Mouse molybdo-flavoenzymes consist of xanthine oxidoreductase, aldehyde oxidase (AOX1), and two recently identified proteins, AOH1 and AOH2 (aldehyde oxidase homologues 1 and 2). Here we demonstrate that CD-1, C57BL/6, 129/Sv, and other mouse strains synthesize high levels of AOH1 in the liver and AOH2 in the skin. By contrast, the DBA/2 and CBA strains are unique, having a selective deficit in the expression of the AOH1 and AOH2 genes. DBA/2 animals synthesize trace amounts of a catalytically active AOH1 protein. However, relative to CD-1 animals, an over 2 log reduction in the steady-state levels of liver AOH1 mRNA, protein, and enzymatic activity is observed in basal conditions and following administration of testosterone. The DBA/2 mouse represents a unique opportunity to purify AOH1 and compare its enzymatic characteristics to those of the AOH1 protein. The spectroscopy and biochemistry of AOX1 are very similar to those of AOH1 except for a differential sensitivity to the non-competitive inhibitory effect of norharmane. AOX1 and AOH1 oxidize an overlapping set of aldehydes and heterocycles. For most compounds, the substrate efficiency ($V_{max}/K_m$) of AOX1 is superior to that of AOH1. Alkylic alcohols and acetaldehyde, the toxic metabolite of ethanol, are poor substrates of both enzymes. Consistent with this, the levels of acetaldehyde in the livers of ethanol administered CD-1 and DBA/2 mice are similar, indicating that neither enzyme is involved in the in vivo biotransformation of acetaldehyde.

Mammalian molybdo-flavoenzymes are a small family of proteins that require molybdenum, in the form of a pterin cofactor (molybdenum cofactor), for their catalytic activity (1). Xanthine oxidoreductase (XOR) (2, 3) and aldehyde oxidase (AOX1) (4, 5) are the two most thoroughly studied members of the family. XOR is involved in the catabolism of purines, oxidizing hypoxanthine into xanthine and xanthine into uric acid (1, 6, 7). However, the enzyme is likely to serve other cell- and tissue-specific functions (8). The homeostatic significance of AOX1 is still obscure, as the enzyme lacks a recognized physiological substrate, although retinaldehyde and hydroxymandelaldehyde, a monoamine catabolite, have been reported to be oxidized to the corresponding acids by the molybdo-protein (1). AOX1 is believed to play a role in the detoxification of exogenous compounds of pharmacological and toxicological interest. The protein catalyzes the oxidation of methotrexate, a widely used anti-neoplastic agent (9), and is purported to play a role in the oxidation of acetaldehyde, the toxic metabolite of ethanol (10, 11).

Recently we demonstrated that the molybdo-flavoenzyme family is larger than previously anticipated (12, 13). In fact, we identified two novel proteins characterized by high structural similarities with AOX1 and XOR (12, 13). Given the closer relationship to AOX1, we named the two proteins aldehyde oxidase homologues 1 and 2 (AOH1 and AOH2). AOH1 and AOH2 are encoded by two distinct genes that map at a very short distance from the AOX1 locus on mouse chromosome 1 (1, 13). The tissue distribution of AOH1 is superimposable to that of AOX1, and the two enzymes are predominantly synthesized in the hepatic, lung, and testicular tissue (12). By contrast the localization of AOH2 is more restricted, as the protein is present only in keratinized epithelia (1, 12). Currently, very little is known regarding the substrate specificity, the enzymatic characteristics, and the physiological function of the AOX1, AOH1, and AOH2 enzymes.

The definition of the enzymatic and biochemical characteristics of AOX1, AOH1, and AOH2 is hampered by the fact that efficient heterologous systems for the expression of molybdo-flavoproteins in their recombinant and catalytically active forms are not available. This has focused our attention on the development of efficient methods for the purification of the enzymes from their native sources (7, 13, 14). Comparison of the enzymatic properties of AOX1 and AOH1 is particularly relevant and difficult. In fact, the two molybdo-proteins cannot...
be separated on common chromatographic supports easily. In addition, the vast excess of AOH1 over AOX1 in mouse liver and lung makes it difficult to obtain the latter enzyme in a pure and AOH1-free form.

The physiopathological function of AOX1, AOH1, and AOH2 is completely obscure. For this reason, it would be useful to identify and characterize natural strains of mice with reduced or enhanced expression of the AOH1 and AOH2 genes. With reference to this last point, several years ago, Holmes and co-workers (15–17) described inbred strains of mice with deficits in the expression of hepatic benzaldehyde oxidase (BOX) activity. Here we follow up on the observation and demonstrate that DBA/2 and CBA inbred mice have a specific deficit in the expression of both AOH1 and AOH2 proteins relative to other common outbred and inbred laboratory strains of mice such as CD-1 and C57BL/6. Thus, the DBA/2 mouse was used to isolate AOX1 in pure form from the hepatic tissue and to compare the biochemical characteristics of the enzyme with those of AOH1. AOH1 and AOX1 oxidize not only aromatic and unsaturated aldehydes but also aliphatic aldehydes, including acetaldehyde, with similar efficiencies. Despite this, the levels of acetaldehyde in ethanol-treated DBA/2 mice are not different from those observed in similarly treated CD-1 mice.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All-trans-retinylalcohol, all-trans-retinoic acid, 2-hydroxypropyrimidine (2-H-2-pyrrolidinone), mannitol, norethandren (β-carboline), allopurinol, benzamide, pthalalzine, potassium ferrozide, testosterone propionate, xanthine, and hypoxanthine were purchased from Sigma. Butyraldehyde, benzaldehyde, and acetaldehyde were from Aldrich. Ethanol was obtained from Carlo Erba Chemicals (Roden, NL, Italy).

**Animals**—CD-1, C57BL/6, CBA, BDF1, SWJL, 129/Sv, C3H/He, and BALB/c mice, weighing 25–30 g, were purchased from Charles River Breeding Laboratories (Calco, Como, Italy) and kept at 25 °C with a 12-h light/dark cycle and given standard chow and water ad libitum in the “Mario Negri” Institute animal house facilities. Animals were treated according to the standards set by Italian Law. All animal experiments were approved by the Internal Animal Care and Use Committee and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996 edition) and European Union directives and guidelines. Western Blot and Zymogram Analyses—The anti-AOH1-, anti-AOX1-, anti-AOH2-specific antibodies have been described previously (12). The polyclonal anti-XOR antibody was raised against the synthetic peptide, NH2-NTMTKQSEFRKH-COOH, according to an established methodology (12). The anti-AOH1, anti-AOX1, anti-AOH2, and anti-XOR rabbit antisera were used for Western blot analyses, which were carried out with a chemiluminescence-based protocol as described previously (12, 13, 18, 19). SDS-PAGE was performed according to standard techniques (20). Proteins were measured with the Bradford method using a commercially available kit (Bio-Rad). Zymogram analysis of BOX and hypoxanthine oxidizing activities was performed following electrophoresis on cellulose acetate plates (Helena Laboratories, Beaumont, TX) as described (12, 13). Determination of aldehyde and alcohol dehydrogenases by zymogram analysis was carried out according to the method of Holmes et al. (21).

In some experiments, BOX activity was determined quantitatively on purified CD-1-derived AOH1 protein or on partially purified preparations of the same enzyme from DBA/2 animals. The protein preparations were electrophoresed on cellulose acetate plates, as above, overlaid with 2% agarose containing 0.3 mM phenazine methosulfate (Sigma), 0.9 mM 3,4,5-tridimethyliazole-2-4,5 2-diphenyltetrazolium bromide (Sigma), and 30 mM benzaldehyde. Following 10 min of incubation, the overlay was detached from the acetate plate, and the spots corresponding to BOX activity were cut with scissors. The purple precipitate due to the reduction of MTT was dissolved along with cellulose acetate in 100 μl of N,N′-dimethyldiamformamide (Sigma), and the solution was read at 540 nm in a Beckman DU-7 spectrophotometer (Beckman Instruments, Palo Alto, CA). Appropriate blanks obtained by cutting an equivalent area of the same cellulose acetate plate and extracted in the same volume of N,N′-dimethyldiamformamide were always run in parallel.

**Northern Blot Analysis, RT-PCR Amplification of the AOH1 cDNAs, PCR Amplification of the 5′-Flanking Regions of the AOH1 Gene, and Sequencing**—Total RNA was extracted from the liver of DBA/2, CBA, and CD-1 mice, and the poly(A)+ fraction of the RNA was selected according to standard protocols (20). Northern blot analysis was performed on the poly(A)+ fraction. The cDNA fragments corresponding to the 5′-untranslated region of mouse XOR (22), AOX1 (4), and AOH1 (13) were generated and cloned with the use of PCR methods (GeneRuler Labeling System (Amersham Biosciences). The coding regions of the AOH1 cDNAs from CD-1 and DBA/2 mice were amplified by RT-PCR using Advantage 2 DNA polymerase (Clontech, Palo Alto, CA) with the two amplifiers described previously (13). The cDNAs were subcloned into the pBluescript plasmid vector (Stratagene, La Jolla, CA) and sequenced in both directions with the use of appropriate oligonucleotide primers and an automatic DNA sequencer model CEQ 8000 (Beckman Instruments). To amplify the 5′-flanking regions of the AOH1 genes of DBA/2 and CD-1 mice, we first amplified the region of genomic DNA contained between exon 35 of AOX1 and exon 1 of AOH1 by PCR using the following two amplifiers: sense primer, 5′-TGGACCTGATGTC- CAGAGTAGCATCTGACTCCTAGAAGGA-3′ (complementary to nucleotides 205/230 of the AOH1 cDNA) (13). The resulting DNA fragments were subjected to a second round of PCR amplification with the same antisense primer as above and the sense primer 5′- CCTACTGTCTATGTGTTGCTG-3′ (nucleotides 894–875 of the AOH1 gene 5′-flanking region) (12). This resulted in the amplification of DNA fragments of ~1.6 kbp from the CD-1 and ~1.1 kbp from the DBA/2 DNAs. These two amplification products were subcloned into pBluescript by the T/A cloning method using a commercially available kit (TA cloning kit, Invitrogen), and the two constructs named CD-1-pBlue and DBA-pBlue. Sequencing was performed in both directions, as above. Aligned cDNAs were custom-synthesized (Invitrogen). Computer analysis of the DNA sequences was performed using the GeneWorks sequence analysis system (IntelliGenetics, San Diego, CA). A search of potential binding sites for transcription factors in the 5′-flanking region of the DBA/2-derived AOH1 gene was performed using the MatInspector algorithm and the TRANSFAC data base (23).

**Functional Characterization of the 5′-Flanking Regions of the AOH1 Genes and Transfection of the Molybdo-flavoenzyme cDNAs**—HEK-293 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen). To determine the promoter activity of the 5′-flanking regions of exon 1 of the CD-1 and DBA-derived AOH1 genes, we constructed plasmids pCD-1-gal and pDBA2-gal. To obtain these constructs, CD-1-pBlue and DBA-pBlue were digested with EcoRI and XhoI, and the resulting fragments were inserted in the plasmid pCMVβ (Strattech) digested with EcoRI and XhoI to release the cytomegalovirus (CMV) promoter-enhancer sequence. These constructs contain the two 5′-flanking regions of the AOH1 genes from CD-1 and DBA/2 mice placed in front of the bacterial β-galactosidase reporter gene. The constructs were transfected in HEK-293 cells along with a plasmid expressing the luciferase reporter gene under control of the Rous sarcoma virus promoter (RSV-Luc) (12) by using cationic liposomes as described previously (24). Forty eight hours following transfection, cells were harvested and lysed, and cell extracts were obtained for the determination of β-galactosidase activity. Invitrogen. The promoter activity associated with the pCD-1-gal and pDBA2-gal constructs was always normalized for the level of luciferase activity in the cell extracts.

The AOX1, AOH1, and AOH2 cDNA constructs in the pCMV-neo (Invitrogen) expression vector have been described previously (13). The construct containing pCMV-XOR was generated by inserting the coding region of the XOR cDNA (EcoRI-PvuII fragment) (22) into the polylinker region of the pCMV-neo plasmid. All the constructs were transfected into HEK-293 cells as above.

**Purification of AOX1 and AOH1 Proteins**—AOH1 was purified from the liver of male CD-1 mice, as described previously (12). AOX1 was isolated from the hepatic tissue of DBA/2 mice. Both enzymes, all the purification steps were carried out at 4 °C. Livers (typically 35–40 g) from male DBA/2 mice were homogenized in 3 volumes of 100 mM sodium phosphate buffer, pH 7.4, with an Ultraturrax homogenizer (IKA, Staufen, Germany). Homogenates were centrifuged at 105,000 ×
g for 45 min to obtain cysteic acid extracts. Extracts were heated at 55 °C for 10 min and centrifuged at 15,000 × g to remove precipitated proteins. An equal volume of saturated ammonium sulfate was added to the supernatant, and the precipitate was collected by centrifugation at 15,000 × g and resuspended in 100 mM Tris-glycine buffer, pH 9.0, containing 100 mM NaCl. Solubilized proteins (equivalent to 10 g of fresh liver) were mixed with 5 ml of benzamidine-Sepharose (Amer- sham Biosciences) pre-equilibrated in 100 mM Tris-glycine buffer, pH 9.0, containing 100 mM NaCl. Following 2 h of incubation, the resin was washed 4 times with 10 ml each of the equilibration buffer to remove unbound proteins. Adsorbed proteins were eluted twice with 5 M aliquots of equilibration buffer containing 10 mM benzamidine (Sigma). The eluate was concentrated to ~1 ml with Centriplus TM-100 (Milli- pore) and diluted for analysis. Chemiluminescence signals corresponding to AOX1 bands were quantitated with a scanning densitometer ( Hoefer Scientific Instruments, San Francisco). The total amount of AOX1-immunoreactive protein in the various experimental samples is expressed in arbitrary units and is calculated on the basis of the intensity of the Western blot signal in optical density multiplied by the total volume of each purification step. One arbitrary unit of immunoreactive protein corresponds to 100 OD of the specific AOX1 band in each experimental sample.

Mass Spectrometry of the Purified AOX1 and AOH1 Proteins—The masses of tryptic peptides obtained from purified AOX1 and AOH1 were determined by MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometric analyses of AOX1 and AOH1 tryptic peptides were performed using a standard protocol involving in situ or in-gel tryptic digestion (26). Briefly, proteins or Coomassie-stained gel slices were incubated with 1 M dithiothreitol in 100 mM ammonium bicarbonate and 12.5 ng/µl of the Western blot signal in optical density multiplied by the total mass of the expected molecular weight (about 150 kDa) correspond- ing to the monomeric subunits of XOR, AOX1, AOH1, and AOH2 and shows reduced expression of AOX1 and AOH1 protein was eluted at 0.5 ml/min with a linear gradient (22.5 ml) from 0 to 1 x NaCl in 50 mM Tris-HCl, pH 7.4. The AOX1 protein was eluted at 0.5 ml/min with a linear gradient (22.5 ml) from 0 to 1 x NaCl in 50 mM Tris-HCl, pH 7.4. The purification of AOX1 was monitored by determination of phthalazine oxidizing activity, as described below. In the case of phthalazine oxidation, 1 unit of enzymatic activity corresponds to 1 nmol of phthalazine oxidized/min.

The purification of AOX1 was also monitored by quantitative Western blot analysis. For this type of experiment, an equivalent amount of protein solution, at each purification step, was loaded onto the same gel and processed for analysis. Chemiluminescence signals corresponding to AOX1 bands were quantitated with a scanning densitometer (Hoefer Scientific Instruments, San Francisco). The total amount of AOX1-immunoreactive protein in the various experimental samples is expressed in arbitrary units and is calculated on the basis of the intensity of the Western blot signal in optical density multiplied by the total volume of each purification step. One arbitrary unit of immunoreactive protein corresponds to 100 OD of the specific AOX1 band in each experimental sample.

Mass Spectrometry of the Purified AOX1 and AOH1 Proteins—The masses of tryptic peptides obtained from purified AOX1 and AOH1 were determined by MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometric analyses of AOX1 and AOH1 tryptic peptides were performed using a standard protocol involving in situ or in-gel tryptic digestion (26). Briefly, proteins or Coomassie-stained gel slices were incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate and 12.5 ng/µl of trypsin (Promega, Madison, WI). Peptides were extracted twice in 50% (v/v) acetonitrile, 5% (v/v) formic acid. The combined extracts were lyophilized and redissolved in 0.1% (v/v) formic acid and desalted using ZipTip (Millipore). Peptides were eluted in 50% methanol, 0.5% formic acid. The eluate was mixed 1:1 (v/v) with a saturated solution of cyanogen bromide in acetonitrile, 0.1% trifluoroacetic acid 1.3 (v/v). Mass mapping of tryptic peptides was performed with a Bruker Biflex™ MALDI-TOF mass spectrometer (Bruker, Bremen, Germany). Data generated were processed with the Mascot program (www.matrixscience.com/) allowing a mass tolerance of ±0.4 Da.

Determination of the Substrate and Inhibitor Specificities of AOX1 and AOH1—For the determination of retinyldehyde oxidizing activity, we followed a slight modification of the method described by Tsujita et al. (27). Purified AOX1 and AOH1 preparations (2 µg in 2–4 µl) were incubated in the dark for 10 min at 37 °C in 100 µl of 10 mM potassium phosphate buffer, pH 7.4, containing all-trans-retinaldehyde. In these conditions, oxygen acts as the final electron acceptor of the reaction. Retinoic acid derivatives were extracted twice with 3 ml of ethyl acetate and analyzed by gas chromatography–mass spectrometry using a Shimadzu GCMS-QP5050 (Shimadzu) equipped with a Carbowax 20M column (3 m × 3.0 mm, Shimadzu) and a quadrupole mass spectrometer (Shimadzu) at an ionizing potential of 70 eV.

The DBA Mouse Strain Has a Selective Deficit in the Expression of AOH1 and AOH2 and Shows Reduced Expression of AOX1—Given the remarkable structural similarity of the various members of the mouse molybdoflavoprotein family, a prerequisite for the determination of the steady-state levels of these proteins in organ and tissue extracts is the development of mono-specific antibodies. For this purpose, we developed polyclonal antibodies by immunization of rabbits with selected synthetic peptides derived from the amino acid sequences of the AOX1, AOH1, AOH2, or XOR proteins. The anti-XOR, anti-AOX1, anti-AOH1, and anti-AOH2 antibodies recognize the corresponding target protein with high selectivity, as demonstrated by the Western blot analyses performed on extracts of HEK-293 cells transfected with the cDNAs coding for the four known mouse molybdoflavoenzymes (Fig. 1A). The four anti-molybdoflavoenzyme antibodies highlight specific bands of the expected molecular weight (about 150 kDa) corresponding to the monomeric subunits of XOR, AOX1, AOH1, and AOH2. Specific protein bands are not recognized by any of the antibodies in extracts of empty vector-transfected or untransfected HEK-293 cells. The anti-XOR, anti-AOX1, and anti-AOH1 antibodies were used to determine the levels of the corresponding proteins in the liver of a number of common laboratory mouse strains of outbred and inbred origin (Fig. 1B). Although the livers of C57Bl/6, BDF1, SW/J, CD-1, 129/Sv, C3H, and BALB/c mice contain significant amounts of AOH1, AOX1, and XOR proteins, DBA2 and CBA mice are selectively deficient in the synthesis of AOH1 and express relatively low levels of AOX1.

In additional experiments, we focused our attention on the
DBA/2 and CBA mice and used the CD-1 outbred strain for all comparisons. As illustrated in Fig. 2A, the cytosolic fraction of livers extracted from CD-1 mice contain two BOX activities that can be separated in the form of discrete bands following cellulose acetate electrophoresis (13, 15–17). The faster and slower migrating bands correspond to the molybdo-flavoenzymes AOH1 and AOX1, respectively (13). Consistent with the results reported in Fig. 1B, livers from the inbred mouse strains, DBA/2 and CBA, do not show detectable amounts of the BOX activity associated with AOH1. In these two strains, a significant reduction in the AOX1-dependent BOX band is also evident. Similar quantities of XOR activity, as measured with the specific substrate hypoxanthine, are present in the liver homogenates of the three mouse strains considered. Fig. 2B confirms that the deficit of AOH1-associated BOX activity in DBA/2 and CBA mice is the result of a dramatic decrease in the steady-state levels of the AOH1 protein, as assessed by Western blot analysis. By contrast, no significant variations in the amounts of liver XOR protein are visible in any of the mouse strains considered, and a significant decrease in the levels of AOX1 is observed in the DBA/2 and CBA animals.

FIG. 1. Mono-specificity of the anti-molybdo-flavoenzyme antibodies. A, HEK-293 cells were mock-transfected (NT) and transfected with a void vector (pCMV) or expression vectors containing full-length cDNAs encoding mouse XOR (pCMV-XOR), AOX1 (pCMV-AOX1), AOH1 (pCMV-AOH1), and AOH2 (pCMV-AOH2). Forty eight hours following transfection, cells were harvested, homogenized, and centrifuged to obtain cytosolic extracts. Identical aliquots of the cytosolic extracts (100 μg of protein) were subjected to Western blot analysis with the indicated polyclonal antibodies. Control (C) = cytosolic extracts from the liver (XOR, AOX1, and AOH1) or skin (AOH2) of CD-1 mice. B, cytosolic extracts of livers (100 μg of protein/each experimental sample) from male mice of the indicated strains were subjected to Western blot analysis with anti-XOR, anti-AOX1, anti-AOH1, and anti-β-actin polyclonal antibodies. The position of relevant molecular weight markers is indicated on the left.

Surprisingly, the XOR transcript is also slightly down-regulated in DBA/2 and CBA relative to what was observed in CD-1 mice. This suggests that the steady-state levels of the XOR protein are controlled not only by the rate of gene expression but also by translational or post-translational events, as observed in other experimental conditions (18, 19). All these results were obtained with probes encompassing the 3′-untranslated regions of the AOH1, AOX1, and XOR cDNAs; however, similar data were generated with the use of probes corresponding to the coding regions (data not shown). The phenomena observed on the AOH1, AOX1, and XOR mRNAs are specific, as demonstrated by the constant amounts of the control glyceraldehyde-3-phosphate dehydrogenase transcript observed across the lanes of the gel.

To evaluate whether lack of expression of the AOH1 and down-regulation of the AOX1 genes in DBA/2 mice are tissue-specific phenomena, the levels of the corresponding proteins were measured in the lung, the second richest source of the two enzymes in adult animals (13). As indicated by Fig. 3A, the lung of CD-1 animals contains detectable amounts of the AOH1 and AOX1 proteins, although the levels of the two enzymes are much lower than those observed in liver. DBA/2 mice do not synthesize significant quantities of AOH1 in the respiratory organ. The lung of the DBA/2 strain contains smaller quantities of AOX1 than those observed in the corresponding organ of CD-1 animals. However, the reduction of AOX1 is less evident...
than that of AOH1, as indicated by longer exposures of the Western blot presented in Fig. 3A.

AOH2, a third gene of the molybdo-flavoenzyme cluster located on mouse chromosome 1 is expressed almost exclusively in the epidermis and in other keratinized epithelia (13). As shown in Fig. 3B, a specific AOH2 band of the same apparent size as that of the corresponding recombinant protein is present in skin extracts of CD-1 animals. A similar band is not detectable in extracts obtained from DBA/2 and CBA mice.

Testosterone Up-regulates the Expression of the AOH1 Gene and the Corresponding Protein in CD-1 but Not in DBA/2 Mice—In CD-1 mice, the AOH1 and AOX1 proteins are synthesized at much higher levels in the hepatic tissue of male than female animals (5, 13). Treatment of female mice with testosterone results in increased synthesis of the two proteins (5, 13). Consistent with these data, Fig. 4A demonstrates that the BOX activities associated with AOX1 and AOH1 are more elevated in male than female CD-1 animals, whereas the hypoxanthine oxidase activity associated with XOR is similar in the liver of both genders. Administration of testosterone to female mice results in a consistent increase in the levels of AOH1- and AOX1-dependent BOX activities. In DBA/2 mice, the male steroid hormone has an inducing action on AOX1-dependent BOX activity in both female and male animals. By contrast, testosterone treatment does not result in the appearance of AOH1-dependent BOX activity in either male or female mice. The results obtained at the enzymatic level are entirely confirmed by the Western blot experiments presented in Fig. 4B. As expected, the steady-state levels of the AOH1 and AOX1 immunoreactive protein are increased by testosterone in female but not in male CD-1 livers, and the amounts of XOR protein are similar in all the experimental conditions described. By contrast, the cytosol of DBA/2 livers is devoid of AOH1 immunoreactivity regardless of the gender considered and the presence of testosterone. As to AOX1, in this mouse strain, the protein is always induced by testosterone.

Fig. 4C indicates that testosterone does not induce the AOH1 protein in female and male DBA/2 mice because it does not lead to a significant accumulation of the corresponding transcript. This is different from what was observed in female CD-1 mice, where the steroid hormone causes a ~3-fold increase in the
levels of the AOH1 mRNA. In the case of AOX1, we observed an almost 3-fold increase in the steady state levels of the transcript following treatment of female CD-1 and DBA/2 mice with testosterone. Testosterone up-regulates the expression of the AOX1 transcript in male CD-1 mice by 1.5-fold, whereas it has a small though significant down-regulatory action on the mRNA in the DBA/2 strain. The mRNA effects documented in testosterone-treated CD-1 and DBA/2 animals suggest that both transcriptional and translational mechanisms concur to the definition of the steady-state levels of the protein also in the case of AOX1.

The AOH1 Gene of DBA/2 Mice Codes for Very Low Levels of a Catalytically Active Protein—The deficit of AOH1 mRNA expression observed in the liver of DBA/2 mice is not absolute. In fact, it is possible to amplify an AOH1 cDNA from the poly(A⁺) fraction of hepatic RNA by the sensitive RT-PCR
TABLE I

Nucleotide and amino acid polymorphism in AOH1 isolated from the liver of different mouse strains

The coding regions of AOH1 mRNAs from CD-1 and DBA2 livers were amplified by RT-PCR and subcloned in pBluescript. Inserts were sequenced in both directions with the use of an automatic sequencer. The table indicates the nucleotide polymorphisms (in boldface) observed in CD-1 and DBA2 mice relative to what determined and published for the C57BL/6 strain (13). Amino acid residues are indicated in italics.

| Nucleotide/amino acid | AOH1 (C57Bl) | AOH1 (CD-1) | AOH1 (DBA2) |
|-----------------------|--------------|-------------|-------------|
| 708                   | AGT          | AGT         | ATT         |
| 170                   | Ser          | Ser         | Ile         |
| 827                   | CTC          | CTC         | TCT         |
| 210                   | Leu          | Leu         | Leu         |
| 847                   | CTT          | CTC         | CTT         |
| 217                   | Leu          | Leu         | Leu         |
| 991                   | CTC          | CTC         | CTC         |
| 264                   | Leu          | Leu         | Leu         |
| 1123                  | GCG          | GGA         | GGC         |
| 398                   | Ala           | Ala         | Ala         |
| 1345                  | GCA          | GCA         | GCC         |
| 382                   | Ala           | Ala         | Ala         |
| 1479                  | GCC          | GTC         | GTC         |
| 427                   | Ala           | Val         | Val         |
| 1565                  | ATT           | ATT         | GTT         |
| 456                   | Ile           | Ile         | Val         |
| 1765                  | TCT          | TCT         | TCT         |
| 522                   | Phe           | Phe         | Phe         |
| 1787                  | TTG          | GTG         | GTG         |
| 530                   | Leu           | Val         | Val         |
| 1974                  | CAC           | CTC         | CAC         |
| 592                   | His           | Leu         | His         |
| 2077                  | TCT           | TCT         | TCT         |
| 626                   | Ser           | Ser         | Ser         |
| 2253                  | GCC           | GTC         | GGC         |
| 685                   | Ala           | Val         | Ala         |
| 2475                  | CAA           | CAA         | CAG         |
| 752                   | Gln           | Gln         | Gln         |
| 2860                  | TAT           | TAC         | TAT         |
| 869                   | Tyr           | Tyr         | Tyr         |
| 3782                  | GCT           | GCT         | ACT         |
| 1195                  | Ala           | Ala         | Thr         |
| 3839                  | CTA           | CTA         | TTA         |
| 1214                  | Leu           | Leu         | Leu         |
| 3874                  | CTA           | CTA         | CTA         |
| 1225                  | Leu           | Leu         | Leu         |
| 3881                  | CTT           | CTT         | CTT         |
| 1228                  | Arg           | Arg         | Cys         |
| 3967                  | CCA           | CCA         | CCC         |
| 1256                  | Pro           | Pro         | Pro         |
| 3976                  | ATC           | ATC         | ATT         |
| 1259                  | Ile           | Ile         | Ile         |
| 3981                  | TCT           | TCT         | TCT         |
| 1261                  | Ser           | Ser         | Ser         |
| 3984                  | TCT           | TCT         | TCT         |
| 1262                  | Ser           | Ser         | Phe         |
| 4006                  | GGA           | GGA         | GGA         |
| 1269                  | Gly           | Gly         | Gly         |
| 4013                  | CTG           | CTG         | TGT         |
| 1272                  | Leu           | Leu         | Leu         |
| 4030                  | TTC           | TTC         | TTC         |
| 1277                  | Phe           | Phe         | Phe         |
| 4114                  | GCA           | GCG         | GCA         |
| 1305                  | Ala           | Ala         | Ala         |

techique. Thus, we cloned the entire coding region of the AOH1 cDNA amplified from the DBA2 liver and compared the deduced amino acid sequence with that of the CD-1 animal. The CD-1- and DBA2-derived AOH1 cDNAs demonstrate the presence of a number of different codons relative to the published AOH1 sequences, which were obtained in the C57BL/6 strain (12, 13) (Table I). Significantly, however, the DBA2-derived AOH1 cDNA does not contain missense or nonsense mutations that alter the open reading frame of the corresponding transcript or lead to the appearance of in-frame STOP codons. This demonstrates that the AOH1 deficit observed in DBA2 mice is not the consequence of the synthesis of a truncated protein that does not accumulate in the cells. Rather, the sequence data suggest that the AOH1 gene present in the DBA2 genome maintains the potential to synthesize residual amounts of a catalytically active protein that can be identified and isolated from the hepatic tissue. We tested this hypothesis and evaluated whether the amino acid differences observed in the DBA2-derived AOH1 sequence have any effect on the catalytic activity of the protein. For this purpose, we used the same purification method described for AOX1 (see below), and we isolated a small amount of semi-purified AOH1 enzyme from DBA2 liver extracts. In some fractions of the last chromatographic step, we demonstrated the presence of AOH1-associated BOX activity incompletely separated from the predominant AOX1 counterpart. As illustrated in Fig. 5, we quantitated the amounts of AOH1-derived BOX activity (as determined by the quantity of MTT reduced in the presence of benzaldehyde), and we related it to the amount of the associated immunoreactivity (as determined by quantitative Western blot analysis with anti-AOH1 antibodies), obtaining a numeric value that represents the specific activity of the enzyme in arbitrary units. The specific activity of the DBA2-derived AOH1 enzyme is 3.9 ± 0.6 arbitrary units (mean ± S.D. of the two independent preparations shown in Fig. 5). This is in the same range of the specific activity values calculated for the purified AOH1 from the liver of CD-1 mice (7.6 ± 0.6 A.U.).

These experiments were conducted under conditions of linearity relative to AOH1-associated BOX activity or immunoreactivity, as determined by appropriate calibration curves obtained with highly purified AOH1 protein from the liver of CD-1 mice. All these data demonstrate that DBA2 animals maintain the potential to synthesize trace amounts of a catalytically active AOH1 enzyme. In addition, they indicate that the observed variations in the amino acid sequence of the DBA2-derived AOH1 protein have only minor (if any) effects on the catalytic activity of the enzyme. Finally, they suggest that the observed deficit in AOH1 activity must be sought for in structural or regulatory abnormalities at the level of the AOH1 gene.

The Structure of the Promoter Region of the AOH1 Gene in DBA2 Is Different from That of the CD-1 Mouse but This Has No Functional Consequences—As a first step in the definition of the mechanisms underlying the functional deficit of these AOH1 gene in DBA2 mice, we looked for structural alterations. Southern blot experiments with probes corresponding to different portions of the AOH1 cDNA indicate that the general structure of the corresponding gene in CD-1 and DBA2 is identical, except for a few restriction site polymorphisms (data not shown). The only consistent structural difference between the CD-1 and DBA2 mice is at the level of the 5'-flanking region of exon 1, where we previously demonstrated the presence of an active promoter region (12). Starting from position −1496 relative to the first ATG of exon 1, the 5'-flanking region of the CD-1-derived AOH1 gene is characterized by the presence of an ~500-bp-long insertion, as illustrated in Fig. 6A. However, comparison of the constitutive promoter activity of the 5'-flanking regions of the CD-1- and DBA2-derived AOH1 genes does not show any significant difference in the ability to modulate the expression of a reporter gene such as bacterial β-galactosidase (Fig. 6B).

The Virtual Absence of AOH1 in the DBA2 Mouse Permits the Isolation of AOX1 in Pure Form—From a structural point of view, AOH1 and AOX1 are very similar proteins (1, 12, 13). This and the vast excess of AOH1 over AOX1 in the liver and lung of common outbred and inbred mouse strains, such as
CD-1 and C57/Bl, have always hampered any effort to purify the latter enzyme free from substantial AOH1 contamination. To compare the biochemical characteristics of AOH1 and AOX1, we thought to devise a method for the isolation of AOX1 from the liver of DBA/2 mice. Table II illustrates the protocol used for the purification of AOX1 in its catalytically active form and summarizes typical results. AOX1 was purified to homogeneity from the cytosolic fraction of liver following ammonium sulfate precipitation, affinity chromatography on benzamidine-Sepharose, and anion exchange chromatography on FPLC Mono-Q columns. The overall yield of purified AOX1 is 2.0–3.0%, as determined by measuring the enzymatic activity with phthalazine as the substrate (similar results were obtained with another AOX1 substrate like 2-hydroxypyrimidine (data not shown)). This compares very well with the recovery of purified AOX1 and AOH1 from bovine and mouse liver (5, 12, 18). Similar final yields are calculated from the immunological data described under “Experimental Procedures” and reported in Table II. This indicates that almost all DBA/2 liver phthalazine oxidizing activity can be accounted for by AOX1 and suggests that the majority of the enzyme is in its catalytically active form.

Fig. 7A shows a typical chromatogram of the last purification step demonstrating that AOX1 elutes in six fractions, as determined by the measurement of BOX activity and Western blot analysis. As already mentioned, the AOX1 purification experiments demonstrate that AOH1 is not completely absent in the livers of DBA/2 mice. In fact, residual amounts of AOH1 immuno-reactive protein and associated BOX activity can be eluted from the Mono-Q column in fractions partially overlapping those containing AOX1. However, in repeated experiments, at least 70% of the purified AOX1 can be eluted from the Mono-Q columns.
Fig. 6. Sequence and functional activity of the 5’-flanking regions of the CD-1 and DBA/2 AOH1 genes. A, the sequence of the 129/Sv AOH1 gene (12) is shown in lowercase letters and serves as a reference. The sequence presented in boldface letters within two vertical lines represents the DNA insertion observed upon sequencing of the 5’-flanking region of the CD-1 AOH1 gene. The nucleotides above the sequence indicate polymorphic sites identified in the AOH1 gene of the CD-1 mouse, whereas those below the sequence indicate similar sites identified in the corresponding gene of the DBA/2 animal. The boxed nucleotides marked with vertical arrowheads indicate residues that are not present in the DBA/2 sequence. An extra adenine nucleotide at position 867 was sequenced in the CD-1 gene and is indicated. The stretch of nucleotides at positions 605–571 is substituted by the indicated sequence in CD-1 and DBA/2 animals. The nucleotide corresponding to the determined transcription start site (dot above the sequences) is numbered as +1. Upstream sequences are indicated by negative numbers, and exon sequences are underlined. Consensus sequences for the binding of known transcription factors are indicated by arrows above the sequence.

B, constructs with the bacterial β-galactosidase reporter gene under the control of no promoter sequence (NO), the cytomegalovirus enhancer/promoter (CMV), or the 5’-flanking regions of the AOH1 genes from DBA/2 (DBA-AOH1) and CD-1 (CD1-AOH1) mice were transfected along with a firefly luciferase expression plasmid in HEK-293 cells. β-Galactosidase and luciferase activities were measured in cell extracts 48 h following transfection. The results are the mean ± S.D. of three replicate culture dishes and are expressed in arbitrary units following normalization of the data for the luciferase activity, which serves as an indicator of the transfection efficiency. The results shown are representative of four independent experiments.
Liver (34 g) from DBA/2 mice were homogenized in 142 ml of buffer and ultracentrifuged to obtain a cytosolic extract (Ultra), which was processed as indicated. Enzymatic activity was measured as the ability of the various purification fractions to oxidize phthalazine and contemporaneously reduce potassium ferricyanide. The reduction of potassium ferricyanide was monitored at 420 nm. One unit of enzymatic activity is defined as 1 nmol of phthalazine oxidized per min. The amount of AOX1 immunoreactive protein was determined by quantitative Western blot and is defined in relative units (see “Experimental Procedures”). The results are representative of at least four separate AOX1 preparations. S.A., specific activity; P.F., purification factor.

**Table II**

Purification scheme of mouse liver AOX1

| Step                  | Volume (ml) | Protein (mg) | Total Units | S.A. (units/mg) | P.F. | Yield (%) |
|-----------------------|-------------|--------------|-------------|-----------------|------|-----------|
| Ultra                 | 142         | 4300         | 13,432      | 3.1             | 1.0  | 100.0     |
| 55 °C                 | 138         | 3600         | 10,070      | 2.8             | 0.9  | 74.9      |
| Ammonium sulfate      | 138         | 2500         | 5546        | 2.2             | 0.7  | 41.2      |
| Benzimidine-Sepharose | 6.2         | 7.7          | 569.2       | 73.9            | 23.8 | 4.2       |
| Mono Q                | 1.0         | 0.4          | 324.0       | 753.5           | 243.1| 2.4       |

**Fig. 7. Purification of AOX1 from the livers of male DBA/2 mice.** The left panel of A shows a representative chromatogram of the last purification step (Mono-Q chromatography) of mouse liver AOX1. The grid indicates the fractions used for the SDS-PAGE with subsequent Coomassie Blue staining (Coomassie), the zymogram analysis of AOX1- and AOH1-associated BOX activities, as well as the Western blot analysis of AOX1 or AOH1 immunoreactivity showed on the right panel. B shows a representative MALDI-TOF analysis of the tryptic peptides deriving from the 1 AOH1 and AOX1 proteins purified from the liver of CD-1 and DBA/2 mice, respectively. Adjusted intensity = a.i.; mass/charge = m/z.

column free from significant AOH1 contamination. Upon gel electrophoresis in reducing conditions, purified AOX1 appears in the form of a single band with an apparent molecular mass of 150 kDa, which is consistent with the calculated mass of the monomeric subunit of the enzyme. Fig. 7B shows the MALDI-TOF mass spectrometric analysis of the tryptic digest of puri-
AOX1 and AO1 proteins purified from the liver of CD-1 and DBA/2 mice were trypsinized and subjected to MALDI-MS analysis. For MALDI-MS, the analysis was limited to tryptic peptides with a molecular mass equal to or superior to 800 Da. The data in the 1st column are the mass values obtained experimentally ($M^+ / H^{+}$). The results in the 2nd column are those calculated from the tryptic fragmentation of the AO1 and AOX1 gene products ($M^+ / H^{+} +$ cal). The 3rd column indicates the number of the first and last amino acid of the identified AO1 and AOX1 peptides. The 4th column shows the corresponding amino acid sequences.

| $M^+ / H^{+}$ | $M^+ / H^{+}$ | Identification | Sequence |
|---------------|---------------|----------------|----------|
| AO1           |               |                |          |
| 861.3         | 861.4         | 979–984        | CSYFESR  |
| 892.5         | 892.5         | 98–104         | LHPIQGER |
| 921.4         | 921.4         | 967–973        | ALSECNR  |
| 995.4         | 995.5         | 991–998        | FNAENSKW |
| 1045.5        | 1045.5        | 938–946        | CGLSPQVR |
| 1057.5        | 1057.5        | 1060–1068      | PMSSVHLR |
| 1096.5        | 1096.6        | 907–916        | TNLPSNTALR|
| 1169.5        | 1169.6        | 420–428        | KWEFVSAR |
| 1259.5        | 1259.7        | 388–397        | RIPLSEEFLR|
| 1401.6        | 1401.7        | 433–445        | QNIALAVNSGMR|
| 1453.6        | 1453.7        | 150–161        | CTGVRYPPYDACK|
| 1590.7        | 1590.8        | 19–31          | NVDPEMLLPLYLR|
| 1669.9        | 1670.0        | 3137–1333      | MIRDEPQSIVFVPINPV|
| 1821.0        | 1822.1        | 130–149        | NHPETPLQDLTDALGGNLCR|
| 1847.2        | 1847.3        | 399–419        | CPEADLKPQEVLVSVNIPWSSR|
| 2048.3        | 2048.4        | 278–300        | GVNPNIISPHEREELGVISQAR|
| 2094.5        | 2094.6        | 399–420        | CPEADLKPQEVLVSVNIPWSSR|

AOX1 and AO1 Oxidize the Same Substrates with Different Efficiencies—We determined the ability of AO1 and AOX1 to oxidize a number of endogenous and exogenous compounds that had been shown previously (28–34) to represent aldehyde oxidase and/or aldehyde dehydrogenase substrates. The majority of the compounds considered is of physiological, pharmacological, or toxicological importance. In particular, acetaldehyde is the oxidation product of ethanol and is involved in the liver damage observed in individuals chronically exposed to alcohol (35, 36). Butyraldehyde is a catabolite of butyryl-CoA (www.expcase.org/cgi-bin/search-biochem-index), and retinaldehyde is the direct precursor of the vitamin A active metabolite retinoic acid (37, 38). As shown in Table IV, upon incubation with purified AO1 and AOX1, benzaldehyde and retinaldehyde are rapidly oxidized and reduce the electron acceptor potassium ferricyanide. Similarly the simple alkyl-aldehyde butyric acid and the heterocyclic compound 2-hydroxypyrimidine are oxidized by both AOX1 and AOH1. With the exception of retinaldehyde, AOX1 is more efficient than AOH1 in oxidizing the various substrates, as indicated by the higher $V_{max}$ value. All these substrates have an affinity for the two enzymes that is in the low micromolar range, as demonstrated by the relative $K_n$ values. Benzaldehyde has the highest affinity, whereas retinaldehyde shows the lowest affinity for AOX1 and AOH1. By far the compound with the lowest affinity for the two oxidases is acetaldehyde, whereas a number of alcohols, such as ethanol, propanol, isopropyl alcohol, and butanol, are not metabolized by either AOX1 or AOH1 to a significant level.
the case of AOX1. In fact, 0.5 μM menadione inhibits AOH1 by over 80%, whereas only an approximate 50% inhibition is observed in the case of AOX1. As expected, menadione is a poor inhibitor of bovine XOR, when the chemical is used at concentrations ranging from 0.5 to 5 μM. High concentrations of benzamidine (1 mM) are necessary to inhibit both AOH1 and AOX1 to a significant level. However, even under these conditions, the degrees of inhibition are ~50 and 30% in the case of AOH1 and AOX1, respectively. At neither concentration considered does benzamidine inhibit XOR activity. Consistent with what is reported in the literature (43), allopurinol is a relatively strong inhibitor of bovine XOR, when the chemical is used at concentrations ranging from 0.5 to 5 μM. Inhibitory effect of norharmane on the two aldehyde dehydrogenases, a group of enzymatic activities known to oxidize ethanol to acetaldehyde and acetaldehyde to acetic acid, respectively. To determine aldehyde dehydrogenase activities, a group of enzymatic activities known to oxidize ethanol to acetaldehyde and acetaldehyde to acetic acid, respectively. To determine aldehyde dehydrogenase activities, a group of enzymatic activities known to oxidize ethanol to acetaldehyde and acetaldehyde to acetic acid, respectively.

**TABLE IV**

Substrate specificity of AOH1 and AOX1

| Substrate       | V_max (μmol/min/mg) | K_m (μM) | V_max/K_m (μmol/min/mg) |
|-----------------|---------------------|----------|-------------------------|
| Benzaldehyde    | 1.96 ± 0.11         | 12 ± 2   | 163                     |
| Butyraldehyde   | 2.85 ± 0.27         | 21 ± 6   | 136                     |
| Acetaldehyde    | 0.25 ± 0.03         | 818 ± 309| 0.3                     |
| Retinaldehyde   | 0.40 ± 0.03         | 70 ± 17  | 5.8                     |
| 2-OH-pyrimidine | 1.71 ± 0.18         | 173 ± 7  | 10                      |
| Ethanol         | ND                  | ND       | ND                      |

**TABLE V**

Inhibition of AOH1 and AOX1 by established molybdo-flavoenzymes inhibitors

| Inhibitor      | Concentration | % inhibition |
|----------------|---------------|--------------|
| Menadione      | 0.5 μM        | 83 ± 1       |
|                | 1 μM          | 86 ± 1       |
|                | 5 μM          | 87 ± 1       |
| Norharmane     | 5 μM          | 78 ± 2       |
|                | 20 μM         | 87 ± 1       |
| Benzamidine    | 1000 μM       | 58 ± 7       |
| Allopurinol    | 100 μM        | <5           |

**Fig. 8**

For this reason, the inhibitory effect of norharmane on the two aldehyde oxidases was studied in greater detail. As shown in Fig. 8A, the inhibition curves of norharmane on 2-hydroxypropyrimidine oxidizing activities of AOH1 and AOX1 have typical sigmoidal shapes. On the basis of these data, we calculated K_v values for AOH1 and AOX1 that differ by approximately 2 orders of magnitude (the K_v for AOH1 is 0.7 μM (interval of confidence 0.5–1.0, p < 0.05, n = 3) and for AOX1 is 32 μM (32–81, p < 0.05, n = 3). Fig. 8B indicates that norharmane is a non-competitive inhibitor of AOH1. In fact the double-reciprocal plot presented demonstrates that incubation of the enzyme with norharmane (1 μM) decreases the V_max of AOH1, when 2-hydroxypropyrimidine is used as a substrate. By contrast, under the same experimental conditions, the K_v of AOH1 for the substrate is left unaltered. Thus, our data suggest that norharmane binds to a site of AOH1 that is different from that of the pyrimidine substrate.

**AOH1 and AOX1 Do Not Play a Significant Role in the Liver Metabolism of Acetaldehyde in Vivo**—Given the purported role of AOX1 in the metabolism of acetaldehyde and the potential of both AOH1 and AOX1 to oxidize the chemical in vitro, albeit relatively inefficiently, we deemed it interesting to perform an in vivo study on CD-1 and DBA/2 mice treated with ethanol.

For this purpose, we made sure that the two strains of mice have comparable amounts of alcohol dehydrogenases and aldehyde dehydrogenases, a group of enzymatic activities known to oxidize ethanol to acetaldehyde and acetaldehyde to acetic acid, respectively. To determine aldehyde dehydrogenase activities, hexanaldehyde was used, as it is more stable than acetaldehyde and an equally good substrate for this type of enzymes. In the case of alcohol dehydrogenase activities, we used ethanol. As shown in Fig. 9A, upon zymogram analysis, liver extracts from four CD-1 and DBA/2 mice do not show specific bands characterized by hexanaldehyde or ethanol oxidizing activity in the absence of exogenously added NAD. Addition of NAD to the agarose overlay leads to the appearance of at least three bands characterized by hexanaldehyde oxidizing activity in both CD-1 and DBA/2 livers. Two prominent cathodic bands and one anodic band are separable under these electrophoretic conditions. The three bands have comparable intensity regardless of the fact that they originate from CD-1 or DBA/2 liver extracts. A single anodic band with ethanol oxidizing activity is evident upon addition of the nicotinamide cofactor. Again, the band is of similar intensity in all the liver extracts, regardless of the
mouse strain considered. The comparable strength of the hypoxanthine-oxidizing bands associated with XOR activity observed in all samples indicate that similar amounts of protein were loaded in each lane of the cellulose acetate plate. Altogether, the results indicate that DBA/2 and CD-1 mice do not differ significantly for the content of hepatic alcohol and aldehyde dehydrogenases.

Next, we evaluated whether the level of acetaldehyde varies significantly in the liver of DBA/2 and CD-1 mice treated with a single dose of ethanol intraperitoneally. As shown in Fig. 9B, 15 min following ethanol administration, the levels of acetaldehyde are 6–8-fold higher than those observed in saline-administered control animals regardless of the strain considered. No statistically significant difference in the basal or post-ethanol-associated levels of acetaldehyde was evident between CD-1 and DBA/2 mice. These data do not support a role for either AOH1 or AOX1 in the metabolism of acetaldehyde following an ethanol overload.

**DISCUSSION**

AOX1 and AOH1 are structurally similar molybdo-flavoenzymes, have an almost identical pattern of tissue- and cell-specific expression, and are regulated in the same fashion by testosterone (4, 12, 13). So far, AOH1 has been described only in the mouse, although it is also present in the rat4 and possibly in other animal species. With reference to this last point, it is unclear whether humans synthesize a protein orthologous to AOH1, although they are known to produce an AOX1 orthologue (1). The amino acid sequences of mouse AOX1 and AOH1 are more than 60% identical, and the two proteins are synthesized in the hepatocytic component of the livers of adult mice in large quantities. Smaller but significant amounts of both AOX1 and AOH1 are synthesized also in the lung and in the spermatogonia (13). In the adult liver of common laboratory mouse strains such as CD-1 and C57BL/6, AOH1 is quantitatively the prevalent enzyme.

Very little is known regarding the comparative biochemistry, enzymology, and substrate specificity of mouse AOX1 and AOH1. Indeed, most of the past literature reporting on the biochemical characteristics of rodent AOX1s is biased by the fact that authors are likely to have worked with mixtures of AOH1 and AOX1. All this is the result of a number of problems associated with the study of the two enzymes: first, AOH1 is a recently identified protein (12, 13); second, AOH1 and AOX1 are very difficult to separate from each other; third, it is hard to express recombinant AOH1 and AOX1 in their catalytically active forms with the use of common heterologous systems. The homeostatic functions and the endogenous substrate(s) of AOX1 and AOH1 are equally ill defined.

In this report, we demonstrate that two commonly available mouse strains like DBA/2 and CBA are characterized by an almost complete functional deficit in the expression of AOH1. This deficit was studied accurately in DBA/2 mice. These inbred animals synthesize amounts of liver AOH1 that are at least 2 orders of magnitude lower than those observed in the outbred CD-1 and the inbred C57BL/6 strains. The deficit is not the result of a non-sense mutation leading to an arrest of the translation of the AOH1 protein. In fact, trace amounts of catalytically active AOH1 can be isolated from the liver of DBA/2 mice. In DBA/2 livers, the decrease in the AOH1 enzymatic activity correlates with the decrease in AOH1 immunoreactivity. Our data demonstrate that the DBA/2 animal synthesizes dramatically reduced amounts of an AOH1 protein that has a specific activity very similar to that synthesized by the CD-1 or the C57BL/6 strain of mice. Sequencing of the cDNAs isolated from the liver of DBA/2 and CD-1 mice documents the presence of a few amino acid differences in the two protein products. However, these differences do not significantly affect the catalytic activity of the DBA/2-derived oxidase, as transient transfection of the corresponding cDNA in a suitable host cell line results in expression of AOH1 enzymatic activity. Interestingly, molecular modeling of AOH1 against the known XOR crystal structure (44) indicates that the amino acid differences observed fall within domains that are predicted to have minor (if any) effects on the overall structure of the substrate binding domain.  

Although we cannot formally exclude post-transcriptional events, the DBA/2 deficit of AOH1 is likely to be the consequence of a deficient transcription of the corresponding gene that leads to a dramatic decrease in the steady-state levels of the mature mRNA. Obvious areas where to look for structural alterations that may affect the transcriptional activity of the AOH1 gene are the two 5′-flanking regions of exons 1 and 1bis,

4 M. Terao and E. Garattini, unpublished results.
containing distinct and functionally active promoter sequences (12). The position of exon 1bis within intron 26 is very unusual, and equally unusual is the transcriptional direction of the very exon that is opposite that of all the other elements of the AOH1 gene (12). However, no sequence differences in the DNA regions contained between exon 27 and exon 1bis of DBA/2 and CD-1 mice were observed. Thus, it is likely that the promoter upstream of exon 1bis is equally active in the two strains of mice. The situation is different in the 5’-flanking region of exon 1. In CD-1 mice the region is ~500 bp longer than in DBA/2 mice, and this is the consequence of the presence of an insertion within a highly repetitive sequence located upstream of the core promoter. The insertion contains a number of potential binding sites for transcription factors. However, it does not seem to modulate the basal transcriptional activity of the promoter, at least on the basis of experiments performed on constructs containing appropriate reporter genes. All these results suggest that sequence variations at the level of the two AOH1 promoters are not at the basis of the reduced expression of the gene in DBA/2 mice, although it is possible that structural alterations of other yet uncharacterized regulatory elements located within the context of the long and complex AOH1 locus explain the observed inter-strain differences of expression. However, it is more likely that suppression of AOH1 transcription is the result of a still unidentified gene-silencing effect due to DNA or chromatin modification events, such as methylation and/or deacetylation (45). With respect to this, Southern blotting experiments conducted with methylation-sensitive enzymes on CD1, 129/Sv, DBA/2 and CBA genomic DNA, with the use of a probe recognizing the AOH1 promoter region upstream of exon 1, indicate a different level of DNA methylation in the four strains of mice considered. In fact, the DNA from the AOH1-positive CD1 and 129/Sv strains is cleaved with the methylation-sensitive HpaII enzyme, whereas the DNA of the AOH1-negative DBA/2 and CBA mice is resistant to cleavage with the restriction enzyme. This suggests a higher level of methylation in the AOH1 promoter region of the DBA/2 and CBA mice relative to the CD1 and 129/Sv counterparts. Thus DNA methylation effects may underlie the observed AOH1 deficit in DBA/2 and CBA animals. Currently, we are conducting more refined experiments aimed at supporting these observations. Nevertheless, it must be emphasized that the situation may be rather complex. In fact, the expression deficit of AOH1 in the liver of DBA/2 and CBA mice is accompanied by an equivalent suppression of the synthesis of AOH2, another structurally related molybdo-flavoprotein, which is present in the epidermal component of the skin. Furthermore, a much less dramatic but significant reduction in the levels of liver AOX1 in DBA/2 and CBA animals has also been documented in this report. As AOH1, AOX1, and AOH2 are coded for by distinct genes clustered at a very short distance from one another on mouse chromosome 1, it is possible that DBA/2 mice are characterized by a complex deficit differentially affecting the expression of the various members of the gene cluster. This suggests that the transcription of the whole aldehyde oxidase gene cluster on mouse chromosome 1 is finely tuned by common regulatory elements. In DBA/2 mice, it is interesting to notice that not only is the basal level of transcriptional activity reduced but also the response of the AOH1 gene to an external stimulus like testosterone.

The almost complete deficit in the expression of AOH1 (and AOH2) in the DBA/2 and CBA mouse is not accompanied by an overt phenotype. This indicates that either the expression of AOH1 is dispensable for the homeostasis of the two strains of mouse or the still unrecognized metabolic pathway in which the enzyme is involved is robust and can withstand the deficit. With respect to this, it is entirely possible that a significant function of the enzyme can be unmasked only under conditions that are stressful for the animal and/or different from those of an animal house facility. It is also plausible that the minute amounts of AOH1 observed in the livers of DBA/2 and CBA mice are enough to prevent a functional deficit. Finally, these
mouse strains may have developed efficient compensatory circuitry to overcome the almost absolute deficit of AOH1 activity. Most interesting, in DBA/2 mice, we did not observe a compensatory up-regulation of the expression of the AOX1 gene, which is indeed less active than in the control CD-1 or C57BL/6 animals. Nevertheless, our results raise the question as to why certain strains of mice express high quantities of AOH1 whereas others do not. In this context, it would be relevant to establish whether the most common phenotype in the wild population of mice is the AOH1-high typical of the CD-1 and C57BL/6 or the AOH1-low typical of the DBA/2 and CBA experimental animals.

Regardless of the reasons underlying the presence of distinct mouse populations characterized by different levels of AOH1 expression, the identification of the molybdo-flavoprotein-related phenotype in DBA/2 mice was useful in at least two respects.

First, the very low level of AOH1 allowed us to purify and to compare the substrate specificities and the inhibition profiles of AOH1 and AOX1. Our data demonstrate that the two enzymes are not only structurally (12, 13) but also biochemically very similar. In fact, AOH1 and AOX1 oxidize various exogenous and endogenous compounds at similar rates and with similar affinities. The observed differences in terms of $K_m$ and $V_{max}$ for the substrates tested are relatively minor, although, in general, AOX1 seems to be a more efficient oxidase than AOH1. However, these results should be interpreted cautiously, as the activities of these enzymes are dependent on the molybdenum cofactor content and the level of molybdenum cofactor sulfuration (1). With respect to the last two points, it is worthwhile noticing that the $A_{280}/A_{450}$ ratio and the UV-visible spectra of our AOH1 and AOX1 protein preparations indicate that the two purified enzymes have at least a comparable molybdenum cofactor content. Both AOH1 and AOX1 have reasonable affinities for and efficiently oxidize two substrates of potential physiological significance, like retinaldehyde and butyraldehyde.

The possibility that retinaldehyde is a physiological substrate for AOH1 is intriguing, as the enzyme, unlike AOX1, is expressed in the liver during the development of the fetus (12, 13), and the retinoid is the direct precursor of retinoic acid, a well known morphogen (46). In certain cell types, such as the hepatocyte, it is conceivable that AOH1 competes for retinaldehyde with certain isomers of the aldehyde dehydrogenase family of proteins (47, 48) and plays a role in the control of the local levels of retinoic acid. Another interesting aspect of our comparison relates to the results obtained with norharman.

Our data demonstrate that the compound is a much more efficient inhibitor of AOH1 than of AOX1. This has two practical consequences. Norharmane may represent a useful tool in future studies aimed at defining the relative functional importance of AOH1 and AOX1. Most important, our data demonstrate that it is possible to identify selective inhibitors of AOH1 despite the remarkable similarity of this protein to AOX1 and other members of the molybdo-flavoenzyme family. By the same token, it can be envisaged that specific substrates of AOH1 and AOX1 may be available in the near future.

Second, DBA/2 mice allowed us to demonstrate that AOH1 and AOX1 do not play a significant role, in vivo, in the metabolism of acetaldehyde following an ethanol load. This suggests some caution as to the in vitro data reported in the literature, suggesting that aldehyde oxidases are involved in the liver damage consequence to chronic ingestion of ethanol (10, 11). In fact, given the in vitro and in vivo data discussed in this report, it is unlikely that AOH1 and/or AOX1 are responsible for the purported production of toxic superoxide from acetaldehyde during ethanol metabolism and subsequent lipid peroxidation as well as liver damage (10, 11). Nevertheless, our data do not rule out the possibility that AOH1 and/or AOX1 produce toxic radical species from the NADH generated by the oxidation of ethanol and acetaldehyde by the corresponding dehydrogenase, as suggested by Mira et al. (11).

In conclusion, the DBA/2 and CBA phenotypes described in this report are likely to be instrumental in the future definition of the functional role of AOH1 and AOH2 in vivo. Furthermore, given the virtual absence of AOH1 from the liver of the DBA/2 and CBA mice, the two strains of experimental animals are likely to represent unique models of the human situation in drug metabolism studies.

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Molybdo-protein Deficiencies in DBA/2 Mice

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