Kinetics and Specificity of Human Liver Aldehyde Dehydrogenases toward Aliphatic, Aromatic, and Fused Polycyclic Aldehydes†

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ABSTRACT: Human mitochondrial aldehyde dehydrogenase (ALDH-2) has a K_m for acetaldehyde that is 900-fold lower than that for the cytosolic isozyme, ALDH-1. An increase in aliphatic aldehyde chain length decreases the ALDH-2 K_m by up to 10-fold but decreases that of ALDH-1 by 5 orders of magnitude. As a consequence, the K_m of ALDH-1 for decanal is 8 times lower than that of ALDH-2, i.e. 2.9 ± 0.4 and 22 ± 3 nM, respectively. Determination of these low K_m values required kinetic analysis of the simultaneous enzymatic conversion of two aldehyde substrates, an approach also applied to aromatic and fused polycyclic aldehydes. For most of these substrates, maximum velocities are 5−100 times lower than those for acetaldehyde. Addition of one of these tight-binding, slow-turnover substrates to a reaction mixture containing ALDH, NAD^+, and a "reference" aldehyde substrate (e.g. acetaldehyde) blocks the principal (reference) enzymatic reaction temporarily and reversibly. Once the first substrate is converted to product, the enzyme can act on the reference substrate. In terms of apparent affinity and blocking capacity, naphthalene and phenanthrene aldehydes were the most potent effectors. Other aromatic and fused polycyclic and heterocyclic aldehydes, as well as derivatives of coumarin, quinoline, indole, and pyridine, are tight-binding, slow-turnover substrates for ALDH-2 and relatively weak inhibitors of ALDH-1. The hydrophobicity of substituents of benzaldehydes, and particularly of naphthaldehydes, correlates with their binding constants toward ALDH-2. Vitamin A1 aldehydes are specific natural substrates for ALDH-1; at pH 7.5, for *all-trans*- and 13-*cis*-retinal, K_m = 1.1 and 0.37 μM, respectively, and k_{cat}/K_m is 50−100 times higher than that for acetaldehyde. At the same time, the retinals are inhibitors of ALDH-2, *all-trans*-retinal being a particularly potent inhibitor (competitive K_i = 43 nM, noncompetitive K_i = 316 nM). These properties suggest that *all-trans*-retinal is a possible regulatory compound for ALDH-2 *in vivo*. The data in general point to specialized roles for both major human liver ALDH isozymes in the oxidation of bulky/hydrophobic natural compounds, with K_m values in the low nanomolar range.

The physiological roles of alcohol (ADH) and aldehyde dehydrogenase (ALDH)[1] remain ambiguous. Ethanol and acetaldehyde are thought to be their primary substrates, but neither of these enzymes nor any of their isozymes seem to be particularly specific for them. Indeed, NAD^+-linked ALDHs (EC 1.2.1.3) catalyze the oxidation of a wide variety of aldehydes, but acetaldehyde has been thought to be the only one of physiological significance (Ambroziak & Pietruszko, 1991). While the major ALDH isozymes of mammalian liver oxidize acetaldehyde readily, they do so with strikingly different K_m values. Acetaldehyde can hardly be the natural substrate for human cytosolic ALDH, since its K_m, 180 μM, far exceeds physiological concentrations, typically 0.4−2.5 μM (Lieber, 1988; Hatake et al., 1990; Inoue et al., 1984; Harada et al., 1981). Indeed, the preceding paper shows that, to compensate for such a difference in K_m relative to the physiological acetaldehyde concentration, the amount of cytosolic ALDH in the liver should be 100−300 times higher than that of mitochondrial ALDH, while in fact it is 3−5 times lower (Zorzano & Herrera, 1990; Rashkovetsky et al., 1994).

Few quantitative data for the K_m values of aromatic and long chain aliphatic aldehyde substrates of ALDH have been reported (Pietruszko, 1989; Ambroziak & Pietruszko, 1991). This is no doubt due to the fact that such K_m values are below 0.5−1.0 μM (Ambroziak & Pietruszko, 1991) and cannot be determined readily by conventional kinetic methods. Similarly, benzaldehyde, cinnamaldehyde, and a few of their derivatives have been reported to be substrates for mammalian liver ALDHs (Pietruszko, 1989) but with K_m values too low to allow accurate determination (<0.1 μM).

We have employed an approach to the determination of kinetic constants of ALDH-catalyzed oxidation of tight-binding substrates that allows the identification of important structural elements involved in specificity and should aid in the recognition of specific native ALDH substrates.

MATERIALS AND METHODS

Materials. The source of biological tissues and their treatment have been described (Klyosov et al., 1996). Aliphatic and aromatic aldehydes were obtained from Aldrich Chemical Co., Eastman Organic Chemicals, ICN Biochemicals, Inc., and Pfaltz & Bauer, Inc. 7-Acetoxycoumarin-4-carboxaldehyde was from Molecular Probes, Inc. 5-Bromo-1-naphthaldehyde (needle crystals from 2:1 acetonitrile−water, mp 102−103 °C), 6-(dimethylamino)-2-naphthaldehyde

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; ALDH-1, human cytosolic ALDH; ALDH-2, human mitochondrial ALDH.

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(canary yellow crystals from 1:2 acetonitrile−water, mp 116.5−117.5 °C), 6-(heptanedioic acid monoether)-2-naphthaldehyde (prisms from 2:1 acetonitrile−water), and 7-(dimethylamino)coumarin-4-carboxaldehyde (dark crystals) were provided by Dr. Jacek Wierzchowski of this laboratory. Other chemicals and their suppliers have been cited previously (Klyosov et al., 1996). The concentration of aldehydes was measured either spectrophotometrically at 340 nm by employing purified human liver ALDH from cytosol or mitochondria (ALDH-1 or ALDH-2, respectively) and determining the amount of NADH generated ($\epsilon = 6.22$ mM^{-1} cm^{-1}) upon complete reaction or by weight (when the reactivity of the aldehyde was too low to reach completion or when there were problems in measuring the absorbance of the compound and/or a product of its conversion).

Preparation of the Enzymes. The purification of ALDH-1 and ALDH-2 has been described (Klyosov et al., 1996). Homogeneity was confirmed by sodium dodecyl sulfate (SDS) and nondenaturing polyacrylamide gel electrophoresis (PAGE) and starch gel electrophoresis and by kinetic behavior toward acetaldehyde. The characteristics of the enzymes, e.g. molecular weight, p*I* value, amino acid composition, and electrophoretic patterns of crude and purified preparations, have been described (Rashkovetsky et al., 1994; Klyosov et al., 1996). Protein content was determined with Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard (Bradford, 1976). The k_{cat} values are based on molecular weights for the tetrameric enzymes of 230 000 (ALDH-1) and 240 000 (ALDH-2), given in the preceding article. Specific activities of purified ALDH-1 and -2 from five different batches were 3.4 ± 0.6 and 4.9 ± 0.8 μmol min^{-1} (mg of protein)$^{-1}$, respectively, at pH 9.5. This corresponds to k_{cat} values of 782 ± 138 min^{-1} for ALDH-1 and 1176 ± 192 min^{-1} for ALDH-2.

Assay of ALDH Activity. Substrates and inhibitors of ALDH were dissolved in methanol (acetonitrile significantly activates some ALDHs), except acetaldehyde, propionaldehyde, valeraldehyde, and benzaldehyde, which were dissolved in water. When added to the assay mixture, the final concentration of methanol did not exceed 0.1−1.0% (v:v). These concentrations of methanol do not affect the enzymatic reaction.

K_m and V_m for the ALDH-catalyzed oxidation of substrates whose K_m values were higher than 10 μM were determined from initial velocities, using both Lineweaver−Burke plots and numerical calculations. Since K_m values in the low to submicromolar range cannot be obtained from initial velocities, they were derived from progress curves (Klyosov & Berezin, 1972). It was assumed that the conversion of the aldehydes under study into their respective carboxylic acids was essentially irreversible under the experimental conditions. Indeed, sets of progress curves, typically for several initial substrate concentrations, yield nearly identical kinetic parameters, verifying the irreversibility and absence of product inhibition. In instances in which K_m and/or V_m could be determined by both methods, the same values were obtained within the limits of experimental error.

The single reaction progress curve method (Klyosov & Berezin, 1972) employs relative rather than absolute values of the reaction parameters (initial substrate concentration, initial reaction time, absolute concentration of the reaction product, etc.), which are typical of more conventional graphic methods for computing parameters from an enzyme reaction time course. This significantly increases the accuracy of the kinetic parameters.

Kinetic curves were recorded spectrophotometrically by measurement of the change in absorbance at 340 nm due to formation of NADH at 25 °C with a Varian Cary 219 spectrophotometer. With vitamin A1 aldehydes (*all-trans*- and 13-*cis*-retinals) as ALDH substrates the change in absorbance at 340 nm resulted from formation of NADH ($\epsilon = 6.22$ mM^{-1}), disappearance of retinal ($\epsilon = 22.8$ mM^{-1} cm^{-1}), and appearance of retinoic acid ($\epsilon = 39.2$ mM^{-1} cm^{-1}), with the resulting apparent $\epsilon = 22.6$ mM^{-1} cm^{-1} (Elder & Topper, 1962). The assay mixture contained 3 mM NAD^+ (pH 7.5 or 9.5). It was shown before (Klyosov et al., 1996) and confirmed in this study (see Results and Discussion) that K_m values for ALDH-1 and -2 are essentially the same at pH 7.5 and 9.5. In a few instances, progress curves were recorded by changes in fluorescence in the ratio mode using a Perkin-Elmer MPF3 spectrofluorimeter equipped with an Osram XBO 150 W xenon lamp and an R446 photomultiplier with the following excitation and emission settings: for 4-methoxy-1-naphthaldehyde, 316 and 370 nm; for 6-(dimethylamino)-2-naphthaldehyde, 330 and 430 nm; for *p*-(dimethylamino)benzaldehyde, 340 and 444 nm; and for fluorene-2-carboxaldehyde, 330 (360 nm, for the product of enzymatic oxidation) and 410 nm (440 nm, for the product of enzymatic oxidation).

Measurements of K_m for Substrates with Extremely High Apparent Affinity for ALDH (K_m in the Low Nanomolar Range). These were performed by using an additional "reference" substrate (having very low K_m and low V_m values) as the second aldehyde substrate in the same reaction system. The two substrates (reference and "target") compete for binding with the enzyme. Consequently, each substrate (S) will be displaced partially from complexing with the enzyme, and the "total" initial velocity of the enzymatic reaction will be determined by a number of parameters related to both of them. In the simplest case, this will be determined by the ratios of $V_m[S]/K_m$ and $[S]/K_m$ for the two substrates, according to eq 1

$$v_{tot} = \frac{\frac{V_1 S_1}{K_1} + \frac{V_2 S_2}{K_2}}{1 + \frac{S_1}{K_1} + \frac{S_2}{K_2}} \quad (1)$$

where v_{tot} is the total velocity, V_1 and K_1 are the maximum velocity and the Michaelis constant, respectively, for the first (target) substrate, S_1 is the concentration of the target substrate in the reaction mixture, and V_2, K_2, and S_2 are the respective parameters and concentrations of the second (reference) substrate in the reaction mixture. V_1, K_2, and V_2 are obtained from separate (control) experiments, where S_1, S_2, and v_{tot} are the experimental values. The maximum velocity (V_2) for the reference substrate as well as V_1 for the target substrate essentially can be obtained as the initial velocity (v_0) of substrate conversion in separate experiments, because their K_m values are very low ($K_m \ll [S]_0$). Thus, only K_1 is unknown and can be calculated using the above equation. Because eq 1 is symmetrical with regard to target and reference substrates, in specific situations, these names can be used interchangeably.

Table 1: Substrate Specificity of ALDH-2 and ALDH-1 toward Aliphatic Aldehydes (pH 9.5)

	ALDH-2		ALDH-1	
	k_{cat} (min^{-1})	K_m (nM)	k_{cat} (min^{-1})	K_m (nM)
formaldehyde	4050 ± 500	320000 ± 80000		
acetaldehyde	1180 ± 80	200 ± 20	790 ± 60	180000 ± 10000
propanal	1180 ± 160	95 ± 5	700 ± 47	4500 ± 300
pentanal	1370 ± 60	34 ± 2	490 ± 11	160 ± 30
hexanal	1710 ± 44	30 ± 5	250 ± 8	41 ± 2
heptanal	1360 ± 34	27 ± 4	260 ± 24	18 ± 2
octanal	900 ± 30	28 ± 4	250 ± 10	12 ± 2
decanal	700 ± 34	22 ± 3	230 ± 16	2.9 ± 0.4

Aliphatic aldehydes were studied as target substrates using *p*-tolualdehyde with ALDH-2 ($K_m = K_2 = 14 \pm 2$ nM) and 5-bromo-1-naphthaldehyde with ALDH-1 ($K_m = K_2 = 2.5 \pm 0.3$ nM; see Results and Discussion) as reference substrates. These Michaelis constants were obtained from eq 1 using acetaldehyde as a temporary reference substrate (K_m for acetaldehyde = 0.2 and 180 μM with ALDH-2 and ALDH-1, respectively). V_m values for these reference substrates (V_2 in eq 1) were significantly lower than those for acetaldehyde: 5.5-fold lower for *p*-tolualdehyde with ALDH-2 and 82-fold lower for 5-bromo-1-naphthaldehyde with ALDH-1.

Aromatic aldehydes were studied as target substrates using acetaldehyde as a reference substrate with both ALDH-1 and -2. Values of $K_m \pm$ standard deviation (SD), calculated from eq 1 for duplicate determinations of initial velocities (v_{tot}), are reported for four to six initial substrate concentrations. Initial velocities, at $[S] \gg K_m$, were used to determine V_m for the substrates listed in Tables 1 and 2 (except formaldehyde with ALDH-2 and acetaldehyde with ALDH-1) as well as for the reference substrates, *p*-tolualdehyde and 5-bromo-1-naphthaldehyde.

RESULTS AND DISCUSSION

The ALDH literature contains many conflicting reports on the kinetics of such a "simple" substrate as acetaldehyde, with reported K_m values varying by as much as 20-fold for ALDH-1 and by more than 100-fold for ALDH-2 (see the preceding article). With more tightly binding ALDH substrates, the current knowledge on their kinetic behavior is no better, though Pietruszko (1989) has reported kinetic parameters for a number of "biogenic aldehydes" such as 5-hydroxyindolacetaldehyde (K_m = 2.4 and 0.8 μM for ALDH-1 and -2, respectively), 3,4-dihydroxyphenylacetaldehyde (K_m = 0.4 and 1.0 μM for ALDH-1 and -2, respectively), and phenylacetaldehyde (K_m = 1.5 and 0.6 μM for ALDH-1 and -2, respectively), with the k_{cat} values practically equal to each other in each case. For the ALDH-catalyzed oxidation of retinal, for example, there are various data. Ambroziak and Pietruszko (1991) reported that both ALDH-1 and -2 are active toward *all-trans*- and 13-*cis*-retinal, though K_m values could no be determined, while Yoshida et al. (1992, 1993) reported that only ALDH-1 is active toward *all-trans*-retinal (*cis*-retinal was not tested), with K_m equal to 0.06 μM (600-fold lower than that with acetaldehyde) and k_{cat} equal to that of acetaldehyde.

Our kinetic data, based on both initial velocities and single progress curves, confirm that Michaelis constants for the ALDH-catalyzed oxidation of most aliphatic and aromatic aldehydes are too low to be determined by conventional kinetic approaches. The lowest value of K_m for an NAD^+-linked ALDH-catalyzed reaction that is measurable by a kinetic progress curve is about 0.1 μM, for the oxidation of propanal by ALDH-2 and oxidation of pentanal by ALDH-1 (Table 1). Kinetics and substrate specificity of ALDHs toward longer-chain aliphatic and aromatic aldehydes were studied in the presence of a concurrent (reference) substrate as described in Materials and Methods (see eq 1). Cornish-Bowden (1979) contended that it is unusual to carry out kinetic experiments in which two substrates compete for the same enzyme since this complicates the analysis and does not provide more information than would be obtained by studying the substrates separately. However, this is not always valid. The procedure does prove useful when one of the substrates has a very low K_m value in the low nanomolar and even subnanomolar range which would be difficult or impossible to measure otherwise.

Kinetic of Two Competitive Substrates To Obtain Low-Nanomolar Range K_m Values. Aliphatic Aldehydes as ALDH Substrates. Preliminary experiments led to the choice of *p*-tolualdehyde (*p*-methylbenzaldehyde) as the reference substrate for ALDH-2. Its k_{cat}, 215 min^{-1} (pH 9.5), is 5.5-fold lower than the k_{cat} for acetaldehyde conversion by the same enzyme; its K_m is less than 0.1 μM. Initial velocities for concurrent oxidation of acetaldehyde (16 μM) and *p*-tolualdehyde (14, 28, 42, and 70 μM) by ALDH-2, determined by eq 1, give a K_2 value, i.e. K_m for *p*-tolualdehyde conversion, of 14 ± 2 nM. A reverse (control) experiment to determine the K_m for *n*-propanal conversion by ALDH-2 using *p*-tolualdehydes as a reference substrate gives a value of 95 ± 5 nM, essentially the same as that determined directly from a progress curve, i.e. 0.1 μM. This approach served to determine K_m values for aliphatic aldehydes ranging from pentanal to decanal as substrates of ALDH-2 (Table 1).

With cytosolic ALDH-1, *p*-tolualdehyde gives strong substrate inhibition and therefore cannot be used as a reference substrate with this isozyme; 5-bromo-1-naphthaldehyde was therefore used instead. Its k_{cat} is 9.6 min^{-1} at pH 9.5 (82 times less than that for acetaldehyde); its K_m is less than 0.1 μM. A kinetic study of the concurrent conversion of acetaldehyde and 5-bromo-1-naphthaldehyde by ALDH-1, using eq 1, gives a K_m for the latter (reference) substrate of 2.5 ± 0.3 nM at pH 9.5 and 3.2 ± 1.0 nM at pH 7.5. Again, a control experiment, using 5-bromo-1-naphthaldehyde as the ALDH-1 reference substrate, concurrent with *n*-propanal, gives a K_m for the latter of 4.5 ± 0.3 μM (pH 9.5), a value virtually identical to that for *n*-propanal (4.2 ± 0.6 μM) obtained from initial reaction velocities. Thus, these data provide additional proof for the validity of the approach which served further to determine K_m values

Table 2: Substrate Specificity of ALDH-2 toward Aromatic and Fused Polycyclic Hydrocarbon and Heterocyclic Aldehydes

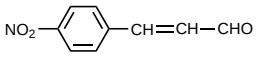
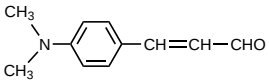
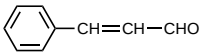
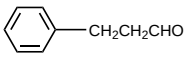
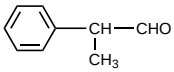
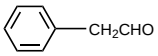
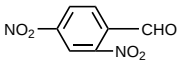
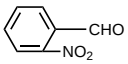
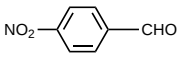
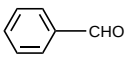
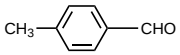
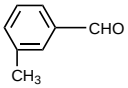
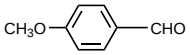
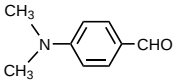
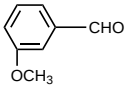
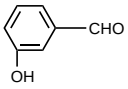
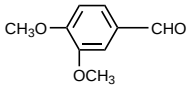
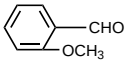
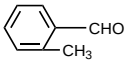
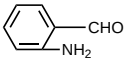
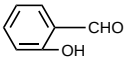
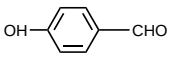
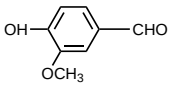
		V_m,rel[a] (%)	K_m (nM)	k_{cat} (min^{-1})
	Cinnamoyl and Hydrocinnamoyl Aldehydes			
p-nitrocinnamaldehyde		2.3 ± 0.5	0.7 ± 0.2	27 ± 7
p-(dimethylamino)cinnamaldehyde		8 ± 1.5	5 ± 2	90 ± 20
trans-cinnamaldehyde		13 ± 3	35 ± 8	150 ± 40
hydrocinnamaldehyde[b]		63 ± 3	500 ± 30	740 ± 50
α-phenylpropionaldehyde[b]		77 ± 4	930 ± 80	910 ± 90
phenylacetaldehyde		153 ± 20	29 ± 4	1800 ± 200
	Benzaldehydes			
2,4-dinitro-		3.4 ± 0.3	3.2 ± 0.6	40 ± 7
o-nitro-		6.3 ± 0.6	6.3 ± 0.1	74 ± 9
p-nitro-		36 ± 1	7 ± 2	430 ± 30
benzaldehyde		30 ± 2	18 ± 2	350 ± 30
p-methyl-		15 ± 3	17 ± 4	180 ± 40
m-methyl-		23 ± 2	18 ± 2	270 ± 30
p-methoxy-		13 ± 1	18 ± 2	150 ± 20
p-(dimethylamino)-[b,c,e]		11 ± 3	20 ± 2	140 ± 40
m-methoxy-		30 ± 2	90 ± 5	350 ± 30 350 ± 60[b]
m-hydroxy-		5.1 ± 0.3	240 ± 10	60 ± 6
3,4-dimethoxy-		7.2 ± 0.4	330 ± 60	85 ± 7
o-methoxy-		1.9 ± 0.2	800 ± 70	22 ± 3
o-methyl-		14 ± 3	1300 ± 400	165 ± 37
o-amino-[b,c]		<2	5100 ± 900	not a substrate
o-hydroxy-[d]		50 ± 5	320000 ± 40000	590 ± 70
p-hydroxy-[b,c]		not an inhibitor/substrate up to 60 μM		
3-methoxy-4-hydroxy-[b,c]		not an inhibitor/substrate up to 20 μM		

Table 2 (Continued)

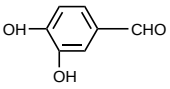
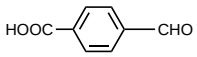
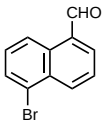
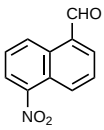
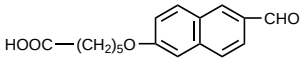
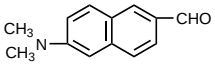
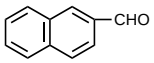
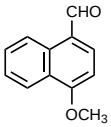
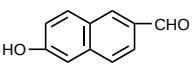
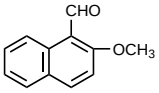
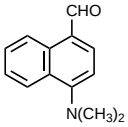
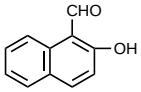
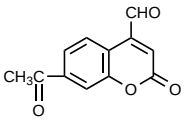
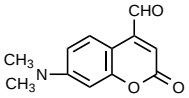
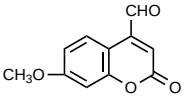
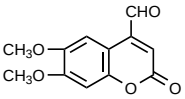
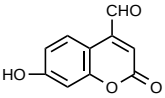
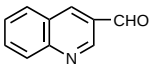
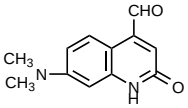
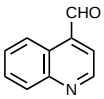
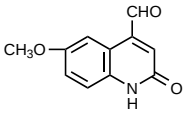
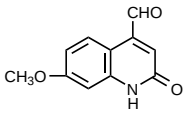
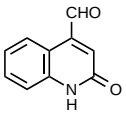
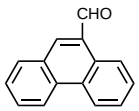
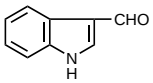
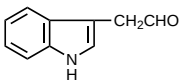
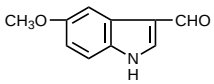
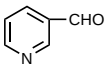
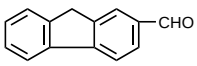
		V_m,rel[a] (%)	K_m (nM)	k_{cat} (min^{-1})
	Benzaldehydes			
3,4-dihydroxy-[b,c]		not an inhibitor/substrate up to 56 μM		
p-carboxy-		noncompetitive activator at 30−180 μM (15−30% activation of acetaldehyde conversion)		
	Naphthaldehydes			
5-bromo-1-		1.3 ± 0.2	0.40 ± 0.09	15 ± 3
5-nitro-1-		4.2 ± 1.7	0.40 ± 0.20	50 ± 20
6-[O-$(CH_2)_5$-COOH]-2-		2.4 ± 0.4	0.9 ± 0.4	28 ± 6
6-(dimethylamino)-2-		3.0 ± 0.5	2.3 ± 1.2	37 ± 2
2-naphthaldehyde		20 ± 3	8 ± 3	240 ± 50
4-methoxy-1-[e]		<0.06	65	<0.7
6-hydroxy-2-[b,c]		<2	160 ± 40	not a substrate
2-methoxy-1-[b,c]		<1	940 ± 90	not a substrate
4-(dimethylamino)-1-[b,c]		<2	1300 ± 500	not a substrate
2-hydroxy-1-[b,c]		<1	2300 ± 300	not a substrate
	Coumarin-4-carboxaldehydes			
7-acetoxy-		5.5 ± 2.5	60 ± 30	65 ± 34
7-(dimethylamino)-		6.6 ± 0.8	62 ± 9	78 ± 10
7-methoxy-		16 ± 2	280 ± 35	190 ± 30
6,7-dimethoxy-		5.0 ± 0.5	690 ± 100	59 ± 7
7-hydroxy-[d]		42 ± 3	150000 ± 20000	500 ± 40

Table 2 (Continued)

		V_m,rel[a] (%)	K_m (nM)	k_{cat} (min^{-1})
	Quinoline- and Quinolinonecarboxaldehydes			
quinoline-3-[b]		57 ± 2	330 ± 20	670 ± 50
7-(dimethylamino)-2-quinolinone-4-		11 ± 1.5	2130 ± 370	130 ± 20
quinoline-4-[b]		24 ± 2	2800 ± 100	280 ± 30
6-methoxy-2-quinolinone-4-[d]		5.0 ± 0.5	5400 ± 400	59 ± 7
7-methoxy-2-quinolinone-4-[d]		27 ± 3	50000 ± 10000	320 ± 40
2-quinolinone-4-[d]		8 ± 2	nd[f]	94 ± 20
	Others			
phenanthrene-9-carboxaldehyde		1.5 ± 0.4	4 ± 3	18 ± 6
indole-3-aldehyde		<0.2	10000−20000	<2.4
indole-3-acetaldehyde[b]		47 ± 3	150 ± 50	560 ± 40
5-methoxyindole-3-carboxaldehyde[d,e]		<0.2	18900	<2
3-pyridinecarboxaldehyde[b,d]		141 ± 5	1960 ± 110	1660 ± 140
fluorene-2-carboxaldehyde		14 ± 2	nd	170 ± 30

[a] V_m, rel is shown in percent in relation to V_m of acetaldehyde (k_{cat} = 1180 ± 80 min^{-1} at pH 9.5). [b] Kinetic data were obtained from progress curves. [c] From inhibitory action in relation to acetaldehyde conversion. [d] Kinetic data were obtained from initial velocities. [e] Kinetics studied spectrofluorimetrically. [f] nd is not determined.

for other aliphatic (Table 1) and aromatic (Tables 2 and 3) aldehydes as substrates of ALDHs.

A direct spectrofluorimetric, kinetic study of the ALDH-1-catalyzed oxidation of 6-(dimethylamino)-2-naphthaldehyde, employing progress curves at an initial substrate concentration of 12 nM, gives K_m values of 2.4 ± 0.2 nM (pH 9.5) and 1.9 ± 0.3 nM (pH 7.5). Thus, the K_m values for naphthaldehydes as ALDH substrates are in the single nanomolar range.

The second substrate in the ALDH-1 reactions, 5-bromo-1-naphthaldehyde, affects the initial velocities of aliphatic aldehyde conversion (Figure 1). In each case in the presence of the reference substrate, the initial velocity is less than in its absence. With increasing chain length of the substrate the inhibitory effect of the reference substrate decreases (with the exception of propanal; see below). Thus, the displacement effect of the reference substrate in relation to longer aliphatic aldehydes is less pronounced, indicating that the apparent affinity of the longer aldehydes for the enzyme is higher, consistent with the respective K_m values listed in Table 1.

Figure 1 demonstrates that a second substrate with a high apparent affinity and a relatively low maximum velocity is an inhibitor of the enzymatic reaction. It displaces the specific substrate from the enzyme and slows the overall velocity of the reaction. When the concentration of the added reference substrate is relatively high, the total velocity of the enzymatic reaction will be reduced to the V_m value for this slow substrate. This is shown by the black bar for n = 2 in Figure 1, where the propanal concentration was relatively

Table 3: Substrate Specificity of ALDH-1 toward Aromatic and Fused Polycyclic Hydrocarbon and Heterocyclic Aldehydes[a]

	V_m,rel (%)[b]	K_m (nM)	k_{cat} (min^{-1})
5-bromo-1-naphthaldehyde	2 ± 0.2	2.5 ± 0.3	9.5 ± 1.6
6-(dimethylamino)-2-naphthaldehyde[c]	20 ± 5	6.3 ± 1.8	160 ± 40
5-nitro-1-naphthaldehyde	4.2 ± 0.6	11 ± 5	33 ± 5
fluorene-2-carboxaldehyde	46 ± 5	54 ± 6	360 ± 40
p-(dimethylamino)benzaldehyde	16 ± 2	60 ± 20	128 ± 16
4-methoxy-1-naphthaldehyde	34 ± 5	200 ± 20	265 ± 45
indole-3-acetaldehyde[c]	86 ± 2	310 ± 20	680 ± 20
trans-cinnamaldehyde[c]	59 ± 5	400 ± 40	470 ± 40
p-(dimethylamino)cinnamaldehyde[c]	62 ± 5	900 ± 200	490 ± 40
7-(dimethylamino)coumarin-4-carboxaldehyde[c]	60 ± 10	1420 ± 230	474 ± 80
phenylacetaldehyde	380 ± 40	5500 ± 1200	3000 ± 340
phenanthrene-9-carboxaldehyde	<4	9400 ± 800 (vs acetaldehyde) 2700 ± 700 (vs cinnamaldehyde)	
2,4-dinitrobenzaldehyde[c]	35 ± 5	2730	substrate inhibition
p-nitrocinnamaldehyde	145 ± 15	<100	substrate inhibition
2-naphthaldehyde	116 ± 12	<100	substrate inhibition
6-[O-$(CH_2)_5$-COOH]-2-naphthaldehyde	74 ± 5	<100	substrate inhibition
m-methylbenzaldehyde	100 ± 10	<100	substrate inhibition
p-methylbenzaldehyde	100 ± 20	<100	substrate inhibition
p-nitrobenzaldehyde	>50	<100	strong substrate inhibition
o-nitrobenzaldehyde	75 ± 8	<100	substrate inhibition

[a] Structures are included in Table 2. [b] V_m, rel is shown in percent in relation to V_m of acetaldehyde (k_{cat} = 790 ± 60 min^{-1} at pH 9.5). [c] Kinetics studied spectrofluorimetrically.

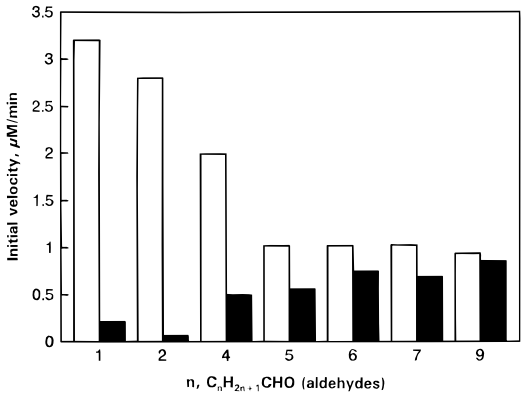


FIGURE 1: Effect of 5-bromo-1-naphthaldehyde (a reference substrate) on the initial velocity of aliphatic aldehyde oxidation by ALDH-1: open bars, in the absence of 5-bromo-1-naphthaldehyde; solid bars, in the presence of 2 μM 5-bromo-1-naphthaldehyde. The reaction conditions were pH 9.5 with an ALDH-1 concentration of 8.9 nM. The substrate concentrations (in micromolar) were as follows: acetaldehyde, 3200; propanal, 13.6; pentanal, 15.4; hexanal, 14.4; heptanal, 14.4; octanal, 7.2; and decanal, 9.6.

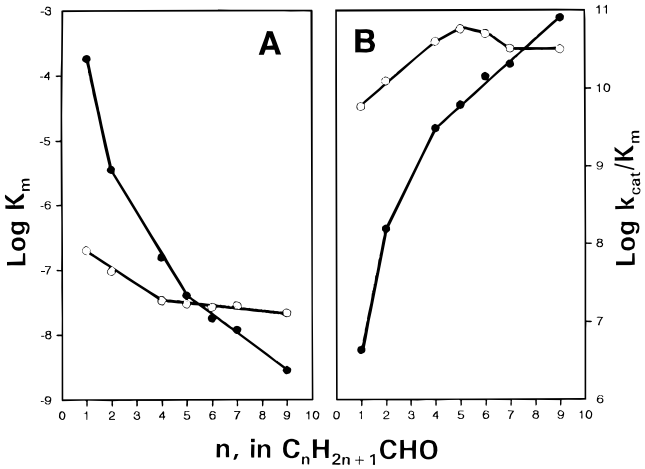


FIGURE 2: Effect of chain length of aliphatic aldehydes (C_nH_{2n+1}-CHO) on (A) apparent affinity (in terms of log K_m) and (B) reactivity [in terms of log (k_{cat}/K_m)] toward ALDH-1 (closed circles) and ALDH-2 (open circles). The data are from Table 1.

low ($[S]_0/K_m$ was the lowest ratio in the series).

Substrate Specificity of Human Mitochondrial and Cytosolic ALDHs toward Aliphatic Aldehydes. With an increase in chain length, the apparent affinity of both ALDH-1 and ALDH-2 for aliphatic aldehydes increases significantly (Table 1). The K_m values for the longest aldehyde tested, *n*-decanal, reach the low nanomolar range: 2.9 ± 0.4 and 22 ± 3 nM for ALDH-1 and -2, respectively. ALDH-1 is particularly substrate-specific (Figure 2). The transition from acetaldehyde to *n*-pentanal decreases K_m 1000-fold (from 180 to 0.16 μM). For ALDH-2, the corresponding change in K_m is only 6-fold, from 0.2 to 0.034 μM. The data suggest that, in addition to acetaldehyde oxidation, ALDH-2 could also have a specialized role involving metabolism of bulky hydrophobic compounds at low concentrations. Because the K_m for acetaldehyde is much higher compared to its physiological concentration, and due to a relatively low amount of ALDH-1 in human liver compared to that of ALDH-2 (see above), acetaldehyde is almost certainly a minor substrate for ALDH-1 which clearly prefers large hydrophobic compounds. It could be said as well that ALDH-1 did not evolve to optimize acetaldehyde oxidation.

Retinals as Substrates for ALDH-1 and Inhibitors of ALDH-2. ALDH-1 is able to oxidize vitamin A1 aldehydes, i.e. both *all-trans*- and 13-*cis*-retinals, in accord with the finding of Yoshida et al. (1992, 1993), who also reported that *all-trans*-retinal is not a substrate for ALDH-2. The present data show that ALDH-1 oxidizes *all-trans*- and 13-*cis*-retinal with K_m equal to 1.1 ± 0.2 and 0.37 ± 0.05 μM, respectively, at pH 7.5. The k_{cat} values (119 ± 11 and 75 ± 8 min^{-1}, respectively) are lower than those for acetaldehyde oxidation (380 ± 30 min^{-1}). Thus, their k_{cat}/K_m values are 108 ± 20 and 203 ± 30 μM^{-1} min^{-1} compared with 2.1 ± 0.3 μM^1 min^{-1} for acetaldehyde, which is 50−100-fold higher, rather close to the recalculated 60-fold value from Yoshida et al. (1992, 1993).

At pH 9.5, kinetics of ALDH-1 toward *all-trans*- and 13-*cis*-retinal is complicated by both substrate activation and product inhibition. Owing to product inhibition, both ap-

parent K_m and k_{cat} values derived from progress kinetics were very high (initial retinal concentrations of 2.5−7.0 μM were used). There was also a strong substrate activation above 2 μM retinals, making it difficult to employ initial velocities for kinetic evaluations at pH 9.5. Taking all of this into consideration, the estimated kinetic parameters (using both progress kinetics and initial velocities at lower substrate concentrations) at pH 9.5 are as follows: for *all-trans*-retinal, $K_m = 0.99 \pm 0.11$ μM and $k_{cat} = 460 \pm 50$ min^{-1}; for 13-*cis*-retinal, $K_m = 1.3 \pm 0.2$ μM and $k_{cat} = 200 \pm 30$ min^{-1}; an for acetaldehyde, 180 ± 10 μM and $k_{cat} = 790 \pm 60$ min^{-1}. Again, k_{cat}/K_m values for ALDH-1 toward *all-trans*- and 13-*cis*-retinal at pH 9.5, 470 ± 70 and 155 ± 25 μM^{-1} min^{-1}, respectively, are 40−100-fold higher compared to that for acetaldehyde (4.4 ± 0.5 μM^{-1} min^{-1}).

With respect to ALDH-2, both *all-trans*- and 13-*cis*-retinal are inhibitors, the former one being much more potent than the latter. *all-trans*-Retinal in concentrations of 75−750 nM is a mixed inhibitor of ALDH-2, with a competitive inhibition constant of 43 ± 15 nM and a noncompetitive inhibition constant of 316 ± 102 nM. At an increased *all-trans*-retinal concentration (3.7, 7.4, and 18.7 μM), the degree of competitive inhibition remains the same but the noncompetitive inhibition weakens. This might reflect an incomplete noncompetitive inhibition of ALDH-2 by *all-trans*-retinal.

13-*cis*-Retinal was a significantly less potent inhibitor of ALDH-2. It was also a mixed inhibitor (decreasing V_m and increasing K_m at the same time) but with competitive and noncompetitive inhibition constants each equal to 5 μM.

These data indicate that in terms of reactivity and apparent affinity both retinals are good but not outstanding substrates for ALDH-1; they are comparable to propanal and pentanal (Table 1). Still, compared to acetaldehyde, the retinals are much better natural substrates. It is quite possible that cytosolic ALDH exercises a specialized function in their metabolism. At the same time, *all-trans*-retinal turns out to be a strikingly potent inhibitor of mitochondrial ALDH, able to affect binding at low nanomolar concentrations and turnover at submicromolar concentrations of the inhibitor. Those properties make *all-trans*-retinal a good candidate to be a specialized regulatory compound for ALDH-2 *in vivo*.

Kinetics and Substrate Specificity of Human Mitochondrial and Cytosolic ALDHs toward Aromatic Aldehydes. Many of the naphthaldehydes, cinnamaldehydes, and other aromatic aldehydes examined (Tables 2 and 3) almost completely saturate the ALDHs at the substrate concentrations that had to be used in kinetic studies. In most cases (except for *ortho*-substituted benzaldehydes and some aromatic aldehydes with bulky substituents), K_m values were below 100 nM and could not be determined by conventional kinetic analysis using either initial velocities or progress curves.

When both aldehyde substrates (the reference and the target) were added to the enzymatic assay system, there were two principal kinetic patterns.

(1) If S_1/K_1 and S_2/K_2 (see eq 1) are of the same order of magnitude, the total velocity of the enzymatic reaction is generally intermediate between V_1 and V_2. The kinetics of the concurrent enzymatic oxidation of acetaldehyde and *trans*-cinnamaldehyde (Figure 3) illustrate this case.

(2) If the ratio S_2/K_2 for the tight-binding substrate is 2 (or more) orders of magnitude higher than S_1/K_1 for the reference substrate, the former would almost completely displace the reference substrate. Until the tight-binding and slow substrate is converted to product, the kinetics of the total reaction essentially reflect the slow conversion (Figures 4−6 for the kinetics of acetaldehyde oxidation by ALDH-1 and -2 in the presence of phenanthrene-9-carboxaldehyde, 5-bromo-1-naphthaldehyde, and 5-nitro-1-naphthaldehyde). Phenanthrene-9-carboxaldehyde is a simple, competitive inhibitor of ALDH-1 (Figure 4B).

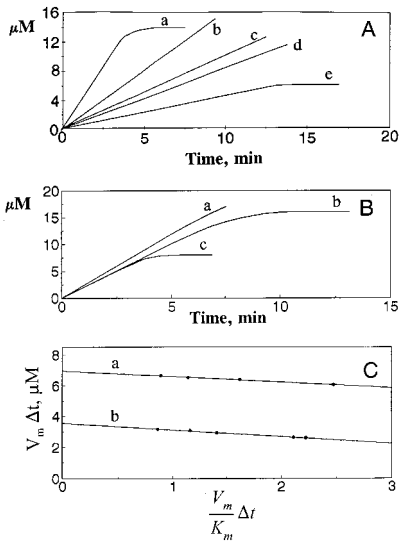


FIGURE 3: *trans*-Cinnamaldehyde (t-CA) as (A) a potent inhibitor of acetaldehyde (AA) oxidation by ALDH-2 and (B) a specific substrate for ALDH-1. The conditions for panel A were as follows: pH 9.5, 14 μM AA, and (a) no t-CA, (b) 4 μM t-CA, (c) 10 μM t-CA, (d) 16 μM t-CA, or (e) no AA and 6 μM t-CA. The conditions for panel B were as follows: 500 μM AA and (a) no t-CA, or no AA and (b) 16 μM t-CA, and (c) 8 μM t-CA. The kinetic parameters for the reactions are as follows: (A) $V_1 = 3.6$ μM/min, $S_1 = 14$ μM, $K_1 = 200$ nM, $V_2 = 0.45$ μM/min, $S_2 = 4$, 10, and 16 μM, and $K_2 = 35$ nM; (B) $V_1 = 3.3$ μM/min, $S_1 = 500$ μM, $K_1 = 180$ μM, $V_2 = 2.1$ μM/min, $S_2 = 8$ and 16 μM, and $K_2 = 400$ nM. (C) Linearization of the progress curves shown in panel B with 16 μM t-CA (a) and 8 μM t-CA (b). The intersection point at the ordinate corresponds to the maximum velocity of the reaction (2.1 ± 0.3 μM/min in this case), and the slope corresponds to the Michaelis constant (400 nM). Δt is a time period related to a half-life of the enzymatic reaction. The linearization method is described in detail in Klyosov and Berezin (1972) and Rashkovetsky et al. (1994).

All of the aldehyde substrates or inhibitors studied are competitive with acetaldehyde in binding to ALDH-1 and -2. Within experimental error, their kinetic behavior can be described by eq 1, where $V_2 > 0$ for substrates and $V_2 = 0$ for inhibitors.

Figures 4−6 show that acetaldehyde begins to be oxidized only after the tight-binding, slow substrate is converted to product. The length of the lag phase corresponds to the reactivity (essentially V_m) of the substrate (eq 1), and the same equation (eq 1) describes the slope of the slower part of the kinetic curve. Tables 2 and 3 list both the experimental and calculated kinetic constants (see Materials and Methods). Whenever it was possible to obtain K_2 values directly, from either initial velocities or kinetic progress curves, within experimental error, the values were the same as calculated by eq 1. Thus, the K_m for 6-(dimethylamino)-2-naphthaldehyde oxidation by ALDH-2 was 2.3 ± 1.2 nM (calculated using kinetic data obtained spectrophotometrically) and 2.6 ± 0.3 nM (by directly recording a spectrofluorimetric progress curve, at initial substrate concentrations of 10, 12, and 15 nM).

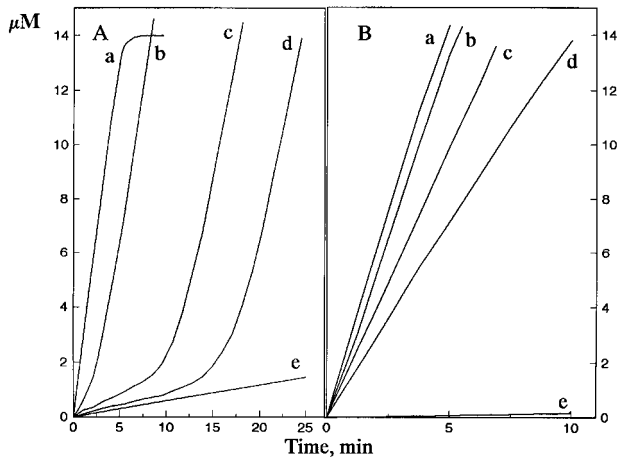


FIGURE 4: Effect of phenanthrene-9-carboxaldehyde (PC) on acetaldehyde (AA) oxidation by (A) ALDH-2 and (B) ALDH-1. The conditions for panel A were as follows: pH 9.5, 14 μM AA, and (a) no PC, (b) 2.08 μM PC, (c) 6.24 μM PC, (d) 10.4 μM PC, or (e) no AA and 10.4 μM PC. The conditions for panel B were as follows: 500 μM AA and (a) no PC, (b) 16.2 μM PC, or 200 μM AA and (c) no PC, (d) 16.2 μM PC, or (e) no AA and 16.2 μM PC. The kinetic parameters for the reactions are as follows: (A) $V_1 = 2.96$ μM/min, $S_1 = 14$ μM, $K_1 = 200$ nM, $V_2 = 0.056$ μM/min, $S_2 = 2.08$, 6.24, and 10.4 μM, and $K_2 = 4.1$ nM; (B) $V_1 = 4.55$ μM/min, $S_1 = 200$ and 500 μM, $K_1 = 180$ μM, $V_2 = 0$, $S_2 = 16.2$ μM, and $K_2 = 9.4$ μM.

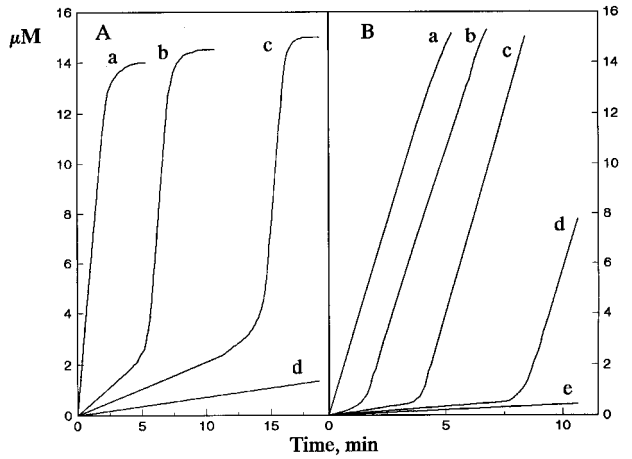


FIGURE 5: Effect of 5-bromo-1-naphthaldehyde (5-Br-NA) on acetaldehyde (AA) oxidation by (A) ALDH-2 and (B) ALDH-1. The conditions for panel A were as follows: pH 9.5, 14 μM AA, and (a) no 5-Br-NA, (b) 0.37 μM 5-Br-NA, (c) 0.74 μM 5-Br-NA, or (d) no AA and 22.1 μM 5-Br-NA. The conditions for panel B were as follows: 500 μM AA and (a) no 5-Br-NA, (b) 0.1 μM 5-Br-NA, (c) 0.2 μM 5-Br-NA, (d) 0.3 μM 5-Br-NA, or (e) no AA and 1.1 μM 5-Br-NA. The kinetic parameters for the reactions are as follows: (A) $V_1 = 5.33$ μM/min, $S_1 = 14$ μM, $K_1 = 200$ nM, $V_2 = 0.071$ μM/min, $S_2 = 370$ and 740 nM, and $K_2 = 0.4$ nM; (B) $V_1 = 4.55$ μM/min, $S_1 = 500$ μM, $K_1 = 180$ μM, $V_2 = 0.046$ μM/min, $S_2 = 100$, 200, 300, and 1100 nM, and $K_2 = 2.5$ nM.

The kinetics of acetaldehyde (the reference substrate) conversion after the second, tight-binding substrate is oxidized completely are virtually identical to those obtained in the absence of the target substrate. Thus, the products of enzymatic oxidation of all aldehydes studied affect neither the V_m nor the K_m of acetaldehyde oxidation by ALDH-2. Apparently, this also pertains to ALDH-1-catalyzed reactions, but the concentrations of acetaldehyde used as the reference substrate in this case were too high to follow the enzymatic reaction to completion.

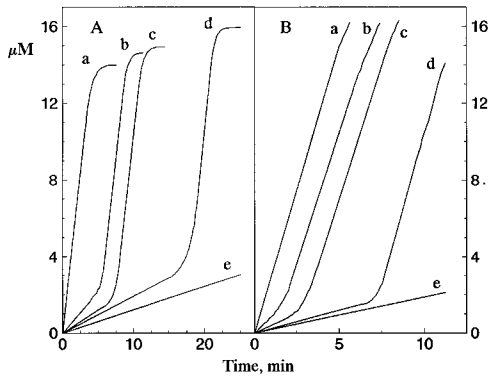


FIGURE 6: Effect of 5-nitro-1-naphthaldehyde (5-NO_2-NA) on acetaldehyde (AA) oxidation by (A) ALDH-2 and (B) ALDH-1. The conditions for panel A were as follows: pH 9.5, 14 μM AA, and (a) no 5-NO_2-NA, (b) 0.63 μM 5-NO_2-NA, (c) 0.94 μM 5-NO_2-NA, (d) 1.57 μM 5-NO_2-NA, or (e) no AA and 3.13 μM 5-NO_2-NA. The conditions for panel B were as follows: 500 μM AA and (a) no 5-NO_2-NA, (b) 0.32 μM 5-NO_2-NA, (c) 0.64 μM 5-NO_2-NA, (d) 1.07 μM 5-NO_2-NA, or (e) no AA and 3.13 μM 5-NO_2-NA. The kinetic parameters for the reactions are as follows: (A) $V_1 = 3.4$ μM/min, $S_1 = 14$ μM, $K_1 = 200$ nM, $V_2 = 0.14$ μM/min, $S_2 = 0.626$, 0.940, 1.57, and 3.13 μM, and $K_2 = 0.4$ nM; (B) $V_1 = 4.55$ μM/min, $S_1 = 500$ μM, $K_1 = 180$ μM, $V_2 = 0.19$ μM/min, $S_2 = 0.32$, 0.64, and 1.07 μM, and $K_2 = 11$ nM.

The time lag in the enzymatic conversion of acetaldehyde in the presence of a slow, tight-binding substrate (as in Figures 4−6) increases linearly with substrate concentration and is a function of the amount of tight substrate added (specifically by its V_m and K_m). Table 2 lists an entire family of such ALDH-2 effectors, covering a wide range of reactivities (V_m) and apparent affinities (K_m). In some cases [*p*-nitrocinnamaldehyde, 5-nitro-1-naphthaldehyde, or 6-[O-$(CH_2)_5$-COOH]-2-naphthaldehyde], concentration of as little as 3 μM supresses acetaldehyde oxidation by 97% and delays it for 30 min even though the enzyme (ALDH-2) is almost completely saturated with acetaldehyde (e.g. 70K_m). Thereafter, enzyme activity is restored completely and rapidly.

Phenanthrene and naphthalene aldehydes are the most potent effectors (Table 2). Some of them, although tight-binding slow substrates for ALDH-2, are only weak inhibitors for ALDH-1. Phenanthrene-9-carboxaldehyde is an example; it binds to ALDH-1 almost 1000 times more weakly than it does to ALDH-2 (Tables 2 and 3, Figure 5). Others, like 5-nitro-1-naphthaldehyde, are tight-binding, slow substrates for both ALDH-1 and ALDH-2 (Tables 2 and 3, Figure 6). Yet others are tight-binding, slow substrates for ALDH-2 and rather good substrates for ALDH-1, with a maximum velocity up to 45% higher than that for acetaldehyde (Table 3).

Electronic and Hydrophobic Effects in ALDH Catalysis. The electronic effects of the substituents in both benzaldehydes and naphthaldehydes, for which a series of data is available, do not influence their K_m values for ALDH-2 (Table 2). Thus, the potent electronegative *p*-nitro group has about the same effect on the apparent affinity as does the electropositive *p*-dimethylamino group [the Hammett σ substituent constants for these two groups are +0.78 and −0.83, respectively (*Handbook of Practical Data, Techniques, and References*, 1972)]. The K_m values for these compounds are 7 nM (*p*-nitrobenzaldehyde) and 19.5 nM (*p*-dimethylamino)benzaldehyde); that for benzaldehyde itself is similar, 18 nM (Table 2). In naphthaldehydes, both substituents, NO_2 and $(CH_3)_2N$, decrease the K_m values

Table 4: Effect of Naphthaldehyde Substituent Hydrophobicity on K_m for ALDH-2

substituent	hydrophobicity coefficient (π)[a]	K_m (nM)
6-OH	−0.61	160
4-CH_3O	−0.04	65
H	0	8
6-$(CH_3)_2N$	0.18	2.3
5-NO_2	0.24	0.4
5-Br	1.02	0.4

[a] From Hansch and Leo (1979).

compared with that for unsubstituted naphthaldehyde (0.4, 2.3, and 8.0 nM, respectively), despite the significant difference in their electronic properties. Overall, no correlation has been found between electronic properties of substituents and the K_m values of the respective aldehydes toward ALDH.

There are some correlations between the apparent affinity of the aldehydes and their hydrophobicity (as expressed in terms of Hansch constants, π; see Table 4). Naphthaldehydes and other fused polycyclic aldehydes generally have lower K_m values with ALDH-2 than do their less hydrophobic benzaldehyde analogs (Table 2). This corresponds to the general trend toward better binding of longer-chain aliphatic aldehydes, described above. The hydrophobicity of substituents of naphthaldehydes and their K_m values also correlate (Table 4).

This correlation is not so obvious for substituted benzaldehydes. However, the transition from rather hydrophobic substituents such as *p*-CH_3, *m*-CH_3, *p*-NO_2, and *p*-$(CH_3)_2N$ (K_m for the respective aldehydes = 7−20 nM) to less hydrophobic ones, such as *m*-CH_3O, *m*-OH, and *p*-OH, leads to a significant increase in K_m, to 90 nM, 240 nM, and even higher (Table 2).

These structure−function relationships indicate that naphthaldehydes with rather hydrophobic substituents, such as *p*-CH_3 (π = 0.93), *p*-C_2H_5 (π = 1.02−1.22), *p*-C_3H_7 (π = 1.40−1.43), or *p*-C_4H_9 (π = 1.90), as well as Cl and I (substituents (π = 0.70−0.76 and 1.15−1.26, respectively), and phenyl and benzyl substituents (π = 1.9−2.0 and 2.38, respectively) might have even greater apparent affinity for the enzyme.

There is a parallel between "apparent binding" and catalysis (in terms of K_m and k_{cat}, or V_m) for a number of substrate series in Table 2. Thus, the data for all cinnamoyl- and hydrocinnamoylaldehydes, the first few benzaldehydes, naphthaldehydes (without hydroxy- and methoxy-substituted compounds), coumarincarboxaldehydes, and some others show that the lower their K_m value with ALDH-2, the slower their oxidation. In terms of energy profiles, this suggests that in each case the transition state is essentially the same and that better binding (if the K_m value is a measure of binding) does not lower the energy level of the transition state and, hence, that better binding is largely nonproductive.

Substitution of aromatic aldehydes in the second position (*ortho* in benzaldehydes) generally leads to a higher K_m value for ALDH-2 (Table 5), except for *o*-nitrobenzaldehyde, the K_m of which is 3 times lower than that of unsubstituted benzaldehyde (6.3 and 18 nM, respectively). All other 2-substituents decrease apparent binding, particularly 2-hydroxy groups, until in the case of *o*-hydroxybenzaldehyde it is virtually abolished (K_m = 320 vs 0.018 μM for benzaldehyde).

Table 5: Effect of 2-Substitution of Aromatic Aldehydes on Binding to ALDH-2

	K_i (μM)	R[a]
o-nitrobenzaldehyde	0.0063	0.35
o-methoxybenzaldehyde	0.8	44
o-methylbenzaldehyde	1.3	72
2-methoxy-1-naphthaldehyde	0.94	118
2-hydroxy-1-naphthaldehyde	2.3	288
o-aminobenzaldehyde	5.1	300
o-hydroxybenzaldehyde	320	18000

[a] Ratio of the binding constants with the respective unsubstituted compound.

Some aromatic substrates are potent inhibitors of ALDH-1 (Table 3). All of them are tight-binding substrates for ALDH-2. Moreover, the greater the degree of substrate inhibition with ALDH-1, the greater the apparent affinity for ALDH-2 (benzaldehydes > nitrocinnamaldehydes > naphthaldehydes). This effect might indicate an evolutionary link between a substrate inhibitory (secondary) site in ALDH-1 and a primary substrate binding site in ALDH-2.

In conclusion, it should be noted that the extremely low K_m values for both isozymes in relation to their hydrophobic substrates are in the range of binding constants for ligand−receptor interactions. This suggests a potential specialized (regulatory) function for human liver aldehyde dehydrogenases *in vivo*, beyond that of the catalytic oxidation of aldehydes, when their substrates are present in extremely low i.e. nanomolar, concentrations. This as yet unknown biological function might relate to the hydrophobicity of such compounds. Vitamin A1 aldehydes, described in this paper as substrates for ALDH-1 and inhibitors of ALDH-2, can serve as specific examples of possible regulatory compounds; *all-trans*- and 13-*cis*-retinal are much better natural substrates of ALDH-1 than acetaldehyde, but they are not oxidized by ALDH-2. Instead, *all-trans*-retinal is a very potent ALDH-2 inhibitor, capable of affecting binding to the enzyme at low molecular concentrations and turnover at submicromolar concentrations of the inhibitor.

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