Human Liver Alcohol Dehydrogenase Isozymes: Reduction of Aldehydes and Ketones†

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ABSTRACT: The class I (α, β_1, γ_1, and γ_2), II (π), and III (χ) isozymes of human liver alcohol dehydrogenase (ADH) were isolated as electrophoretically homogeneous preparations to examine their kinetics of aldehyde and ketone reduction. While the oxidation of a wide variety of alcohols by ADH has been investigated extensively, the reduction of aldehydes and ketones has received much less attention even though the equilibrium favors the latter process. For each isozyme, the K_m and k_{cat} values were measured at pH 7.0 with acetaldehyde, pentanal, octanal, benzaldehyde, and cyclohexanone as substrates. Activity could not be detected with succinic semialdehyde and betaine aldehyde for any of the isozymes. The nonenzymatic hydration, oxidation, and aldol condensation of aldehydes in aqueous solutions present serious experimental obstacles in determining the isozymes' kinetic constants. The effects of these reactions on the enzymatic parameters were studied and compensated for. Michaelis constants for all class I and II isozymes vary by more than 8000-fold, from less than 1 μM for $\beta_1\gamma_1$ and $\beta_1\beta_1$ with octanal to 8.3 mM for π-ADH for acetaldehyde. However, with any given aldehyde, these values vary by less than 40-fold, and the constants are approximately equal to K_m values reported previously for the corresponding alcohols. In contrast, K_m values for χ-ADH are extremely high and could be determined accurately only for octanal (75 μM). The k_{cat} values for class I and II isozymes range from 21 min^{-1} for $\beta_1\beta_1$ with cyclohexanone to 3400 min^{-1} for $\beta_1\gamma_2$ with octanal and are generally an order of magnitude greater than those for the alcohols. However, the $\beta_1\beta_1$ isozyme catalyzes both the reduction of aldehydes and the oxidation of alcohols at turnover rates significantly lower than those of other class I forms. Hence, in principle, poor oxidase activity does not necessarily imply good reductase activity.

Ethanol oxidation has been accepted as the primary function of yeast alcohol dehydrogenase since the enzyme was isolated and crystallized by Negelein & Wulff (1937). This assumed role has been extended to the more recently isolated horse (Bonnichsen & Wasseñ, 1948) and human liver (von Wartburg et al., 1964) forms and has persisted virtually unaltered for 40 years. In large measure, this somewhat uncritical acceptance of an experimentally unproven premise may be attributed both to the remarkable role that ethanol consumption plays in human affairs and to the equally remarkable physiological consequences. Together the historical interest in fermentation processes and the interest in alcoholism have contributed to a preoccupation with the metabolism of ethanol as the probable target of such enzyme systems.

The substrate specificity of any of these and other alcohol dehydrogenases has never been settled convincingly. This problem has now been propelled to center stage by the emerging polymorphism of human alcohol dehydrogenases, as expressed by the remarkable number of isozymes in human liver (Strydom & Vallee, 1982; Vallee & Bazzone, 1983) and their broad specificities for the oxidation and reduction of large numbers of alcohols and aldehydes, respectively (Pietruszko, 1979; Wagner et al., 1983; Vallee & Bazzone, 1983).

All human liver alcohol dehydrogenase isozymes characterized to date are dimeric zinc metalloenzymes with subunits of molecular weight 40 000 (Bosron et al., 1979; Lange & Vallee, 1976; Parês & Vallee, 1981). The isozymes, whose distribution varies from individual to individual and among population groups, can be resolved into three classes on the basis of their kinetic, physical, and immunological characteristics (Strydom & Vallee, 1982; Vallee & Bazzone, 1983). The class I isozymes are composed of α, β_1, γ_1, and γ_2 subunits as described by Smith et al. (1973). They migrate cathodically during electrophoresis at pH 8.2 and are strongly inhibited by 4-methylpyrazole with a K_i of 0.2 μM (Li & Theorell, 1969). Electrophoretically, the class II isozyme (π-ADH)[1] exhibits less cathodic mobility and is inhibited by 4-methylpyrazole with a K_i of 2 mM (Bosron et al., 1979). In contrast, the class III isozyme (χ-ADH) displays anodic mobility at pH 8.2 and is virtually insensitive to 4-methylpyrazole inhibition (Parês & Vallee, 1981).

The classification of ADH isozymes with respect to inhibition constants for 4-substituted pyrazoles coincides with markedly different specificities of each class toward different groups of alcohols. All three isozyme classes catalyze the oxidation of ethanol; remarkably, ethanol does not saturate χ-ADH (class III) at concentrations as high as 2.5 M (Parês & Vallee, 1981). Similarly, the K_m for π-ADH (class II) with ethanol is much higher than the respective values of class I isozymes (Bosron et al., 1977; Wagner et al., 1984). In contrast to class I, neither the class II nor the class III isozymes oxidize methanol, ethylene glycol, or the genins of the cardiac glycosides to an appreciable extent, but they do oxidize a variety of longer chain aliphatic alcohols quite effectively (Vallee & Bazzone, 1983). Indeed, within a given class of ADH, individual isozymes may be kinetically distinct. These results underscore the need for a thorough kinetic characterization of individual, homogeneous isozymes of all three classes to discern any further differences which in turn may

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[1] Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CapGapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]-pyrazole; AMP–agarose, agarose–hexane–adenosine 5′-phosphate (type 2); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NAD^+, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; CM, carboxymethyl; HPLC, high-pressure liquid chromatography.

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provide clues to their possible or probable metabolic functions.

Immunological data further assist in these differentiations. Antibodies raised against the class I isozymes cross-react by immunoprecipitation with all the members of this class but not with the class II or III isozymes. Similarly, anti-class II antibodies do not cross-react with class I or III isozymes, and anti-class III antibodies do not cross-react with class I and II isozymes (Vallee & Bazzone, 1983). The amino acid compositions and sequences of the subunits of classes II and III are currently under study, but the sequence information necessary for an analysis of their possible genetic origin is not yet available. However, the earlier genetic hypothesis of Smith et al. (1973) clearly does not encompass classes II and III.

The optimal activity of ethanol oxidation for all three classes occurs between pH 9 and 11 (Lutstorf et al., 1970; Wagner et al., 1984; Ditlow et al., 1984). In contrast, the maximal activity for the reduction of acetaldehyde to ethanol by class I isozymes is between pH 6 and 7 (Blair & Vallee, 1966; Lange et al., 1976). In general, at physiological pH, the turnover rate for the reduction of aldehydes is much greater than that for the oxidation of the corresponding alcohols (Pietruszko, 1979). Such results could imply that the physiological roles of these isozymes might involve the reduction of endogenous aldehydes and ketones rather than the oxidation of alcohols.

We have examined the reduction of several carbonyl compounds, i.e., aldehydes and ketones, by the individual class I, II, and III ADH isozymes. Relatively little information is available for this direction of ADH catalysis even for unresolved mixtures of human liver alcohol dehydrogenases. This may be due, in part, to the fact that such carbonyl compounds can undergo several nonenzymatic reactions in aqueous solution, e.g., oxidation, polymerization, and hydration, all of which may generate anomalies and, hence, questionable results. We have therefore established the occurrence and extent of these processes for each of the substrates investigated under the conditions employed for their enzymatic assay, eliminated them where possible, and corrected for them both in the experimental design and in the final calculation of the kinetic constants.

Experimental Procedures

Materials. Benzaldehyde (Sigma Chemical Co.), cyclohexanone (Eastman Kodak), and acetaldehyde, pentanal, and octanal (Aldrich) were distilled and stored under nitrogen. HPLC-grade acetonitrile was obtained from J. T. Baker. Horse liver alcohol dehydrogenase was purchased as a crystalline suspension from Boehringer Mannheim Co. and was used only in the characterization of aldehyde hydration. NADH and NAD^+ (both grade III) were obtained from Sigma Chemical Co. All grades of other reagents and buffers were the best available and used without further purification.

Purification and Storage of ADH Isozymes. Human livers were obtained at autopsy within 12-h postmortem and stored at −70 °C. The class I isozymes were isolated as a group by chromatography on DEAE-cellulose and the CapGapp–Sepharose affinity resin (Lange et al., 1976). The individual class I isozymes were then separated from one another by CM-cellulose chromatography (Wagner et al., 1983). The class III (χ-ADH) isozyme was separated from both class I and class II isozymes on DEAE-cellulose and purified to homogeneity on AMP–agarose (Wagner et al., 1984). The class II enzyme (π-ADH) exhibits little affinity for CapGapp–Sepharose and was separated from the class I forms by means of this resin. A homogeneous preparation of π-ADH was obtained by chromatography on AMP–agarose (Ditlow et al., 1984). The purity of each isozyme was demonstrated by $NaDodSO_4$–polyacrylamide (Laemmli & Favre, 1973, urea–polyacrylamide (W. M. Keung, C. C. Ditlow, and B. L. Vallee, unpublished results), and starch gel electrophoresis (Bosron et al., 1979).

All class I isozymes prepared for this study were very stable and retained virtually full activity toward ethanol over a period of 1 month when stored in 5 mM Tris-HCl, pH 7.3, with 0.5 mM NAD^+ and 0.1 mM dithiothreitol (DTT). The class II isozyme was stored in 50 mM phosphate, pH 7.3, containing 0.1 mM DTT. The class III isozyme was stored in 100 mM HEPES, pH 8, in the presence of 1 mM DTT and approximately 1 μM NADH. The class II and III isozymes were used within 1–2 weeks of isolation and retained at least 75% of their initial activity toward ethanol.

Protein concentrations of the human liver ADH isozymes were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard. The protein concentration of the horse liver ADH (EE) was determined by using the A_{280} value with a molar absorptivity of 36 400 M^{-1} cm^{-1}.

Enzyme Assays. ADH activity was determined by measuring the production or utilization of NADH in the presence of ethanol or aldehyde based on an A_{340} value of 6220 M^{-1} cm^{-1}. The spectrophotometric assay was performed on a Cary 219 spectrophotometer, and the data were stored on an Apple II+ computer using software supplied by Varian Associates. The specific activities (units per milligram of protein, 1 unit = 1 μmol of product/min) of the ADH isozymes with respect to ethanol oxidation were determined in 0.1 M glycine, pH 10.0 at 25 °C. The assay contained 2.5 mM NAD^+ and either 33 mM ethanol for the class I and II isozymes or 0.5 M ethanol for the class III isozyme. ADH activities for the reduction of the carbonyl compounds were measured in 50 mM TES, pH 7.0, in the presence of 213 μM NADH at 25 °C. Under these conditions, NADH saturates all three ADH classes (Lange et al., 1976; Bosron et al., 1979; Wagner et al., 1984). The enzyme concentrations in the assays were adjusted to result in rates from 0.2 to 20 μM NADH consumed/min, for efficient data collection and analysis.

Stock solutions of the hydrate-free aldehydes were prepared in acetonitrile. Stock solutions of "prehydrated" aldehydes were obtained by a 5-fold dilution of the aldehyde–acetonitrile solution with TES buffer. Before use, they were allowed to stand for at least 5 half-lives of the hydration reaction but not for longer than 30 min in order to prevent extensive degradation (see Results). All aldehyde assays contained a final concentration of 2% acetonitrile. The addition of aldehyde to the reaction mixture initiated the enzymatic assays.

Measurements: Hydration–Dehydration and Attendant Calculations. Both the extent and rate of aldehyde hydration were determined enzymatically. The aldehyde in question (≈30 μM) was first hydrated and then added to a cuvette containing horse liver ADH (2 μM) and NADH (213 μM) in 50 mM TES, pH 7.0. The large amount of ADH present very rapidly catalyzed the reduction of any unhydrated aldehyde, resulting in an initial burst in the absorbance change at 340 nm. After reduction of all unhydrated aldehyde, the rate slowed, and the limiting step for the reduction of the remaining aldehyde corresponds to the first-order rate of aldehyde dehydration. The equilibrium constant for the hydration reaction (K_{eq} = [unhydrated aldehyde]/[*gem*-diol]) was calculated from the concentrations of unhydrated and hydrated aldehydes. The rate constant for the dehydration was determined from an analysis of the single exponential decay of the *gem*-diol. Rate constants for the hydration step were then calculated from the relationship $K_{eq} = k(\text{dehydration})/k(\text{hydration})$.

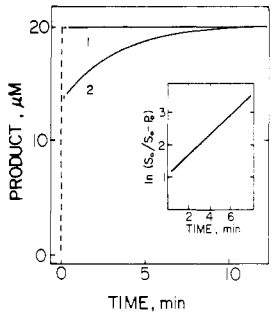

FIGURE 1: Effect of hydration on the reduction of pentanal with horse liver alcohol dehydrogenase. The reaction was initiated by the addition of pentanal (20 μM) to a cuvette containing 214 μM NADH and 1.4 μM ADH in 50 mM TES buffer, pH 7.0 at 25 °C. The stock solution of pentanal was prepared in either anhydrous acetonitrile (curve 1) or 50 mM TES buffer, pH 7.0 (curve 2). The concentration of product was calculated from the change in NADH concentration. The insert is a first-order plot of curve 2 where S_0 is the initial pentanal concentration and P is the product concentration at time t.

Data Analysis. Absorbance vs. time data were fit to either a first-order or a third-order linear equation of the type [product] = $a + bt + ct^2 + dt^3$ (Orsi & Tipton, 1979; Cornish-Bowden, 1976; Philo & Selwyn, 1973). A least-squares curvilinear regression procedure (Carnahan et al., 1969; Cleland, 1967) was used to determine initial velocities corresponding to the "b" term in the above equation. The resultant pairs of velocity vs. substrate concentration data were then fit to the Michaelis–Menten equation by using the least-squares iterative method of Cleland (1967, 1979) to estimate the kinetic parameters. At least eight pairs of velocity vs. substrate concentration data were used for each K_m and k_{cat} determination, and substrate concentrations were from 0.5 to 10 times the value of K_m except where substrate inhibition interfered with the use of such high concentrations. Values of k_{cat} were determined from the maximal velocity estimate and are based on a molecular weight of 80000 for all isozymes.

Results

Purification and Identification of Isozymes. All the alcohol dehydrogenase isozymes exhibited single bands on NaDodSO$_4$–polyacrylamide gel electrophoresis corresponding to an approximate subunit molecular weight of 40000. They were identified by starch gel electrophoresis under conditions where each isozyme migrated as a single band of activity at a rate characteristic of its subunit composition. The identities of class I isozymes were verified further by urea–polyacrylamide gel electrophoresis (W. M. Keung et al., unpublished results). On the basis of these criteria, the isozymes used in this study are $\alpha\alpha$, $\alpha\beta_1$, $\alpha\gamma_1$, $\alpha\gamma_2$, $\beta_1\gamma_1$, $\beta_1\gamma_2$, $\beta_1\beta_1$, π, and χ. The specific activities of all isozymes agree with previously published values: $\beta_1\beta_1$, 0.2 unit/mg of protein; all other class I and II forms, 0.6–1.2 units/mg of protein; class III, 0.4–0.6 unit/mg of protein.

Hydration–Dehydration of Aldehydes. Upon addition to aqueous solutions, the carbonyl group of aldehydes may become appreciably hydrated. An example of the hydration reaction is shown in Figure 1. The extent of pentanal hydration is determined by using horse liver ADH, although any other ADH isozyme can be used. When an aliquot of hydrate-free pentanal is added to a curvette containing an excess of NADH and a large amount of ADH activity, there is a rapid decrease in absorbance at 340 nm that is complete within the time of mixing (Figure 1, curve 1). However, when pentanal is hydrated before addition to the cuvette, this rapid initial decrease in absorbance is less and is followed by a slower first-order process (Figure 1, curve 2). In both experiments,

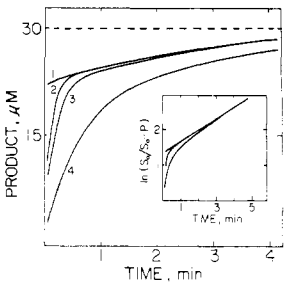

FIGURE 2: Effect of enzyme concentration on the reduction of pentanal. Pentanal (30 μM from an aqueous stock solution) was added to a cuvette containing 214 μM NADH in 50 mM TES buffer, pH 7.0 at 25 °C. The concentration of horse liver ADH in cuvettes 1, 2, 3, and 4 was 1.4 μM, 14 nM, 8.2 nM, and 4.1 nM, respectively. The final product concentration for all curves was 30 μM (dashed line). The insert is a first-order plot of curves 1–3, where S_0 is the initial pentanal concentration and P is the product concentration at time t.

Table I: Hydration Constants for Aldehydes in Aqueous Solution[a]

aldehyde	$k_{dehydration}$ (min^{-1})	$k_{hydration}$[b] (min^{-1})	K_{eq}	$t_{1/10}$(hydration) (min)
acetaldehyde	0.66	0.55	1.2	0.20
pentanal	0.49	0.25	2.0	0.48
octanal	0.45	0.28	1.6	0.42
benzaldehyde			N.H.[c]	
cyclohexanone			N.H.	

[a] 50 mM TES at pH 7, 25 °C, 213 μM NADH, ≈2.1 μM horse ADH, and ≈30 μM aldehyde or ketone. [b] Calculated as described under Experimental Procedures. [c] N.H. = no measurable hydration (less than 0.5% hydrated).

the total absorbance change corresponds to the complete reduction to pentanol. Thus, prior exposure of pentanal to water decreases the amount of aldehyde initially available for enzymatic reduction. Figure 2 shows the effect of horse liver ADH concentration on the rate of reduction of 30 μM "prehydrated" pentanal. While the rate of the initial decrease in absorbance is directly proportional to enzyme concentration, the subsequent first-order process is independent of enzyme concentration (Figure 2, insert). The initial burst corresponds to the enzymatic reduction of unhydrated aldehyde, while the subsequent first-order rate corresponds to the dehydration of the *gem*-diol. Both the extent and rate of the hydration process agree with values derived from spectroscopic studies (Bell, 1966). The independence of the first-order rate with respect to enzyme concentration further demonstrates that the hydrated form of the aldehyde is not a substrate for ADH.

Table I shows rates of hydration and dehydration for all carbonyl compounds used in this study and the calculated equilibrium constants. The hydration rates at pH 7.0 for pentanal and octanal, 0.25 and 0.28 min^{-1}, respectively, are about half the rate of acetaldehyde hydration (0.55 min^{-1}). These three aldehydes are hydrated approximately to the same extent with equilibrium constants ranging from 1.2 for acetaldehyde to 2.0 for pentanal. In contrast, benzaldehyde and cyclohexanone are not hydrated to a detectable amount, i.e., less than 0.5%. Assuming a reversible unimolecular reaction, [carbonyl compound] $\rightleftharpoons$ [*gem*-diol], the time required to hydrate a given aldehyde is calculated as follows:

$$t = \ln\left[\frac{k_1 A_0}{(k_1 + k_2)A - k_2 A_0}\right] \Big/ (k_1 + k_2) \qquad (1)$$

where k_1 and k_2 are the first-order rate constants for the hydration and dehydration processes, respectively, A_0 is the initial unhydrated aldehyde concentration, and A is the con-

Table II: K_m Values (μM) for ADH Isozymes

substrate	class I isozymes							class II isozyme, π	class III isozyme, χ
	$\alpha\alpha$	$\alpha\gamma_1$	$\alpha\gamma_2$	$\alpha\beta_1$	$\beta_1\gamma_1$	$\beta_1\gamma_2$	$\beta_1\beta_1$		
acetaldehyde	2000	1300	700	150	1100	280	76	8300	*a*
pentanal	8	23	16	11	21	13	16	53	*a*
octanal		1–2	5.8	3.8	<1[b]	6.1	<1[b]	6.8	75
benzaldehyde	45	30	39	52	250	65	380	10	*a*
cyclohexanone	230	130	140	140	150	260	110	100	N.A.[c]

[a] Cannot approach saturation due to substrate solubility limits. [b] Detection limits do not permit a small enough concentration to determine K_m accurately. [c] N.A. = no activity (<30 nM/min at 200 mM cyclohexanone with 30 nM χ-ADH).

Table III: k_{cat} Values (min^{-1}) for ADH Isozymes

substrate	class I isozymes							class II isozyme, π	class III isozyme, χ
	$\alpha\alpha$	$\alpha\gamma_1$	$\alpha\gamma_2$	$\alpha\beta_1$	$\beta_1\gamma_1$	$\beta_1\gamma_2$	$\beta_1\beta_1$		
acetaldehyde	350	1100	890	630	860	1100	380	650	*a*
pentanal	450	1800	1800	690	1500	2000	280	920	*a*
octanal		1300	2100	1100	670	3400	300	1200	73
benzaldehyde	670	280	420	500	560	320	190	450	*a*
cyclohexanone	590	230	270	800	100	74	21	20	N.A.[b]

[a] Cannot approach saturation due to substrate solubility limits. [b] N.A. = no activity (<30 nM/min at 200 mM cyclohexanone with 30 nM χ-ADH).

Table IV: k_{cat}/K_m Values (μM^{-1} min^{-1}) for ADH Isozymes

substrate	class I isozymes							class II isozyme, π	class III isozyme, χ
	$\alpha\alpha$	$\alpha\gamma_1$	$\alpha\gamma_2$	$\alpha\beta_1$	$\beta_1\gamma_1$	$\beta_1\gamma_2$	$\beta_1\beta_1$		
acetaldehyde	0.18	0.85	1.3	4.2	0.78	3.9	4.9	0.08	0.005
pentanal	56	78	110	63	71	150	17	17	0.014
octanal		870	360	290	>670[a]	560	>300[a]	180	0.97
benzaldehyde	15	9.3	11	9.6	2.2	4.8	0.5	45	0.0002
cyclohexanone	2.6	1.8	2	5.7	0.67	0.28	0.2	0.2	N.A.[b]

[a] Detection limits do not permit a small enough concentration to determine K_m accurately. [b] N.A. = no activity (<30 nM/min at 200 mM cyclohexanone with 30 nM χ-ADH).

centration of the unhydrated aldehyde at t seconds (Frost & Pearson, 1961).

Figure 3A,B demonstrates the effect of hydration on the reduction of aldehydes by ADH. Although these curves are generated with $\alpha\gamma_1$ isozyme, similar results are obtained with all other human liver ADH isozymes. Figure 3A shows a reaction initiated by the addition of anhydrous acetaldehyde in acetonitrile. The observed nonlinearity is due to the nonenzymatic formation of the *gem*-diol which reduces the effective concentration of the substrate available to ADH. A tangent to the data at zero minutes would represent the initial enzymatic velocity. When acetaldehyde is allowed to hydrate before addition to an otherwise identical reaction mixture, the rate of reduction by ADH is linear (Figure 3B). Thus, under conditions where the hydration of the aldehyde is slow with respect to the mixing time, e.g., pentanal and octanal, addition of the hydrate-free aldehyde to the enzymatic assay generates accurate initial velocities. However, accurate initial velocities cannot be obtained in this manner for acetaldehyde where the hydration rate is rapid with respect to the time of mixing. In such instances, it is necessary to prehydrate the aldehyde and then add it to the enzymatic assay as described under Experimental Procedures.

Anhydrous acetonitrile was selected to serve as the organic carrier for all the aldehydes; it is inert with respect to them, is not a substrate for ADH, and minimally affects the kinetics of ADH. An acetonitrile concentration of 5% or less does not affect K_m or k_{cat} by more than a factor of 2. Hence, all assays contain 2% acetonitrile except those employing cyclohexanone, since it does not undergo significant hydration. When assays are initiated with an aliquot of pentanal which is either non-

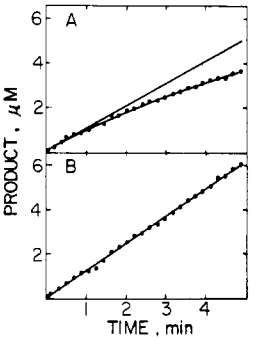

FIGURE 3: Representative time courses for the reduction of acetaldehyde by ADH. The reaction was catalyzed in this case by $\alpha\gamma_1$ (24 nM) in the presence of 214 μM NADH and 50 mM TES, pH 7.0 at 25 °C. (A) The reaction was initiated by the addition of an aliquot of acetaldehyde in acetonitrile. The data were fit to a third-order polynomial equation, and the tangent at zero time represents the initial velocity. (B) The reaction was initiated by the addition of an aliquot of acetaldehyde previously hydrated by 5-fold dilution with buffer. The initial velocity can be calculated from a linear fit of the data. The initial concentration of unhydrated acetaldehyde in (A) and (B) was 50 and 55 μM, respectively.

hydrated in acetonitrile or hydrated in an aqueous solution, the kinetic parameters are identical if the pentanal concentration of the "prehydrated" solution is corrected by using the equilibrium constant (Table I). Apparently the hydrate is neither a substrate nor an inhibitor for the human liver ADH isozymes.

Aldehydes and Isozymes. Tables II, III, and IV show the K_m, k_{cat}, and k_{cat}/K_m values, respectively, for class I, II, and III ADH isozymes with five carbonyl substrates. K_m values

for the class I and II isozymes vary approximately 8000-fold, i.e., from less than 1 μM for $\beta_1\gamma_1$ and $\beta_1\beta_1$ with octanal to 8.3 mM for π-ADH with acetaldehyde. However, with any given aldehyde, the K_m values for different class I and II isozymes vary less than 40-fold. Increasing the chain length of the aliphatic aldehydes lowers K_m values for all class I and II isozymes, i.e., octanal < pentanal < acetaldehyde. The K_m with acetaldehyde for π-ADH is 4–50-fold greater than for any of the class I isozymes; however, the K_m with octanal for π-ADH is comparable to those for $\alpha\gamma_1$ and $\beta_1\gamma_2$. The K_m values with octanal and benzaldehyde for π-ADH are approximately equal whereas in these terms the class I isozymes clearly prefer octanal. The K_m values for acetaldehyde, pentanal, and benzaldehyde for the class III isozyme are extremely high, and the concentration of substrate which corresponds to half V_{max} exceeds their solubility limits. χ-ADH has a relatively low K_m with octanal which suggests that, similar to the class I and II isozymes, hydrophobicity enhances substrate binding.

Overall, the k_{cat} values for carbonyl reduction by class I and II isozymes vary 160-fold, i.e., from 21 min^{-1} for $\beta_1\beta_1$ with cyclohexanone to 3400 min^{-1} for $\beta_1\gamma_2$ with octanal. Notably, the k_{cat} value with all aldehydes is lowest with the $\beta_1\beta_1$ isozyme. Although for class I isozymes the K_m values with cyclohexanone are relatively constant, the k_{cat} values vary 40-fold. Hence, in this study, cyclohexanone is kinetically distinct since the k_{cat} values with all the aldehydes vary less than 10-fold.

Values of k_{cat}/K_m most obviously reflect the specificities of the purified ADH isozymes (Table IV). Octanal is the "best" substrate for all isozymes examined; they all prefer pentanal over acetaldehyde. Compared to the class I isozymes, π-ADH is less effective in catalyzing the reduction of acetaldehyde, but more effective with benzaldehyde. χ-ADH has the lowest k_{cat}/K_m values with all substrates examined. Two aldehydes not listed in Tables I–IV, succinic semialdehyde and betaine aldehyde, show no detectable activity for any of the three classes of ADH.

Discussion

Aldehydes may undergo nonenzymatic reactions, e.g., hydration, oxidation, and aldol condensations, upon addition to an aqueous solution, thereby potentially complicating the enzymatic study of aldehyde reduction. The chemistry of these reactions has previously been studied selectively but not necessarily in relation to the enzymology here under consideration. Again, enzymological investigations have considered any one of these possible complications, but none have integrated them all. Certainly many investigators have been aware of these sources of potential conflicts, and the anticipated experimental difficulties may account for the relative paucity of data on aldehyde reduction by ADH in the literature. Hence, it seems prudent to summarize these features here and the corrections which must be made to account for them.

Aldehydes are subject to oxidation in aqueous solutions, yielding the corresponding acids. Hastings et al. (1963) reported a half-life of approximately 2 h for the oxidation of decanal, consistent with our observations for both octanal and pentanal. This oxidation reaction alters the concentrations of aldehyde stock solutions with time and introduces potential artifacts to the enzymatic reactions. In addition to this oxidation, short-chain alkyl aldehydes can undergo a base-catalyzed aldol condensation (Naylor & Fridovich, 1968). Since both of these side reactions are relatively slow, they can be minimized if the aldehydes are stored in an anhydrous state and exposed to water only at the start of the enzymatic assay or, when prehydration is necessary, if hydration is allowed to proceed only to 95% completion.

Alkyl aldehydes are hydrated reversibly to their respective *gem*-diols in aqueous solution. These hydrated aldehydes appear to be neither substrates nor inhibitors of the human liver ADH isozymes. Indeed, identical kinetic parameters are obtained for aldehydes in either the absence or the presence of the corresponding *gem*-diols. The enzyme-independent first-order rate of Figure 2 also indicates that the hydrates are not substrates. These findings are consistent with those of Abdallah et al. (1979) for horse liver ADH with acetaldehyde.

When the rate of *gem*-diol formation is significant within the time course of the assay, the concentration of substrate is effectively altered which results in nonlinear velocities (Figure 3A). This complicates the graphic determination of the initial rates, though initial velocities can be obtained from a fit of the data to a third-order polynomial equation, as described under Experimental Procedures. This analysis was applied to all substrates except acetaldehyde, which has twice the hydration rate of pentanal and octanal (Table I). In this case, more than 10% of the acetaldehyde is hydrated within 20 s, the time necessary to obtain a reliable measurement of the initial velocity. Hence, acetaldehyde was hydrated (4–30 min) before addition to the reaction mixture, and the initial concentration of the free aldehyde was calculated from K_{eq}. It is important to allow sufficient time to prehydrate acetaldehyde, i.e., 5 half-lives of the hydration reaction, but insufficient time for either oxidation or aldol condensation to significantly affect results. The absorbance traces are then linear, and a good estimate of the initial velocity is obtained.

The concentrations of the aldehydes and ketones in stock solutions were determined enzymatically by using horse liver ADH as described under Experimental Procedures. This procedure requires only that the aldehyde or ketone be a substrate for ADH; it is not dependent on the characteristics of each carbonyl compound. Under the present assay conditions, the reduction of the aldehyde to the alcohol is essentially complete as calculated from the redox potentials (Jones & Beck, 1976). This method eliminates the need for secondary derivatization techniques (Knapp, 1979) and the reliance on gravimetric measurements which may be inaccurate due to the volatility of some of the substrates. In addition, the quantities of both unhydrated and hydrated forms of the aldehydes can be calculated as described under Results. The equilibrium constants thus obtained (Table I) coincide quite well with literature values determined by UV and NMR spectroscopy, or polarography (Bell, 1966; Bodley & Blair, 1971).

The human livers used in the preparation of the purified isozymes predominantly contained the heterodimers, the $\beta_1\beta_1$ homodimer, and traces of the $\alpha\alpha$ homodimer. The $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ homodimers were also present but were not isolated in quantities sufficient to allow inclusion in this study. Substrates were selected to complement a study of the individual class I isozymes with respect to alcohols (Wagner et al., 1983).

The K_m values with the more hydrophobic aldehydes are consistently low for all ADH isozymes listed in Table II. In this respect, octanal, the most hydrophobic one among these, is the only reasonably good substrate for χ-ADH. In fact, the K_m values for χ-ADH with the other aldehydes exceed their solubility limits in aqueous buffers. In contrast, none of the three classes of ADH reduce succinic semialdehyde or betaine aldehyde at a detectable rate. This preference for uncharged hydrophobic aldehydes and alcohols suggests that the substrate binding sites of all three classes of human liver ADH may consist of a hydrophobic channel, in accord with observations

on the horse liver EE isozyme by X-ray crystallography (Eklund, 1983).

The k_{cat} values for the individual class I and II isozymes are relatively independent of the structure of the aldehydes. Cyclohexanone, the only ketone examined, exhibits values of k_{cat} for $\beta_1\gamma_1$, $\beta_1\gamma_2$, $\beta_1\beta_1$, and π-ADH which are significantly lower than the average values obtained with the aldehydes. If the dissociation of coenzyme is the rate-limiting step in the conversion of aldehydes to alcohols, then the structure of the substrate should not affect the value of k_{cat}. These present data suggest that the release of NAD^+ is indeed the rate-limiting step in the reduction of the aldehydes. However, when cyclohexanone is the substrate, another step in the catalytic mechanism may be slower than the dissociation of coenzyme. It is of potential interest that the dimers which contain an α subunit turn over cyclohexanone at rates comparable to those of the aldehydes, indicating that in these cases the dissociation of coenzyme may also be the rate-limiting step. The k_{cat} values for the $\beta_1\beta_1$ isozyme are markedly lower than the corresponding ones for the other class I or class II isozymes. If the differences in the maximal rates of the individual isozymes are due to changes in coenzyme dissociation rates, then $\beta_1\beta_1$ may be distinct from the other class I forms with respect to the structure of the coenzyme binding site. In this respect, Jörnvall et al. (1984) have shown that a His/Arg substitution in the coenzyme binding sites of an allelic variant of the ADH_2 locus explains the functional differences between two β forms. Given the limited data in Table II, it is not possible to extend these arguments to the class III isozyme; however, its k_{cat} value with octanal is 4-fold lower than that for $\beta_1\beta_1$ and 46 times lower than that for $\beta_1\gamma_2$. Again, this may indicate that here the coenzyme binding site is significantly different from that of the other forms.

Under optimal conditions, pH 7 and 10 for aldehydes and alcohols, respectively, the turnover rates for the individual class I isozymes with aldehydes (Table III) are generally an order of magnitude greater than those with the corresponding alcohols (Wagner et al., 1983). Their K_m values, however, are comparable. Previous studies showed that both a mixture of the class I forms at pH 7.0 (Pietruszko et al., 1973) and the class II isozyme at pH 7.5 (Bosron et al., 1979) yield k_{cat} values with aldehydes 30–100-fold greater than those with the corresponding alcohols. Again, the values of K_m did not differ significantly. Thus, the dramatic differences in the k_{cat}/K_m values between alcohols and aldehydes result more from differences in their turnover rates than in their capacities to bind the enzymes. These data are consistent with the general assumptions that the rate-limiting step in catalysis is the dissociation of the enzyme–coenzyme complex and that the dissociation rate of the enzyme·NAD^+ complex is faster than that of the enzyme·NADH complex. Indeed, the coenzyme releasing step for the horse EE isozyme is rate limiting with a dissociation rate of >7200 min^{-1} at pH 7 for the E·NAD^+ complex and 450 min^{-1} at pH 10 for the E·NADH complex (Kvassman & Petersson, 1979). Comparison of these kinetic constants also suggests that a given aldehyde which is a good substrate for a particular ADH isozyme will correspond to an alcohol which will be a good substrate as well.

The class I and II isozymes appear to be more closely related with respect to their substrate specificities than class III ADH (Table IV). Without exception, the k_{cat}/K_m values for χ-ADH are extremely low relative to those of other isozymes; hence, this isozyme is the most distinctive one. Compared to the class I isozymes, the k_{cat}/K_m for π-ADH with acetaldehyde is relatively low, but that with benzaldehyde is high. The $\alpha\alpha$ homodimer is the form most similar to class II with respect to acetaldehyde and benzaldehyde. Differences among the class I isozymes with respect to pentanal and octanal are less pronounced. Jointly, these data are consistent with the classification scheme proposed by Strydom & Vallee (1982) [see also Vallee & Bazzone (1983)] which organizes the isozymes into three classes based on their activities toward alcohols and on their physicochemical and immunological characteristics.

If the subunits of the dimers do not interact in a manner which alters either K_m or k_{cat} values, then the kinetics of the heterodimers could be deduced from the kinetics of the homodimers. Dimers would be kinetically equivalent to "two enzyme systems" in which both enzymes catalyze the same reaction with a velocity defined by

$$v = [S]E_{t1}k_{cat,1}/(K_{m,1} + [S]) + [S]E_{t2}k_{cat,2}/(K_{m,2} + [S]) \quad (2)$$

under saturating coenzyme conditions (Segel, 1975). Any significant difference between $K_{m,1}$ and $K_{m,2}$ would result in a nonlinear Lineweaver–Burk plot. However, in the present study, substrate concentrations were limited both by the slight solubilities of the aldehydes and by substrate inhibition which interfered with the detection of any potential curvature. Equation 2 also predicts that the K_m and k_{cat} values of the heterodimers should fall within the range of values for the corresponding homodimers, although these need not be the average. The K_m values for $\alpha\beta_1$ are within this range (Table II). However, the k_{cat} values are consistently higher than would be predicted for a heterodimer consisting of subunits which act independently; e.g., experimentally, a value of 630 min^{-1} is obtained with acetaldehyde, whereas the average of the $\alpha\alpha$ and $\beta_1\beta_1$ k_{cat} values is 360 min^{-1}. This is also true with cyclohexanone, where a value of 800 min^{-1} was measured for $\alpha\beta_1$ compared to 310 min^{-1} which would be predicted. Hence, the data for $\alpha\beta_1$ suggest that α and β_1 subunits interact specifically, resulting in a heterodimer with turnover rates significantly greater than those of either the $\alpha\alpha$ or the $\beta_1\beta_1$ homodimers, and imply that these interactions are most pronounced in the coenzyme binding domain. Thus, the heterodimers must be studied individually as unique ADH forms.

Broad substrate specificity is characteristic of all mammalian liver alcohol dehydrogenases. The k_{cat}/K_m values for short-chain alcohols are relatively poor while those for hydrophobic substrates are much better (Brändén et al., 1975; Wagner et al., 1983). Such observations have generated considerable speculation about the existence and identity of "physiological" ADH substrate(s) and have challenged the unproven premise that ethanol oxidation is the primary function of ADH (Wagner et al., 1983, 1984). Although the microbial flora of the gastrointestinal tract and endogenous metabolism may produce substantial amounts of ethanol (Krebs & Perkins, 1970; Lundquist & Wolthers, 1958; Blomstrand, 1971), the exceptionally broad substrate specificity and the multiplicity of ADH isozymes, including forms with extremely high K_m values for ethanol, i.e., classes II and III (π-ADH and χ-ADH), argue for a much more diverse role for ADH in the metabolism of other alcohols and/or aldehydes. In this regard, it is important to note that ethanol is one of the poorer substrates for all classes of human ADH isozymes (Wagner et al., 1983).

In the absence of clearly identifiable physiological substrate(s), in vivo studies have centered on the role of these enzymes in the elimination of ingested ethanol. However, the equilibrium for the alcohol dehydrogenase reaction is unfavorable for the oxidation of ethanol. It is the efficient removal

of acetaldehyde by aldehyde dehydrogenases (ALDHs) in vivo which allows the process of ethanol oxidation to proceed (Li, 1977). Thus, if ADH were to exhibit catalytic activity with a carbonyl compound which is a poor substrate for the ALDHs or other carbonyl oxidases, then ADH could function in vivo primarily as a carbonyl reductase for that substrate. Since ALDH cannot oxidize ketones, cyclohexanone could serve as an example of such a substrate (Tables II–IV). Cyclohexanone also serves as a model for the A ring of steroids in several enzymatic systems; hence, this may be potentially significant.

While the physiological substrates of the individual human liver ADH isozymes are not yet known, a variety of carbonyl compounds are excellent substrates. Although the equilibrium constant for the ADH reaction strongly favors reduction, the ingestion of intoxicating quantities of ethanol may compete with endogenous substrates and thereby interfere with the physiological functions of the individual isozymes. This could account for some of the multiple, deleterious effects of chronic ethanol abuse.

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Registry No. ADH, 9031-72-5; benzaldehyde, 100-52-7; cyclohexanone, 108-94-1; acetaldehyde, 75-07-0; pentanal, 110-62-3; octanal, 124-13-0.

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