Physical and Enzymatic Properties of a Class III Isozyme of Human Liver Alcohol Dehydrogenase: χ-ADH†

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ABSTRACT: χ-Alcohol dehydrogenase (χ-ADH), a class III isozyme characterized by its anodic electrophoretic mobility and lack of inhibition by 4-methylpyrazole, has been isolated from human liver and purified to homogeneity in a reducing medium. χ-ADH resembles other human liver ADH isozymes of classes I and II with respect to its molecular weight, dimeric structure, stoichiometry of zinc and NADH binding, and pH optima for the oxidation of alcohols. This homodimer exhibits subtle differences in its absorption spectrum and amino acid composition relative to those of other human isozymes but differs markedly from their specificity toward alcohols and aldehydes. χ-ADH oxidizes ethanol very poorly. The reaction is bimolecular, and an apparent K_m cannot be discerned up to 2.3 M ethanol. The enzyme is inactive toward methanol, ethylene glycol, digitoxigenin, digoxigenin, and gitoxigenin, but alcohols with carbon chain lengths greater than four are oxidized rapidly with K_m values decreasing with increasing carbon chain length. Taken jointly, the composition, structure, and enzymatic properties of the ADH isozymes purified and studied so far strongly imply that their metabolic roles, yet to be discovered, will give a new perspective to ethanol metabolism and pathology.

More than 20 isozymes of alcohol dehydrogenase (ADH, EC 1.1.1.1)[1] (Blair & Vallee, 1966; Smith et al., 1973) have now been detected in and isolated from human liver, and the kinetic properties of many have been characterized to various degrees (Bosron et al., 1980; Parés & Vallee, 1981; Wagner et al., 1983). While ethanol is assumed to be their preferred substrate, in actuality most of them oxidize it relatively poorly (Pietruszko, 1979; Wagner et al., 1983). In fact, screening of liver extracts by means of starch gel electrophoresis followed by staining with an alcohol of longer chain length, i.e., pentanol, led to the identification of an isozyme which hardly oxidizes ethanol at all (Parés & Vallee, 1981). The substrate specificity and the kinetic and physicochemical behavior of this isozyme proved to differ significantly from those of all others known thus far. It is anionic at neutral to slightly alkaline pH values and is not saturated by ethanol at concentrations up to 2 M. Similar ADH isozymes have now been detected in and isolated from liver extracts of species other than the human and have been named provisionally χ-ADH (Parés & Vallee, 1981; Dafeldecker & Vallee, 1982).

The ADH isozymes of liver extracts have been categorized into three classes on the basis of their functional and physicochemical properties (Strydom & Vallee, 1982; Vallee & Bazzone, 1983). Class I (α, β, γ) ADH isozymes are cationic and sensitive to inhibition by pyrazole. Class II (π) ADHs are pyrazole-insensitive cationic proteins which have been purified and characterized (Bosron et al., 1980); Wagner et al., 1983). Class III (χ) ADH comprises the anionic isozymes whose purification and characterization are the subject of this report.

Parés & Vallee (1981) have partially purified human class III (χ-ADH) from liver. Some of its catalytic features but none of its physical and chemical characteristics have been described previously. The latter and additional kinetic properties have been investigated further with χ-ADH purified to complete homogeneity.

Experimental Procedures

Materials. NAD^+ (grade III and grade AA1) and DTE were purchased from Sigma Chemical Co., St. Louis, MO. Ethanol was obtained from U.S. Industrial Chemicals Co., New York, NY. Other alcohols were obtained from Fisher Scientific Co. and used without further purification. Aldehydes were distilled just prior to use. Ion-exchange resins were obtained from Whatman Chemical Separation, Inc., Clifton, NJ, and AMP–agarose (agarose–hexane–adenosine 5′-phosphate; AGAMP type 2) was from P-L Biochemicals, Milwaukee, WI.

Purification of χ-ADH. Human livers were obtained at autopsy within 12-h postmortem and stored at −70 °C.

The present procedure is a modification of that described by Parés & Vallee (1981). Approximately 200 g of liver was added to 400 mL of 1 mM ascorbate dissolved in degassed water. Subsequent to thawing for 10–15 min, livers were homogenized first in a Waring blender and then in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 27300*g* for 45 min. The supernatant fluid was decanted and filtered immediately through a filter cake (600-mL total volume) of DEAE-cellulose equilibrated with 10 mM Hepes–1 mM ascorbate, pH 7.9. After the sample had entered the filter cake, it was washed with 1 L of the same buffer to elute the class I and II isozymes of ADH (Bosron et al., 1980; Wagner et al., 1983). The resin was then washed with 1 L of 10 mM Hepes, 1 mM ascorbate, and 0.1 M NaCl, pH 7.9, and 200-mL fractions were collected. Fractions containing χ-ADH activity were pooled and concentrated to 100 mL by means of an Amicon concentrator

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[1] Abbreviations: ADH, alcohol dehydrogenase; 12-HDA, 12-hydroxydodecanoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; OP, 1,10-phenanthroline; DTE, dithioerythritol; CD, circular dichroism; MCD, magnetic circular dichroism.

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equipped with a UM-50 membrane. The reslting concentrate was dialyzed against two, 1-L changes of 10 mM Hepes–0.1 mM DTE, pH 7.9, and applied to a column of DEAE-cellulose (2.5 × 48 cm) equilibrated in 10 mM Hepes–0.1 mM DTE, pH 7.9. The column was eluted at 60 mL/h with 200 mL of the same buffer followed by a linear gradient of increasing NaCl concentration (0–75 mM). Fractions of 10 mL were collected and assayed for χ-ADH activity which eluted as a major peak with a minor shoulder. The active fractions were pooled, concentrated as before, and then dialyzed against 5 mM Hepes–0.1 mM DTE, pH 7.2. This preparation was applied to an AMP–agarose column (1.2 × 30 cm) equilibrated in the same buffer. The column was washed with the same buffer, then with 10 mM Hepes–0.1 mM DTE, pH 8.0, 10 mM Hepes, 0.1 mM DTE, and 20 mM galactose, pH 8.0, and 10 mM Hepes–0.1 mM DTE, pH 8.0, and finally with 500 mL of a gradient from 0 to 25 μM NADH in the same buffer. Fractions of 5 mL were collected and assayed as described below.

Enzyme Assays. ADH activity toward alcohols was measured spectrophotometrically as the increase in absorbance at 340 nm due to the reduction of NAD^+. Assays were performed at 25 °C in 0.1 M glycine buffer–2.4 mM NAD^+, pH 10.0, unless otherwise stated. The activity of χ-ADH in partially purified fractions was calculated as the difference in velocity in separate assays using 33 mM ethanol and 0.5 M ethanol as substrate. One unit of ADH activity is defined as the amount of enzyme required to catalyze the reduction of 1 μmol of NAD^+ per min under the conditions of the assay.

When octanal served as substrate, ADH activity was measured as the decrease in absorbance at 340 nm due to the oxidation of NADH (see Table V). Assays were performed in 0.1 M sodium phosphate–58 μM NADH, pH 6.8 or 7.5 and at 25 °C. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of NADH per min under the conditions of the assay.

Electrophoresis. Electrophoresis in starch gels was performed as described earlier (Bosron et al., 1979). Gel slices were stained for ADH activity by using ethanol or pentanol as the substrate. Electrophoresis in $NaDodSO_4$–polyacrylamide gels followed the procedure of Laemmli & Favre (1973), and the gels were stained with Coomassie blue and then destained with acetic acid–methanol–water (1:2:8). Isoelectric focusing was performed with ampholine AG plates (LKB Instruments), over a range of pHs from 3.5 to 9.5 standardized with ferritin, bovine serum albumin, β-lactoglobulin, and conalbumin. Details of the procedure were those supplied with the ampholine plates.

Metals were measured with a Perkin-Elmer 2280 atomic absorption spectrophotometer by using standard procedures. Enzyme was prepared for metal analysis by dialysis against 50 mM Hepes, pH 7.5, extracted with 0.01% dithizone in CCl_4. Other procedures to avoid contamination by trace elements were those of Thiers (1957).

Spectral Measurements. Absorption spectra were measured on a Cary 219 spectrophotometer and circular dichroic and magnetic circular dichroic spectra on a Cary 61 spectropolarimeter equipped with a Varian Model 4145 superconducting magnet and a V4106 power supply. MCD measurements were at a field of 4 T.

Molecular Weight and Molar Absorptivity Determinations. The molecular weight of purified χ-ADH was determined by the sedimentation equilibrium method of Yphantis (1964). Centrifuge measurements were performed in a Spinco Model E ultracentrifuge equipped with interference optics. χ-ADH, 0.4 mg/mL, was dialyzed for 48 h against 10 mM Tris, pH 7.9, and then loaded into a double-sector cell equipped with an aluminum-filled epon centerpiece. Centrifugation was performed at 21 740 rpm at 20 °C for 36 h when equilibrium had been achieved. The interference pattern was recorded photographically, and the negatives were measured subsequently for the coordinates of a single fringe. The plot of the natural logarithm of the fringe displacement vs. r^2 was linear, and the molecular weight was calculated from its slope $[M_r = 2RT(\text{slope})/[(1 - \bar{v}\rho)\omega^2]]$ where ω is the angular velocity, ρ the density of the solvent, $\bar{v}$ the partial specific volume of χ-ADH assumed to be 0.74 mg/mL, T the temperature, and R the gas constant.

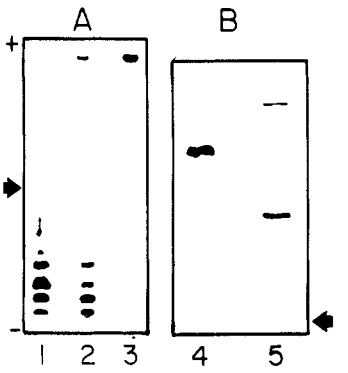

FIGURE 1: Electrophoresis of χ-ADH preparations. (A) Starch gel electrophoresis, pH 8.2, of crude liver extracts and purified preparations: lane 1, supernatant fluid from a liver extract stained by using ethanol as the substrate; lane 2, same as lane 1 except pentanol was the substrate; lane 3, purified χ-ADH; lane 3 was stained with pentanol as the substrate. (B) $NaDodSO_4$–polyacrylamide gel electrophoresis: lane 4, purified χ-ADH; lane 5, standard sample containing bovine serum albumin (slower moving component) and carbonic anhydrase (each added to 20 μg/mL). Origins are indicated by the arrows.

The molar absorptivity of χ-ADH was calculated on the basis of the spectrum of a solution whose concentration was measured in the ultracentrifuge by using a synthetic boundary cell (Klainer & Kegeles, 1955). A displacement of 4 fringes was equivalent to a protein concentration of 1.0 mg/mL. Protein estimations were performed in the course of purification procedures by the method of Lowry et al. (1951), with bovine serum albumin as the standard. The Lowry procedure correlated directly with the concentration of purified χ-ADH as measured spectrophotometrically.

Amino Acid Analyses. Quantitative amino acid analysis of χ-ADH was performed with a Durrum D-500 amino acid analyzer. χ-ADH (1 mg/mL), dialyzed against two changes of glass-distilled water (1000 volumes), was sulfopropylated exhaustively by the method of Ruegg & Rudinger (1974), dissolved in 1 mL of 6 N HCl, degassed at 50 μmHg pressure (Moore & Stein, 1963), and hydrolyzed in vacuo for up to 89 h at 110 °C. Samples were analyzed in triplicate. Values for tyrosine were calculated by extrapolating a first-order plot of log amino acid concentration vs. time to zero time, while those of valine and isoleucine were calculated from the analysis of the 72-h hydrolysate. Half-cysteine was calculated from the measurement of sulfopropylcysteine. Tryptophan was measured by magnetic circular dichroism (Holmquist & Vallee, 1973).

Results

Electrophoresis of human liver extracts resolves three classes of ADH. Class III (χ-ADH), the only anodic form, stains with pentanol but poorly or not at all with ethanol (Figure 1, lanes 1 and 2). In solution, χ-ADH readily oxidizes only very

Table I: Purification of Human Liver χ-ADH[a]

step	volume (mL)	[protein] (mg/mL)	activity (units/mL)	sp act. (units/mg)	yield (%)
crude extract	1010	0.93	0.015	0.016	100
DEAE-cellulose	168		0.048		60
AMP-agarose	106	0.077	0.054	0.6	38

[a] Human liver (200 g) was homogenized (crude extract) and passed through DEAE-cellulose, and the pooled active fractions were chromatographed on AMP-agarose. See Experimental Procedures for detailed conditions.

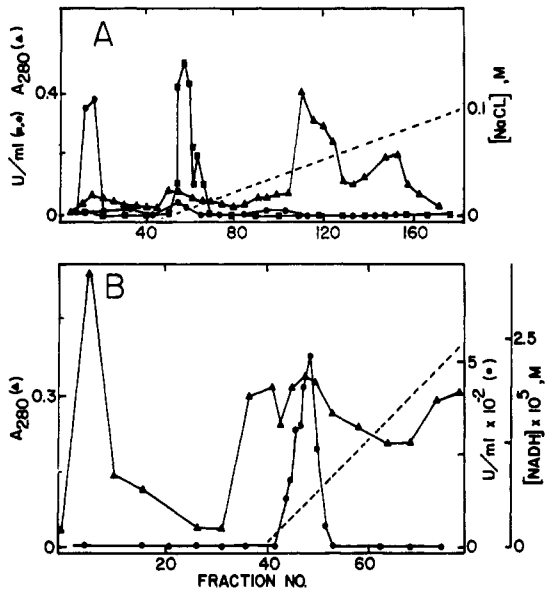

FIGURE 2: Purification of χ-ADH from human liver extracts. (A) Chromatography of χ-ADH containing fractions adsorbed to DEAE-cellulose and eluted with a 0–0.1 M NaCl gradient (---): (●) activity with 33 mM ethanol; (■) activity with 0.5 M ethanol; (▲) absorbance at 280 nm. Fractions are 10 mL. (B) Fractions 53–65 from the DEAE step were pooled, concentrated, and rechromatographed on AMP-agarose. The column was washed with buffer as described under Experimental Procedures and then eluted with a 500-mL gradient (0–25 μM) of increasing NADH concentration (---) in 0.1 M Hepes–0.1 M DTE, pH 8.0: (●) activity with 0.5 M ethanol; (▲) absorbance at 280 nm. No activity was detected with 33 mM ethanol. Fractions are 5 mL.

high concentrations of ethanol (Parês & Vallee, 1981); hence, at the ethanol concentrations employed conventionally to detect other known ADH forms (33 mM), its activity is readily missed. This characteristic differentiates χ-ADH from all other ADH isozymes. To facilitate isolation, the anodic class III and the cathodic classes I and II ADH isozymes are separated first from one another by passage of crude liver homogenate through a filter cake of DEAE-cellulose. Class III (χ-ADH) is adsorbed, but both classes I and II are not. The class III isozyme is then eluted from the DEAE-cellulose filter cake with 0.1 M NaCl in 10 mM Hepes containing 1 mM ascorbate, pH 7.9. This and subsequent steps in the purification contained 1 mM ascorbate which served as an antioxidant to prevent final product heterogeneity (see below). For the final purification steps, DTE was found more suitable for this purpose than ascorbate. Table I summarizes the results of purification.

Rechromatography of χ-ADH on DEAE-cellulose separates heme pigments from the isozyme which elutes from the column as a major single peak with a trailing shoulder (Figure 2). Active fractions were pooled, dialyzed, and adsorbed onto an AMP–agarose column (Figure 2). All the remaining heme pigments elute from the column with 10 mM Hepes–1 mM DTE, pH 7.3. χ-ADH activity which adheres to the affinity resin is then eluted as a single component with a gradient of NADH (0–25 μM).

Table II: Physical Constants of χ-ADH

parameter	value
mol wt	
dimer	82 700
monomer	~41 000
ϵ_{280} (M^{-1} cm^{-1})	77 600
mol of Zn/mol of χ-ADH	3.8 ± 0.3
NADH/mol of χ-ADH	2
isoelectric pH	6.4
pH optimum, octanol oxidation	11.0

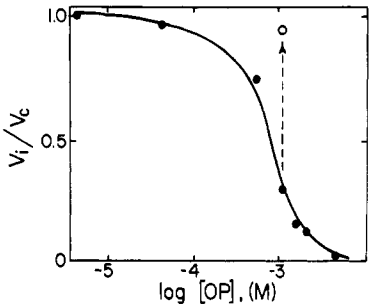

FIGURE 3: Inhibition of χ-ADH by 1,10-phenanthroline. OP (20 mM) was added to both the assay solution and the enzyme solution (0.01 unit/mL in 10 mM Hepes–1 mM DTE, pH 7.5) to give the desired concentration: (●) assays performed by the addition of 20 μL of χ-ADH solution to 0.58 mL of a solution containing 25 μM 12-HDA and 10 μM NAD^+ as substrates in 0.1 M glycine, pH 10.0; (---) reversibility of OP inhibition in which enzyme (20 μL) was incubated with 1 mM OP and then diluted into an assay containing substrate to a final OP concentration of 33 μM. Activity was expressed as the velocity in the presence of inhibitor, v_i, relative to that of the control, v_c.

The specific activity of the final product was 0.6 unit/mg of protein. When subjected to electrophoresis on a starch gel (stained for enzymatic activity; Figure 1, lane 3) or a $NaDodSO_4$–polyacrylamide gel (Figure 1), a single, major component accounts for virtually all detectable activity and protein.

The final preparation of χ-ADH, homogeneous by electrophoresis and ultracentrifugation, is stable for 2 weeks when stored at 4 °C in 10 mM Hepes buffer–1 mM DTE, pH 7.5, but slowly loses activity upon extended storage under these conditions. Electrophoresis of preparations after 7–8 weeks of storage results in additional bands with minor staining activity, presumably the result of the oxidation of χ-ADH.

Physical and Chemical Characteristics. Table II shows physical constants of χ-ADH. Molecular weight determinations for the dimer were performed by sedimentation equilibrium centrifugation. A plot of the natural logarithm of fringe displacement vs. r^2 resulted in a straight line, indicative of a single molecular species with a calculated molecular weight of 82 700. $NaDodSO_4$–polyacrylamide gel electrophoresis of χ-ADH (Figure 1), when bovine serum albumin and carbonic anhydrase serve as standards, gave an apparent molecular weight for the denatured enzyme of ~41 000, confirming its dimeric nature. Isoelectric focusing experiments in polyacrylamide yielded an isoelectric point of pH 6.4 for the pure enzyme (data not shown).

Two preparations of ADH from different livers contained 3.8 ± 0.3 mol of Zn/mol of protein, i.e., 2 mol of Zn/mol of subunit (Table II).

Characteristic of many zinc metalloenzymes, χ-ADH is inhibited instantaneously and reversibly with 1,10 phenanthroline (OP); the K_i^{app} is 9×10^{-4} M (Figure 3). Preincu-

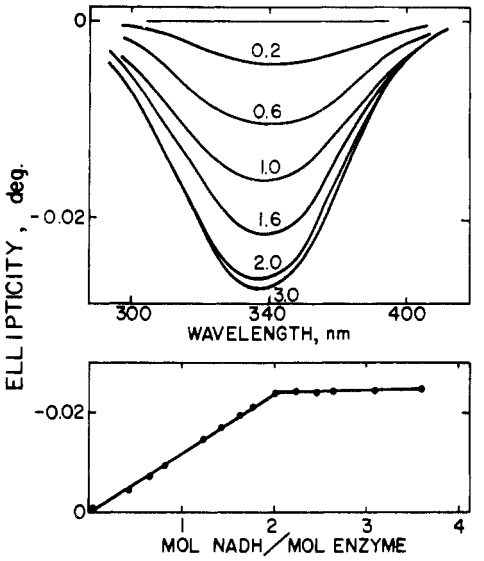

FIGURE 4: Stoichiometry of NADH binding by χ-ADH. Purified enzyme (2 mg/mL) was dialyzed against 10 mM Hepes–1 mM DTE at pH 7.5. Aliquots of NADH (1 mM) were added to the enzyme (1 mL), and the CD spectrum was measured. (Upper panel) CD spectra for (in descending order) 0, 0.2, 0.6, 1.0, 1.6, 2.0, and 3.0 equiv of NADH. (Lower panel) Stoichiometry of NADH association by ellipticity titration at 338 nm.

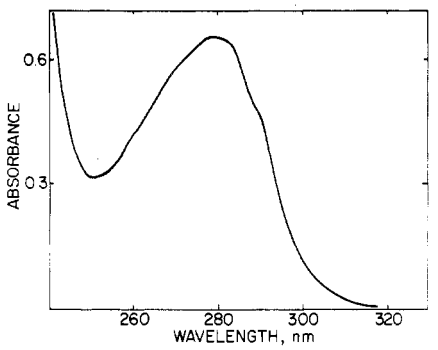

FIGURE 5: Absorption spectrum of χ-ADH. χ-ADH (ca. 0.6 mg/mL) was dialyzed 3 times against 500 volumes of 10 mM Hepes–1 mM DTE, pH 7.5, to remove excess NADH.

bation of χ-ADH with 1.0 mM OP results in 70% inhibition which is reversed by 30-fold dilution to yield a final inhibition of only 6%.

The stoichiometry of NADH binding was determined by CD titration measuring the change in ellipticity at 338 nm (Dafeldecker et al., 1981). The enzyme is saturated completely by 2 mol of NADH/mol of enzyme, i.e., 1 mol of NADH/subunit (Figure 4).

Table III compares the amino acid composition of human liver χ-ADH as prepared from two different livers with class I ADH, class II (π-ADH), and horse liver ADH. The amino acid composition of human class III (χ-ADH) differs from that of the class I isozymes or that of the horse ADH by more than two residues for eight different amino acids: Asp, Ser, Glu, Pro, Gly, Val, Leu, and Phe.

The spectral features of χ-ADH differ significantly from those of horse ADH. Its absorption maximum is at 280.5 nm (Figure 5), a bathochromic shift of 2.5 nm compared with horse ADH (Drum & Vallee, 1970). However, the fine structure characteristic of phenylalanine near 260 nm is much less apparent in χ-ADH than in the horse enzyme, in accord with a lower Phe/Trp ratio. Finally, the shoulder at 292 nm, characteristic of tryptophan, is hyperchromic, indicating a relatively high tryptophan/tyrosine ratio, further consistent with the amino acid composition.

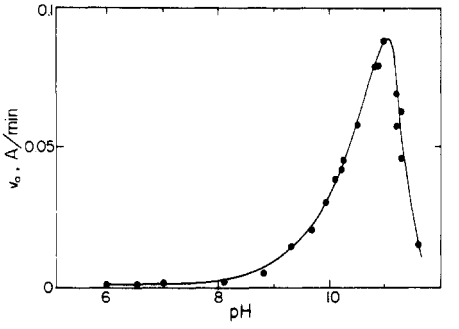

FIGURE 6: pH–activity profile of χ-ADH toward 1-octanol. Assays were performed by using 2.5 mM NAD^+ and 0.4 mM octanol in 0.1 M glycine adjusted to the appropriate pH with 1 M NaOH or 1 M HCl.

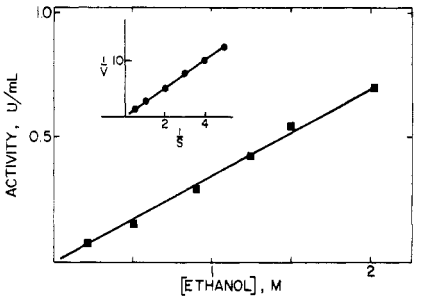

FIGURE 7: Ethanol concentration dependence for the reduction of NAD^+ (1 mM) by χ-ADH at pH 10.5, 50 mM glycine. Inset: Lineweaver–Burk plot of the same data.

The molar absorptivity of χ-ADH is 77 600 M^{-1} cm^{-1}, based on the protein concentration determined in the analytical ultracentrifuge by counting the interference fringe shift across a synthetic boundary of protein and buffer (Klainer & Kegeles, 1955) and a molecular weight of 82 700.

Enzymatic Characteristics. pH Optima. 1-Octanol is oxidized optimally at pH 11.0. At pH 6.8, the rate of 1-octanol oxidation is only 5% of that at pH 11.0 (Figure 6).

Table IV illustrates the broad specificity of χ-ADH as exemplified by the oxidation of a number of alcohols relative to that of 5 mM ethanol. The kinetic parameters of alcohol oxidation and aldehyde reduction were assessed quantitatively in the usual manner. As reported previously (Parês & Vallee, 1981), ethanol does not saturate χ-ADH, even when the ethanol concentration is as high as 2 M (Figure 7). A double-reciprocal plot of these data pass through the origin when extrapolated to infinite substrate concentration, as would be expected for a first-order reaction. Even though K_m cannot be evaluated, the k_{cat}/K_m value can be estimated from the slope of the plot of velocity vs. substrate concentration: $v = (k_{cat}E_t/K_m)$ S, where E_t is the enzyme concentration. Longer chained aliphatic alcohols do saturate χ-ADH, allowing kinetic parameters to be determined for these and additional alcohols (Table V). The affinity of NADH for χ-ADH is an order of magnitude greater than that for NAD^+, i.e., K_m values of 2.5 vs. 25 μM, respectively. $NADP^+$ is not a cofactor for the oxidation of ethanol, confirming earlier results (Parês & Vallee, 1981); however, it is reduced when coupled with 12-HDA, but at a rate one-third that observed with NAD^+. On the basis of values for k_{cat}/K_m, primary alcohols with aliphatic chains of five or more carbons are good substrates. 4-Methylpyrazole inhibits χ-ADH poorly, unlike the class I isozymes. Oxidation is inhibited 30% when the 12-HDA concentration is equal to K_m, the NAD^+ concentration is saturating, and 4-methylpyrazole concentration is 50 mM. Isobutyramide inhibits the reduction of 1-octanal with a K_i of 0.125 mM.

Table III: Amino Acid Composition of Human Class III (χ) ADH Compared to Class I (α, β, γ), Class II (π), and Horse Liver ADH

residue	class III (χ-ADH) sample 1 (mean[a] ± SE)	class III (χ-ADH) sample 2 (mean[e] ± SE)	integer value	class I[g]	class II[k] (π-ADH)	horse liver ADH[i]
half-cystine	15 ± 0.14	12.4 ± 0.58	14	14	14	14
aspartic acid	24.2 ± 0.08	25.5 ± 0.13	25	31	31	25
threonine	24[b]	23	24	23	23	24
serine	27[b]	31	29	24	21	26
glutamic acid	35.4 ± 0.5	36 ± 0.12	36	29	26	29
proline	17.7 ± 0.32	17.6 ± 0.31	18	21	22	20
glycine	44.6 ± 0.24	45.0 ± 0.15	45	40	37	38
alanine	36.6 ± 0.20	31.4 ± 0.06	34	33	32	28
valine	35.6 ± 0.56	38[f] ± 0.50	37	40	28	39
methionine	5.9 ± 0.12	5.7 ± 0.15	6	7	6	9
isoleucine	24.5 ± 0.13	23 ± 0.13	24	22	25	24
leucine	21.1 ± 0.12	20.8 ± 0.08	21	29	30	25
tyrosine	6.8 ± 0.15[c]	7.5 ± 0.04	7	6	8	4
phenylalanine	11.9 ± 0.15	12.87 ± 0.06	12	17	17	18
histidine	8.3 ± 0.09	6.75 ± 0.06	8	6	7	7
lysine	33.5 ± 0.08	27.88 ± 0.14	31	33	28	30
arginine	8.8 ± 0.16	8.8 ± 0.12	9	11	10	12
tryptophan	3.0[d]	3.0[d]	3	3	3	2

[a] Mean ± standard error of duplicate analyses of samples hydrolyzed for 41, 56, and 89 h. [b] Corrected for destruction during hydrolysis. [c] Phenol added to prevent destruction of Tyr during hydrolysis. [d] By MCD (Holmquist & Vallee, 1973). [e] Mean ± standard error of duplicates hydrolyzed for 24, 48, and 72 h. [f] Mean of sample hydrolyzed for 72 h. [g] From Lange et al. (1976). [h] From Bosron et al. (1979). [i] Calculated from the primary sequence (Jörnvall, 1970).

Table IV: Oxidation of Alcohols by χ-ADH

substrate[a]	v_s/v_{EtOH}[b]	substrate[a]	v_s/v_{EtOH}[b]
ethanol	1	digitose	3
12-HDA	524	DL-synephrine	2
vanillyl alcohol	34	cyclohexanol	2
furfuryl alcohol	8	tryptophol	1
benzyl alcohol	5	ethylene glycol	0.4
2-octanol	4	butanol	58
17β-hydroxyetiocholan-3-one	4	pentanol	6
phenylalaninol	3		

[a] All substrates were 1 mM in the reaction mixture, except ethanol (5 mM), butanol (50 mM), and pentanol (10 mM). Alcohols not detectably oxidized were methanol, norepinephrine, digitoxigenin, digoxigenin, gitoxigenin, and 3β-hydroxyetiocholan-17-one. [b] Ratio of the initial velocity of the substrate, v_s, to that of ethanol, v_{EtOH}.

Table V: Kinetic Constants of χ-ADH

substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
NAD^+[a]	0.025		
NADH[b]	0.0025		
ethanol			45
butanol			466
pentanol	40	14.7	368
octanol	1.2	440.0	367 000
12-hydroxydodecanoic acid	0.10	182.0	1 820 000
vanillyl alcohol	11	28.0	2 540
octanal[c]	2.4	200	83 300

[a] Using 0.4 mM 12-HDA. [b] Using 4 mM octanal. [c] At pH 6.8.

Discussion

The ethanol dehydrogenase activity of human liver led to the recognition and subsequent isolation of alcohol dehydrogenase (Von Wartburg et al., 1964). At that time, there was no reason to question the premise that ethanol is the native substrate. Even when the substrate specificity of the liver enzymes from many species became progressively known to be broad, this did little to alter this perspective. Subsequently, isozymes of the horse enzyme were described to be "specific" for ethanol and sterols (Pietruszko et al., 1966), much as that specificity has turned out to be relative rather than absolute (Bränden et al., 1975).

Similarly, the substrate specificity of unresolved human liver ADH is also broad, but meaningful comparisons of isozyme specificities had to await the relatively recent identification and isolation of the cathodic isozymes (Bosron et al., 1980; Wagner et al., 1983). The eight predominant cathodic class I human liver isozymes, $\alpha\gamma_2$, $\gamma_2\gamma_2$, $\alpha\gamma_1$, $\alpha\beta_1$, $\beta_1\gamma_2$, $\gamma_1\gamma_1$, $\beta_1\gamma_1$, and $\beta_1\beta_1$, all oxidize ethanol, ethylene glycol, methanol, benzyl alcohol, octanol, cyclohexanol, and 16-hydroxyhexadecanoic acid at the pH optimum, 10.0. However, as judged by kinetic criteria, in no case is ethanol the best substrate for any of the class I isozymes.

These findings pertain directly both to the detection and to the characterization as well as the potential enzymological and physiological implications of χ-ADH. In the course of identification and resolution of ADH isozymes, the choice of ethanol as the test substrate has proven limiting. Ethanol is such a poor substrate for χ-ADH that gels stained with this substrate do not reveal its presence. To complicate matters further, conventional electrophoretic methods for identification of mammalian ADH isozymes were designed to resolve only cathodic proteins, and the need for the detection of anodic proteins such as χ-ADH was not even envisioned. Jointly, this procedural happenstance combined with the expectation that ethanol is suitable as a universal substrate for all ADH isozymes most likely accounted for the past failure to detect, identify, and purify this isozyme. Electrophoretic resolution of both anodic and cathodic proteins and staining with *pentanol* as the substrate solve this problem and detect both χ-ADH and all other ADH isozymes known so far (Figure 1).

The original procedure for the isolation of χ-ADH (Parés & Vallee, 1981) yielded purified preparations which contained two χ-ADH isozymes, though they proved unstable at 4 °C (half-life ≃ 7 days). This instability appears to be due to a mild oxidizing environment which fails to keep the large number of cysteine residues of ADH reduced. When ascorbic acid or DTE is added throughout, purification results in a single, anodic χ-ADH isozyme, with good yields (40%) and

stable for up to 10 days at 4 °C with less than 20% loss of specific activity. As a consequence, we have found it effective to employ ascorbic acid in purification steps requiring large volumes of buffer while DTE is more suitable for storage since its adventitious zinc content is lower than that of ascorbic acid. There are good indications, moreover, that simultaneously class II (π-ADH) benefits from these precautionary measures; it, too, is stabilized by the inclusion of reducing agents resulting in higher yields (C. C. Ditlow, M. M. Morelock, and B. L. Vallee, unpublished results). While class I (α, β, γ) isozymes are purified in good yields even in the absence of reducing agents (Lange & Vallee, 1976; Wagner et al., 1983), some of the members of this calss also seem more stable when stored in DTE.

The relative and the absolute adsorption of the three classes of ADH isozymes onto DEAE-cellulose and CapGapp affinity resin is critical to their isolation and separation. Under conditions where class III (χ-ADH) is adsorbed to DEAE-cellulose, classes I and II are not. Rechromatography on DEAE-cellulose effectively removes trace amounts of class I and II isozymes from the class III fraction obtained by batchwise DEAE-cellulose separation (Figure 2) (Lange et al., 1976). An immobilized 4-methylpyrazole affinity resin completely separates classes I and II from one another, owing to their differential inhibition by this agent (K_i = 2 μM and 2 mM, respectively) (Lange et al., 1976). This affinity resin, however, does not retard χ-ADH, since its K_i for 4-methylpyrazole is >>5 mM. When used in series, these procedures allow the isolation of χ-ADH in 40% yield, resulting in complete separation of the three classes of ADH without cross-contamination. Hence, when present, all currently known ADH isozymes can be purified from a single liver (Li et al., 1977; Wagner et al., 1983).

The molecular weight of χ-ADH, essentially homogeneous by electrophoretic and sedimentation criteria, is 82 700, and that of the monomer is ~41 000, values close to those for all other ADH isozymes. The isoelectric point, 6.4, is 4 pH units lower than that of classes I or II, in accord with both the unique electrophoretic and the chromatographic behavior of Class III, likely related to a charge difference of amino acid residues (see below).

Values of zinc content and stoichiometry of NADH binding coincide with the corresponding data for the horse liver enzyme (EE-ADH) and with those of the various human class I and II isozymes. The absorption spectrum of χ-ADH reflects known differences in its phenylalanine and tyrosine content, and the shoulder at 292 nm reflects tryptophan. The distinct identities and dissimilarities of the amino acid composition (Table III) compared with that of classes I and II are sufficient to account for many of the differences of charge, structure, and potential function. Clearly, the basis of the differences between ADH isozyme classes awaits further structural and kinetic analysis.

The amino acid composition of χ-ADH from human liver more nearly resembles that of the analogous isoenzymes from horse and primate livers (unpublished observations) than those of human class I or class II. This suggests that χ-ADH is genetically distinct from other ADH isozyme classes, a proposition supported by the fact that antibodies prepared against $\beta\gamma_2$, $\beta\gamma_1$, and $\beta\beta$ (class I) and π-ADH (class II) do not cross-react with human χ-ADH. However, antibodies toward both horse and human liver χ-ADH *do* cross-react with each other (B. L. Vallee et al., unpublished results). In addition, the peptide fingerprint maps of class I, II, and III ADH isozymes are distinctive while those of individual isozymes of class I are quite similar to one another (Strydom & Vallee, 1982), perhaps reflecting structural features of each class of isozyme ultimately related to their unique substrate specificities.

To our knowledge, the χ-ADH isozyme is the first example of a mammalian ADH enzyme for which ethanol is one of the poorest substrates. The failure of as much as 2.3 M ethanol to saturate χADH is unprecedented for an enzyme which oxidizes other aliphatic alcohols such as pentanol, octanol, and 12-HDA. Yet its K_m values for long-chain alcohols are typical of those of other ADH isozymes. Not too surprisingly, the presence of 2.3 M ethanol, equivalent to ~11% ethanol, in the assay mixture adversely affects the stability of the enzyme, precluding satisfactory kinetic studies.

The low or nonexistent activity of χ-ADH toward methanol, ethylene glycol, digitoxigenin, digoxigenin, and gitoxigenin resembles the substrate specificity of π-ADH. Thus, compared to class I, the specificity of class II and class III is curtailed. On the basis of these findings and those on horse χ-ADH (Dafeldecker & Vallee, 1982), χ-ADH prefers aliphatic alcohol substrates. Studies of additional alcohols of more complex structures and physiological and metabolic significance as well as those of aldehydes in general are not yet complete. However, present indications are that the substrate specificity of each class of ADH isozyme is distinct and likely significant metabolically.

Whatever the primary function of χ-ADH, it seems most unlikely that ethanol oxidation is the role. Taken jointly, the composition, structure, and enzymatic properties of the ADH isoenzymes purified and studied so far strongly imply that their metabolic roles, yet to be discovered, will give new perspective to ethanol metabolism and pathology.

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Registry No. χ-ADH, 9031-72-5; NAD, 53-84-9; NADH, 58-68-4; ethanol, 64-17-5; butanol, 71-36-3; pentanol, 71-41-0; octanol, 111-87-5; 12-hydroxydodecanoic acid, 505-95-3; vanillyl alcohol, 100-51-6; octanal, 124-13-0.

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Bound-Cation Exchange Affects the Lag Phase in Actin Polymerization†

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ABSTRACT: The delay or lag phase at the onset of polymerization of actin by neutral salt is generally attributed to an actin nucleation reaction. However, when nucleation is circumvented by the use of phalloidin-stabilized nuclei, a lag phase persists when Ca^{2+}-containing actin is polymerized with $MgCl_2$. Pretreatment of actin with ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and/or Mg^{2+} shortens or eliminates this lag phase, suggesting that exchange of the actin-bound divalent cation occurs during this nucleation-independent lag phase. Measurement of the actin-bound cation initially and after brief incubation with EGTA/Mg^{2+} directly verifies that Mg^{2+} has replaced Ca^{2+} as the actin-bound cation, producing a highly polymerizable Mg^{2+}-actin species. Bound-cation exchange prolongs the lag phase in actin polymerization and probably explains what has been termed the monomer activation step in actin polymerization.

The early time course in the polymerization of monomeric actin, initiated by the addition of neutral salt, is usually characterized by a delay. This lag phase was first attributed to an actin nucleation step by Oosawa and his colleagues (Kasai et al., 1962; Oosawa & Asakura, 1975), and it is now generally considered that the nucleation process is mainly responsible for the lag phase during the spontaneous polymerization of actin. Recently, a number of investigations (Tobacman & Korn, 1983; Pollard et al., 1982; Cooper et al., 1983; Gilbert & Frieden, 1983; Gershman et al., 1983; Brenner et al., 1983) have suggested that monomer activation and/or exchange of the tightly bound cation on monomeric actin may also be part of the lag phase, particularly when actin containing bound Ca^{2+} is polymerized with $MgCl_2$ (a frequently employed condition for in vitro polymerization studies). However, there has been no direct experimental demonstration of the effect of cation exchange in monomeric actin on the kinetics of polymerization. Such experiments are difficult because of interference from the simultaneously occurring nucleation step. In experiments reported here, the nucleation step was circumvented by adding phalloidin-stabilized nuclei ("seeds") to initiate the polymerization of fluorescent-labeled actin, and the fluorescence intensity was monitored as a measure of polymer formation. Interestingly, when low concentrations of $MgCl_2$ are added to G-actin containing Ca^{2+} as the bound cation, a lag phase still occurs before polymer formation even when seeds are added with $MgCl_2$. If polymerization is initiated with EGTA also present to facilitate removal of the actin-bound Ca^{2+} or if actin is preincubated with a low concentration of $MgCl_2$ before seeds are added, the lag phase is shortened considerably or eliminated. Measurements of the bound-cation content of actin prior to Mg^{2+} addition and again after incubation with EGTA/Mg^{2+} indicate that Mg^{2+} has replaced Ca^{2+} as the bound cation within a time interval comparable to the duration of the nucleation-independent lag phase.

Experimental Procedures

All reagents were analytical grade with ATP and EGTA being purchased from Sigma Chemical Co., phalloidin from Boehringer-Mannheim, ^{45}Ca from New England Nuclear, and N-P[1] from Molecular Probes. All solutions were prepared with doubly distilled water.

Actin was extracted from rabbit acetone powder (Szent-Gyorgyi, 1951) and purified by previously published procedures (Estes et al., 1981), except a Sephacryl S-300 column was used for the column purification step. To label actin with N-P, column-purified actin (ca. 20 μM) was reacted with equimolar N-P dissolved in dimethylformamide (0.1% final concentration) at room temperature overnight in the presence

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[1] Abbreviations: N-P, *N*-(1-pyrenyl)iodoacetamide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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