Rat Liver Constitutive and Phenobarbital-Inducible Cytosolic Aldehyde Dehydrogenases Are Highly Homologous Proteins That Function as Distinct Isozymes†

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ABSTRACT: Rat liver contains two class 1 aldehyde dehydrogenases (ALDHs): a constitutive isozyme (ALDH1) and a phenobarbital-inducible isozyme (ALDH-PB). Defining characteristics of mammalian class 1 ALDHs include a homotetrameric structure, high expression in liver, sensitivity to the inhibitor disulfiram, and high activity for the oxidation of retinal. It is often presumed that ALDH-PB is the rat ortholog of mammalian ALDH1, and the identity of rat ALDH-PB is commonly interchanged with ALDH1. In this study, we characterized recombinant rat liver cytosolic ALDH1 and ALDH-PB. Previous reports indicate that ALDH-PB is a homodimer; however, we found by mass spectrometry and gel electrophoresis that it is a homotetramer. ALDH1 mRNA was highly expressed in untreated rat liver, while ALDH-PB had very weak expression, in contrast to a previous report that ALDH-PB mRNA is expressed in untreated rat liver. Rat liver ALDH1 had a high affinity for retinal ($K_m = 0.6$ μM), while no oxidation by ALDH-PB could be detected with 20 μM retinal. ALDH1 was more efficient at oxidizing acetaldehyde, propionaldehyde, and benzaldehyde and was more sensitive to disulfiram inhibition. We conclude that rat liver ALDH1 is the ortholog of mammalian liver ALDH1. Furthermore, despite a high level of sequence identity and classification as a class 1 ALDH, ALDH-PB does not function like ALDH1. ALDH-PB is not merely an inducible ALDH1 isozyme; it is a distinct ALDH isozyme.

The aldehyde dehydrogenase (ALDH,[1] EC 1.2.1.3) family consists of enzymes that catalyze the NAD-dependent irreversible oxidation of a wide range of aldehyde substrates to the corresponding carboxylic acids (*1*−*3*). Numerous ALDH isozymes can be distinguished on the basis of tissue and subcellular distribution, physicochemical properties, substrate specificity, disulfiram sensitivity, and amino acid sequence (*2*). The ALDH isozymes are traditionally divided into three classes. Class 1 contains the homotetrameric constitutive (ALDH1) and phenobarbital-inducible (ALDH-PB) cytosolic forms; class 2 (ALDH2) consists of the homotetrameric constitutive mitochondrial forms, and class 3 (ALDH3) includes the homodimeric constitutive and inducible cytosolic and microsomal forms (*1*). As new ALDH isozymes are identified, many of them do not fit into any of the three ALDH classes (*3*−*6*) and a new nomenclature system has recently been proposed (*7*).

The sequences of ALDH1 isozymes from various species are more than 85% identical, and the isozymes share definitive characteristics. ALDH1 is a cytosolic enzyme with high activity in liver (*2*). The active form is a homotetramer composed of 55 kDa subunits (*8*, *9*). ALDH1 activity is very sensitive in vitro to disulfiram inhibition (*10*−*12*). The K_m values of ALDH1 for the prototypical substrates acetaldehyde and propionaldehyde are in the micromolar range (*2*, *13*, *14*). ALDH1 has high activity for the oxidation of retinal to the differentiation factor retinoic acid (*6*), which regulates the transcription of genes involved in cell differentiation and embryonic development (*15*).

The rat liver ALDH-PB isozyme is also classified as a class 1 ALDH (*1*). ALDH-PB is induced by phenobarbital treatment in responsive rats, causing an up to 10-fold increase in liver supernatant ALDH activity (*16*). Rat ALDH-PB is 83% identical to human ALDH1 and is speculated to be functionally similar to ALDH1 (*17*). Many investigators have found little or no basal acetaldehyde oxidizing activity in the cytosolic fraction of untreated rat liver (*18*−*21*). Thus,

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; ALDH1, class 1 constitutive cytosolic aldehyde dehydrogenase encoded by the *ALDH1A1* gene (*ALDH1A2* gene in rats and mice); ALDH-PB, class 1 phenobarbital-inducible cytosolic aldehyde dehydrogenase encoded by the *ALDH1A3* gene; ALDH2, class 2 mitochondrial aldehyde dehydrogenase encoded by the *ALDH2* gene; ALDH3, class 3 tumor-associated aldehyde dehydrogenase encoded by the *ALDH3A1* gene; UTR, untranslated region; PAGE, polyacrylamide gel electrophoresis; PATRIC, position- and time-resolved ion counter; μESI, microelectrospray ionization; *m*/*z*, mass-to-charge ratio; ϵ, extinction coefficient; MeDTC, *S*-methyl *N*,*N*-diethylthiocarbamate; IC_{50}, inhibitor concentration causing 50% inhibition.

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rat liver ALDH-PB is often considered the rat ortholog of mammalian ALDH1, and its identity is commonly interchanged with ALDH1 (*5*, *22*−*26*), despite ALDH-PB having high K_m values (in the millimolar range) for acetaldehyde and propionaldehyde (*27*, *28*). About 25 years after rat liver ALDH-PB was described, the cDNA encoding ALDH1 was isolated from rat kidney (*23*), rat testis (*29*), and rat liver (*30*). The rat ALDH1 sequence is 89% identical to that of rat ALDH-PB and 87% identical to that of human ALDH1. The recombinant rat testis ALDH1 has high activity for the oxidation of retinal with a K_m of 1.4 μM (*29*). On the basis of sequence homology and retinal activity, ALDH1 appears to be a good candidate for the rat ortholog of mammalian ALDH1.

To clarify functional differences between rat ALDH1 and ALDH-PB, we characterized the two recombinant rat liver proteins. We found that rat liver ALDH1 and ALDH-PB have different expression patterns, substrate preferences, and sensitivities to disulfiram. We also report for the first time to our knowledge that ALDH-PB is a homotetramer. Rat liver ALDH1 shares the definitive characteristics with mammalian ALDH1, and we conclude that rat ALDH1 is the ortholog of mammalian ALDH1. Furthermore, the functional differences between ALDH1 and ALDH-PB distinguish the two proteins as distinct isozymes.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis. A Sprague-Dawley rat multiple-tissue Northern blot (Clontech, Palo Alto, CA) was probed with rat ALDH1 and ALDH-PB specific probes. The blot contained approximately 2 μg of mRNA per lane. A portion of the ALDH1 3′-UTR corresponding to nucleotides 1660−1905 (relative to the adenosine of the ATG translation initiation codon) and nucleotides 1496−1884 of the ALDH-PB 3′-UTR were randomly labeled with [α-^{32}P]dCTP using the Oligolabelling Kit (Amersham Pharmacia Biotech, Piscataway, NJ). The blot was hybridized with the ALDH1 probe using ExpressHyb Hybridization Solution (Clontech) following the supplied protocol. The blot was then stripped and reprobed with the ALDH-PB probe. Film exposure times were 20 h for the ALDH1 probe and 93 h for the ALDH-PB probe.

Recombinant ALDH Expression and Purification. The open reading frame of the rat liver ALDH1 cDNA (*30*) was cloned into the pET-14b expression vector (Novagen, Madison, WI). The ALDH-PB open reading frame was amplified by the polymerase chain reaction from Sprague-Dawley rat liver Marathon-Ready cDNA (Clontech) with primers designed from the cDNA sequence (*17*). The clone was inserted into the pET-14b vector and sequenced on both DNA strands. Both ALDH clones were transformed into *Escherichia coli* BL21(DE3) and expressed with a six-residue histidine fusion tag at the amino terminus following the protocol in the *pET System Manual* (Novagen, 7th ed.). The expressed proteins were purified by metal chelation chromatography by employing a nickel column followed by removal of the histidine tag by cleavage with thrombin. The purified recombinant proteins were dialyzed against 20 mM sodium phosphate (pH 7.4), 0.1 mM dithiothreitol, and 1 mM sodium EDTA (pH 8.0) and stored in small aliquots at −80 °C.

Rat liver ALDH2 was expressed and purified as described for the human recombinant protein by Lam et al. (*12*). All protein concentrations were determined with the BCA Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Tissue Preparation. Adult male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were anesthetized with carbon dioxide and sacrificed by decapitation. Liver and kidney mitochondria were prepared in H-medium [0.07 M sucrose, 0.21 M D-mannitol, 2 mM HEPES, and 0.05% bovine serum albumin (pH 7.4)] as described by Pedersen et al. (*31*). The supernatants from the mitochondrial preparations were centrifuged at 100000*g* for 1 h to obtain the cytosolic fractions.

Western Blot Analysis. Rabbits were immunized (Cocalico Biologicals, Reamstown, PA) with a keyhole limpet hemocyanin-conjugated peptide (AQPAVPAPLANL) corresponding to residues 5−16 of the rat ALDH1 sequence. Recombinant rat liver proteins (0.3 μg of ALDH1, ALDH-PB, and ALDH2) and rat liver and kidney fractions (50 μg of cytosol and mitochondria) were subjected to SDS−PAGE and transferred to nitrocellulose. The rabbit antiserum was used at a dilution of 1:500. Chemiluminescent detection was performed with the ECL Western Blotting Analysis System (Amersham Pharmacia Biotech).

Mass Spectrometry Analysis. Protein sample solutions were prepared for mass spectrometric analysis by buffer exchange into 5 mM ammonium acetate using a Chroma Spin-10 gel filtration column (Clontech). All mass spectrometric analyses were performed on a Finnigan MAT 900 mass spectrometer (Bremen, Germany) consisting of an electrostatic analyzer (E) and magnet (B) with a position- and time-resolved ion counter (PATRIC) detector. All microelectrospray ionization (μESI)-mass spectrometry measurements were performed in the positive ion mode using a home-built source. Flow rates of 0.3 μL/min were used in conjunction with SF_6 introduced through the auxiliary port to prevent source corona discharge. The μESI source voltage was 3.9 kV with a capillary temperature of 180 °C. The magnet (B) was scanned from *m*/*z* 1000 to 8000 at a rate of 20 s/decade. The PATRIC detector was used for ion detection typically at about 850 V. Multiple scans were collected and summed, and the multiply charged spectra were transformed to give molecular mass values using the Finnigan MAT software.

Native Gel Electrophoresis. Recombinant rat liver ALDH1 (0.5 μg), ALDH-PB (0.5 μg), and ALDH3 (0.1 μg) were analyzed by native PAGE. The sample and running buffers contained no SDS or β-mercaptoethanol, and samples were not heated. Proteins underwent electrophoresis for 1 h at 200 V in a 4 to 15% linear gradient polyacrylamide gel (Bio-Rad, Hercules, CA). Separated proteins were stained with silver (*32*).

ALDH Activity Assays. ALDH activity toward acetaldehyde and propionaldehyde was assayed spectrophotometrically as the reduction of NAD at 340 nm in a microtiter plate reader using the method of Nelson and Lipsky (*33*). Reactions were performed at 24 °C in 0.05 M sodium pyrophosphate buffer (pH 8.8) with 0.5 mM NAD. Protein concentrations in the reaction mixtures were 13−25 μg/mL for ALDH1 and 19−25 μg/mL for ALDH-PB. Kinetics for the oxidation of acetaldehyde and propionaldehyde were measured from initial velocities utilizing both hyperbolic

plots and double-reciprocal linear plots. The ALDH monomeric molecular masses were used for calculating turnover numbers and catalytic efficiencies.

The initial velocities method could not be used to estimate the K_ms for retinal and benzaldehyde due to their low values. Therefore, the single-reaction progress curve method was employed using the simplified calculation formula for irreversible reactions (*34*). Assays with *all-trans*-retinal were performed at 24 °C in 0.02 M HEPES and 0.15 M potassium chloride (pH 8.5) with 0.5 mM NAD and 2−20 μM *all-trans*-retinal. *all-trans*-Retinal was dissolved in dimethyl sulfoxide which made up 1.3% of the final reaction volume. The enzyme concentrations were 7−9 μg/mL for ALDH1 and 5−25 μg/mL for ALDH-PB. The ALDH activity was assayed spectrophotometrically in a microplate reader by measuring the change in absorbance at 340 nm resulting from the formation of NADH ($\epsilon = 5657\ M^{-1}\ cm^{-1}$), the formation of retinoic acid ($\epsilon = 37\ 250\ M^{-1}\ cm^{-1}$), and the disappearance of retinal ($\epsilon = 17\ 911\ M^{-1}\ cm^{-1}$) with an apparent ϵ of 24 996 $M^{-1}\ cm^{-1}$. Extinction coefficients were measured in a Molecular Devices Thermomax microplate reader (Menlo Park, CA) in 225 μL of 0.02 M HEPES and 0.15 M potassium chloride (pH 8.5).

Benzaldehyde was dissolved in an 80:20 solution of 0.05 M sodium pyrophosphate buffer (pH 8.8) and methanol (methanol made up less than 1% of the final reaction volume). Reactions including ALDH-PB (5 μg/mL) were performed at 24 °C in 0.05 M sodium pyrophosphate buffer (pH 8.8) with 0.5 mM NAD and 9−30 μM benzaldehyde. The formation of NADH was followed spectrophotometrically for 20 min at 340 nm using a microplate reader. Reactions employing ALDH1 (25 μg/mL) were performed at 22 °C in 0.05 M sodium pyrophosphate buffer (pH 8.8) with 0.5 mM NAD and 0.5−1.8 μM benzaldehyde. NADH production was monitored using an Applied Photophysics model SX.18MV stopped-flow instrument (Leatherhead, U.K.) by measuring absorbance at 340 nm in a 1 cm optical cell.

Inhibition Assays. $MgCl_2$ was dissolved in 50 mM Tris-HCl (pH 7.5). $MgCl_2$ was mixed with ALDH (0.033 mg/mL), and reactions were initiated with the addition of propionaldehyde (1 mM) and NAD (0.5 mM). The formation of NADH was followed spectrophotometrically at 340 nm.

S-Methyl *N*,*N*-diethylthiocarbamate sulfoxide (MeDTC sulfoxide) was prepared as described by Mays et al. (*35*). Disulfiram and MeDTC sulfoxide were dissolved in methanol. The recombinant ALDHs were incubated with inhibitor (0.04−1 μM) or methanol (vehicle) in 0.05 M sodium pyrophosphate (pH 8.8) for 15 min at room temperature. The ALDH activity was measured spectrophotometrically as the production of NADH at 340 nm. The assay mixtures contained 0.9% methanol, 0.5 mM NAD, 23 μg/mL ALDH1 or 13 μg/mL ALDH-PB, and 1 mM acetaldehyde (for ALDH1) or 8 mM propionaldehyde (for ALDH-PB). IC_{50} values were calculated from concentration−inhibition curves.

RESULTS

Distribution of ALDH1 and ALDH-PB mRNAs in Untreated Rat Tissues. As we have described previously (*36*), Northern blot analyses with probes specific for a portion of the rat ALDH1 or ALDH-PB 3′-UTR show that the mRNAs have different distributions in untreated rat tissues. The ALDH1 mRNA was expressed strongly in lung, kidney, and liver, moderately in skeletal muscle and testis, and weakly in heart and brain and was not expressed in spleen (Figure 1A). In comparison, ALDH-PB mRNA was expressed very weakly in lung and liver and was not expressed in the other tissues that were examined (Figure 1B).

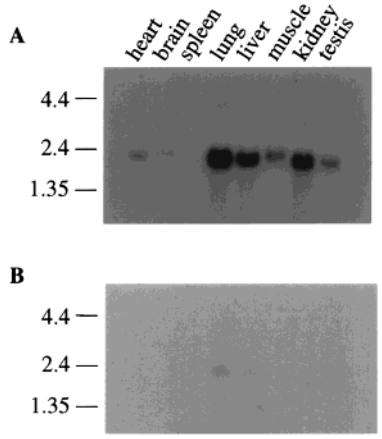

FIGURE 1: Tissue distribution of rat ALDH1 and ALDH-PB. (A) A rat multiple-tissue Northern blot (Clontech) was hybridized with a rat ALDH1 3′-UTR specific probe. The film was exposed for 20 h. (B) The blot was stripped and hybridized with a rat ALDH-PB 3′-UTR specific probe. The film was exposed for 93 h. Molecular size indicators are given on the left in kilobases. This figure was reprinted from ref *37* with the permission of Kluwer Academic/Plenum Publishers.

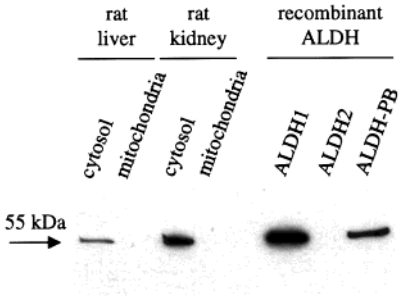

FIGURE 2: Expression of rat liver class 1 ALDH in rat liver and kidney cytosol. Cytosolic and mitochondrial fractions (50 μg) from untreated rat liver and kidney and recombinant ALDH proteins (0.3 μg) were separated by SDS−PAGE and transferred to nitrocellulose. Proteins were immunoblotted with antibodies raised against a rat ALDH1 amino-terminal peptide.

Detection of Class 1 ALDH in Untreated Rat Liver. Due to the questionable presence of ALDH1 in untreated rat liver (*18*−*21*), Western blot analysis of liver cytosol from an untreated rat was performed. Rat kidney was used as a control since it is known to contain ALDH1 (*23*, *37*). Several attempts to produce rabbit antiserum that could distinguish between recombinant rat liver ALDH1 and ALDH-PB were unsuccessful due to the 90% degree of amino acid identity between them. The antiserum used for Western blot analysis recognized recombinant ALDH1 and ALDH-PB, but did not recognize the recombinant ALDH2 (Figure 2). Both ALDH1 and ALDH-PB had molecular masses of about 55 000 Da. A protein with a similar molecular mass was detected in both rat liver and kidney cytosol, but not in rat liver or kidney mitochondria (Figure 2). Rat kidney cytosol contains more immunoreactive class 1 ALDH than rat liver cytosol. These results, in conjunction with the mRNA tissue expression

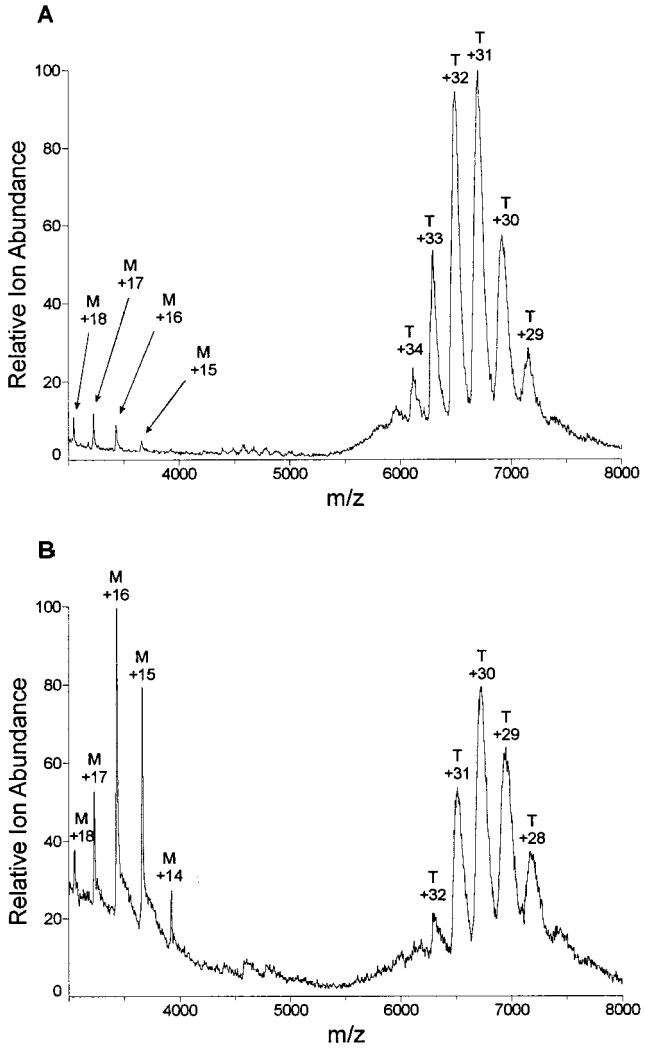

FIGURE 3: Mass spectrometry analyses of recombinant rat liver class 1 ALDH proteins. Positive ion μESI-mass spectrometry analyses of (A) recombinant rat liver ALDH1 and (B) recombinant rat liver ALDH-PB. M represents multiply charged ion species, accompanied by the charge state (e.g., +17), corresponding to the monomeric protein. T represents multiply charged ion species, accompanied by the charge state (e.g., +31), corresponding to the homotetrameric protein.

pattern, indicate that the ALDH1 protein is present in rat liver cytosol.

Mass Spectrometry Analysis. The μESI-mass spectrometry analyses of the recombinant rat liver ALDH1 and ALDH-PB both revealed two distinct multiply charged ion series labeled M and T corresponding to monomer and homotetramer, respectively (Figure 3). The transformed raw data (labeled M) for rat liver ALDH1 (Figure 3A) revealed a subunit molecular mass of 54 861 Da, whereas the transformed raw data (labeled M) for ALDH-PB afforded a molecular mass of 54 962 Da (Figure 3B). This compares with predicted molecular mass values of 54 871 Da for ALDH1 and 54 972 Da for ALDH-PB, which in both cases is within the experimental expectations of the ~0.02% error value. The predicted values for both proteins contain four additional amino acid residues (GSHM) that remained at the amino terminus after thrombin cleavage of the histidine tag.

Both proteins also afforded distinct multiply charged ion series at higher *m*/*z* values labeled as T in Figure 3. Transformation of the ion series revealed a molecular mass

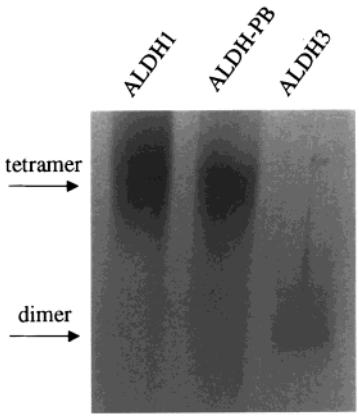

FIGURE 4: Native PAGE of recombinant rat liver class 1 and 3 ALDH proteins. ALDH1 (0.5 μg), ALDH-PB (0.5 μg), and ALDH3 (0.1 μg) were electrophoresed under nondenaturing conditions and stained with silver.

of ~219 440 Da for ALDH1 and a molecular mass of ~219 840 Da for ALDH-PB. Both values corresponded to homotetrameric protein complexes. There was no detectable presence of homodimeric complexes. Finally, the T:M ratio for ALDH1 was significantly greater than for ALDH-PB, indicating a relatively higher-affinity binding capability for the ALDH1 homotetramer. However, this may be due to the conditions employed during mass spectrometry analysis, as heat denaturation studies with 0.3 mg/mL protein at 46 °C showed no clear difference in stability between ALDH1 and ALDH-PB (data not shown).

Since it was previously reported that ALDH-PB was a homodimer (*1*, *38*), native PAGE was also performed on both ALDH1 and ALDH-PB protein samples. The homodimeric rat liver ALDH3 (*39*) was used as a reference. There were no apparent ALDH1 or ALDH-PB protein bands at positions similar to that of the ALDH3 dimer (Figure 4). The ALDH-PB protein migrated to the same position as ALDH1 (Figure 4), which is a homotetramer as determined by X-ray crystallography (*9*). These results support the mass spectrometry results which show that ALDH-PB is indeed a homotetramer.

Substrate Specificities. We examined the ability of rat liver ALDH1 and ALDH-PB to oxidize the substrates acetaldehyde, propionaldehyde, *all-trans*-retinal, and benzaldehyde. Table 1 presents the estimated K_m, the turnover number (k_{cat}), and the catalytic efficiency (k_{cat}/K_m) of ALDH1 and ALDH-PB for each substrate. Turnover numbers were calculated with the molecular masses of the ALDH subunits. ALDH1 is more efficient than ALDH-PB at oxidizing all four substrates. The catalytic efficiencies of ALDH1 for acetaldehyde, propionaldehyde, and benzaldehyde were 23-, 48-, and 12-fold higher, respectively, than that of ALDH-PB. While rat ALDH1 had a high affinity for retinal (K_m = 0.6 μM), no oxidation of up to 20 μM retinal by ALDH-PB could be detected.

Inhibition Assays. Magnesium inhibits cytosolic class 1 ALDH activity (*40*−*43*), while it enhances mitochondrial class 2 ALDH activity (*41*, *44*). The effect of magnesium on ALDH-PB is unknown. Therefore, we examined the effect of $MgCl_2$ on recombinant rat liver ALDH1 and ALDH-PB activity. $MgCl_2$ inhibited the activity of both ALDH1 and ALDH-PB (Figure 5). The inhibition was more pronounced for ALDH1 with $MgCl_2$ concentrations of $\geq$0.25 mM.

Table 1: Kinetic Constants of Recombinant Rat Liver Class 1 Aldehyde Dehydrogenase Isozymes[a]

enzyme	substrate	K_m	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
ALDH1	acetaldehyde	41 μM	0.16	3900
	propionaldehyde	5.5 μM	0.14	25500
	all-trans-retinal	0.6 μM	0.03	50000
	benzaldehyde	0.12 μM	0.12	1.0×10^6
ALDH-PB	acetaldehyde	2.4 mM	0.41	170
	propionaldehyde	1.6 mM	0.85	530
	all-trans-retinal	NA[b]	–	–
	benzaldehyde	4.7 μM	0.39	83000

[a] Enzyme activity was measured spectrophotometrically as the change in absorbance at 340 nm. Reactions were performed at pH 8.8 (acetaldehyde, propionaldehyde, and benzaldehyde) or pH 8.5 (retinal) at 24 °C. Kinetic values for the oxidation of acetaldehyde and propionaldehyde were measured from initial velocities, while those for retinal and benzaldehyde were measured from single-reaction progress curves. The molecular masses of the ALDH monomers were used to calculate k_{cat}. Values are the average of two or more independent determinations, where each point in the curve of each determination is the average of three replicates. [b] No activity was detected with retinal concentrations of $\leq$20 μM.

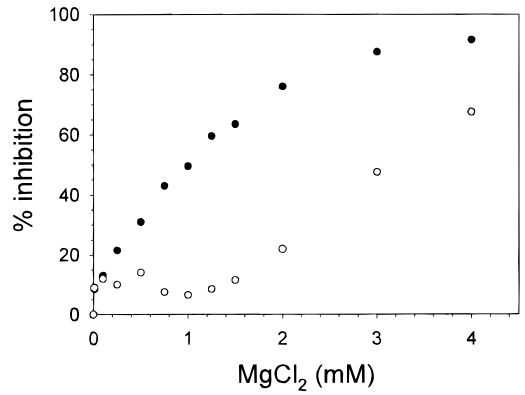

FIGURE 5: Inhibitory effect of $MgCl_2$ on recombinant rat ALDH1 (●) and ALDH-PB (○) activity. The assays consisted of ALDH (0.033 mg/mL), NAD (0.5 mM), and propionaldehyde (1 mM) at pH 7.5. Points are the average of two experiments, each carried out in triplicate.

We examined the inhibition of the recombinant ALDH1 and ALDH-PB enzymes by disulfiram and its metabolite MeDTC sulfoxide, both potent inhibitors of recombinant human ALDH1 (*12*). Disulfiram and MeDTC sulfoxide inhibited rat liver ALDH1 and ALDH-PB enzymes, under preincubation conditions, with IC_{50} values ranging from 0.13 to 0.40 μM (Table 2). ALDH1 was more sensitive than ALDH-PB to inhibition by disulfiram, while both enzymes were inhibited nearly equally by MeDTC sulfoxide. The potencies of both inhibitors were about equal toward ALDH1, while MeDTC sulfoxide was more potent than disulfiram toward ALDH-PB.

Table 2: Inhibition of Recombinant Rat Liver Class 1 Aldehyde Dehydrogenase Isozymes[a]

		IC_{50} (μM)	
enzyme	inhibitor	experiment 1	experiment 2
ALDH1	disulfiram	0.15	0.15
	MeDTC sulfoxide	0.16	0.24
ALDH-PB	disulfiram	0.38	0.40
	MeDTC sulfoxide	0.16	0.13

[a] Recombinant ALDH was incubated with inhibitor or methanol for 15 min. Reactions were initiated by the addition of NAD and acetaldehyde (for ALDH1) or propionaldehyde (for ALDH-PB). Enzyme activity was measured spectrophotometrically as the formation of NADH at 340 nm. IC_{50} values were calculated from concentration–inhibition curves. Enzyme inhibition was assessed in triplicate for eight or nine concentrations of inhibitor in the range of 0.04–1 μM.

DISCUSSION

The basal ALDH activity in untreated rat liver cytosol has been reported by many investigators as none or very little (*18*–*21*). The induction of ALDH activity in the liver cytosol of phenobarbital-treated rats was described in 1971 (*16*). The cDNA encoding liver ALDH-PB was cloned in 1989, and strong conservation was seen between the deduced amino acid sequence and human liver ALDH1, indicating that these proteins may be functionally similar (*17*). ALDH-PB is often presumed to be the rat ortholog of mammalian ALDH1, and the identity of ALDH-PB is commonly confused with ALDH1 (*5*, *22*–*26*). The recent cloning of a cDNA encoding ALDH1 from rat liver (*30*) has allowed us to compare the characteristics of the two recombinant rat liver class 1 ALDHs to clarify the confusion surrounding their identities.

We have previously shown that the distributions of ALDH1 and ALDH-PB mRNAs are different in untreated rat tissues (*36*). Rat liver exhibited strong expression of ALDH1 mRNA, while ALDH-PB was very weakly expressed (Figure 1). These results differ from those of Dunn et al. (*17*), who reported that ALDH-PB mRNA is expressed in hepatic as well as other tissues of untreated control rats. At the time of their study, the rat liver ALDH1 sequence was not known. The probes they used for Northern blot analysis could not distinguish between ALDH-PB and ALDH1. Therefore, it is possible that the mRNA they detected in untreated rat tissue was ALDH1 rather than ALDH-PB.

The patterns of ALDH1 mRNA expression in humans (*24*, *45*) and mice (*46*) resemble the distribution of rat ALDH1 more closely than the distribution of rat ALDH-PB. While rat ALDH-PB mRNA is only very weakly expressed in liver and lung (Figure 1B), ALDH1 mRNA is strongly expressed in rat, mouse, and human liver and in rat and mouse lung (human lung has low-level expression). Thus, expression patterns indicate that ALDH1 is more closely related than ALDH-PB to the human and mouse ALDH1 genes.

The tissue distribution of rat and mouse ALDH-PB mRNA in untreated animals is drastically different. We detected only a very low level of expression in rat liver and lung (Figure 1B); mouse liver and lung have high levels of expression and testis and kidney moderate levels of expression, and brain has a low level of expression (*46*). The 5′-flanking regions of rat and mouse ALDH-PB genes may explain these differences. When compared to human, mouse, and marmoset ALDH1 genes, the rat ALDH-PB 5′-flanking region has a 13-nucleotide deletion, part of which is contained in an octameric binding motif in the other genes (*25*). When these 13 nucleotides were deleted from the human ALDH1 5′-flanking region, the ability of the promoter region to drive transcription was severely diminished (*25*). Thus, the 13-nucleotide deletion, which does not appear in the mouse ALDH-PB gene (*46*), may explain our finding that rat ALDH-PB is not constitutively expressed.

We have shown that the class 1 ALDH protein is present in rat liver cytosolic preparations. The antiserum used for

Western blot analysis was unable to differentiate between ALDH1 and ALDH-PB (Figure 2). However, since the level of expression of ALDH-PB mRNA was very low in untreated rat liver compared to that of ALDH1 (Figure 1), it is likely that the class 1 ALDH detected in rat liver cytosol (Figure 2) is the ALDH1 protein. These data, in addition to the isolation of an ALDH1 cDNA clone from rat liver (*30*), clarify the controversy over the existence of this protein. An explanation for low or no measurable acetaldehyde oxidizing activity in rat liver cytosol preparations reported by other investigators (*18*−*21*) remains to be elucidated.

The purified recombinant rat liver ALDH1 and ALDH-PB were analyzed by mass spectrometry. ALDH1 and ALDH-PB were observed primarily in the monomeric and tetrameric forms (Figure 3). The crystal structure of ALDH1 reveals that it is a homotetramer (*9*). However, the multimeric nature of ALDH-PB is questionable. Rat liver ALDH-PB has been reported to be a homodimer (*1*, *38*) or possibly a homotrimer or larger oligomeric structure (*28*). To our knowledge, this is the first report that ALDH-PB is a homotetramer. Mammalian ALDH isozymes that terminate at position 500 (with respect to class 1 and 2 numbering) exist as tetramers, while the dimeric ALDH3 contains additional residues beyond position 500. ALDH-PB terminates at position 500, which suggests that ALDH-PB is a tetramer. Both our mass spectrometry and native PAGE results show that this is indeed the case.

The kinetic properties of the two rat liver class 1 ALDH enzymes were very different. ALDH1 had a greater catalytic efficiency than ALDH-PB for acetaldehyde, propionaldehyde, retinal, and benzaldehyde (Table 1). On the basis of kinetic studies, it has been suggested that retinal may be a physiological substrate of ALDH1 and a biological role of the enzyme may be the generation of retinoic acid, thereby modulating cell differentiation and embryonic development (*47*, *48*). Human, mouse, and sheep liver ALDH1 have K_m values of 0.06 (*47*, *48*), 0.7 (*49*), and 0.14 μM (*48*), respectively, for *all-trans*-retinal. Similarly, rat liver ALDH1 had a K_m for *all-trans*-retinal of 0.6 μM, while no oxidation by ALDH-PB could be detected with up to 20 μM *all-trans*-retinal (Table 1). Our results suggest that retinal is not a physiological substrate for ALDH-PB. Similar findings were recently reported for the mouse liver ALDH-PB enzyme, which was found to have no role in retinoic acid synthesis under physiological conditions (*46*). The biological role of ALDH-PB remains to be determined, as does the identification of a human ortholog of ALDH-PB.

Magnesium was seen to have an inhibitory effect on both recombinant rat ALDH1 and ALDH-PB (Figure 5). These results are consistent with NADH release as the rate-limiting step for ALDH-PB, as it is for ALDH1 (*43*). In contrast, magnesium enhances the activity of mitochondrial ALDH2 (*41*, *44*), for which the rate-limiting step is deacylation (*50*−*52*).

Disulfiram and its metabolite MeDTC sulfoxide were potent inhibitors of recombinant rat liver ALDH1 and ALDH-PB (Table 2). Rat liver ALDH1 was more sensitive than ALDH-PB to inhibition by disulfiram (Table 2). ALDH1 is also more sensitive than mitochondrial ALDH2 to the inhibitory effects of disulfiram (*12*). This has been explained by the three-dimensional structures of the two enzymes (*9*). The substrate entrance tunnel of ALDH1 is more open than that of ALDH2, allowing ALDH1 to interact more readily with the large, bulky disulfiram molecule. Our results suggest that the substrate entrance tunnel of ALDH-PB may be more narrow than that of ALDH1. This could also explain the difference observed between ALDH1 and ALDH-PB for retinal oxidation (Table 1) if the substrate entrance tunnel of ALDH-PB were too narrow to accommodate the bulky retinal molecule. This is further supported by the observation that ALDH1 had a greater catalytic efficiency than ALDH-PB for the aromatic substrate benzaldehyde.

Table 3: Comparison of Amino Acid Residues Expected To Line the Substrate Entrance Tunnel[a]

Enzyme	Amino Acid Sequence[b]	Number of Non-Hydrogen Side Chain Atoms
ALDH1	115 FANAYLSDLGGSIKA 129	50
ALDH-PB	115 FTHAYLLDTEVSIKA 129	64
ALDH1	170 NFPLLMFIW 178	47
ALDH-PB	170 NGPLILFIW 178	40
ALDH1	293 HGVFYHQGQCCV...L 428	51
ALDH-PB	293 QGVFFHQGQICV...L 428	51
ALDH1	455 NCYMILSAQCPF...EL 478	55
ALDH-PB	455 NCYLTLSVQCPF...EM 478	56

[a] Based on those reported by Lamb and Newcomer (*55*). [b] The numbering is relative to the initiator methionine residue.

Disulfiram, which is rapidly metabolized, is not the inhibitor of ALDH in vivo (*53*). The active metabolite of disulfiram is believed to be MeDTC sulfoxide (*54*). MeDTC sulfoxide was equally potent toward rat liver ALDH1 and ALDH-PB (Table 2). Inhibition of cytosolic ALDH1 and ALDH-PB by MeDTC sulfoxide could contribute to the pharmacological actions of disulfiram, in addition to the well-known inhibition of mitochondrial ALDH2. MeDTC sulfoxide was a more potent inhibitor than disulfiram toward ALDH-PB (Table 2). MeDTC sulfoxide is smaller and less bulky than the parent compound disulfiram. Therefore, it may have easier access than disulfiram to the active site of ALDH-PB, possibly because the substrate entrance tunnel is too narrow to readily accommodate disulfiram.

Table 3 shows the alignment of the residues predicted to project into the substrate entrance tunnel of rat liver ALDH1 and ALDH-PB. As a simple estimate of the size difference, the number of non-hydrogen side chain atoms of the residues lining the substrate entrance tunnel can be counted (*55*). By this measure, it appears that the substrate entrance tunnel of rat ALDH1 (203 non-hydrogen atoms) would be slightly larger than that of rat ALDH-PB (211 non-hydrogen atoms). This estimation supports our substrate and inhibitor data which suggest that ALDH1 has a more open substrate entrance tunnel than ALDH-PB, conferring differences in the substrate specificity of the two isozymes. This is only a prediction; the features accounting for differences in substrate preferences and inhibitor sensitivities can only be determined upon elucidating the three-dimensional structure of ALDH-PB.

We have demonstrated that despite an 89% extent of sequence identity, rat liver ALDH1 and ALDH-PB are functionally distinct isozymes; ALDH-PB is not simply phenobarbital-induced ALDH1. We conclude that rat liver

ALDH1 is the ortholog of mammalian liver ALDH1. Rat liver ALDH1 and ALDH-PB are examples of two very closely related enzymes that have diverged with very different functionalities.

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