum cofactor that prevents the assembly of the apoforms of AO and the other molybdoflavoproteins into the respective holo-enzymes (14). The deficit leads to impairment in the development of the central nervous system, which is accompanied by severe neurologic symptoms (15). This suggests a possible role for AO in the development or the homeostasis of the brain.

In this report, we describe the purification of bovine liver AO, the sequencing of peptides obtained by proteolytic cleavage of the purified protein, and the molecular cloning of the respective cDNA. The deduced primary structure of AO shows a high degree of similarity between this protein and XDs isolated from various animal species. A cDNA probe and a monospecific antibody raised against the purified enzyme were used to study the tissue distribution of bovine AO mRNA and protein.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X87251.

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[1] The abbreviations used are: AO, aldehyde oxidase; XD, xanthine oxidoreductase; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; RACE, rapid amplification of cDNA ends; aa, amino acid(s).

MATERIALS AND METHODS

Determination of AO and XD Enzymatic Activity—AO enzymatic activity was determined by the spectrophotometric method outlined by Taylor *et al*. (16), which monitors the increase in absorbance at 322 nm caused by the production of 6-phenantridone from phenantridine in aerobic conditions at 25 °C (17). One unit of enzyme activity was defined as 1 nmol of 6-phenantridone produced/min. Total XD activity was measured by the method of Stirpe and Della Corte (18), as modified by Carpani *et al*. (19). One unit of XD enzyme activity was defined as 1 nmol of uric acid produced/min in aerobic conditions and in the presence of NAD^+ as a cofactor. Proteins were measured according to the Bradford method using a commercially available kit (Bio-Rad).

Purification of Bovine Liver AO and Sequencing of Proteolytic Peptides—Freshly isolated bovine liver (300 g) obtained from a local abattoir was homogenized in 600 ml of 0.15 M KCl containing 0.1 mM EDTA, then centrifuged at 15,000 × *g* for 60 min at 4 °C. AO was precipitated from the resulting supernatant by addition of ammonium sulfate to 40% saturation. The protein pellet was resuspended in 200 ml of 50 mM Tris/HCl, pH 7.4, containing 0.1 mM EDTA. Following overnight dialysis against 5 liters of the Tris buffer, the AO-containing solution was heated at 60 °C for 10 min. The clear supernatant, obtained following centrifugation at 15,000 × *g* for 20 min, was chromatographed on a Fast-Q anion exchange column (2.5 × 35 cm) (Pharmacia Biotech Inc.), equilibrated in 50 mM Tris/HCl, pH 7.4, and run at a flow rate of 2 ml/min. AO activity was recovered in six fractions (total volume of 18 ml) following elution with a linear gradient of NaCl (0–350 mM) superimposed to the equilibration buffer. Fractions containing the active enzyme were pooled and concentrated by precipitation following addition of ammonium sulfate to 80% saturation. The AO-containing pellet was resuspended in 5 ml of 100 mM glycine buffer, pH 9.0, containing 100 mM NaCl and loaded on a Sephacryl S-300 gel permeation column (2.5 × 90 cm) (Pharmacia) equilibrated in the glycine buffer and run at a flow rate of 2 ml/min. The enzyme with an apparent molecular mass of 300 kDa was eluted in 14 fractions of 3 ml each. Active fractions were pooled and loaded on a benzamidine-Sepharose 6B column (1.5 × 15 cm) (Pharmacia) equilibrated in glycine buffer. Following extensive washing with equilibration buffer, AO was eluted (in three fractions of 3 ml each) with 6 mM benzamidine in glycine buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions according to standard procedure (20). Proteins were stained with silver nitrate using a commercially available kit (Bio-Rad).

The highly purified AO preparation (2.5 mg) obtained following chromatography on benzamidine-Sepharose 6B (Pharmacia) was subjected to buffer exchange into 0.1 mM $CaCl_2$, 100 mM ammonium bicarbonate, pH 8.0, by three rounds of concentration and dilution using a Centricon 30 apparatus (Amicon, Danvers, MA). β-Mercaptoethanol (2.5 μl) was added to 150 μg of AO in 50 μl of buffer. The solution was bubbled with N_2 and incubated for 18 h at room temperature. Following elimination of the reducing agent by three rounds of concentration and dilution, AO was cleaved with either 1 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated bovine pancreas trypsin (Sigma) at 37 °C for 18 h or with 500 μg of cyanogen bromide (Sigma) at room temperature for 24 h. Tryptic or cyanogen bromide peptides were separated by one or two passages on a PEP-RPC FPLC column (Pharmacia) using appropriate linear gradients of acetonitrile in 0.1% trifluoroacetic acid. Six peptides were subjected to amino-terminal sequencing by the automated Edman degradation using a Milligen model 6625 automated protein sequencer (Milligen, Bedford, MA).

Preparation of the Anti-AO Polyclonal Antibody and Western Blot Analysis—The anti-AO polyclonal antibody was obtained from rabbits immunized with the same highly purified preparation of bovine AO shown in Fig. 1, following injection of the antigen into the popliteal lymph nodes of the animals. For Western blot analysis, freshly isolated bovine organs were homogenized in 10 volumes of 50 mM Tris/HCl buffer containing EDTA (0.1 mM), phenylmethylsulfonyl fluoride (0.1 mM), leupeptin (1 mM), and aprotinin (0.15 μM) and the homogenates were ultracentrifuged at 100,000 × *g* for 1 h. Cytosolic supernatants were heated at 60 °C for 10 min, briefly centrifuged to eliminate the protein precipitate, resuspended in SDS-PAGE buffer, and subjected to Western blot analysis (21). In the case of samples used for the determination of anti-AO antibody specificity, Western blot analysis was also performed on fractions obtained from Mono-Q chromatography (Pharmacia). Electro-transfer onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) was as already described (8). Membranes were sequentially incubated with a 1–3,000 dilution of the rabbit anti-AO antiserum followed by a 1–3,000 dilution of goat anti-rabbit IgG secondary antibody linked to horse-radish peroxidase (Bio-Rad) in standard conditions (20). AO specific bands were visualized by autoradiography on X-Omat films (Eastman Kodak Co.), using a commercially available kit (ECL, Amersham, Little Chalfont, United Kingdom).

Molecular Cloning and Sequencing of the cDNA Encoding AO and Northern Blot Analysis—On the basis of the sequence data obtained from the AO tryptic and cyanogen bromide-generated peptides, the following oligonucleotide mixtures, containing inosines (I) in positions where a degeneracy of 4 nucleotides is possible, were synthesized.

1: 5′-TT(C/T)(A/T)(C/G)IGA(A/G)GA(A/G)GA(A/G)TT(C/T)(C/T)T-3′

2: 5′-TA(C/T)CA(A/G)AA(C/T)GCIGA(C/T)(C/T)TIAA-3′

3: 5′-TT(C/T)GA(A/G)GA(A/G)GGIAA(C/T)GA(A/G)AC-3′

1′: 5′-A(A/G)(A/G)AA(C/T)TC(C/T)TCC/TTCI(C/G)(A/T)(A/G)AA-3′

2′: 5′-TTIA(A/G)(A/G)TCIGC(A/G)TT(C/T)TG(A/G)TA-3′

3′: 5′-GT(C/T)TC(A/G)TTICC(C/T)TC(C/T)TC(A/G)AA-3′

Oligonucleotide mixtures 1–3 correspond to possible sequences coding for the peptides H_2N-Phe-Ser-Glu-Glu-Glu-Phe-Leu-COOH, H_2N-Tyr-Gln-Asn-Ala-Asp-Leu-Lys-COOH and H_2N-Phe-Glu-Glu-Gly-Asn-Glu-Thr-COOH, respectively, whereas oligonucleotide mixtures 1′–3′ correspond to the respective complementary sequences. RNA was extracted from bovine liver, and the poly$(A)^+$ fraction was selected according to standard procedures (20). Polymerase chain reaction amplifications were carried out using poly $(A)^+$ RNA following reverse transcription with the Gene-AMP kit (Perkin Elmer) according to the recommended protocol. The samples were subjected to 35 cycles of amplification (94 °C for 1 min, 50 °C for 2 min, and 72 °C for 3 min). The 1.1-kilobase pair cDNA band, obtained following amplification in the presence of the two oligonucleotide mixtures 3 and 2′, was subcloned (bAO1) in the plasmid vector pCR™ using the T/A cloning kit (Invitrogen, San Diego, CA) following the recommended protocol.The AO insert was sequenced in both directions by the dideoxynucleotide-chain termination method (22), using double-stranded DNA as templates and T7 DNA polymerase (Pharmacia) according to the instruction of the manufacturer. Nucleotide sequencing of bAO1 demonstrated that the cDNA was a *bona fide* copy of part of the AO transcript, since it showed an uninterrupted open reading frame, which encodes amino acid sequences that match all the sequences of the tryptic and cyanogen bromide peptides determined. The clone bAO1 was labeled with [^{32}P]dCTP and used to screen a random-primed bovine liver cDNA library constructed in the phage vector λgt10 from bovine liver poly$(A)^+$ RNA according to standard procedures (20). Screening of the library with bAO1 and subsequently isolated cDNA clones as hybridization probes resulted in the isolation of overlapping cDNA fragments, which were subcloned in pBluescript (bAO2, bAO3 and bAO4). Screening of an oligo(dT)-primed library constructed in λgt10 with the use of bAO4 as a probe resulted in the isolation of two hybridizing phages whose inserts were subcloned in pBluescript (bAO5 and bAO6). Since neither insert contained a polyadenylation signal and a corresponding poly$(A)^+$ tail, the sequence of the most 3′ portion of the AO cDNA (bAO7) was isolated with the use of a commercially available 3′-RACE kit (Clontech, Palo Alto, CA) using an appropriate synthetic oligonucleotide as a primer (5′-CATAAGAA-CATCAGAACAGACA-3′) obtained from clone bAO5. The most 5′ region of the cDNA (bAO8) was isolated with a commercially available 5′-RACE kit (Clontech) with the oligonucleotide 5′-CTCGGGAG-GAAATATCAGCTCTTGAGTTGGATCCA-3′ synthesized on the basis of the nucleotide sequence of clone bAO1. Hybridization and washing of the plaque lifts obtained from the random-primed and oligo(dT)-primed libraries was performed in stringent conditions according to standard protocols (20). Each clone was sequenced in both directions using either vector primers or specific oligonucleotides synthesized based on the information obtained from former sequence analysis. Oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). Computer analysis of the DNA sequences was performed with the Genwork software package (IntelliGenetics, Mountain View, CA). Northern blot analysis was performed according to standard conditions (20), using ^{32}P-radiolabeled bAO1 cDNA as a probe.

RESULTS AND DISCUSSION

Purification of Bovine Liver AO and Cloning of the Respective cDNA—Typical results for the purification of AO from bovine liver are illustrated in Table I. Following the various purification steps, the yield of active enzyme is between 3 and 5% of the

TABLE I
Purification of bovine liver AO

$(NH_4)_2$ SO_4, ammonium sulfate precipitation step at 40% saturation; FAST-Q, anion exchange chromatography; Sephacryl, Sephacryl S-300 gel permeation chromatography; Benz. Seph., benzamidine-Sepharose 6B affinity chromatography.

Step	Volume	Activity	Protein	Specific activity	Yield	Purification factor
	ml	*units*	*mg*	*units/mg*	%	
Cytosol	570	135,000	22,530	6	100	1.0
$(NH_4)_2$ SO_4	200	108,000	5,061	21	80	3.5
60 °C heating	88	52,800	1,232	43	39	7.2
FAST-Q	18	22,300	95.4	234	16	39.0
Sephacryl	42	20,200	60.3	335	15	55.8
Benz. Seph.	9	4,600	2.5	1,840	3	306.7

amount originally present in the homogenate (as determined by three independent purifications) and this corresponds to approximately 0.8 mg of pure AO/100 g of tissue. Enrichment of AO following each purification step is indicated by an increase in the specific activity of the enzyme. The final specific activity of pure AO is around 1,800 units/mg protein (three experiments), which is similar to that of highly purified enzyme preparations obtained from other sources (23). Pure bovine AO has an apparent molecular mass of 300 kDa, as assessed by size-exclusion chromatography, and it shows an absorption maximum at 450 nm, typical of flavoproteins. The isoelectric point of AO is 4.6, as determined by chromatofocusing on a Mono-P column (Pharmacia). As illustrated in Fig. 1, the denatured and reduced protein consists of a single 150-kDa protein band, which corresponds to the monomeric subunit of the homodimeric active enzyme. Sequencing of the amino terminus of the AO protein was attempted on three independent highly purified AO preparations with negative results. This suggests that the amino-terminal amino acid of AO was blocked as a result of unknown post-translational modifications, which are either physiological or the result of the purification procedure.

Partial sequence information on AO was obtained from protein subjected to cleavage with trypsin and cyanogen bromide. Both digestions resulted in the production of many peptides, which were separated by reverse phase chromatography. Six of the quantitatively most prominent peptides were sequenced, allowing the molecular cloning of overlapping cDNA fragments corresponding to the bovine liver AO mRNA. The structure of the various clones along with a partial physical map of the bovine liver AO cDNA is shown in Fig. 2.

As documented in Fig. 3, the AO cDNA consists of a 102-nucleotide 5′-untranslated region, followed by a 4017-nucleotide open reading frame and a 511-nucleotide 3′-untranslated sequence. A single polyadenylation signal (AATAAA), located 23–28 nucleotides upstream of the poly(A) tail, is evident. The AO cDNA is the copy of a full-length transcript, as indicated by the sequence of several independent clones obtained by 5′- and 3′-RACE experiments, which never went beyond the sequence determined for bAO8 and bAO7, respectively. The open reading frame of the cDNA predicts a protein of 1339 amino acids with a calculated molecular weight of 147,441. This is in line with the molecular mass of the enzyme monomeric subunit obtained by SDS-PAGE in reducing conditions (see Fig. 1). The assignment of the first coding amino acid to the first in-frame methionine is presumptive, owing to the lack of amino-terminal sequence data on the protein. However, the codon for the putative first methionine is within the context of sequences that are very often found around the translation initiation site on a variety of proteins (24). The open reading frame of the cDNA predicts a translation product that contains the sequence of the six peptides obtained by tryptic or cyanogen bromide cleavage of the AO protein. The AO transcript is devoid of a nucleotide sequence coding for a hydrophobic signal peptide, which is

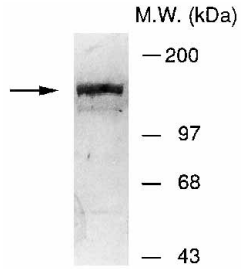

FIG. 1. **SDS-PAGE of bovine liver AO.** SDS-PAGE of pure bovine AO was performed in reducing conditions using 7.5% acrylamide slab gel. The gel was stained for proteins with silver nitrate. Molecular mass standards are ovalbumin (43 kDa), bovine serum albumin (68 kDa), phosphorylase *b* (97 kDa), and myosin heavy chain (200 kDa). An *arrow* indicates the position of the 150-kDa protein band corresponding to the purified AO.

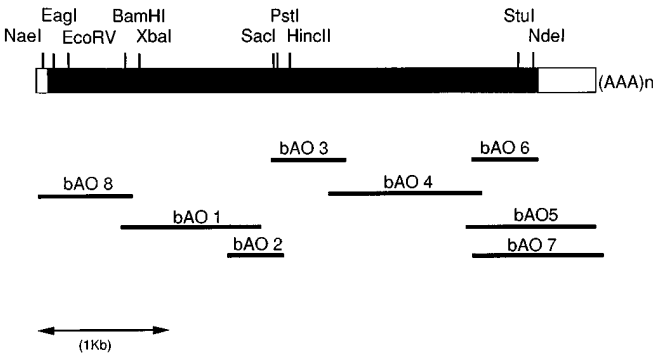

FIG. 2. **Structural organization and physical map of bovine liver AO cDNA.** The *upper part* of the figure shows the physical map of bovine liver AO cDNA from the 5′- to the 3′-end (*left* to *right*). Selected restriction endonucleases that cut once only inside the cDNA are indicated. The *black* and *white* boxes indicate the protein coding region and the 5′- or 3′-untranslated region, respectively. The poly(A)$^+$ tail is indicated by the symbol $(AAA)_n$. The *thin lines* shown in the *lower part* of the figure represent inserts of recombinant λ phages and polymerase chain reaction products. All the clones were sequenced completely in both directions.

consistent with the generally accepted cytoplasmic localization of the AO protein. Near the amino- and the carboxyl-terminal regions of the putative translation product, two consensus sequences for the binding of NAD (aa 42–47 and 1268–1273) are observed. As AO does not require the cofactor for its catalytic activity, it is unlikely that these structural elements have functional significance. Between amino acid residues 44 and 74, the fingerprint of an Fe-S center is evident and is of the same type observed in a number of ferredoxin proteins, in bacterial fumarate reductase, and in eukaryotic succinic dehydrogenase (25, 26). Two tyrosine kinase recognition sequences are observed between amino acids 535 and 542 and between 958 and 965; however, it remains to be established whether the two sites are really phosphorylated *in vivo* by tyrosine kinases

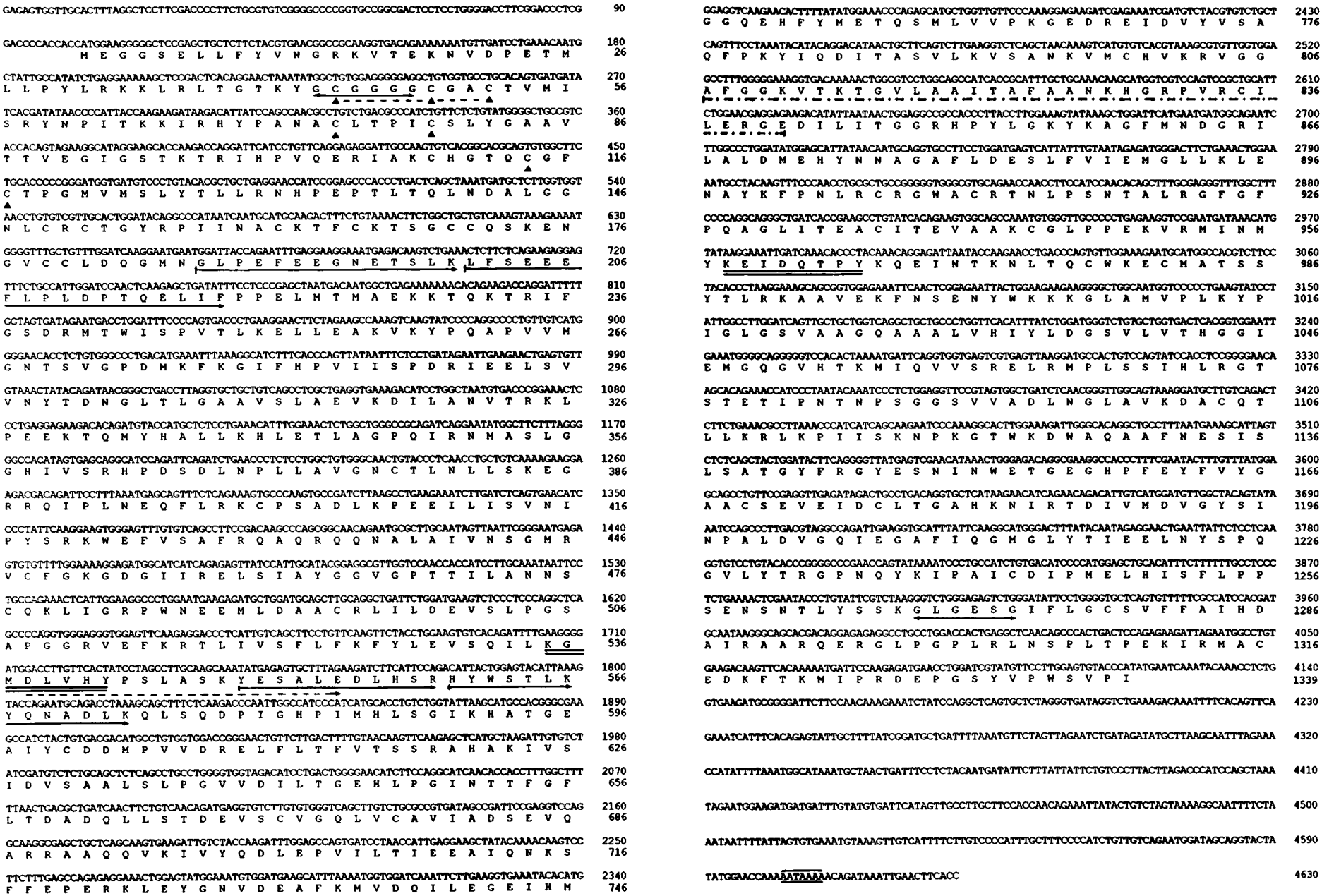

FIG. 3. **Nucleotide sequence and deduced amino acid sequence of bovine liver AO.** The nucleotide sequence of bovine liver AO cDNA (*upper line*) was obtained from the overlapped cDNA clones shown in Fig. 2 and is presented with the deduced amino acid sequence (*lower line*). Nucleotide residues are numbered in the 5′ to 3′ direction, whereas amino acid residues are numbered from the NH_2 terminus to the COOH terminus starting from the putative first methionine residue. The tryptic peptides and the cyanogen bromide peptide whose sequence has been determined are *underlined* with *solid arrows* and a *dashed arrow*, respectively. Two consensus sequences coding for Gly-Xaa-Gly-Xaa-Xaa-Gly essential for NAD^+ binding are *underlined* with a *two-headed arrow*. The cysteine residues of AO that are conserved with those proposed to participate in the iron-sulfur center of rat XD are indicated with a *solid triangle* below the protein sequence. Consensus sequences for putative tyrosine kinase phosphorylation sites are *doubly underlined*. The consensus sequence pattern of the eukaryotic molybdopterin-binding site is indicated with a *dotted line* below the protein sequence. The polyadenylation signal AATAAA is *under-* and *overlined*.

and whether this has any relevance for the catalytic activity of AO. Between amino acids 829 and 863, AO contains a sequence that conforms to the consensus for the molybdenum cofactor binding site (27). Although AO is a flavoprotein, a FAD binding consensus sequence of the type described by Correll *et al.* (28) cannot be determined. Nevertheless, the flavin nucleotide binding site of the oxidase must be near the molybdenum cofactor center, by analogy with what reported for the related (see below) molybdoflavoprotein XD (27).

Similarity between AO and XDs—A search in the Swiss-Prot and EMBL data banks demonstrates that the amino acid sequence derived from the bAO cDNA has a significant level of similarity with that of XDs from various sources. The overall level of identity is 19% to *Aspergillus nidulans* (27), 44% to *Calliphora vicina* (29), 45% to *Drosophila melanogaster* (30), 50% to mouse (31, 32), 50% to rat (33), 53% to chicken (34), and 50% to human XD (deduced from a recently isolated cDNA (35, 36), which we will name hXD-1 for simplicity). This is interesting, given the fact that AO and XD are both homodimeric molybdoflavoproteins, have a very similar molecular weight, contain two Fe-S centers/subunit (1, 6, 31), and show overlapping substrate specificity (37). As shown in Fig. 4, bAO can be easily aligned with all the known XDs along the whole length of the protein. However, two large regions show a particularly high degree of identity. The first region is located at the amino terminus of bAO and corresponds to the amino-terminal XD domain containing the two Fe-S centers (aa 5–170). In this region, we observe a cluster of 16 cysteine residues. Thirteen of these cysteines are conserved in both AO and XDs, and 8 of them must contribute to the structure of the two Fe-S centers. As already discussed in the preceding section, in bAO, cysteine residues 44, 49, 52, and 74 are likely to be part of the first Fe-S cluster. By analogy with what is proposed for XDs (27), two other amino-terminal cysteines (aa 114 and 117) are probably involved in the formation of the second Fe-S center present in AO, since they are conserved in the two classes of molybdoflavoproteins. It is unlikely that the cysteine at position 79 of the bovine AO sequence is critical for the formation of the second Fe-S redox center, since an analogous cysteine residue is not observed in the very recently cloned *A. nidulans* XD (27). By contrast, we propose that one (or both) of the two cysteines (aa 149 and 151) located in a strictly conserved stretch of amino acids are involved in chelating iron in AO and consequently in XD. Another region with a particularly high level of convergence between bAO and XDs is near the carboxyl terminus from amino acid 1172 to amino acid 1219. It is a hydrophobic region; however, no functional role can be ascribed to this stretch of amino acids. The substrate binding of AO is believed to be in close proximity with the molybdenum cofactor binding site although it has not yet been defined. Interestingly, the Glu-Arg-Xaa-Xaa-Xaa-His (position 910–915 of *A. nidulans* XD) motif reported by Glatigny and Scazzocchio (27) to be involved in substrate binding in XDs does not align to a similar sequence in bovine AO. Thus, the substrate binding pocket must be different in the two enzymes, in spite of the fact that AO and XD show a certain degree of overlapping substrate specificity.

As illustrated in Fig. 4, bAO has a surprisingly high degree of conservation (86%) with the predicted translation product of a second putative human XD cDNA clone recently reported by Wright *et al.* (which we will name hXD-2 for clarity) (38). Although these authors reported that hXD-2 is a cDNA clone coding for human XD, partial or complete sequencing data obtained from the purified human XD protein, which support this contention, are not yet available. We propose that the translation product of this cDNA is indeed the human homolog

FIG. 4. **Amino acid sequence comparison between bovine AO and various eukaryotic XDs.** The amino acid sequence of bovine AO (*bAO*) is aligned with XDs of human (*hXD-2*, *hXD-1*), mouse (*mXD*), rat (*rXD*), chicken (*cXD*), *D. melanogaster* (*D.m.XD*), *C. vicina* (*C.v.XD*), and *A. nidulans* (*A.n.XD*) origin. Amino acid residues are *numbered* from the NH_2 terminus to the COOH terminus from the putative first methionine and *boxed* if conserved in all the sequences. Symbols above the sequences are as follows: *asterisks*, cysteines conserved in all the sequences; *open squares*, cysteines common to bAO and hXD-2; *solid triangles*, cysteines reported to be involved in the formation of the iron-sulfur centers of XDs; *solid circles*, cysteines that we propose to be involved in the chelation of iron atoms in AO; *double line*, consensus sequences for putative tyrosine kinase-dependent phosphorylation sites in bAO. Symbols below the sequences are as follows: *two-headed arrow*, consensus sequence for putative NAD binding site; *double line* (*NAD*), putative NAD binding site determined for chicken XD; *dotted line* (*Mo*), putative molybdopterin-cofactor binding site; *double-headed arrow* pointing inward (*S*) = amino acid residues important for the binding of the substrate to *A. nidulans* XD.

of AO on the basis of the following considerations. First, like bAO, the protein sequence deduced from h-XD2 has a significant but low level of similarity with that of mouse (49% identity), rat (49%), *Drosophila* (43% identity), *Calliphora* (43% identity), and *Aspergillus* (39% identity) XDs. Second, the protein encoded by hXD-2 has a low degree of similarity, *i.e.* 49%, with hXD-1 (29, 31), which, in turn, is extremely similar to the chicken (71% identity), mouse (89% identity), and rat (90% identity) homologs (31, 33). Third, unlike what is observed in all the XD species homologs, bAO and hXD-2 are devoid of a sequence similar to that known to bind NAD analogs in chicken XD (aa 415–427 in cXD; Ref. 39). This is consistent with the fact that bAO is devoid of dehydrogenase activity and suggest that the same is true also for the hXD-2 translation product. Fourth, the high degree of conservation at the amino acid level between bAO and hXD-2 is retained at the nucleotide level, where the overall similarity is 80%. Finally, convergence between bovine AO and the human XD-2 nucleotide sequences is not only limited to the coding region, it is also observed in the 5′-untranslated region (45% identity) and more evidently in the 3′-untranslated region (72% identity). These portions of the AO mRNA are highly divergent from those of the other mammalian XDs.

Regardless of the relationship between bAO and hXD-2, our data clearly demonstrate a remarkable level of similarity between AO and XDs. This strongly suggests that the two enzymes evolved from a common precursor protein. The most primitive species in which AO has been found is the coelenterate *Segartia luciae* (7), whereas XD is present in the fungus *A. nidulans* (27). This indicates that, of this enzyme pair, XD is probably primordial. Bovine AO has also a low but significant level of similarity (17%) with the putative translation product of a recently cloned *Desulfovibrio gigas* gene coding for a molybdoprotein, which does not bind FAD, but it is capable of oxidizing purines (40). At present, however, the phylogenetic relationship between bAO and this protein is unclear.

Tissue Distribution of AO Protein and mRNA—To study the tissue distribution of the AO protein, we developed an anti-AO polyclonal antibody. Given the similarity between AO and XDs, we first evaluated the specificity of our antibody by establishing its cross-reactivity with the latter protein. Bovine liver cytosolic fractions were loaded on an anion exchange column to separate AO from XD. As shown in Fig. 5, AO and XD bind to the column and elute as two partially separated chromatographic peaks when a NaCl gradient is superimposed to the column equilibration buffer. Our antibody specifically recognizes a 150-kDa protein only in AO-containing fractions (fractions 24–29). Intensity of the band visualized correlated with the amount of AO. No immunoreactivity was observed in any other region of the chromatogram. Particularly, fractions 19–23, which contained the highest amounts of XD enzymatic activity, did not contain proteins recognized by the anti-AO antibody preparation. Given the fact that AO and XD are present in roughly equivalent amounts, as determined by results obtained with the use of an anti-XD antibody,[2] these data demonstrate that the anti-AO antibody is monospecific.

Western blot analysis performed on cytosolic extracts of various bovine organs with the anti-AO antibody is shown in the *upper panel* of Fig. 6. In reducing conditions, a single band of approximately 150 kDa is present in the majority of the tissues examined, although the liver shows a significant proportion (approximately 30%) of a 135-kDa AO fragment of proteolytic origin, which is probably generated by a relatively specific

[2] M. Li Calzi, C. Raviolo, E. Ghibaudi, L. De Gioia, M. Salmona, G. Cazzaniga, M. Kurosaki, M. Terao, and E. Garattini, unpublished observations.

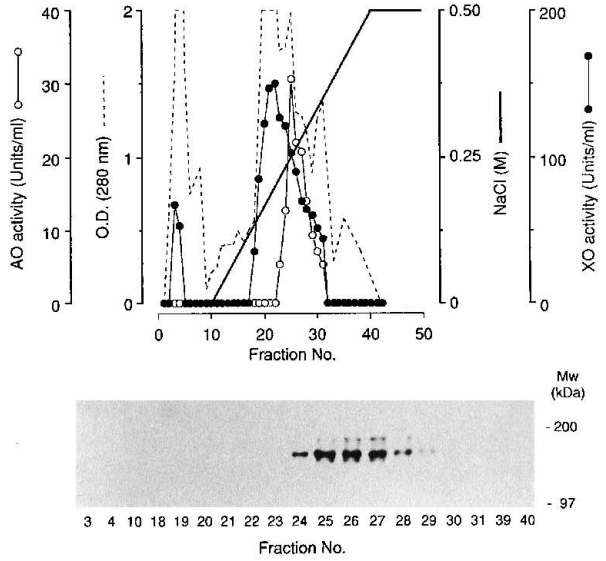

FIG. 5. **Chromatographic separation of bovine liver AO and XD enzymatic activities and determination of the specificity of the anti-AO antibody.** *Upper panel*, ammonium sulfate cuts of bovine liver cytosols were loaded on a Mono-Q anion exchange FPLC column equilibrated with 50 mM Tris/HCl (pH. 7.0) to separate AO (*open circles*) from XD (*solid circles*) enzymatic activities. The two enzymatic activities were eluted with a 30-min linear gradient of NaCl superimposed to the equilibration buffer. The total protein elution profile was monitored at 280 nm with an on-line UV spectrophotometer. One-ml fractions were collected, and a portion of each fraction was used for the determination of XD and AO activities. *Lower panel*, an aliquot of the indicated fractions was subjected to Western blot analysis using the anti-AO antibody. The positions of the molecular mass markers are indicated (myosin heavy chain, 200 kDa; phosphorylase *b*, 97 kDa).

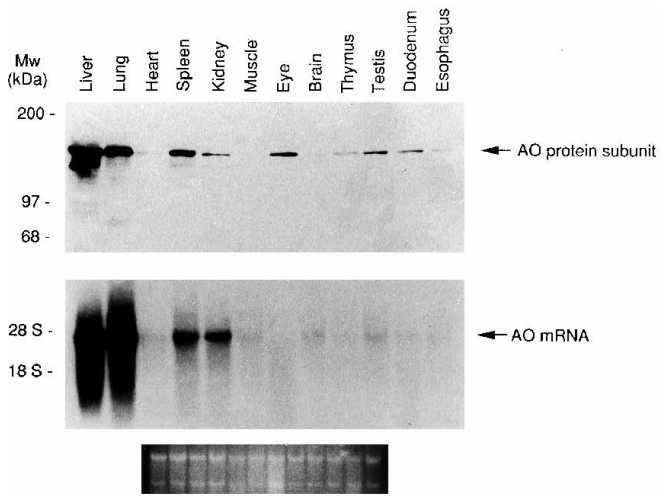

FIG. 6. **Tissue distribution of AO protein and mRNA.** *Upper panel*, homogenates obtained from the indicated organs were ultracentrifuged at 100,000 × *g*, heated at 60 °C, and a fraction of the resulting cytosolic supernatants (100 μg) were subjected to Western blot analysis using the AO-specific polyclonal antibody. The positions of the molecular mass markers are indicated (myosin heavy chain, 200 kDa; phosphorylase *b*, 97 kDa). *Lower panel*, total RNA was obtained from the indicated tissues. RNA (30 μg/lane) was loaded on a 1% formaldehyde/agarose gel and subjected to Northern blot analysis. After transfer, the nylon membrane was hybridized with the bovine AO cDNA clone bA01. The positions of 28 and 18 S rRNAs are shown on the *left*. An ethidium bromide staining of the agarose gel is shown at the bottom of the figure to illustrate that equal amounts of RNA were loaded in each lane.

protease present in high concentrations in this organ. The highest levels of AO expression are observed in the hepatic tissue and in the lung, followed by the spleen. These are the only organs where significant amounts of AO enzymatic activity are reproducibly measurable in the 100,000 × *g* supernatant following ammonium sulfate precipitation (16.0, 8.5, and 3.2 units/mg protein, for liver, lung, and spleen, respectively).In all the other tissues, the levels of phenantridine oxidizing activity are at the limit of detection of the assay or below it, preventing determination of AO activity. However, the eye, kidney, thymus, testis, duodenum, heart, and esophagus show detectable amounts of an AO-immunoreactive band, which is not observed in the striated muscle and the pancreas (the latter tissue is not shown). Interestingly, high concentrations of AO protein were determined in the liver and in the lung. These sites provide barriers against foreign compounds and are the main organs where xenobiotics are metabolized. This observation supports the hypothesis that AO is important in the detoxification processes of certain types of environmental pollutants. The presence of AO-immunoreactive protein in the eye supports a possible involvement of the enzyme in the metabolism of retinal, although it remains to be established whether the protein is enzymatically active.

AO is a very complex enzyme, whose expression is potentially regulated at various levels. To investigate the relationship between the amounts of the AO protein and the steady-state levels of the respective transcript, Northern blot experiments on RNA extracted from the same tissue preparations used for Western blot analysis were performed. As shown in the *lower panel* of Fig. 6, a single AO mRNA, migrating slightly faster than the 28 S ribosomal RNA is readily detected in liver, lung, spleen, and kidney, and a similar transcript of much lower abundance is observed in all the other tissues considered. With respect to this, notice that the AO transcript in the eye is visible only upon higher exposures of the autoradiogram. The mobility of the AO transcript in the various tissues is consistent with the length of the corresponding cDNA. A correlation between AO mRNA and protein levels is not always evident. Liver and lung show the highest amounts of mRNA, as expected on the basis of the Western blot data. However, the levels of AO mRNA in the spleen and the eye are lower, whereas in the kidney, they are higher than expected. In addition, the striated muscle contains detectable quantities of the AO transcript despite the absence of the respective protein. Taken together, these data suggest that both transcriptional and translational events control the expression of AO in a tissue-specific fashion.

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Purification, cDNA Cloning, and Tissue Distribution of Bovine Liver Aldehyde Oxidase

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