Possible Role of Liver Cytosolic and Mitochondrial Aldehyde Dehydrogenases in Acetaldehyde Metabolism†

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ABSTRACT: To provide a molecular basis for understanding the possible mechanism of action of antidipsotropic agents in laboratory animals, aldehyde dehydrogenase (ALDH) isozymes were purified and characterized from the livers of hamsters and rats and compared with those from humans. The mitochondrial ALDHs from these species exhibit virtually identical kinetic properties in the oxidation and hydrolysis reactions. However, the cytosolic ALDH of human origin differs significantly from those of the rodents. Thus, for human ALDH-1, the K_m value for acetaldehyde is 180 ± 10 μM, whereas those for hamster ALDH-1 and rat ALDH-1 are 12 ± 3 and 15 ± 3 μM, respectively. K_m values determined at pH 9.5 are virtually identical to those measured at pH 7.5. *In vitro* human ALDH-1 is 10 times less sensitive to disulfiram inhibition than are the hamster and rat cytosolic ALDHs. Competition between acetaldehyde and aromatic aldehydes or naphthaldehydes for the binding and catalytic sites of ALDHs shows their topography to be complex with more than one binding site. This also follows from data on substrate inhibition and activation, effects of NAD^+ on ALDH-catalyzed hydrolysis of *p*-nitrophenyl esters, substrate specificity toward aldehydes and *p*-nitrophenyl esters, and inhibition by disulfiram in relation to oxidation and hydrolysis catalyzed by the ALDHs. The data further suggest that acetaldehyde cannot be considered as a "standard" ALDH substrate for studies aimed at aromatic ALDH substrates, e.g. biogenic aldehydes. Apparently, in human liver, only mitochondrial ALDH oxidizes acetaldehyde at physiological concentrations, whereas in hamster or rat liver, both the mitochondrial and cytosolic isozymes will do so.

NAD-linked aldehyde dehydrogenases (ALDHs,[1] EC 1.2.1.3) catalyze the irreversible oxidation of a broad variety of aldehydes to carboxylic acids and are known to be involved in ethanol metabolism *in vivo*. They are also believed to be involved in the control of ethanol consumption by humans and by laboratory animals. Hence, alcohol research investigations of humans and rodents commonly compare ALDHs with the expectation that the kinetic properties of their individual isozymes *in vitro* and *in vivo* might provide insight regarding biochemical mechanisms underlying the use and abuse of ethanol.

Isozymes of ALDH from human, rat, mouse, horse, cow, and sheep liver have been purified and examined kinetically (Senior & Tsai, 1988; Pietruszko, 1989; Goedde & Agarwal, 1990; Ehrig et al., 1990; Yoshida et al., 1991; Rashkovetsky et al., 1994). However, the extent to which those individual isozymes contribute to the oxidation of acetaldehyde derived from ingested ethanol still remains to be defined.

ALDH-catalyzed reactions exhibit extremely low (often submicromolar) K_m values and, hence, require analysis by nonconventional kinetic means. One approach which we have described employs the single kinetic progress curve for ALDH-catalyzed reactions (Rashkovetsky et al., 1994). This and the following paper (Klyosov, 1996) also use an approach based on the kinetics of concurrent reactions of two ALDH substrates.

The characteristics of the major human liver ALDH isozymes are generally assumed to resemble those of rodent isozymes, much as only rat and mouse ALDHs have been studied thus far. Further, the kinetic properties and subcellular distribution of the major mitochondrial and cytosolic ALDH isozymes isolated from different species are reported to be similar (Yoshida et al., 1991). The action of the mitochondrial (low-K_m) ALDH is widely believed to be the major contributor to the clearance of ethanol-derived acetaldehyde in both man and rat (Svanas & Weiner, 1985; Farves et al., 1989; Smolen et al., 1990; Huang & Lindahl, 1990; Mitchell & Petersen, 1991; Tsai & Senior, 1991), a premise that has led to the adoption of a rodent "model" for acetaldehyde metabolism, as well as for metabolic and behavioral studies of ethanol.

We have examined ethanol metabolism as part of our interest in the biochemical basis underlying excessive ethanol consumption in man. For this purpose, we have chosen the Syrian golden hamster as an experimental animal. When given a free-choice regimen, it consumes large and predictable amounts of ethanol (Keung & Vallee, 1993a,b). The total daily intake under the conditions established is ∼15 g/kg of body weight, i.e. at least 10 times greater than that consumed by an average human heavy drinker (Stibler, 1991). To our knowledge, the isolation and characterization of hamster ALDH isozymes have not been reported, and hence, the biochemical mechanisms for acetaldehyde oxidation in hamsters are unknown.

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; ALDH-1, cytosolic ALDH (human, hamster, and rat); ALDH-2, mitochondrial ALDH (human, hamster, and rat); ALDH-3, a high-K_m (millimolar range) hamster cytosolic ALDH; 6-DMA-2-NA, 6-(dimethylamino)-2-naphthaldehyde; p-NPA, *p*-nitrophenyl acetate.

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The aim of this research was to purify the major ALDH isozymes in human, hamster, and rat liver and determine their kinetics to provide a biochemical basis for the comparison and interpretation of the role of these enzymes in acetaldehyde metabolism in these species. These isozymes were also examined on the basis of the kinetics of acetaldehyde and aromatic aldehydes when competing for the same active center, in a search for the mode of binding of other potential physiological ALDH substrates, e.g. biogenic aldehydes. The results would be pertinent to subsequent investigations and comparison of the major patterns for oxidation of acetaldehyde and other substrates in the liver of man and laboratory animals. They should also provide a biochemical rationale for the modulation of this conversion by isozyme-specific and tight-binding ALDH inhibitors and for examination of the proposition that the production of acetaldehyde is the primary basis for drug-induced aversion to ethanol.

MATERIALS AND METHODS

Materials

Human liver was obtained at autopsy within 12 h postmortem and was stored at −70 °C. A 50 g sample of liver tissue was used for each ALDH purification.

Male LVG Syrian golden hamsters (Sasco, Omaha, NE), weighing 120−140 g, were anaesthetized by inhalation of methoxyflurane and killed by cervical dislocation. The livers were either used as such or perfused *in situ* via a portal vein cannula with ice-cold 0.25 M sucrose solution. The weight of fresh or frozen hamster liver was typically in the range of 4.4−5.7 g (4.9 ± 0.4 g).

Male Sprague−Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 200−225 g, were anaesthetized and killed by cervical dislocation. The weight of fresh or frozen rat liver ranged from 11.7 to 14.8 g (12.7 ± 1.5 g).

Chemicals. α-Cyano 4′-hydroxycinnamate, disulfiram (tetraethylthiuram disulfide), and AMP-Sepharose 4B were from Sigma (St. Louis, MO); agarose, molecular biology grade, was from International Biotechnologies, Inc. (New Haven, CT). Diethylaminoethyl (DEAE)-cellulose (DE-53) was from Whatman Lab Sales (Hillsboro, OR). α-Cyano cinnamate−Sepharose 6B was prepared according to Poole and Halestrap (1989). 2-Mercaptoethanol, Coomassie Brilliant Blue R-250, and Bromophenol Blue were from Bio-Rad. Precast sodium dodecyl sulfate (SDS) and nondenaturing polyacrylamide gradient Daiichi gels were from Integrated Separation Systems (Hyde Park, MA). Ultrafiltration materials were from Amicon (Danvers, MA). Isoelectric-focusing (code no. 17-0473-01) and electrophoresis (code no. 17-0446-01) calibration kits were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). 6-DMA-2-NA (crystals from 1:2 acetonitrile−water, mp 116.5−117.5 °C) and 6-(heptanedioic acid monoether)-2-naphthaldehyde (prisms from 2:1 acetonitrile−water) were provided by Dr. Jacek Wierzchowski. Other substituted benzaldehyde, cinnamaldehyde, and naphthaldehyde derivatives were from Aldrich Chemical Co., Pfaltz & Bauer, Inc., and Eastman Organic Chemicals. *p*-Nitrophenyl acetate, propionate, butyrate, and laurate were from Sigma. All other chemicals were standard commercial products of the highest purity available.

Buffers. Buffer A was 30 mM K_2HPO_4 and 70 mM MES [2-(*N*-morpholino)ethanesulfonic acid] at pH 6.0. Buffer B was 10 mM KH_2PO_4 at pH 7.3. Buffer C was 500 mM K_2HPO_4 and 70 mM MES at pH 8.0. Buffer D was 0.1 M sodium phosphate at pH 7.5. Buffer E was 0.1 M sodium pyrophosphate at pH 9.5.

Purification Procedures

All preparations were performed at 4 °C; buffer solutions were saturated with nitrogen and contained 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 2 mM benzamidine. Protein content was determined with Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard (Bradford, 1976).

Human Liver ALDH Isozymes. The procedure for purification of ALDH-1 and -2 has been detailed (Rashkovetsky et al., 1994). Briefly, the first step of purification eliminates the bulk amount of protein from a human liver homogenate by negative dye−ligand chromatography on Green A−Sepharose 4B. α-Cyano cinnamate−Sepharose 6B chromatography then separates ALDH-1 and ALDH-2. ALDH-1 is eluted by increasing pH from 6.0 to 7.6. ALDH-2 is then eluted with 2 mM α-cyano-4-hydroxycinnamic acid at pH 6.0. The resultant ALDH-2, which still contains trace amounts of ALDH-1 [SDS−polyacrylamide gel electrophoresis (SDS−PAGE)], is purified to homogeneity by rechromatography on the same column and by elution with 50 μM testosterone, which has a higher affinity for ALDH-2.

Hamster Liver ALDH Isozymes. *Homogenization*. Perfused hamster livers were homogenized with a Teflon−Glass Potter-Elvehjem homogenizer in 28 mL of 0.25 M sucrose. Six livers were sufficient to purify cytosolic ALDHs, whereas mitochondrial fractions from 12 livers were required for purification of ALDH-2.

Separation of Mitochondria from Cytosol. Hamster liver homogenate was layered carefully over an equal volume of 0.34 M sucrose and centrifuged for 10 min at 700*g*. The supernatant was then centrifuged for 10 min at 5000*g*. The second supernatant was centrifuged at 45 000 rpm in a Beckman L8-80M ultracentrifuge (60 Ti rotor, 45 min) and then used for purification of cytosolic ALDHs.

The residue from the second centrifugation (mitochondrial fraction) was suspended in 120 mL of 0.25 M sucrose and centrifuged for 10 min at 24000*g*. Mitochondria were resuspended, centrifuged twice at 24000*g*, resuspended in 30 mL of buffer A containing 1% Triton X-100, homogenized, and centrifuged for 10 min at 24000*g*. Activities of ALDH (total) and succinate cytochrome *c* reductase as a mitochondrial marker enzyme (Mackler et al., 1962) were determined in the supernatant. Marker enzyme was found in the mitochondrial fraction only.

Hamster Cytosolic ALDH-1. Cytosol was loaded onto a 5′-AMP−Sepharose 4B column (1.2 × 3.5 cm) equilibrated with buffer A. The flow-through fraction was loaded directly onto an α-cyanocinnamate Sepharose 6B column (1.5 × 12.5 cm) equilibrated with buffer A and eluted with 2 mM α-cyano 4-hydroxycinnamate in the same buffer (pH 6.0). The flow-through fraction was rechromatographed, concentrated in a stirred Amicon ultrafiltration cell (XM-50 Diaflo ultrafiltration membrane), and applied to a DE-53 column (1.6 × 22 cm) equilibrated with buffer B. The flow-through fraction was collected, and 2 mL of the concentrated

preparation was passed through a Sephadex G-100 column (2.6 × 28 cm) equilibrated with buffer B.

Hamster Cytosolic ALDH-3. After separation from mitochondria, the cytosol was chromatographed twice on α-cyano cinnamate−Sepharose 6B as described above for ALDH-1 and then applied to a 5′-AMP−Sepharose 4B column equilibrated with buffer A. The bound material was eluted with a linear pH gradient (150 mL of buffer A:150 mL of buffer C). It was unstable and not treated further. ALDH-3 gave a single enzymatically active band on agarose gel electrophoresis and electrofocusing and one major and a few minor protein bands on SDS−PAGE.

Hamster ALDH-2. Mitochondrial lysate (66 mL) was loaded onto a Sephadex G-25 column (3.2 × 36 cm) equilibrated with buffer B, and the drop-through fraction was applied to an α-cyano cinnamate−Sepharose 6B column equilibrated with the same buffer. The affinity column was washed with buffer C and then with buffer B, and ALDH-2 was eluted with 2 mM α-cyano 4-hydroxycinnamate in buffer B (pH 7.3).

Rat Liver ALDH Isozymes. These were purified by essentially the same procedure as employed for hamster ALDH isozymes. After elution from α-cyano cinnamate−Sepharose 6B, rat ALDH-2 was additionally passed through DEAE-cellulose. The resulting material appeared homogeneous by both SDS−PAGE and agarose gel electrofocusing. ALDH-1 was eluted from a 5′-AMP−Sepharose column with 0.75 mM NAD^+ and gave a single band on both SDS−PAGE and agarose gel electrofocusing.

Assay of ALDH Activity

Absorbance. Dehydrogenase activity was followed by measuring the change in absorbance at 340 nm due to formation of NADH (ϵ_{340} = 6.22 mM^{-1} cm^{-1}) at 25 °C in a Varian Cary 219 spectrophotometer under initial velocity conditions. Assays were performed in buffer D (pH 7.5) or E (pH 9.5) containing 3.2 mM acetaldehyde for high-K_m (cytosolic) human ALDH-1 and hamster ALDH-3, 300 μM acetaldehyde for hamster and rat ALDH-1, or 10−14 μM acetaldehyde for low-K_m mitochondrial ALDHs and 3 mM NAD^+. ALDH hydrolytic activity with *p*-nitrophenyl acetate was followed at 400 nm (*p*-nitrophenolate formation, ϵ_{400} = 16 mM^{-1} cm^{-1}) at 25 °C (pH 7.5) (buffer D), with a substrate concentration of 5 μM, to which NAD^+ was not added unless indicated. Most substrates and inhibitors of ALDH were dissolved in methanol, which when added to the assay mixture was present at a final concentration of 0.1−1.0% (v:v). These concentrations of methanol do not adversely affect the enzymatic reactions. In contrast, acetonitrile significantly activates some ALDHs; e.g. 1% acetonitrile (v:v) activates human ALDH-2 by 20−50%.

Fluorescence. The presence of other NAD-linked dehydrogenases in crude enzyme preparations and liver homogenate supernatants interferes with the determination of ALDH activity by the spectrophotometric method. This problem was overcome by measuring ALDH activity with 6-DMA-2-NA in a Perkin-Elmer MPF3 spectrofluorimeter (excitation at 330 nm and emission at 430 nm). An increase in fluorescence as a result of 6-DMA-2-NA hydrolysis into 6-(dimethylamino)-2-naphthoic acid was monitored (Rashkovetsky et al., 1994). The concomitant reduction of NAD^+ did not interfere as was shown for different NAD^+ concentrations, between 6 and 750 μM. Total ALDH activity was determined in buffer D (pH 7.5) or E (pH 9.5) containing 0.75 mM NAD^+ and 50 nM 6-DMA-2-NA. The effect of acetaldehyde on the kinetics of oxidation of 6-DMA-2-NA by ALDH isozymes was also monitored by spectrofluorimetry. Both initial velocities and kinetic progress curves were recorded.

Table 1: ALDH Isozymes Purified from Human, Hamster, and Rat Liver

source	isozyme	p*I*	molecular weight (subunit)
human	ALDH-1	5.2	230 000 (57 700)
	ALDH-2	4.9	240 000 (52 800)
hamster	ALDH-1	7.0	150 000 (58 200)
	ALDH-2	5.5	230 000 (55 600)
	ALDH-3	6.2	190 000
rat	ALDH-1	6.9	120 000 (55 000)
	ALDH-2	5.4	240 000 (53 000)

Determination of Kinetic Constants. The human ALDH-1- and hamster ALDH-3-catalyzed oxidation of acetaldehyde have a relatively high K_m as determined from initial velocities, using both Lineweaver−Burke plots and numerical calculations. In contrast, the K_m values for the reactions catalyzed by mitochondrial ALDH from all three sources are submicromolar, precluding the use of initial velocities. Hence, kinetic parameters were obtained from progress curves (Klyosov & Berezin, 1972; Rashkovetsky et al., 1994). Where it was possible to determine K_m and/or V_m by both methods (e.g. oxidation of acetaldehyde catalyzed by low-K_m cytosolic ALDH-1 from hamster and rat liver), the values were identical within the limits of experimental error. Sets of progress curves, recorded from several initial substrate concentrations, yielded virtually identical kinetic parameters, indicating the absence of product inhibition in all cases. Substrate inhibition and activation were deduced from initial velocities in triplicate determinations.

The k_{cat} values are based on protein content, determined with Coomassie protein assay reagent (see above) and the native enzyme molecular weights listed in Table 1.

Polyacrylamide gel electrophoresis, agarose gel electrophoresis, and isoelectric focusing were performed as described previously (Rashkovetsky et al., 1994).

Molecular weight determinations were made under nondenaturing conditions by gel filtration on Sephacryl S-300HR (68 × 0.5 cm) equilibrated with 0.01 M ammonium bicarbonate buffer, containing 0.15 M NaCl.

RESULTS AND DISCUSSION

Analysis of Liver Homogenates. Agarose gel electrophoresis yields different ALDH patterns for human, hamster, and rat liver homogenates upon activity staining with acetaldehyde (Figure 1). Human liver gives two major bands, ALDH-1 (cytosolic) and ALDH-2 (mitochondrial), and hamster liver gives four, ALDH-1, ALDH-3 and one more cytosolic ALDH, and ALDH-2 (mitochondrial). Staining patterns with different acetaldehyde concentrations (not shown) indicate that, among hamster ALDHs, ALDH-2 has the lowest K_m for acetaldehyde, followed by ALDH-1 and one more (unidentified) hamster cytosilic ALDH, which are similar, and finally ALDH-3, which has the highest. Rat liver also exhibits four ALDH activity bands (Figure 1). The origin of the two middle bands (one strong, one weak) is unknown. The mobility of the upper, rat ALDH-2 band is

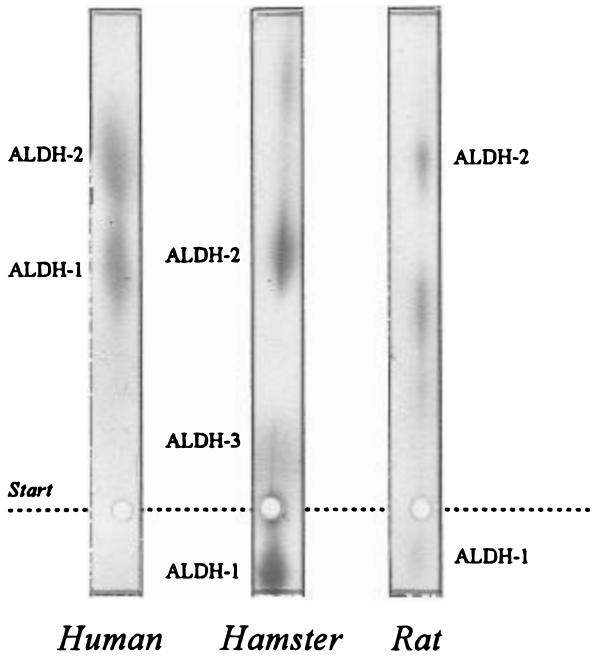

FIGURE 1: Agarose gel electrophoresis of human, hamster, and rat liver homogenate supernatants. The gels were stained in the presence of 72 mM acetaldehyde. The ALDH isozymes are identified on the lanes.

the same as that of human ALDH-2, while rat ALDH-1 migrates much like hamster ALDH-1 (Figure 1). These data indicate, first, that the ALDH isozymes from all three sources are not identical and, second, that the major differences are seen with the cytosolic isozymes.

Purification and Characterization of ALDHs. Seven of the principal cytosolic and mitochondrial ALDH isozymes were purified to examine their characteristics further, i.e. human, hamster, and rat ALDH-1, human, hamster, and rat ALDH-2, and hamster ALDH-3. We have given particular attention to the hamster ALDH isozymes since they have not been purified previously, and this species is the principal model in our studies of ethanol metabolism and its modulation.

Human cytosolic and mitochondrial ALDH isozymes can be separated readily by α-cyano cinnamate affinity chromatography. Unlike the human ALDHs, *both* major mitochondrial and cytosolic hamster ALDHs bind tightly to the affinity resin between pH 6 and 8, and both can be eluted with 2 mM α-cyano 4-hydroxycinnamate. The purity and total enzymatic activity of the hamster ALDH isozymes are much greater when the mitochondrial and cytosolic fractions of the liver homogenate are first separated by differential centrifugation and then applied separately to the α-cyano cinnamate affinity column. This single purification step affords virtually homogeneous hamster ALDH-2, whereas hamster ALDH-1 required two additional steps, i.e. affinity chromatography on 5′-AMP−Sepharose 4B and negative chromatography on DEAE-cellulose. Yet another hamster cytosolic ALDH, ALDH-3, was purified by a linear pH gradient elution from 5′-AMP−Sepharose 4B, as described in detail in Materials and Methods.

The preparations of human ALDH-1 and -2, hamster ALDH-1 and -2, and rat ALDH-1 and -2 all are homogeneous, as judged by SDS-PAGE, agarose gel electrophoresis, and electrofocusing (not shown). Hamster ALDH-3 is a single enzymatically active isozyme (one band on agarose gel stained with acetaldehyde) with a few minor extraneous proteins (SDS−PAGE) that apparently lack ALDH activity. It does not deviate from Michaelis−Menten behavior and acts kinetically as a single enzyme.

Table 1 lists the p*I* values for the purified ALDH isozymes. The major cytosolic enzymes (ALDH-1) of the rodents are significantly more basic (p*I* 7.0 and 6.9) than human cytosolic ALDH (p*I* 5.2), whereas all three mitochondrial ALDHs have similar p*I* values (Table 1).

All mitochondrial ALDH isozymes (human, hamster, and rat), as well as the human cytosolic isozyme, have the same molecular weight (230000−240000, Table 1) and are known (human ALDH-1 and -2, rat ALDH-2) or appear (hamster ALDH-2) to be tetrameric. The low-K_m cytosolic ALDH-1 of hamster and rat liver exhibit significantly lower molecular weights (120000−150000) and are presumed to be dimeric. There are apparently no data in the literature regarding the oligomeric composition of hamster and "normal" rat liver (not treated with carcinogenic or chemically related compounds) cytosolic ALDH. A few years ago, it was pointed out (Pietruszko, 1989) that cytosolic ALDHs from normal rat liver were not purified to homogeneity. It appears that since then no new data were published on whether the rat cytosolic ALDH from normal liver is dimeric. Rat testis cytosolic ALDH (Bedino & Testore, 1993) is a dimer, but it has a much higher isoelectric point, 9.5, compared with 6.9 for the rat liver ALDH-1 (Table 1). Besides, the rat testis cytosolic ALDH has a much higher K_m toward acetaldehyde, 246 μM (Bedino et al., 1990), compared with 15−17 μM for the rat ALDH-1 (Table 2). Further, the rat testis mitochondrial ALDH has a K_m value toward acetaldehyde, 0.17 μM (Bedino et al., 1992), almost identical with that reported in this work for the rat liver mitochondrial ALDH (0.2 μM, Table 2). Phenobarbital-inducible cytosolic ALDH from rat liver (Koivula & Koivusalo, 1975; Petersen et al., 1977; Lindahl et al., 1982; Lindahl & Evces, 1984; Simpson et al., 1985; Dunn et al., 1989) is a dimer with a molecular weight between 90000−118000 and 165000 (Koivula & Koivusalo, 1982; Lindahl & Evces, 1984; Simpson et al., 1985). However, phenobarbital-inducible rat liver cytosolic ALDH is known to have a very high K_m toward acetaldehyde, in the millimolar range (Koivula & Koivusalo, 1982; Lindahl & Evces, 1984; Simpson et al., 1985; Tank et al., 1986), and certainly differs from the rat liver ALDH-1 described in this work.

Kinetic Properties of Purified ALDHs. The "kinetic homogeneity" of hamster ALDH-3 activity was further verified by assays carried out at high enzyme concentrations (Berezin & Klyosov, 1976). To monitor such kinetics, the activity of the enzyme must be very low; otherwise, the reaction will be complete in seconds or even faster. The catalytic activity of hamster ALDH-3 with 6-DMA-2-NA ($k_{cat} = 0.29$ min^{-1}, or only 1% of that with acetaldehyde at pH 7.5, Table 2) meets this criterion. The enzyme (E) concentration in this assay is 11.4 nM, and the 6-DMA-2-NA concentration is 1.3−6.4 nM (Figure 2). The dependence of the initial velocity on substrate (S) concentration in this range is linear (Figure 2), in accord with the equation for $[E]_0 > [S]_0$ (non-Michaelis−Menten kinetics):

$$v = \frac{k_{cat}[E]_0[S]_0}{K_m + [E]_0} \quad (1)$$

Table 2: Kinetic Parameters for ALDH Isozymes from Human, Hamster, and Rat Liver[a]

			substrates					
			acetaldehyde[b]		6-DMA-2-NA[c]		p-NPA[d]	
source	enzyme	pH	K_m	k_{cat} (min^{-1})	K_m (nM)	k_{cat} (min^{-1})	K_m	k_{cat} (min^{-1})
cytosol								
human	ALDH-1	7.5	180 μM	380	1.9	53 (14%)	0.63 μM	34 (16%)
		9.5	180 μM	790				
hamster	ALDH-1	7.5	12 μM	108	1.5	18 (17%)	1.5 μM	305 (280%)
		9.5	8.8 μM	140	1.6	31 (22%)		
	ALDH-3	7.5	3.6 mM	29	1.3	0.29 (1.0%)	3.8 μM	28 (100%)
		9.5	3.0 mM	36	1.5	0.66 (1.8%)		
rat	ALDH-1	7.5	17 μM	46	2.2	9.2 (20%)	1.9 μM	148 (320%)
		9.5	15 μM	60				
mitochondria								
human	ALDH-2	7.5	200 nM	280	2.1	11 (4%)	78 nM	53 (24%)
		9.5	200 nM	1180	2.6	37 (3%)		
					4.0[e]			
hamster	ALDH-2	7.5	200 nM	170	3.3	3.4 (2%)	80 nM	23 (26%)
		9.5	200 nM	960	1.6	38 (4%)		
rat	ALDH-2	7.5	200 nM	96	2.4[e]	2.9 (3%)	72 nM	47 (27%)
		9.5	200 nM	645				

[a] Coefficients of deviation for the K_m and k_{cat} values are within 10−20%. In parentheses is the k_{cat} value relative to that with acetaldehyde as a substrate, in percent. [b] $[NAD^+] = 3$ mM. [c] $[NAD^+] = 0.75$ mM, unless indicated. [d] No NAD^+. [e] $[NAD^+] = 0.06$ mM.

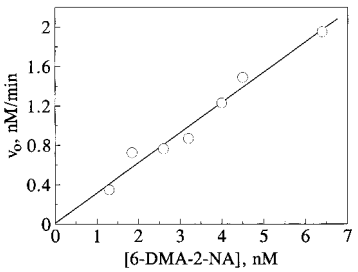

FIGURE 2: Kinetics of 6-DMA-2-NA oxidation by hamster ALDH-3 isozyme at high enzyme and low substrate concentrations. The enzyme concentration was 11.4 nM (60 μM NAD^+, pH 7.5). The straight line fits the constants $k_{cat} = 0.34 \pm 0.03$ min^{-1} and $K_m = 1.3 \pm 0.2$ nM (cf. Table 2).

Further in accord with eq 1, the experimental kinetics of 6-DMA-2-NA oxidation at high enzyme concentrations are essentially first-order. Calculation of k_{cat} from the slope in Figure 2 and using eq 1 results in a value of 0.34 ± 0.03 min^{-1}, almost identical with 0.29 ± 0.04 min^{-1} obtained for the same enzyme preparation by conventional kinetics (Table 2). Thus, in addition to its apparent electrophoretic purity, the hamster ALDH-3 preparation is "kinetically pure".

The kinetic properties of the purified ALDHs toward acetaldehyde, 6-DMA-2-NA, and p-NPA are shown in Table 2. The human, hamster, and rat mitochondrial ALDHs are practically indistinguishable with these substrates; all exhibit very low K_m values toward acetaldehyde (200 ± 20 nM) and p-NPA (~80 nM). Their catalytic activities are also very similar, particularly when compared on the basis of acetaldehyde conversion. For example, the relative reactivity of the mitochondrial ALDHs from all three species for p-NPA vs acetaldehyde is ~25% (Table 2).

In contrast, the kinetic properties of human cytosolic ALDH differ significantly from those of the hamster and rat cytosolic isozymes with respect to acetaldehyde and p-NPA as substrates. All seven ALDH isozymes, however, exhibit very low and similar K_m values (2.2 nM on average) toward 6-DMA-2-NA. We have studied the competition of acetaldehyde with a series of naphthaldehydes to examine whether they interact with the same or different binding sites (see the next section).

Acetaldehyde as a Substrate and an Effector. Particular attention was paid here to K_m values toward acetaldehyde, because these values are considered crucial for interpretation of the physiological functions of ALDHs *in vivo*, especially for assignment of the relative importance of the two isozymes in the oxidation of this substrate. Most reported K_m values for human ALDH-2 which range from <0.1 to 9 μM (Koivula, 1975; Greenfield & Pietruszko, 1977; Ikawa et al., 1983; Henehan et al., 1985; Ambroziak & Pietruszko, 1987, 1991; Johnson et al., 1987; Pietruszko, 1989; Ehrig et al., 1990; Zorzano & Herrera, 1990; Yoshida et al., 1991; Henehan & Tipton, 1991; Dockham et al., 1992) are significantly higher and in one case lower than the value of 0.2 μM determined here (Table 2). Published K_m values for mitochondrial ALDHs from rat testis (Bedino et al., 1992), mouse liver (Algar & Holmes, 1985), and horse liver (Eckfeldt et al., 1976), however, are practically identical with those for mitochondrial ALDHs determined in this work (0.2 μM, Table 2) and equal to 0.17, 0.2, and 0.2 μM, respectively. Taking into account that mitochondrial ALDHs from different sources exhibit a high degree of homology—ALDH-2 from human, horse, rat, and beef liver share 95% sequence identity (Farres et al., 1994, 1995)—we believe that the K_m values, reported in this work, are accurate. Literature K_m values for human ALDH-1 are in the range of 22−483 μM (Greenfield & Pietruszko, 1977; Ikawa et al., 1983; Henehan et al., 1985; Ambroziak & Pietruszko, 1987, 1991; Johnson et al., 1987; Pietruszko, 1989; Ehrig et al., 1990; Zorzano & Herrera, 1990; Yoshida et al., 1991; Dockham et al., 1992) compared to the value of 180 μM reported here. The K_m values for human liver ALDH-1 and -2 have been stated to differ by a factor of ~4 (Zorzano & Herrera, 1990), 6 (Yoshida et al., 1991), 10 (Pietruszko, 1989), or >4800 (Dockham et al., 1992). We find that they differ by a factor of 1000. The basis for this discrepancy is not clear. It could be due to the apparently various degree of purification obtained by different investigators or, we suspect, to difficulties in measurement of K_m values in the submicromolar range using initial velocities of human ALDH-2-catalyzed reactions, as employed in all but one of the above cited papers (Dockham et al., 1992).

The K_m value of acetaldehyde for human liver cytosolic ALDH is more than 1 order of magnitude higher than those of the two rodent cytosolic ALDHs (180 ± 10 vs 9−17 μM, Table 2). This would render human cytosolic ALDH virtually inactive with physiological concentrations of acetaldehyde, typically 0.4−2.5 μM (Lieber, 1988; Hatake et al., 1990; Inoue et al., 1984; Harada et al., 1981). Indeed, taking into account the k_{cat} values for the human cytosolic and mitochondrial ALDHs at pH 7.5 (Table 2), it follows that, to compensate for the difference in their K_m values, the concentration of cytosolic ALDH in liver should be 100−300 times higher than that of mitochondrial ALDH. In fact, the human liver contains 3−5 times less cytosolic ALDH than mitochondrial ALDH (Zorzano & Herrera, 1990; Rashkovetsky et al., 1994). Therefore, in humans, mitochondrial ALDH would be almost exclusively responsible for the clearance of acetaldehyde. The major cytosolic ALDH isozymes in hamster and rat liver, however, have K_m values in the physiological range of acetaldehyde and, hence, would be important for its oxidation under physiological conditions.

There are few studies published on pH effects on ALDH-catalyzed reactions; most of the data were obtained with sheep liver cytosolic ALDH (Dickinson et al., 1981; Dickinson, 1986; Hill et al., 1991a,b; Buckley et al., 1991). Our data show that activity of mitochondrial ALDHs from all three sources toward acetaldehyde has a greater pH dependence than that of cytosolic ALDHs; from pH 9.5 to 7.5, the k_{cat} values for ALDH-2 decrease (5.5 ± 1.2)-fold, while those for ALDH-1 decrease (1.5 ± 0.4)-fold (Table 2). Upon a further decrease to pH 7.0, the respectively k_{cat} value decreases 2.9 times for human ALDH-2 but does not change for human ALDH-1 (Rashkovetsky et al., 1994). K_m values for all seven ALDH isozymes (Table 2) stay the same between pH 7.5 and 9.5. These data are similar to published ones, according to which from pH 9.0 to 7.4 the activity toward acetaldehyde drops 4.5−5.3 and 2.9−3.0 times for human ALDH-2 and ALDH-1, respectively (Ambroziak & Pietruszko, 1987), with K_m values almost unchanged (Ambroziak & Pietruszko, 1987) or changed within experimental error (Zorzano & Herrera, 1990).

To compare the mode of binding of acetaldehyde to ALDHs with that of aromatic aldehydes and naphthaldehydes, we have studied the kinetics of two substrates when they are competing for the same active center:

$$E + S_1 \rightleftharpoons ES_1 \rightarrow E + P_1$$

$$E + S_2 \rightleftharpoons ES_2 \rightarrow E + P_2 \qquad (2)$$

A simple Michaelis−Menten equation describes the velocity of P_1 formation (e.g. a product of 6-DMA-2-NA oxidation, 6-DMA-2-naphthoic acid) in the presence of a second substrate (S_2, acetaldehyde). The second substrate does not affect the maximum velocity but increases the apparent Michaelis constant of the "total" reaction:

$$K_m = K_1\left(1 + \frac{[S_2]}{K_2}\right) \qquad (3)$$

where K_1 and K_2 are Michaelis constants for S_1 and S_2, respectively. The ratio of the velocities in the absence and presence of the second substrate (v_0 and v_i, respectively) is

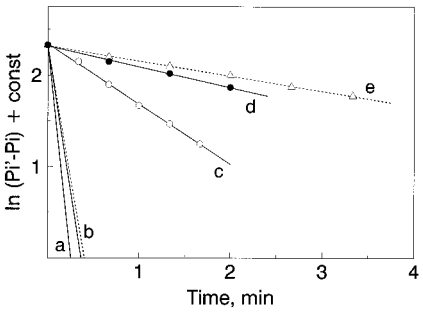

FIGURE 3: Effect of acetaldehyde (AA) on the kinetics of human ALDH-1- (solid lines) and hamster ALDH-1-catalyzed (dashed lines) oxidation of 6-DMA-2-NA. The first-order progress kinetic curves are shown as Guggenheim plots. The concentrations of AA were as follows: (a) 0 ($k = V_m/K_m = 8.4 \pm 1.7$ min^{-1}, determined from separate experiments; experimental points are not shown), (b) 0 ($k = V_m/K_m = 5.9$ min^{-1}, determined from separate experiments; experimental points are not shown), (c) 1.28 mM ($k = 0.67$ min^{-1}), (d) 6.4 mM ($k = 0.23$ min^{-1}), and (e) 1.28 mM ($k = 0.15$ min^{-1}) (pH 7.5, 3 mM NAD^+).

equal to

$$\frac{v_0}{v_i} = 1 + \frac{\dfrac{[S_2]}{K_2}}{1 + \dfrac{[S_1]}{K_1}} \qquad (4)$$

and allows the calculation of K_2 (the Michaelis constant for acetaldehyde).

Cytosolic ALDHs. Acetaldehyde and 6-DMA-2-NA are fully competitive for human ALDH-1 and apparently bind at the same active site. Thus, in the presence of 1.28 and 6.4 mM acetaldehyde (7 and 36 times K_m), the initial velocity of 6-DMA-2-NA conversion decreases 2.4- and 4.3-fold, respectively, and the apparent K_m of the reaction increases so much that the progress curve becomes first-order (Figure 3), in complete accord with eqs 3 and 4. For acetaldehyde, the competition experiments give an apparent dissociation constant of 190 ± 60 μM (initial velocities) and 150 ± 40 μM (kinetic progress curves). These figures are close to the K_m for acetaldehyde, 180 μM, determined in separate experiments (Table 2). On the other hand, the K_m values of NAD^+ for the oxidation of these two substrates are different: 1.0 ± 0.4 μM for oxidation of 6-DMA-2-NA and 7.1 ± 0.4 μM for oxidation of acetaldehyde.

In accord with the above equation and earlier kinetic data (Table 2), Figure 3 shows that 1.28 mM acetaldehyde affects the progress kinetics of hamster ALDH-1 (curve e) more than 6.4 mM acetaldehyde with human ALDH-1 does (curve d). Thus, hamster ALDH-1 also binds both acetaldehyde and 6-DMA-2-NA at the same active site; i.e. these two substrates are fully competitive.

Hamster ALDH-3 Has Two Active Sites. The inhibition pattern of hamster ALDH-3 is different in kind. Although its K_m for acetaldehyde is very high (3.6 mM, Table 2), much lower acetaldehyde concentrations strongly inhibit its activity with 6-DMA-2-NA (Figure 4). Concentrations of acetaldehyde as low as 48 μM decrease the initial velocity, and 1.28 mM decreases it by almost 10-fold. The apparent dissociation constant for acetaldehyde, calculated on the basis of competition with 6-DMA-2-NA as the reference substrate, is 12.6 ± 3.5 μM. Progress kinetics show that acetaldehyde

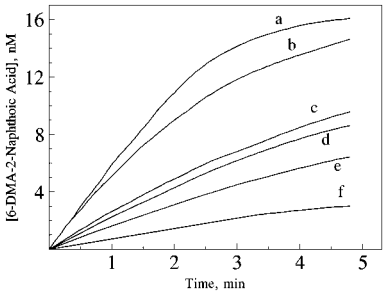

FIGURE 4: Effect of acetaldehyde (AA) on the kinetics of hamster ALDH-3-catalyzed oxidation of 6-DMA-2-NA, monitored by spectrofluorimetry. The concentrations of AA were as follows: (a) 0 and 9.5 μM, (b) 48 μM, (c) 128 μM, (d) 256 μM, (e) 640 μM, and (f) 1.28 mM (pH 7.5, 6 μM NAD^+).

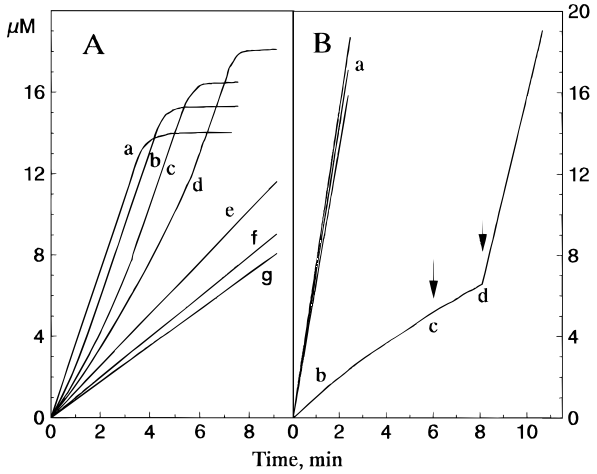

FIGURE 5: Effect of benzaldehyde (BA) on the kinetics of acetaldehyde (AA) oxidation by human ALDH-2 (A) and hamster ALDH-3 (B). (A) The concentration of AA was 14 μM, and the concentrations of BA were as follows: (a) 0, (b) 1.25 μM, (c) 2.5 μM, (d) 4.2 μM, (e) 8.3 μM, (f) 21 μM, and (g) 8.3 and 21 μM (initial velocities are the same) (no AA). (B) The reaction conditions were as follows: (a) multiple experiments, 6.4 mM AA, 0, 16.8, and 84 μM BA (did not affect the kinetics); (b) 16.8 μM BA, no AA; (c) 84 μM BA added to 16.8 μM BA (no effect on the velocity); (d) 6.4 mM AA added to 100.8 μM BA (pH 9.5, 3 mM NAD^+).

increases K_m without any effect on V_m, in complete accord with eqs 3 and 4 and with an apparent dissociation constant of 20 ± 3 μM. Thus, acetaldehyde competes with 6-DMA-2-NA for the same site of hamster ALDH-3. However, it is not oxidized catalytically at this site but instead at one with a much poorer K_m, i.e. 3.6 mM. It could be said that in hamster ALDH-3 one of these two active sites provides a catalytic center for a physiological substrate, which is still unknown, whereas the other might serve a regulatory function(s).

Benzaldehyde binds to hamster ALDH-3 with the same K_m as to human ALDH-2, 19 ± 4 and 18 ± 2 nM, respectively. The first figure was determined from competition experiments with 6-DMA-2-NA (not shown) and the second with acetaldehyde (Figure 5A). Surprisingly, benzaldehyde does not compete with acetaldehyde for binding to hamster ALDH-3 (Figure 5B), even at concentrations 100−500 times higher than those that effectively inhibit 6-DMA-2-NA oxidation. Hence, benzaldehyde and acetaldehyde do not compete with each other for binding to hamster ALDH-3, but they both compete with 6-DMA-2-NA.

Additional experimental data support the conclusion that acetaldehyde and aromatic aldehydes are oxidized at different sites on hamster ALDH-3. (i) 2-Naphthaldehyde and 5-bromo-1-naphthaldehyde, both very tight-binding substrates of hamster ALDH-3, essentially do not inhibit acetaldehyde oxidation by this enzyme, even at concentrations up to 10 μM, i.e. 3−4 orders of magnitude above their K_m values. (ii) The inhibition of the activity of hamster ALDH-3 toward acetaldehyde by disulfiram differs from that toward 6-DMA-2-NA. In both cases, the inhibition is instantaneous, but the respective K_i values are 400 ± 100 nM for acetaldehyde and 2.9 ± 0.3 nM for 6-DMA-2-NA. (iii) The K_m values for NAD^+ in relation to acetaldehyde and 6-DMA-2-NA oxidation vary, 100 ± 20 and 1.4 ± 0.2 μM, respectively (pH 7.5.). Clearly, the catalytic centers for the oxidation of these two substrates must also differ.

Human and Hamster Mitochondrial ALDHs Have Two Active Sites. Human and hamster ALDH-2 exhibit the greatest affinity toward acetaldehyde, and in both cases, K_m is 0.2 μM (Table 2). Acetaldehyde at much higher concentrations (from 9.5 μM to 1.3 mM, i.e. 48−6500 × K_m) has almost no effect on the oxidation of 6-DMA-2-NA by either isozyme; the initial velocities in the presence and absence of all acetaldehyde concentrations varied by 5−8%. With a 500-fold lower NAD^+ concentration (from 3 mM to 6 μM), acetaldehyde inhibits the reaction only slightly, with an apparent dissociation constant of 0.9 ± 0.2 mM; this is a few thousand times higher than the K_m value for acetaldehyde itself. Thus, the human and hamster mitochondrial ALDHs have separate active centers for acetaldehyde and the naphthaldehyde as is the case for hamster cytosolic ALDH-3.

Benzaldehyde inhibition of human ALDH-2 provides further evidence for the existence of two active sites. Acting as a second substrate together with 6-DMA-2-NA, benzaldehyde has an apparent dissociation constant of 110 ± 50 nM as compared with 18 ± 2 nM for competition with acetaldehyde. Apparently, acetaldehyde and benzaldehyde, but not the naphthaldehyde, share the same human ALDH-2 binding site.

p-Nitrophenyl Acetate as a Substrate and an Effector. We examined the kinetics of the ALDHs toward p-NPA, a surrogate substrate, to further extend the characterization of the purified isozymes. Hydrolytic activity of ALDH toward p-NPA has been reported for the enzyme from horse (Feldman & Weiner, 1972) and human livers (Sidhu & Blair, 1975), and various aspects of this reaction have been described (Eckfeldt & Yonetani, 1976; Dunkan, 1977; Kitson, 1982, 1986; MacGibbon et al., 1978; Takahashi & Weiner, 1981; Vallari & Pietruszko, 1981; Blackwell et al., 1983; Tu & Weiner, 1988a,b; Abriola & Pietruszko, 1992). The reported K_m values range from 1.5 to 10 μM. NAD^+ is an activator (up to 8-fold) of hydrolysis (except for rabbit ALDH, where it is an inhibitor).

Table 2 shows that with respect to p-NPA all cytosolic ALDHs have K_m values in the low micromolar to submicromolar range. For hamster and rat cytosolic enzymes, p-NPA is a much better substrate than acetaldehyde (3-fold difference in favor of the ester), whereas the opposite is true for the human cytosolic isozyme (6-fold difference in favor of the aldehyde). This again points to significant differences between human and rodent cytosolic ALDHs, as shown above for acetaldehyde as a substrate.

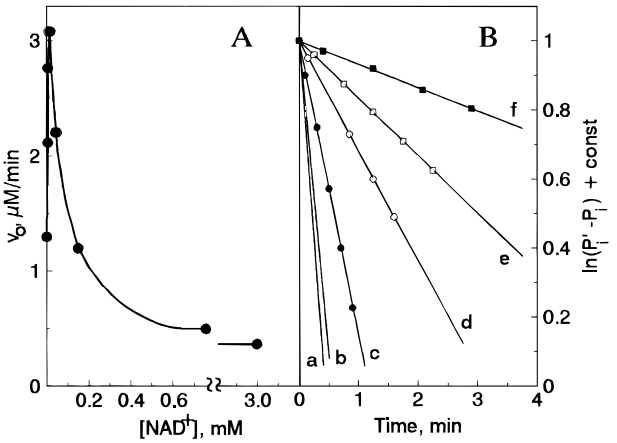

FIGURE 6: Effect of NAD^+ on kinetics of p-NPA hydrolysis, catalyzed by human ALDH-1. (A) Effect of NAD^+ on initial velocities of the enzymatic hydrolysis. (B) Guggenheim plots of first-order kinetic curves of the enzymatic hydrolysis. For curves a and b, slopes equal to V_m/K_m (in min^{-1}) were calculated from progress kinetic curves (mixed-order kinetics). The NAD^+ concentrations (in μM) were as follows: (a) 0 and 3.0 ($V_m/K_m = 2.54$ min^{-1}), (b) 7.5 ($V_m/K_m = 1.8$ min^{-1}), (c) 45 (for a number of experiments, $k = 0.78-0.86$ min^{-1}), (d) 150 ($k = 0.325$ min^{-1}), (e) 750 ($k = 0.165$ min^{-1}), and (f) 3000 (for a number of experiments, $k = 0.00650-0.0875$ min^{-1}).

NAD^+ increases the maximum velocity of p-NPA hydrolysis by ALDHs from various sources (Feldman & Weiner, 1972; Sidhu & Blair, 1975; Eckfeldt & Yonetani, 1976; MacGibbon et al., 1978; Takahashi & Weiner, 1981; Vallari & Pietruszko, 1981; Kitson, 1986; Abriola & Pietruszko, 1992). We show here that NAD^+ in fact has two opposite effects on the hydrolysis of p-NPA by human ALDH-1 (Figure 6); it increases V_m and competes with the substrate. The higher the initial substrate concentration and the lower its K_m, the more the reaction is controlled by V_m and, hence, the higher is the activating effect of NAD^+. When initial velocity begins to be controlled by K_m (above a certain NAD^+ concentration), the coenzyme becomes an inhibitor rather than an activator. The K_m values on the descending part of the curve in Figure 6A are so high that the overall kinetics of the reaction become first-order (Figure 6B).

With respect to the effect of NAD^+ on p-NPA hydrolysis, human and hamster cytosolic ALDHs again behave differently. NAD^+ (0.15−3.0 mM) activates hydrolysis catalyzed by hamster ALDH-1 (not shown) but inhibits that catalyzed by human ALDH-1 (Figure 6); under the same conditions, both human and hamster mitochondrial isozymes are activated by NAD^+ (not shown).

Mitochondrial ALDH isozymes have much lower K_m values than cytosolic ALDHs (Table 2) and are therefore more markedly activated by NAD^+. For instance, 3 mM NAD^+ was an overall inhibitor of human ALDH-1 (Figure 6) but an activator of human ALDH-2; in the latter case, V_m increased at least 2.5-fold and V_m/K_m decreased 10-fold (from 8.66 to 0.84 min^{-1}).

The same interpretation for the inhibition or activation effect of NAD^+ pertains to hamster ALDH isozymes. Hamster ALDH-1 has a lower K_m for acetaldehyde than does human ALDH-1 (12 and 180 μM, respectively). Hence, the same concentration of NAD^+ (150 μM) activates hamster ALDH-1 but inhibits human ALDH-1. Hamster and human mitochondrial ALDHs both have low K_m values; hence, both are activated by NAD^+.

At low concentrations, ≤ 3 μM, NAD^+ increases both V_m and K_m of human cytosolic ALDH in parallel; the ratio, which is determined directly from the kinetic experiment, stays constant (Figure 6B, curve a). At higher NAD^+ concentrations, > 3 μM, the maximum velocity increases less and tends to level off, whereas K_m continues to increase to give a $K_{m(app)}$ of 20 ± 2 μM. This pattern provides a basis for speculation that NAD^+ binds to two different sites on human ALDH-1: occupation of one activates V_m, while occupation of the other competes with p-NPA binding and thereby increases its apparent K_m.

When something causes a parallel increase in V_m and K_m, it generally indicates elimination of nonproductive ("wrong-way") binding of substrate. In this case, it would mean that p-NPA binds to ALDHs in a predominantly nonproductive mode; nonproductive binding appears to be more pronounced in mitochondrial ALDHs. This would account for the very low K_m values and relatively low k_{cat} values of the mitochondrial ALDHs. Binding of NAD^+ to the first (tighter) site specifically displaces p-NPA that is bound nonproductively (leaving the productive one intact) and thereby increases V_m and K_m in parallel (see above). Occupation of the second site by NAD^+ (with $K_{m(app)} = 20 \pm 2$ μM) would then compete with productively bound p-NPA and lead to inhibition of hydrolysis. Indeed, more than one NAD^+ binding site has been found in ALDHs by spectrophotometry (Ambroziak et al., 1989, and references therein).

Further evidence that NAD^+ and p-NPA occupy the same binding site stems from the fact that they both protect human ALDH-2 from rapid, spontaneous inactivation. In the presence of 150 μM NAD^+, the half-life for activity toward acetaldehyde was only 3.6 ± 0.2 min, while with 3 mM NAD^+, it was almost 20 times longer (61 ± 3 min). p-NPA protects human ALDH-2 from inactivation with similar efficiency; the presence of 15−25 μM p-NPA increases the half-life of the enzyme to 48 ± 5 min.

Substrate Specificity of ALDHs in the Hydrolysis of Long-Chain Carboxylic Acid p-Nitrophenyl Esters. Yet additional evidence pointing to the complexity of the ALDH active center topography has been obtained with *p*-nitrophenyl esters of long-chain carboxylic acids. In contrast with the substrate specificity of aliphatic aldehydes (Klyosov, 1996), elongation of the aliphatic chain of carboxylic acid *p*-nitrophenyl esters increases K_m and decreases k_{cat}/K_m (Table 3). With aldehydes, human ALDH-1 has the highest specificity for longer-chain substrates (from acetaldehyde to propanal, K_m decreases 40-fold; with pentanal, K_m decreases 1000-fold while k_{cat}/K_m increases 1000-fold). Remarkably, the reverse is true for esters. K_m increases 17-fold from p-NPA to p-NP propionate and becomes so high for p-NP butyrate that it cannot be measured; in this series, k_{cat}/K_m decreases more than 20-fold (Table 3). Such negative specificity is observed for both the cytosolic and mitochondrial ALDHs of human and hamster liver. Also in contrast with aldehyde oxidation, all ALDH isozymes exhibit characteristic substrate activation for p-NPA hydrolysis (Table 4).

Substrate Inhibition in ALDH Reactions. A distinctive characteristic of human ALDH-1 is its strong substrate inhibition with aromatic aldehydes, whereas human ALDH-2 usually obeys normal Michaelis−Menten kinetics. K_m values for the oxidation of aromatic aldehydes by human ALDH-1

Table 3: Kinetic Parameters for Human and Hamster Liver ALDH Isozymes for Hydrolysis of Carboxylic Acid *p*-Nitrophenyl (p-NP) Esters

		substrates										
		p-NP acetate			p-NP propionate			p-NP butyrate			p-NP laurate	
source	enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)	K_m (μM)	k_{cat}/K_m ($min^{-1}\,\mu M^{-1}$)
cytosol												
human	ALDH-1	0.63	34	54	10.9	278	25	>100	nd[a]	2.5	>50	2.21
hamster	ALDH-1	1.5	305	203	2.6	530	204	9.4	740	79	>50	16
	ALDH-3	3.8	28	7.4	10.1	83	8.2	12.3	81	6.6	>50	0.95
mitochondria												
human	ALDH-2	0.078	53	679	0.2	40	200	3.4	76	22	>50	1.8
hamster	ALDH-2	0.072	47	653	0.2	37	184	3.25	74	23	>50	1.9

[a] Not determined.

Table 4: Substrate Activation in ALDH-Catalyzed Hydrolysis of Carboxylic Acid *p*-Nitrophenyl (P-NP) Esters (pH 7.5)[a]

	cytosolic			mitochondrial	
	human ALDH-1[d]	hamster ALDH-1	hamster ALDH-3	human ALDH-2	hamster ALDH-2
p-NP acetate[b]	158% × V_m at $[S]_0$ = 77K_m	150% × V_m at $[S]_0$ = 36K_m	230% × V_m at $[S]_0$ = 16K_m	slight substrate activation	38% × V_m at $[S]_0$ = 700K_m
p-NP propionate[c]	no substrate activation at 4K_m	143% × V_m at $[S]_0$ = 17K_m	no substrate activation at 6K_m		no substrate activation at 215K_m
p-NP butyrate	no substrate activation	370% × V_m at $[S]_0$ = 11K_m	no substrate activation at 4K_m		no substrate activation at 12K_m

[a] NAD^+ at 7.5 μM activated V_{max} for all substrates of ALDH-2 (human and hamster) by 15−47% and apparently did not change K_m values. [b] NAD^+ activated V_m but inhibited binding (increased K_m) for human ALDH-1-catalyzed hydrolysis of p-NPA. [c] NAD^+ did not effect human ALDH-1-catalyzed hydrolysis of *p*-nitrophenyl propionate. [d] NAD^+ inhibited human ALDH-1-catalyzed hydrolysis of p-nitrophenyl laurate (decreased V_m/K_m).

Table 5: Substrate Inhibition by Some Aldehydes in Reactions Catalyzed by Human ALDH-1

substrate	K_S' (μM)
m-methylbenzaldehyde	26
p-methylbenzaldehyde	15−25
o-nitrobenzaldehyde	15
2,4-dinitrobenzaldehyde	15
p-nitrocinnamaldehyde	5.5
2-naphthaldehyde	0.8
6-[*O*-$(CH_2)_5$COOH]-2-naphthaldehyde	0.56
p-nitrobenzaldehyde	0.5

[a] K_S' is the substrate inhibition constant.

are very low (typically less than 100 nM), and substrate inhibition constants (K_S') are also low (from 0.5 to 26 μM, Table 5). Therefore, the initial velocity vs initial substrate concentration profile is not bell-shaped, as is often observed for substrate inhibition kinetics, but is a descending curve (Figure 7). The substrate inhibition constants were determined from the reciprocal initial velocity vs the initial substrate concentration plot (Figure 8), according to standard equations for substrate inhibition at $[S]_0 \gg K_m$ (Laidler & Bunting, 1973).

Inhibition of ALDHs by Disulfiram. Liver Homogenates. Previous studies of human liver homogenates, with 6-DMA-2-NA as the substrate (Rashkovetsky et al., 1994), revealed "disulfiram-sensitive" and "disulfiram-insensitive" ALDH activities, which turn out to be the major cytosolic (ALDH-1) and mitochondrial (ALDH-2) isozymes, respectively. In hamster and rat liver homogenates, disulfiram inhibits disulfiram-sensitive enzymes (acting on 6-DMA-2-NA) with K_i values of 13 and 15 nM, respectively, whereas in human liver homogenates, the inhibition constant is more than 10 times weaker, i.e. 200 nM (Figure 9). In all cases, the inhibition by disulfiram is rapid, reversible, and noncompetitive.

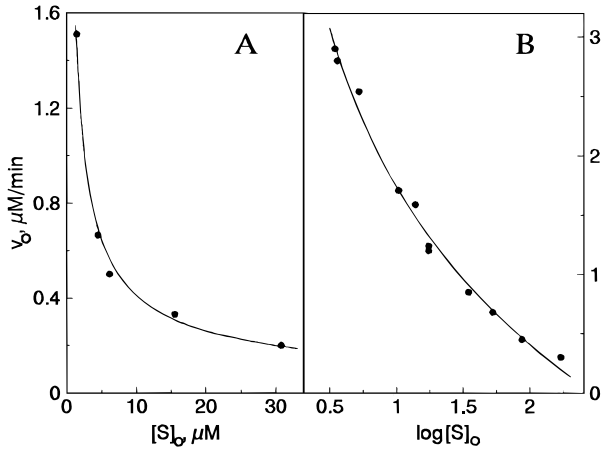

FIGURE 7: Substrate inhibition of human ALDH-1 by (A) 2-naphthaldehyde and (B) *p*-nitrocinnamaldehyde. The ordinate is initial reaction velocity. The abscissa is initial substrate concentration (0.1 M sodium pyrophosphate buffer, pH 9.5, 25 °C).

The presence of disulfiram-insensitive ALDH-2 isozymes in this *in vitro* system does not mean, of course, that these isozymes will remain active *in vivo* in the presence of disulfiram. Quite extensive studies have shown lately that a number of disulfiram metabolites formed *in vivo* are potent irreversible inhibitors of ALDH-2 and are able to suppress its activity along with that of ALDH-1 (MacKerell et al., 1985; Madan et al., 1994; Nagendra et al., 1994; Madan & Faiman, 1994a,b, 1995; Hart & Faiman, 1994, 1995; Mays et al., 1995). This results in the accumulation of acetaldehyde *in vivo*, even when ALDH-1, apparently the most sensitive target for the disulfiram inhibition, does not participate in the metabolism of acetaldehyde.

Purified ALDHs. Disulfiram is a relatively weak inhibitor (both reversible and irreversible, K_i > 12 μM) with respect

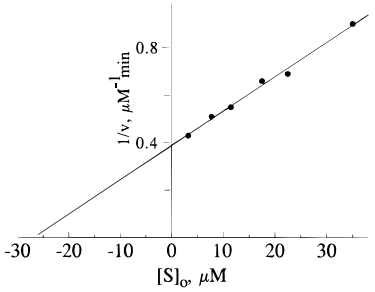

FIGURE 8: Substrate inhibition of human ALDH-1 by *m*-methylbenzaldehyde. Determination of the substrate inhibition constant (K_s') as the abscissa intersection point.

to purified human ALDH-2 and a rather strong reversible inhibitor (K_i = 0.2 μM) of human ALDH-1 toward acetaldehyde oxidation (Table 6). However, disulfiram does not inhibit the hydrolysis of p-NPA catalyzed by human ALDH-2 (K_i > 500 μM).

Hamster ALDH-1 is more sensitive to disulfiram inhibition, with respect to both dehydrogenase (acetaldehyde and 6-DMA-2-NA) and hydrolytic (p-NPA) reactions (K_i = 13 and 12−55 nM, respectively). However, the kinetics of disulfiram inhibition of p-NPA hydrolysis are rather complex and biphasic; the extent of inhbition decreases with time. It apparently reflects the complex topography of the enzyme active center.

Rat ALDH-1 is strongly inhibited by disulfiram; K_i = 15 nM with both acetaldehyde and 6-DMA-2-NA as a substrate (Table 6).

Table 6: Disulfiram Inhibition of ALDH Isozymes

source	isozyme	disulfiram inhibition
human	ALDH-1	$K_i = 200 \pm 30$ nM
	ALDH-2	weak inhibition[a,b]
hamster	ALDH-1	$K_i = 13 \pm 2$ nM[c]
	ALDH-2	very weak inhibition[a,e]
	ALDH-3	$K_i = 2.9 \pm 0.3$ nM[d]
rat	ALDH-1	$K_i = 15 \pm 3$ nM
	ALDH-2	very weak inhibition[a]

[a] Weak reversible and irreversible inhibition with acetaldehyde as a substrate. At pH 9.5, 12 μM disulfiram caused less inhibitory effect on hamster or rat ALDH-2 than 5 μM disulfiram does on human ALDH-2. At pH 7.5, inhibition with 5 μM disulfiram (human ALDH-2) or 40 μM disulfiram (hamster ALDH-2) was undetectable. For the noncompetitive (reversible) inhibition of human ALDH-2, K_i = 9.1 μM (pH 9.5) or 150 μM (pH 7.5). [b] With 12 μM disulfiram (pH 9.5), the half-life for the enzymatic activity was 1.45 ± 0.05 min; with 40 μM disulfiram, the half-life was 0.9 ± 0.1 min. At pH 7.5, the inactivation was much slower; With 40 μM disulfiram, the half-life was 6.7 ± 0.8 min. [c] Noncompetitive inhibition. The K_i value is the same in the presence of various concentrations of acetaldehyde, from 12 μM to 3.2 mM. This and other K_i values listed were determined at pH 7.5. [d] With 6-DMA-2-NA as a substrate. [e] For the noncompetitive (reversible) inhibition, K_i > 50 μM at pH 9.5, undetectable with 40 μM disulfiram at pH 7.5.

For hamster cytosolic ALDH-3, the patterns of inhibition by disulfiram with acetaldehyde and 6-DMA-2-NA as substrates are quite different; K_i = 400 ± 100 and 2.9 ± 0.3 nM, respectively. These data have been discussed above together with others indicating that acetaldehyde and naphthaldehydes are oxidized at different sites on hamster ALDH-3.

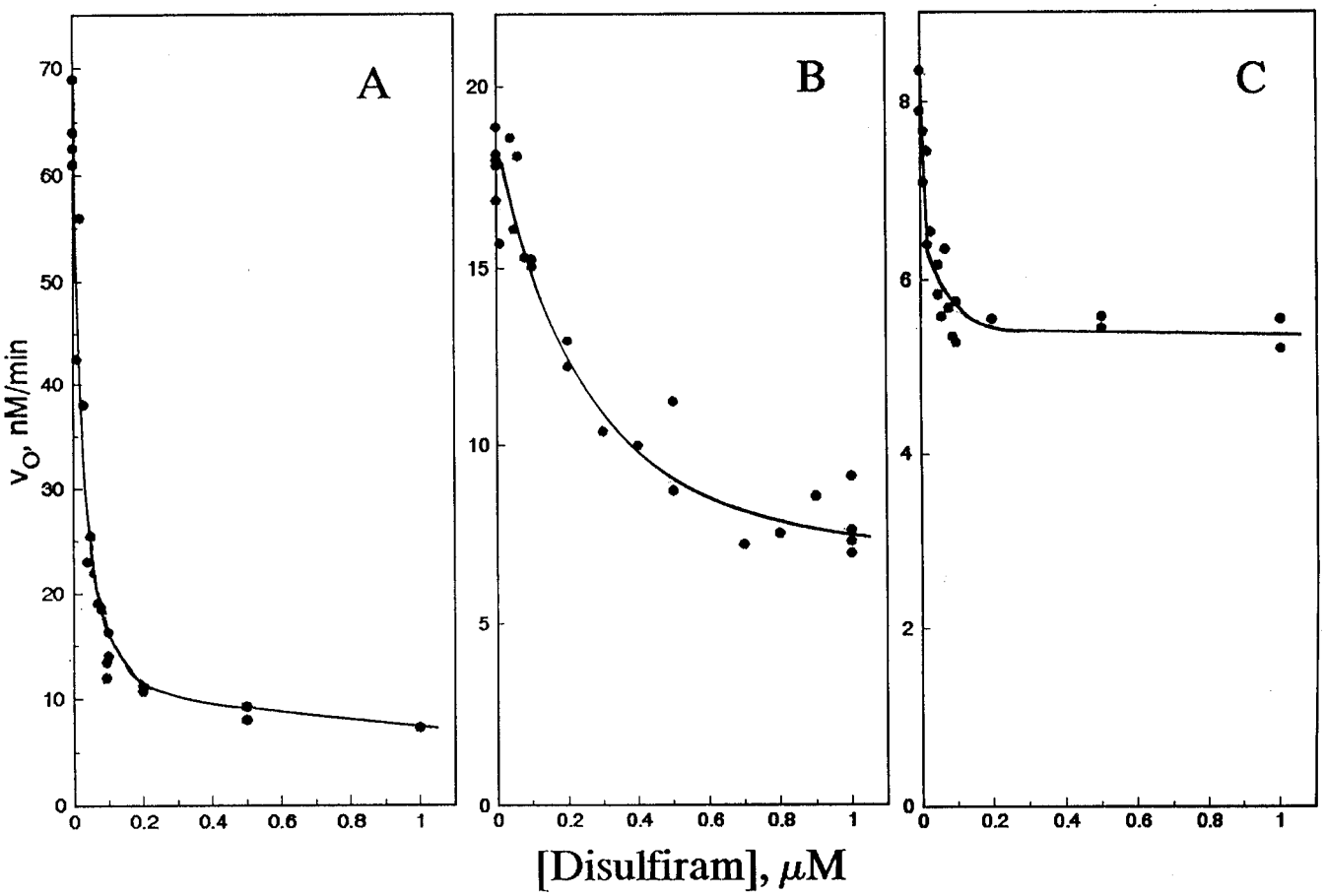

FIGURE 9: Inhibition of ALDHs in hamster (A), human (B), and rat (C) liver homogenates by disulfiram. The substrate is 6-DMA-2-NA (50 nM) (pH 7.5). Curves are calculated for K_i = 13 nM (A), 200 nM (B), and 15 nM (C).

CONCLUSIONS

The present data complement the already huge amount of data published in the ALDH field with respect to purification of ALDH isozymes and their characterization and kinetic behavior. Purification of seven ALDH isozymes, four of which have never been purified and characterized before (rat ALDH-1 from normal liver and the three hamster ALDHs), allows us to highlight several new findings that are important for understanding ALDH systems. First, the major mitochondrial ALDHs from human and rodent liver appear to be very similar to terms of kinetic behavior; as shown earlier, some of them also share a high degree of sequence identity. Second, it is the cytosolic ALDHs that distinguish ALDH systems from different sources in terms of their kinetic behavior and, hence, relative importance in acetaldehyde metabolism. Certainly, the relative amounts of ALDH-1 and -2 in liver is yet another important factor contributing to acetaldehyde metabolism by ALDH systems. Both of these factors, kinetics properties and isozyme composition, differentiate the three ALDH systems studied in this work. Third, the new data presented strongly indicate the existence of several binding and/or catalytic sites in mitochondrial ALDHs that differentiate acetaldehyde from aromatic aldehydes, on the one hand, and those from carboxylic *p*-nitrophenyl esters, on the other. Major cytosolic ALDHs, studied here (exept hamster ALDH-3), provide one active site for both acetaldehyde and aromatic aldehydes. At the same time, some kinetic differences between esterase (with p-NPA) and dehydrogenase (with acetaldehyde) activities, and between esterase activites of ALDHs from different sources, observed earlier can now be explained by largely nonproductive binding of p-NPA with ALDHs (both mitochondrial and cytosolic). These data contribute to the discussion of whether two distinct active sites exist on ALDHs (Tu & Weiner, 1988; Pietruszko, 1989).

Laboratory animals have generally served to simulate aspects of human behavior in response to ethanol consumption. While the applicability of rodent behavioral models in studies of the use and abuse of alcohol has long been accepted, there is no evidence that ethanol metabolism in humans and rodents should follow the same pathways when monitored by similar or identical enzymes. Quite the same, it is generally assumed that the characteristics of the major human liver ALDH isozymes closely resemble those of rodents, though the data supporting this assumption are fragmentary and generally are related to mitochondrial ALDH isozymes only.

The present results demonstrate significant differences between the human, hamster, and rat liver ALDH systems in regard to their apparent subunit composition, inhibition by disulfiram, kinetics toward acetaldehyde and some synthetic substrates, as well as competition between acetaldehyde and aromatic aldehydes. The data suggest that acetaldehyde cannot be considered a "standard" ALDH substrate for studies aimed at aromatic ALDH substrates, e.g. biogenic aldehydes.

The properties of all mitochondrial ALDH isozymes are consistently similar, much as those of the major cytosolic ALDHs of the hamster and rat liver differ significantly from those of the human. Such important characteristics as the K_m values for acetaldehyde are more than 1 order of magnitude higher for human cytosolic ALDH than for rodents. Moreover, both rat and hamster liver cytosolic ALDHs are at least 10 times more sensitive to disulfiram than is human liver cytosolic ALDH. This further underscores the difference between human and rodent ALDH systems.

These observations demonstrate that neither the rat nor the hamster liver ALDH isozyme system is ideal as a model for human acetaldehyde metabolism. Both simulate only certain aspects of the liver ALDH dynamic and/or metabolic behavior. Thus, blocking the cytosolic ALDH pathway for acetaldehyde oxidation in either of the animal models would not completely abolish activity since the mitochondrial pathway will still operate, as it does in humans. On the other hand, blocking mitochondrial ALDH might differentiate the human ALDH system from that of these animal models. Acetaldehyde oxidation in humans would invariably be diminished, but the consequences in rats or hamsters would depend largely on the amount of the major cytosolic ALDH isozymes in the liver. As a result, acetaldehyde metabolic patterns in human, hamster, and rat could differ significantly.

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