form. The preparations of methanol dehydrogenase used in this investigation showed a free-radical signal corresponding to only 1% of the total enzyme. The reason for these discrepancies is not obvious. Possibly, the preparation previously used was contaminated with an endogenous electron acceptor. It must be pointed out that the lack of a radical signal in the enzyme preparation used in these experiments does not rule out the intermediate participation of a methoxatin radical in catalysis. It is possible, for instance, that a radical is generated in the presence of dye and substrate.

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

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ABSTRACT: Homogeneous class II alcohol dehydrogenase (π-ADH) has been isolated from human liver homogenates by chromatography on DE-52 cellulose, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole–Sepharose, SP-Sephadex C-50, and agarose–hexane–AMP, yielding an enzyme that has a significantly higher specific activity and is markedly more stable than that isolated by an earlier procedure. π-ADH is composed of two identical 40 000-dalton subunits, contains 4 mol of zinc/dimer, and is readily inhibited by metal-chelating agents. The purified enzyme binds two molecules of coenzyme per dimer, exhibits an absorption maximum at 280 nm, ϵ_{280} = 57 000, and exhibits an isoelectric point of 8.6. The class II isozyme catalyzes the oxidation of a variety of alcohols with K_m values ranging from 7 μM to 560 mM and with k_{cat} values from 32 min^{-1} to 600 min^{-1} and demonstrates a preference for hydrophobic substrates. The k_{cat}/K_m ratio for ethanol oxidation exhibits a pH maximum at 10.4.

Alcohol dehydrogenase (ADH)[1] (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) is the primary enzyme responsible for the oxidation of ethanol, the first step of ethanol elimination in humans (Li, 1977). This enzyme represents 2–3% of the soluble protein in normal human liver, which suggests that, aside from its action on exogenous ethanol, it may serve an important though presently unknown function(s) in intermediary metabolism. Efforts to elucidate the metabolic role of liver ADH have been frustrated by the diversity of its mammalian isozymes (Brandén et al., 1975). Thus, more than 20 have now been identified in human liver (von Wartburg et al., 1965; Blair & Vallee, 1966; Smith, et al., 1971; Bosron et al., 1979a, 1980; Parés & Vallee, 1981; Wagner et al., 1984).

The ADH isozyme makeup of human liver is apparently a function of the genetic background and state of health of the individual (Smith et al., 1973; Li & Magnes, 1975; Ricciardi et al., 1983). These isozymes have been differentiated into three classes on the basis of their electrophoretic mobilities and inhibition by 4-methylpyrazole (Strydom & Vallee, 1982;

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[1] Abbreviations: ADH, alcohol dehydrogenase; CapGapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole; HPLC, high-performance liquid chromatography; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; SDS, sodium dodecyl sulfate.

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Vallee & Bazzone, 1983). Class I ADH includes those that are composed of α, β_1, β_2, γ_1, and γ_2 subunits described by the genetic model of Smith et al. (1973a). These molecular forms exhibit isoelectric points that range from 9 to 11, are effectively inhibited by 4-methylpyrazole with a $K_i \simeq 0.2$ μM (Li & Theorell, 1969), and are isolated readily as an undifferentiated mixture by chromatography on CapGapp–Sepharose, an immobilized 4-substituted pyrazole affinity resin (Lange & Vallee, 1976). Class II apparently consists solely of one isozyme, i.e., π-ADH (Li et al., 1977). It has an isoelectric point between 8 and 9 and is relatively insensitive to pyrazole inhibition with a K_i for 4-methylpyrazole of $\simeq 2$ mM (Bosron et al., 1979a). Class III ADH also contains but a single major form, χ-ADH, characterized by a low isoelectric point, 6.4, and a nearly complete insensitivity to inhibition by 4-methylpyrazole (Wagner et al., 1984).

Kinetics and peptide mapping of human ADH further support this classification. All human isozymes catalyze the oxidation of ethanol; however, ethanol does not saturate class III ADH even at concentrations as high as 2.5 M (Parés & Vallee, 1981; Wagner et al., 1984). Moreover, the K_m of class II ADH for ethanol oxidation, 140 μM (Bosron et al., 1977), is significantly higher than that of all class I forms (Lange et al., 1976; Wagner et al., 1983). In contrast to class I ADH, neither class II nor class III is able to catalyze the oxidation of methanol. HPLC peptide maps of tryptic digests of human ADH isozymes also exhibit significant class-specific variations (Strydom & Vallee, 1982). Thus, the classifications based on electrophoretic and kinetic properties are reflected in gross differences of the primary structures of the isozymes.

Human class II ADH was originally observed in liver biopsy specimens and was then detected also in livers of healthy individuals who had succumbed to sudden trauma, but not necessarily in the livers of victims of disease (Li & Magnes, 1975). The partial characterization of this isozyme was first reported in 1977 (Bosron et al., 1977). Class II ADH is similar to other human ADH isozymes with two subunits of 40 000 daltons, containing 4 mol of zinc/dimer, and with an amino acid composition similar to that of the class I isozymes (Li et al., 1977; Bosron et al., 1979a). Class II ADH catalyzes the oxidation of ethanol, butanol, pentanol, and 3-pyridinylcarbinol, but it does not oxidize methanol, glycerol, or any of the genins of the cardiac sterols. The catalysis of ethanol oxidation at pH 7.5 follows an ordered bi bi reaction mechanism with the dissociation of NADH from the NADH·ADH complex being the rate-limiting step. Because of its unique kinetic properties, it has been suggested that class II ADH may play a unique role in the elimination of ingested ethanol (Li et al., 1977), calling for further investigation of its kinetic and physical properties.

We here describe the purification of human class II ADH with higher specific activity and significantly greater stability than reported previously. The physical–chemical characterization of this isozyme has been extended by the determination of the absorption spectrum, isoelectric point, pH optimum, number of coenzyme binding sites, and degree of inhibition by metal chelators. In addition, its specificity toward alcohol substrates has been more completely defined.

Experimental Procedures

Materials. Human livers were obtained at autopsy within 12 h postmortem and stored at −70 °C. Horse liver ADH was from Boehringer, Indianapolis, IN. Bovine serum albumin (BSA), Tris base, NAD^+ (grades III and AA-1), hydroxyacetic acid, 4-hydroxybutyric acid, 12-hydroxydodecanoic acid, 16-hydroxyhexadecanoic acid, 2-deoxy-D-ribose, and tryptophol were from Sigma Chemical Co., St. Louis, MO. Methanol, ethylene glycol, 2-propanol, cyclohexanol, and benzyl alcohol were products of the Fisher Scientific Co., Fairlawn, NJ. Pentanol, octanol, 3-phenyl-1-propanol, and 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol) were from Aldrich Chemical Co., Milwaukee, WI. Absolute ethanol was obtained from U.S. Industrial Corp., New York, NY. 4-Pentyl- and 4-octylpyrazole were synthesized by the method of Tolf et al. (1979). All other chemicals were of the highest purity available.

DE-52 cellulose was purchased from Whatman Chemical Separations Inc., Clifton, NJ; SP-Sephadex C-50, Sephadex G-25 fine, and prepacked PD-10 columns were obtained from Pharmacia Fine Chemicals, Piscataway, NJ; agarose–hexane–adenosine 5′-phosphate type 2 (AMP–Sepharose) was from P-L Biochemicals, Milwaukee, WI. CapGapp–Sepharose was prepared as described previously (Lange & Vallee, 1976). Distilled water was used throughout.

Enzyme Assays. Enzyme activities were determined spectrophotometrically at 25.0 ± 0.2 °C on either a Cary 219 or a Gilford 2600 spectrophotometer. Assays were performed in 0.1 M glycine–NaOH buffer, pH 10.0, containing 2.5 mM NAD^+ (grade III) and 33.3 mM ethanol. One unit is defined as the amount of ADH required to produce 1 μmol of NADH/min at 25 °C, a molar absorptivity of 6220 M^{-1} cm^{-1} for NADH at 340 nm being used.

In crude fractions, class II ADH activity was differentiated from class I ADH activity by the use of the potent class I inhibitor 4-methylpyrazole. The enzyme was incubated in assay buffer containing 2.5 mM NAD^+ and 33.3 μM 4-methylpyrazole at 25 °C for 4 min while a background rate of NAD^+ reduction was determined. The assay was then initiated by the addition of 33.3 mM ethanol (Li & Theorell, 1969).

Kinetic measurements were performed on a Cary 219 spectrophotometer interfaced with an Apple II computer. The data were collected and stored with software supplied by Varian Associates (Florham Park, NJ) and analyzed as described elsewhere (C. A. Luehr, J. S. Deetz, and B. L. Vallee, unpublished results).

Enzyme Purification. Human liver class II ADH was purified by a modification of the procedure described previously (Bosron et al., 1977). Approximately 75 g of liver was thawed in 120 mL of 1 mM ascorbic acid. The tissue was homogenized with a Waring Blendor (Dynamics Corp. of America, New Hartford, CT) followed by treatment in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Unless otherwise noted, all subsequent steps were carried out at 4 °C, and all buffers were degassed prior to use. The homogenate was centrifuged at 25000*g* for 45 min and the supernatant filtered through glass wool. The resulting solution (~150 mL) was dialyzed overnight against 10 mM Tris-HCl, pH 7.9, containing 1 mM ascorbate. The deep red dialyzed solution was applied to a 600-mL cake of DE-52 cellulose equilibrated with 10 mM Tris-HCl–1 mM ascorbate, pH 7.9. ADH classes I and II were eluted from the cake with equilibration buffer. The fractions exhibiting ADH activity were pooled and mixed with $^1/_9$ volume of 0.5 M sodium phosphate, pH 7.3. NAD^+ (grade AA-I) was added to a final concentration of 1 mM and the pH adjusted to 7.3. The resulting pale yellow solution was applied to a 5 × 10 cm CapGapp–Sepharose affinity column at a flow rate of 120 mL/h. The column was washed with 50 mM sodium phosphate, containing 1 mM ascorbate, pH 7.3. Under these conditions, class I ADH is specifically bound to CapGapp–Sepharose while class II

Table I: Purification of Human Liver Class II ADH

fraction	vol (mL)	act. (units/mL)[a]	total act. (units)	protein concn (mg/mL)[c]	sp act. (units/mg)	yield (%)
supernatant	140	1.37[b]	192	35	0.04	100
DE-52 cellulose	170	0.55[b]	94	6	0.09	49
CapGapp–Sepharose	435	0.19	81	0.9	0.21	42
SP-Sephadex	60	1.09	65	2.1	0.52	34
AMP–Sepharose	40.5	1.10	43.8	0.75	1.47	23

[a] In 0.1 M glycine–NaOH, pH 10.0, containing 33.3 mM ethanol and 2.4 mM NAD^+. [b] Class I ADH activity was inhibited by the addition of 33 μM 4-methylpyrazole. [c] Determined by the method of Lowry et al. (1951) with BSA as the protein standard.

ADH passes through essentially unretarded (Lange & Vallee, 1976; Bosron et al., 1977). Fractions containing class II ADH were pooled, cooled to 0 °C, and adjusted to pH 8.1 with Tris base, and the ADH was precipitated by the addition of solid ammonium sulfate (476 g/L) while the pH was maintained at 8.1 by the dropwise addition of a saturated Tris base solution. The precipitated ADH was collected by centrifugation, 23000*g* for 45 min.

The pellet was redissolved in a minimum volume of 5 mM Tris–phosphate, 0.5 mM NAD^+ (grade AA-I), and 0.1 mM dithiothreitol, pH 7.3, and gel filtered on Sephadex G-25 fine. The conductivity of the pooled protein fractions was adjusted to 1 mS by the addition of buffer and the pool chromatographed on an SP-Sephadex C-50 column (2.5 × 15 cm) equilibrated in the above Tris–phosphate buffer. The column was washed with equilibration buffer and the bound class II ADH eluted with a linear gradient of NaCl, 0–300 mM, at a flow rate of 100 mL/h. The active fractions were pooled and the proteins precipitated with ammonium sulfate as above. The resulting pellet was redissolved in 50 mM sodium phosphate, pH 7.3, and gel filtered on Sephadex G-25 fine. The desalted protein pool was applied to a 1.5 × 7 cm AMP–Sepharose column equilibrated with sodium phosphate buffer at a flow rate of 11 mL/h. The column was washed overnight with equilibration buffer and the purified class II ADH eluted with a linear gradient of NADH, 0–75 μM.

Electrophoresis. High-voltage starch gel electrophoresis was performed at pH 8.2 (Bosron et al., 1979b). Gel slices were stained in the presence of either 100 mM ethanol or 35 mM pentanol with or without the addition of 40 μM 4-methylpyrazole. Electrophoresis in 10% polyacrylamide gels in the presence of 0.1% SDS was performed according to the method of Laemmli & Favre (1973). Polyacrylamide gel electrophoresis in the presence of 7 M urea was performed by a procedure recently developed (W. M. Keung, C. C. Ditlow, and B. L. Vallee, unpublished results). Isoelectric focusing was conducted in thin polyacrylamide gel slabs over a pH range of 7.5–10 essentially as described elsewhere (Pharmacia Fine Chemicals, 1980) under a nitrogen atmosphere as recommended by Valkonen & Piha (1980). Protein bands in all types of polyacrylamide gels were stained with Coomassie Brilliant Blue R-250. Gels were photographed on Kodak Ektapan 4162 film with a yellow filter.

Protein Determinations. Protein concentrations were estimated by the procedure of Lowry et al. (1951), with bovine serum albumin or horse liver ADH as protein standard. Standard concentrations were determined spectrophotometrically with $A_{280}^{1mg/mL} = 0.66$ for BSA (Fasman, 1976) and 0.45 for horse ADH (Drum et al., 1969). The concentration of purified class II ADH was determined by ultracentrifugation (Klainer & Kegeles, 1955).

Metal Analysis. Buffers for metal analysis were rendered free of metal ion contamination by extraction with 0.01% dithizone in CCl_4 (Thiers, 1957). Prior to analysis, enzyme solutions were gel filtered on a Pharmacia PD-10 column that had been washed with 3 column volumes of 12 mM HCl followed by 20 volumes of dithizone-extracted 5 mM sodium phosphate, pH 7.5. Residual NADH that remains bound to the class II ADH after the final purification step and that interferes with UV spectral measurements was removed by incubating the enzyme solution with 100 μM pentanal for 15 s just prior to gel filtration on the PD-10 column. The specific activity of class II ADH gel filtered in this manner remained unaltered. Zinc was determined with a Perkin-Elmer Model 2280 atomic absorption spectrophotometer.

Spectral Measurements. Absorption spectra were measured on a Cary 219 spectrophotometer and the data recorded by an Apple IIe computer with a Varian spectral scanning program. Circular dichroic spectra were determined on a Cary 61 spectropolarimeter.

Results

Enzyme Purification. Separation of the three classes of ADH from homogenized liver is accomplished by batchwise chromatography on DE-52 cellulose, which removed class III ADH as well as about 80% of the soluble proteins, followed by chromatography on CapGapp–Sepharose, which specifically binds class I ADH (Lange & Vallee, 1976). The fractions from the CapGapp–Sepharose column, containing class II activity, are pooled and concentrated by ammonium sulfate precipitation. The resulting ammonium sulfate pellet can be stored for several weeks at −20 °C without loss of activity.

Chromatography of the desalted pellet on SP-Sephadex C-50 resolved the remaining proteins into two peaks. The proteins of the major peak, which bind to SP-Sephadex and contain nearly all class II ADH activity, are concentrated by ammonium sulfate precipitation. Chromatography of this material on agarose–hexane–AMP, with subsequent development of the column by an NADH gradient, results in the elution of a single major protein peak, at 20–25 μM NADH, which superimposes precisely on class II ADH activity. About 25% of the activity insensitive to 4-methylpyrazole originally present in the liver homogenate is recovered in this purified fraction. Table I summarizes the purification procedure.

The specific activity of purified class II ADH is 1.5 units/mg with protein determined by either the method of Lowry et al. (1951) with BSA as a standard or the ultracentrifugation method of Klainer & Kegeles (1955). With the horse EE isozyme as the protein standard, a value of 1.7 units/mg was found, which may be compared with the 1.3 units/mg reported by Bosron et al. (1977).

Homogeneity. Class II human liver ADH migrates as a single protein band in polyacrylamide gels containing 1% SDS (Figure 1A) with a relative mobility corresponding to a molecular weight of 42 000. Class II ADH also migrates as a single band in polyacrylamide gels containing 7 M urea (W. M. Keung, C. C. Ditlow, and B. L. Vallee, unpublished results). These results are consistent with human liver class II

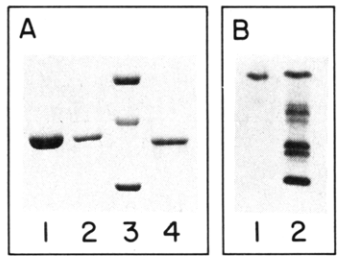

FIGURE 1: Electrophoresis of purified class II ADH. (A) SDS–polyacrylamide gel electrophoresis: (lane 1) 32 μg of class II ADH; (lane 2) 6 μg of class II ADH; (lane 3) a standard mixture composed of bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 30 000) at 5 μg each; (lane 4) 6 μg of an undifferentiated mixture of class I isozymes illustrating the common molecular weight of class I and class II. The gel was stained with Coomassie Brilliant Blue R-250. (B) Starch gel electrophoresis: (lane 1) class II ADH; (lane 2) crude liver homogenate. The gel was stained for ADH activity with ethanol as the substrate.

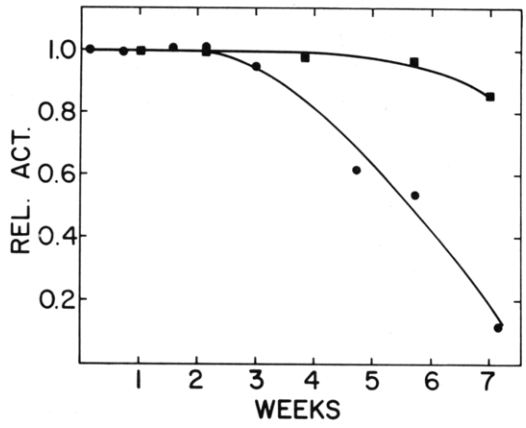

FIGURE 2: Stability of purified class II ADH. ADH activity was determined in 0.1 M glycine–NaOH buffer, pH 10.0, containing 2.5 mM NAD^+ and 33 mM ethanol after storage at 4 °C in 50 mM NaP_i buffer, pH 7.3 (●), plus 0.1 mM dithiothreitol under nitrogen (■).

ADH being a homodimer composed of two subunits of equal size and charge. Starch gel electrophoresis of the purified class II isozyme reveals only a single activity band whose mobility is identical with that of the class II ADH in the original homogenate (Figure 1B); no other ADH forms are detectable in the purified fraction. Isoelectric focusing of class II ADH yields a single protein band with an isoelectric point of 8.6.

The amino acid composition of human class II ADH prepared by this procedure is essentially the same as that reported earlier (Bosron et al., 1979a). Such preparations prepared by this procedure from two different livers contain 3.92 ± 0.05 mol of zinc/dimer, in agreement with an earlier report (Bosron et al., 1979a).

Stability. The extreme lability of purified human class II ADH, with a half-life of 1 day at 4 °C reported by Bosron et al. (1979a), was also observed in the present study with enzyme prepared by the earliest procedure (Bosron et al., 1977). In contrast, enzyme prepared by the present method may be stored for 2–3 weeks in 50 mM NaP_i, pH 7.5, at 4 °C without significant loss of activity (Figure 2). After sterile filtration (Millex HA, 0.45 μm, Millipore Corp., Bedford, MA) and the addition of 1 mM dithiothreitol, the enzyme may be kept under nitrogen in phosphate buffer at 4 °C for 6–8 weeks without significant deterioration.

Inhibition by Metal Chelators. Metal-chelating reagents inhibit the activity of the class II isozyme in a manner characteristic of other forms of ADH. 1,10-Phenanthroline (OP) instantaneously inhibits with a K_i^{app} of 5.6×10^{-5} M. This inhibition is fully reversible by dilution. Thus, exposure of the

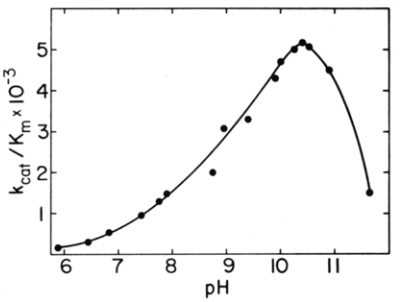

FIGURE 3: pH–activity profile of class II ADH toward ethanol. k_{cat}/K_m values were determined from Lineweaver–Burk plots obtained with 2.5 mM NAD^+ and ethanol in 0.1 M glycine adjusted to the appropriate pH with NaOH.

enzyme to 1×10^{-4} M OP for from 0 to 30 min inhibits activity by 60%; subsequent 10-fold dilution restores activity, and only 15% inhibition remains. This value is exactly the value observed when the enzyme is exposed to 1×10^{-5} M OP directly. EDTA and 2,2′-bipyridine also inhibit class II ADH, but 4,7-phenanthroline, a nonchelating analogue of OP, does not. Thus, as with all other isozymes, zinc is essential for class II ADH activity.

Spectral Features. The ultraviolet absorption spectrum is similar to that of other proteins containing both tryptophan and tyrosine, with a maximum at 280 nm and a shoulder at 290 nm. Maxima of 278 and 280.5 nm have been reported for the horse EE and human class III isozymes, respectively (Drum & Vallee, 1970; Wagner et al., 1984). The fine structural characteristics of phenylalanine near 260 nm, which are particularly evident in the horse EE spectrum but not in the human class III isozyme, are detectable in the spectrum of human class II ADH. Its molar absorptivity is 57 000 on the basis of a molecular weight of 80 000.

NADH binding to class II ADH was examined by circular dichroic spectral titration (Lange et al., 1976). The addition of NADH to the enzyme, 18.7 μM in 5 mM dithizone-extracted sodium phosphate, pH 7.5, reveals a negative extrinsic Cotton effect centered at 328 nm. A plot of the change in ellipticity at 328 nm as a function of moles of NADH added (not shown) breaks at 1.7 mol of NADH/mol of enzyme, demonstrating one coenzyme binding site per subunit.

Enzymatic Characteristics. The optimal pH for ethanol oxidation by human liver class II ADH is 10.4 (Figure 3). at pH 7.4, the rate of oxidation is only 20% of that at pH 10.0. The increased activity of the enzyme at the higher pH is due entirely to an increase in k_{cat}, since the K_m for ethanol at pH 10 is 6-fold higher than the K_m at pH 7.4.

Table II shows the kinetic constants for the oxidation of a number of alcohols by human class II ADH at pH 10.0. The values represent the average of enzyme obtained from at least three liver specimens. Values of K_m and k_{cat} for different preparations did not vary by more than 2-fold. The k_{cat} values for different alcohols are relatively constant, varying by less than 16-fold. In contrast, the values of K_m for different substrates range from 7 μM to 560 mM or 80 000-fold. The large k_{cat}/K_m values for benzyl alcohol and octanol show that human class II ADH favors hydrophobic primary alcohols. Short-chain, hydrophilic, as well as secondary and cyclic, alcohols all exhibit high K_m values and are poor substrates.

While 4-methylpyrazole is a poor inhibitor of class II ADH (Bosron et al., 1979a), hydrophobic side chains in the 4-position of the ring greatly increases the effectiveness of pyrazole as an inhibitor (Tolf et al., 1979). Thus, 4-pentylpyrazole and 4-octylpyrazole inhibit ethanol oxidation by class II ADH, with K_i values of 1 μM and 0.1 μM, respectively. At concentrations

Table II: Kinetic Constants for Human Class II ADH toward Various Alcohols at pH 10.0[a]

substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^{-5}$ M^{-1} min^{-1})
methanol	ND[b]		
ethanol	470	120 000	0.04
pentanol	480	90	50
octanol	500	7	710
benzyl alcohol	550	7	790
3-phenyl-1-propanol	450	32	140
vanillyl alcohol[c]	520	34	150
tryptophol	110	200	5.5
hydroxyacetate	ND		
4-hydroxybutyrate	ND		
12-hydroxydodecanoate	240	230	10
16-hydroxyhexadecanoate	370	60	60
ethylene glycol	45	290 000	0.002
2-propanol	45	560 000	0.001
cyclohexanol	35	210 000	0.002
2-deoxy-D-ribose	170	310 000	0.006

[a] Activities were determined in 0.1 M glycine–NaOH, pH 10.0, in the presence of 2.4 mM NAD^+. [b] ND, no detectable activity at a concentration of 0.1 M, i.e., initial velocities of less than 2% of that obtained with cyclohexanol at its K_m at the same enzyme concentration. [c] Corrected for the increase in absorbance due to formation of vanillin with ΔA_{340} = 23 300 at pH 10.0.

as high as 25 mM, the enzyme does not use $NADP^+$ as a cofactor in ethanol oxidation, confirming earlier results (Bosron et al., 1977).

Discussion

The identification of a previously unknown "anodic" form of ADH, now known as π-ADH (Li et al., 1977) or class II (Vallee & Bazzone, 1983), was initially received with skepticism largely due to both its relative instability and its variable occurrence in liver of different individuals (Li et al., 1977). Although the genetic model of Smith et al. (1973a) does not account for its existence, class II ADH shares many physical and chemical properties with other forms, i.e., the oligomeric structure, subunit molecular weight, stoichiometry of zinc and coenzyme binding, and alkaline pH optimum.

Metal-chelating reagents such as OP inhibit all human ADH isozymes though the degree of inhibition as a function of its concentration varies from class to class, suggesting differences in the accessibility of the enzyme-bound active site zinc atoms to the surrounding medium and perhaps reflecting variations in three-dimensional structure of the isoenzymes. The isoelectric points of human ADH vary from 6.4 to 11 for the class III and class I $\beta\beta$ isozymes, respectively. The amino acid composition of these isozymes is quite similar, including the numbers of positively charged residues (Wagner et al., 1984); hence, the differences in isoelectric point may well be the consequence of variations in the ratios of aspartic and glutamic acid residues to their respective amides.

The history of detection and subsequent work have focused on the extreme lability of class II ADH as compared with that of the other isozymes (see above). However, chromatography with SP-Sephadex C-50 in the course of purification greatly increases the stability of the isozyme. Class II ADH prepared by the earlier procedure (Bosron et al., 1977) consistently contains a minor contaminant of M_r 30 000 when an excess amount of protein is applied to a SDS–polyacrylamide gel. The removal of this contaminant accompanies the increased stability of class II ADH, which is enhanced further by the use of mildly reducing conditions during preparation and storage.

The human class II isozyme follows an ordered bi bi reaction mechanism (Bosron et al., 1979a), as does the horse liver EE form (Wratten & Cleland, 1963; Dworschack & Plapp, 1977). For horse ADH, the dissociation of NADH from the ADH·NADH complex is rate limiting during ethanol oxidation, while for the oxidation of methanol the catalytic step is rate limiting (Brooks & Shore, 1971). Thus, the relatively constant value for k_{cat}, ~500 min^{-1}, for the primary aliphatic alcohols, aromatic alcohols, and long-chain hydroxy fatty acids (Table II) apparently reflects the rate-limiting dissociation of NADH while the lower k_{cat} values for certain other alcohols indicate that an alternative step is rate limiting.

The class II isozyme is not uniquely specific for any given alcohol. In that regard, it does not differ from any of the class I isozymes as is apparent from comparison of K_m and k_{cat}/K_m values with those of class I (Wagner et al., 1983). However, among them, the class II isozyme oxidizes benzyl alcohol and octanol most effectively and ethanol, ethylene glycol, and cyclohexanol most poorly. The wide range of k_{cat}/K_m values exhibited by class II ADH (Table II) is primarily due to variations in K_m, indicating the primary importance of hydrophobic interactions for substrate binding. Other human (Wagner et al., 1983, 1984), horse (Pietrusko, 1975), and mouse ADH isozymes (Holmes et al., 1981) similarly prefer hydrophobic alcohols.

Certain human ADH isozymes are organ specific, as, for example, the class I β subunits that are said to be the only ones in lung, skin, hair roots, and adult kidney (Smith et al., 1971; Goedde et al., 1979, 1980), γ subunits that are the only ones in stomach and infant kidney (Smith et al., 1971, 1973b; Hemple & Pietruszko 1979), and χ or class III ADH is the sole one in placenta (Parês et al., 1984). This could be thought to imply specific physiological roles for these ADH forms in each of these organs. Thus far, class II ADH has only been detected in liver and not in any of the above organs, nor has it been found in spleen or testes (W. P. Dafeldecker, personal communication) while isozymes with similar kinetic properties have been identified in the livers of other primates (Dafeldecker et al., 1981a,b; W. P. Dafeldecker, S.-J. Liang, and B. L. Vallee, unpublished results), of the Chinese hamster (Talbot et al., 1981), and of the horse (J. S. Deetz, personal communication). Thus, class II ADH may well be liver specific but coexist with other ADH forms. Its organ specificity and unique kinetic properties clearly call for further studies of its occurrence and properties.

Registry No. ADH, 9031-72-5; ethanol, 64-17-5; pentanol, 71-41-0; octanol, 111-87-5; benzyl alcohol, 100-51-6; 3-phenyl-1-propanol, 122-97-4; vanillyl alcohol, 498-00-0; tryptophol, 526-55-6; 12-hydroxydodecanoate, 505-95-3; 16-hydroxyhexadecanoate, 506-13-8; ethylene glycol, 107-21-1; 2-propanol, 67-63-0; cyclohexanol, 108-93-0; 2-deoxy-D-ribose, 533-67-5.

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