Human Liver Alcohol Dehydrogenase: Purification, Composition, and Catalytic Features†

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ABSTRACT: Alcohol dehydrogenase has been purified from human liver by affinity chromatography. Ultracentrifugation, Sephadex G-200 chromatography, and amino acid analyses of multiple preparations demonstrate homogeneity of molecular weight. Sodium dodecyl sulfate disc gel electrophoresis reveals a single species of molecular weight 42 000. Based on a molecular weight of 85 000 for the dimer obtained from the amino acid composition and a molar absorptivity of $A_{280nm}^{0.1\%} = 0.58$, the enzyme contains 3.6–4.2 g-atoms of zinc, as determined by emission spectrography, microwave-induced emission, and atomic absorption spectrometry. Inhibition by *o*-phenanthroline, (ethylenedinitrilo)tetraacetic acid, and α,α'-bipyridine demonstrates that zinc is essential to enzymatic function. Detailed kinetic analyses using primary alcohols of the homologous series $CH_3(CH_2)_nOH$, n = 0–5, and the corresponding aldehydes as substrates show that K_M values become smaller as n increases. This suggests that hydrophobic interactions play a role in substrate binding. The availability of well-defined preparations of human liver alcohol dehydrogenase now allows definitive genetic and functional studies of this enzyme to elucidate human ethanol metabolism.

The purification of human liver alcohol dehydrogenase, thought to be the principal ethanol oxidizing enzyme in man, has been problematic and, therefore, knowledge of its properties is limited (von Wartburg et al., 1964; Blair and Vallee, 1966). Although alcohol dehydrogenase has been purified from other species (Racker, 1955; Bonnichsen and Brink, 1955), their properties appear to differ significantly from those of the human enzyme. This limits their value in efforts designed to understand the role of this enzyme in human ethanol metabolism and its pathological derangements.

We have recently developed a ternary complex affinity chromatographic procedure suitable for the isolation and purification of alcohol dehydrogenases in general (Lange and Vallee, 1976). This technique has now been employed to isolate and purify large quantities of human liver alcohol dehydrogenase to homogeneity. The molecular weight, amino acid composition, metal content, and subunit structure of the product have been determined. Further, its catalytic features including substrate specificity, inhibition by chelating agents, and the nature of the substrate and coenzyme binding sites have been investigated. The availability of homogeneous human liver alcohol dehydrogenase represents an important forward step in the study of human ethanol metabolism.

Materials and Methods

Enzymatic Assay. Alcohol dehydrogenase activity was measured with a Gilford Model 240 spectrophotometer equipped with a Heathkit Model IR-18M recorder, thermostated at 25.0 ± 0.2 °C unless otherwise stated. The absorbance at 340 nm was recorded after addition of 25 μl of enzyme to 3 ml of reaction mixture containing 100 μmol of ethanol, 7.6 μmol of NAD^+,[1] and 283 μmol of glycine at pH 10.0. In the reverse reaction, the cuvette contained 99 μmol of sodium phosphate, 27 μmol of acetaldehyde, and 0.77 μmol of NADH, pH 6.8. One unit of activity, U, is defined as a ΔA_{340}/min of 1.0. Protein concentrations were determined using a value of $A_{280nm}^{0.1\%} = 0.58$ (vide infra). Solutions of oxidized and reduced forms of β-diphosphopyridine nucleotide from yeast (Sigma Chemical Co.) were prepared daily and kept at 4 °C.

Inhibition by Metal-Binding Agents. Aliquots of chelating agents in 0.1 M glycine, pH 10.0, were added directly to the assay mixture. All assay components were rendered metal free by either dithizone extraction (Thiers, 1957) or passage over Chelex-100 (Bio-Rad).

pH Measurements. A glass pH electrode (Model GK 2321 c) and a Radiometer (Copenhagen) Model PHM 22, 63, or 64 pH meter were used and standardized at operating temperature with reference buffers, pH 4.00 ± 0.01, 7.00 ± 0.01, and 10.00 ± 0.01 (Harleco).

Spectral Measurements. Continuous absorption and circular dichroic spectra were obtained with a Cary Model 14 spectrophotometer and a Cary Model 61 spectropolarimeter, respectively. Magnetic circular dichroic spectra were obtained with a Cary Model 61 spectropolarimeter equipped with a Varian Model V4145 superconducting magnet, energized by a Varian V4106 power supply. Quartz cells of 1.0 cm path length were employed and thermostated as noted. All spectra were recorded with air as the reference.

Purification of Human Liver Alcohol Dehydrogenase. Fresh human liver, obtained at autopsy and verified to be free of disease by microscopic examination, was ground at 4 °C into 2 l. of water/kg of tissue, and the resultant slurry was stirred for 2 h. After screening through cheesecloth to remove large pieces of debris, the ratio of enzymatic activity at pH 10.0 to

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[1] Abbreviations used are: NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

that at 8.8 was determined and found to be approximately 1.7 to 1.9 in all cases. The crude extract was then aspirated through a bed of DEAE-cellulose (10 × 15 cm) equilibrated with 0.01 M Tris-Cl, pH 7.9. The resulting straw-colored solution was made 50 mM in sodium phosphate and 0.37 mM in NAD^+, pH 7.5, and applied to a Sepharose-4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole column (0.9 × 20 cm) equilibrated with the same buffer. When the effluent activity equaled that of the material applied, the column was washed with equilibrating buffer until the A_{280} of the effluent approached zero, and enzyme finally eluted with buffer containing 500 mM ethanol (Lange and Vallee, 1976).

Protein Concentration. Gravimetric determination of the protein dry weight, performed after trichloroacetic acid precipitation of three 10-mg samples according to the method of Hoch and Vallee (1953), yields a value of $A_{280nm}^{0.1\%} = 0.58$. This was confirmed in the analytical ultracentrifuge (Model E, Beckman Instrument Co.) by counting the interference fringe shift across a synthetic boundary of protein and buffer (Klainer and Kegeles, 1955). The value determined in this manner is 0.59.

Determination of Metals and the Control of External Contamination with Metals. All experiments sensitive to metal contamination were carried out in metal-free glassware cleaned by soaking in nitric and sulfuric acids (1:1) followed by rinsing in glass-distilled water. Buffers and reagents were extracted with 0.01% dithizone in carbon tetrachloride to remove adventitious metal ions (Thiers, 1957).

In the course of purification, triplicate metal analyses were performed by quantitative emission spectrography (Vallee, 1955) both on each fraction of human liver alcohol dehydrogenase and on the enzymes purified from multiple preparations. On the same samples, quantitative zinc measurements were also performed in triplicate both by atomic absorption (Fuwa et al., 1964) and microwave-induced emission spectrometry (Kawaguchi and Vallee, 1975). Prior to such analyses, all samples were dialyzed three times for at least 6 h against a 100-fold volume excess of metal-free 0.01 M Tris-Cl, pH 7.5. For samples to be analyzed by microwave-induced emission spectrometry, 10 mM KCl was included in the dialysate. Buffer blanks analyzed in parallel with each sample were always free of metals.

Molecular Weight Determination: Sephadex Chromatography. A Sephadex G-200 superfine column (2.6 × 87 cm) was equilibrated with 0.1 M Tris-Cl, pH 7.5, at a flow rate of 4.8 ml/h. Five hundred microliter samples (10 mg/ml) of proteins of known molecular weight (ribonuclease A, 13×10^3; chymotrypsinogen A, 25×10^3; ovalbumin, 45×10^3; horse liver alcohol dehydrogenase, 83×10^3; yeast hexokinase, 106×10^3; rabbit muscle aldolase, 158×10^3) were applied to the column and their elution volumes were determined using an Isco Model UA-5 absorbance monitor equipped with a 280-nm filter. The chromatographic parameter, K_{av}, was calculated as the ratio:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e = elution volume, V_t = total column volume, and V_0 = blue dextran elution volume. The values for the standard proteins were 0.950, 0.760, 0.550, 0.370, 0.318, and 0.180, respectively, and a semilog plot of molecular weight for these proteins vs. K_{av} was linear. The elution position of human liver alcohol dehydrogenase was determined both by absorbance at 280 nm and by enzymatic activity.

Sodium Dodecyl Sulfate Disc Gel Electrophoresis. Analytical electrophoresis for molecular weight determination and assessment of molecular weight homogeneity was performed as described by Weber and Osborn (1969). For human liver alcohol dehydrogenase identical results were obtained in the presence and absence of mercaptoethanol. After staining for protein with Amido black or Coomassie blue, the data were assessed by absorption spectroscopy using a Gilford Model 240 spectrophotometer equipped with a gel scanner.

Ultracentrifugation. Both the conventional low speed (Klainer and Kegeles, 1955) and meniscus depletion (Yphantis, 1964) sedimentation equilibrium methods were employed. An aliquot of human liver alcohol dehydrogenase solution, 2.15 and 0.28 mg/ml, respectively, in 0.01 M Tris-0.1 M KCl, pH 7.5, was placed in a double-sector cell (12 mm) with interference window holders, sapphire windows, and an aluminum-filled Epon double-sector centerpiece. For the low speed method, an initial concentration in fringe shifts, C_0, was determined by a synthetic boundary procedure. Operating speeds were 8766 and 21 740 rpm, respectively. Photographs were taken at intervals until equilibrium was achieved. A plot of $\log [Y_{(r)} - Y_0]$ or $\log C$ vs. r^2 was linear, and the molecular weight was calculated from the slope of the line using mol wt = slope$((2RT)2.302/(1 - \bar{v}p)w^2)$ where R = gas constant, T = temperature, $\bar{v}$ = partial specific volume, p = density of the solvent, and w^2 = angular velocity. A partial specific volume of 0.743 ml/g was calculated by the method of Cohn and Edsall (1943).

Sedimentation velocity experiments in a Spinco Model E ultracentrifuge were carried out at 56 000 rpm at 21 °C. Protein concentration was 7.3 mg/ml in 0.01 M Tris-0.1 M KCl, pH 7.5.

Amino Acid Analysis. Total quantitative amino acid analyses of human liver alcohol dehydrogenase were performed using a Durrum 500 amino acid analyzer. One hundred microgram samples of enzyme were transferred to hydrolysis tubes with 5 μl of redistilled phenol, added to prevent chlorination of tyrosine, and 0.1 μmol of norleucine was added as an internal standard. An equal volume of 12 N HCl was added, the tubes were evacuated and sealed, and hydrolysis was carried out for 24, 48, and 72 h at 110 ± 1 °C.

Each analysis was performed at least in triplicate. The final values for serine, methionine, and threonine were extrapolated to zero time, while those for valine and isoleucine were determined after 72 h of hydrolysis. Tryptophan content was determined by the magnetic circular dichroic method of Holmquist and Vallee (1973). A minimum molecular weight was determined from the best fit of data to integral numbers of the most stable amino acids (glycine and phenylalanine). The value agrees closely with that obtained by fitting the data to integral numbers for cysteine determined as cysteic acid (Moore, 1963).

Results

Purification of Human Liver Alcohol Dehydrogenase. The results of a typical affinity chromatographic purification procedure of human liver alcohol dehydrogenase are presented in Table I. Passage of the crude extract over DEAE-cellulose (10 × 15 cm) increases specific activity from 0.02 to 0.21 U/mg (column 5, line 1 and 2), without loss of total activity. Affinity chromatography of the DEAE effluent yields 195 mg of homogeneous human liver alcohol dehydrogenase (column 3, line 3) and the overall purification is 290-fold. The total time for final isolation varies from 6 to 8 h, excluding dialysis time to remove cofactors. In routine preparations, yields have varied from 50 to 80%. However, when making allowances for the

TABLE I: Purification of Human Liver Alcohol Dehydrogenase.[a]

Step	Vol. (ml)	Total Protein (mg)	Act. (U/ml)	Sp Act. (U/mg)	Yield (%)
Crude extract	950	100 000	2.2	0.02	100
DEAE-cellulose	950	10 000	2.2	0.21	100
Affinity chromatography	300	195	4.5	5.7	65

[a] Human liver, 606 g, was ground in 1200 ml of cold H_2O and the mixture was stirred at 4 °C for 2 h. After screening, the crude extract was passed through DEAE-cellulose (10 × 15 cm) equilibrated with 0.01 M Tris-Cl, pH 7.9, and the resultant effluent was affinity chromatographed. See text for other conditions.

TABLE II: Amino Acid Compositions of Alcohol Dehydrogenase: Three Different Preparations From Human Liver.[a]

Residue	1	2	3	4	Horse liver Calcd from the Primary Sequence[b]
Lys	32	33	33	33	30
His	6	6	6	6	7
NH_3	35	38	40	38	16
Arg	11	10	11	11	12
Asp	31	30	31	31	25
Thr[b]	23	22	24	23	24
Ser[a]	24	24	24	24	26
Glu	29	29	30	29	29
Pro	22	20	22	21	20
Gly	40	39	40	40	38
Ala	33	33	34	33	28
Val[c]	39	41	40	40	39
Met*	6	7	7	7	9
Ile[c]	22	22	24	23	24
Leu	30	29	29	29	25
Tyr	6	6	6	6	4
Phe	17	17	17	17	18
Trp[d]	3	3		3	2
CysOH[e]	16	15	14	15	14
Sum[g]	390	386	395[h]	391	374

[a] Columns 1, 2, and 3 are the compositions of different preparations of human alcohol dehydrogenase and column 4 is the average of these. The stable residues are the average of nine analyses; those footnoted are the average of three analyses. Results are expressed as residues per subunit to the nearest whole number. [b] Extrapolated to zero time hydrolysis. [c] Values after 72-h hydrolysis. [d] Determined by magnetic circular dichroism (Holmquist and Vallee, 1973). [e] Determined after performic acid oxidation. [f] Jörnvall (1970). [g] Calculated assuming 3 Trp residues. [h] Excluding line 3.

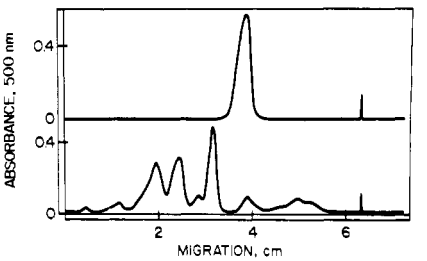

FIGURE 1: Sodium dodecyl sulfate disc gel electrophoresis of human liver alcohol dehydrogenase before (bottom) and after (top) affinity chromatography. Polyacrylamide gels (7.5%) were run at 5 mA/gel (Methods); 50 and 30 μg of protein were loaded, respectively. Amido black was used to stain for protein and the results are presented after scanning at 500 nm. The sharp peak at 6.3 cm represents the bottom of the gel.

state of livers employed as the starting material and when chromatographed under conditions that do not saturate the affinity resin, 100% of the enzymatic activity present initially has been obtained in the final product.

Purity and Molecular Weight. Sodium dodecyl sulfate disc gel electrophoresis of the DEAE effluent resolves multiple species, among which alcohol dehydrogenase is a minor component (Figure 1, bottom). In contrast, after affinity chromatography, only a single species remains (Figure 1, top). Comparison of its mobility with those of standard proteins provides a molecular weight of 42 000, indistinguishable from that of horse liver alcohol dehydrogenase (Boehringer-Mannheim Corp.).

Sedimentation velocity experiments of human liver alcohol dehydrogenase, as isolated in this manner, reveal one boundary, suggesting molecules of but one size, with $s_{obsd} = 4.83$ in 0.01 M Tris-0.1 M KCl, pH 7.5. Sedimentation equilibrium experiments performed both by the Yphantis (1964) and the conventional low-speed methods (Klainer and Kegeles, 1955) yield linear plots of log $[Y(r) - Y_0]$ or log C vs. r^2 with slopes of 0.864 and 0.340, respectively, equivalent to molecular weight values of 78 000 and 79 000.

Sephadex G-200 chromatograms of human alcohol dehydrogenase exhibit coincident and symmetrical peaks of absorbance at 280 nm and of enzymatic activity, but without changes in specific activity. Thus, contaminating proteins of higher or lower molecular weight do not appear to be present. The elution parameter, K_{av}, corresponds to a molecular weight of 83 000.

The amino acid composition of three different preparations of human liver alcohol dehydrogenase studied is virtually identical (Table II). The human contains 50% more tyrosyl and tryptophyl residues than the equine enzyme, as reflected in the correspondingly higher absorbance at 280 nm. Based on 391 amino acid residues/subunit for the human enzyme (Table II, column 4), the molecular weight for the dimer is 85 000, compared to the 374 residues of horse liver alcohol dehydrogenase (Table II, column 5) with a dimeric molecular weight of 83 000 (Jörnvall, 1970). Moreover, for human liver alcohol dehydrogenase, the difference between the sum of the basic residues (including ammonia) and that of the acidic residues is 28, while for the horse liver enzyme it is 11.

Metal Analyses. The metal contents of consecutive fractions obtained during a typical purification are shown in Table III. Zinc, iron, and magnesium are the only stoichiometrically significant metals found in the crude extract (line 1, columns 3, 4, 5, and 6). As specific activity rises progressively from 0.02 U/mg of protein in the crude extract to 5.7 U/mg of protein in the homogeneous enzyme (column 2), the zinc content also rises from 20 to 2900 μg/g of protein, corresponding to 0.02 and 3.9 g-atoms of zinc/85 000 g of protein, respectively (columns 3 and 4). On the other hand, the DEAE step completely removes iron and magnesium.

Zinc was measured by three independent methods on four different preparations of homogeneous enzyme with compa-

TABLE III: Metal Content of Fractions Attending Human Liver Alcohol Dehydrogenase Purification.[a]

Step	Sp Act. (U/mg)	Zinc (μg/g)	Zinc (g-atom/85 000 g of protein)	Fe	Mg (μg/g)	Al
Crude extract	0.02	20	0.02	70	22	Trace
DEAE-cellulose	0.21	530	0.7	0	0	10
Affinity chromatography	5.7	2900	3.9	0	0	4

[a] All samples were dialyzed at 4 °C vs. three changes of 0.01 M Tris-Cl, pH 7.5, under metal-free conditions and then analyzed by emission spectrography as described in the text. Not detected: Ba, Ca, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sn.

TABLE IV: Different Preparations of Human Liver Alcohol Dehydrogenase: Zinc Content by Three Methods.[a]

Prep	Sp Act. (U/mg)	Atomic Absorption		Emission Spectrography		Microwave Excited Emission	
		μg of Zn/g of LADH	g-atoms	μg of Zn/g of LADH	g-atoms	μg of Zn/g of LADH	g-atoms of Zn/mol
1	5.7	3030	4.0	3170	4.1	2990	3.9
2	5.6	2820	3.7	2680	3.6	2880	3.8
3	5.4	3150	4.1	2720	3.6	3140	4.1
4	5.4	3270	4.2	3110	4.1	3050	4.0

[a] All values represent an average of triplicate determinations on human liver alcohol dehydrogenase which had been dialyzed against 0.01 M Tris-Cl, pH 7.5, under metal-free conditions. The amount of protein was determined gravimetrically by the method of Hoch and Vallee (1953), and the molar stoichiometry was calculated based on a molecular weight of 85 000.

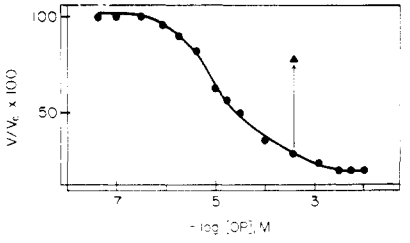

FIGURE 2: Instantaneous inhibition of human liver alcohol dehydrogenase by *o*-phenanthroline added to the assay mixture at 25 °C containing 100 μmol of ethanol, 7.6 μmol of NAD^+, and 283 μmol of glycine at pH 10.0. Reversal of inhibition, the arrow, is obtained by addition of zinc, 6.6×10^{-2} M.

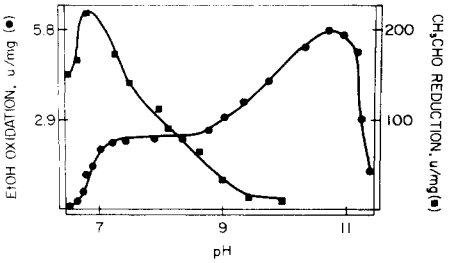

FIGURE 3: pH dependence of ethanol oxidation (●) and acetaldehyde reduction (■) by human liver alcohol dehydrogenase. 24 and 2 μg of enzyme, respectively, were added to the assay mixture buffered at the desired pH by phosphate (0.033 M), pyrophosphate (0.033 M), or glycine (0.1 M), 25 °C.

rable specific activities (Table IV, column 2). Atomic absorption spectrometry detects from 2820 to 3270 μg of zinc/g of protein (Table IV, column 3), corresponding to a molar ratio of metal to protein varying from 3.7 to 4.2 g-atoms/mol based on a molecular weight of 85 000 (column 4). By emission spectrography and microwave-induced emission spectrometry, the stoichiometry ranges from 3.6 to 4.1 and from 3.8 to 4.1 g-atoms/mol, respectively (columns 6 and 8), constituting excellent agreement both between different preparations and analytical methods.

Catalytic Properties.

Inhibition by Metal-Binding Agents. Inhibition with metal-binding agents demonstrates the importance of zinc to the enzymatic activity of human liver alcohol dehydrogenase. *o*-Phenanthroline, α,α′-bipyridine, and EDTA at concentrations of 0.34, 28, and 28 mM, respectively, reduce the activity of the native enzyme to 29, 55, and 65%. The concentration-dependent inhibition with *o*-phenanthroline is instantaneous with a pK_I of 4.8 (Figure 2). Dilution restores activity to the level expected for the *o*-phenanthroline concentration in the final assay mixture. Thus, in an assay mixture containing 0.34 mM *o*-phenanthroline, the enzyme exhibits 29% of native activity. Addition of buffer dilutes the *o*-phenanthroline to 0.0028 mM and simultaneously restores activity to 85% of the native activity, identical to that obtained when 0.0028 mM *o*-phenanthroline is added to the assay mixture directly. Moreover, addition of zinc, 67 mM (Figure 2, arrow), restores activity from 29 to 73% of native activity. The number of moles of inhibitor bound per active site, $\bar{n}$, is 1.05, as calculated by the method of Kistiakowsky and Shaw (1953). These results demonstrate that the enzyme and *o*-phenanthroline form a dissociable, mixed complex, accounting for the reversibility of the inhibition (Vallee et al., 1959).

Stoichiometry of Coenzyme Binding. NADH binding to human liver alcohol dehydrogenase was examined by absorption and circular dichroic spectral titrations. Incremental amounts of NADH, 1 μM, added to human liver alcohol dehydrogenase, 6.8 μM in 50 mM phosphate, pH 7.5, result in increasingly larger differences of absorption at 328 and 351 nm. A plot of $(A_{328} - A_{351})$ vs. mol of NADH (not shown) added breaks sharply at 2.1 mol of NADH/mol of enzyme. The corresponding circular dichroic spectrum reveals a negative extrinsic Cotton effect centered at 325 nm with a molar ellipticity of $[\theta_{325} = -9.20 \times 10^4$ deg cm^2/dmol. A plot of the change of ellipticity at 325 nm vs. mol of NADH added breaks (not shown) at 1.8 mol of NADH/mol of enzyme. Under identical conditions, titrations with the coenzyme analogues, 3-acetylpyridine-NADH and deamino-NADH, result in negative extrema centered at 352 and 330 nm, respectively,

TABLE V: Steady-State Kinetic Parameters For Human Liver Alcohol Dehydrogenase.[a]

n	K_M (mM)	k_{cat} (min^{-1})
	Alcohols	
1	2.9	20
2	2.7	240
3	0.40	250
4	0.033	240
5	0.040	240
6	0.086	250
	Aldehydes	
1	24.0	470
2	1.4	6940
3	0.10	6940
4	0.022	6180
5	0.031	6940
6	0.064	6940

[a] Primary alcohols and aldehydes of increasing chain length from 1 to 6 carbon atoms (n) are all substrates of human liver alcohol dehydrogenase. Assays were carried out in 100 mM glycine, pH 10.0, and 50 mM phosphate, pH 6.8, for alcohol oxidation and aldehyde reduction, respectively. Other conditions are described under Methods.

with molar ellipticities of $[\theta]_{352}$ of -9.15×10^4 and $[\theta]_{330}$ of -9.42×10^4 deg cm^2/dmol.

pH Optima of Catalysis. The human liver alcohol dehydrogenase catalyzed rates of ethanol oxidation and of acetaldehyde reduction were studied as a function of the pH of the assay (Figure 3). The oxidation of ethanol has a pH maximum at about 10.8, is still 40% active at pH 7, but decreases rapidly thereafter; it exhibits little or no activity towards ethanol below pH 6, though the enzyme is still physically stable at pH 5.5. In contrast, the decrease of activity above pH 11.0 reflects rapid, irreversible inactivation of the enzyme. The reduction of acetaldehyde exhibits a sharp pH maximum at 6.8. Activity rapidly falls to virtually zero at pH 9.0.

Steady State Kinetic Parameters. The Michaelis and catalytic constants for the human liver alcohol dehydrogenase catalyzed interconversion of alcohols, aldehydes, and pyridine nucleotides were determined kinetically by the method of Lineweaver and Burk (1934). K_M for NAD^+ is 27 μM at pH 10.0 ([ethanol]/K_M = 10); in the reverse reaction, K_M for NADH is 18 μM at pH 6.8 ([acetaldehyde]/K_M = 10). Employing saturating coenzyme concentrations based on these values of K_M, the kinetic constants for a series of aliphatic primary alcohols and the corresponding aldehydes have been determined (Table V). In both the forward and reverse direction, the one carbon substrates bind weakest, K_M = 2.9 and 24 mM, respectively, and have the lowest k_{cat} values, 20 and 470 min^{-1}. K_M values become progressively lower with increasing length of the substrate, reaching minima at 0.033 and 0.022 mM for butanol and butanal, respectively. On the other hand, for substrates with more than one carbon atom, k_{cat} values remain approximately constant.

Temperature Dependence of K_M (Ethanol). K_M for ethanol was determined over the range from 290 to 317 °K at about 10-degree intervals. The pH of the assay was adjusted to remain constant at 10.0 utilizing a pH/temperature coefficient of -0.0087 ΔpH/°K for glycine. For temperatures of 290, 298, 308, and 317 °K. K_M decreases from 3.5 to 2.5, 1.5, and 1.1 mM, respectively. A plot of reciprocal temperature vs. $-\ln K_M$ is linear with a slope of -4.4 degrees.

Discussion

Human liver alcohol dehydrogenase has been purified only partially so far (von Wartburg et al., 1964; Blair and Vallee, 1966; Mourad and Woronick, 1967) precluding definitive biochemical characterization. The present isolation procedure has overcome past technical limitations and surpasses earlier methods that required many days of work to obtain a few milligrams of impure material. In less than 1 day, affinity chromatography, as here described, results in several hundred milligrams of enzyme which is homogeneous by multiple molecular-weight criteria. Sodium dodecyl sulfate disc gel electrophoresis, Sephadex chromatography, and sedimentation velocity and equilibrium experiments all identify one species as the specific product of this procedure, and the amino acid composition and specific enzymatic activities of several different enzyme preparations are virtually identical.

Ultracentrifugation and gel exclusion chromatography result in molecular weights of 79 000 and 83 000 for human liver alcohol dehydrogenase prepared by affinity chromatography. Together with the value of 42 000 obtained under dissociative conditions, i.e., sodium dodecyl sulfate with or without mercaptoethanol, these results indicate that human liver alcohol dehydrogenase is composed of two subunits with equal or nearly equal molecular weights. The molecular weight calculated directly from the amino acid composition is 85 000 for the dimer. Since this value depends only on the chemical composition of the enzyme, it has been used throughout for calculations of enzyme concentration. The present values agree closely with those reported previously. The molecular weight of the human liver alcohol dehydrogenase dimer has been reported to be 87 000 (von Wartburg et al., 1964) and that of the monomer 40 000 (Jörnvall and Pietruszko, 1972).

Preparations of partially purified human liver alcohol dehydrogenase contained 1.9–2.5 g-atoms of zinc/molecular weight 87 000 (von Wartburg et al., 1964). The zinc content of the material purified by affinity chromatography increases from 20 μg of zinc/g of protein in the crude extract to 2900 μg of zinc/g of protein in the homogeneous enzyme (Table III), while the content of all the other metals decreases. Atomic absorption, emission spectrography, and microwave-induced emission spectrometry independently show that the zinc content of the final product varies from 2720 to 3270 μg of zinc/g of protein, corresponding to a molar ratio of 3.7 to 4.2 g-atoms of zinc/molecular weight of 85 000 (Table IV). Thus, the alcohol dehydrogenase from human liver, like that of the horse, contains 4 g-atoms of zinc/mol of enzyme.

Zinc is essential for enzymatic activity of human liver alcohol dehydrogenase: it is inhibited by EDTA, α,α'-bipyridine, and o-phenanthroline. Moreover, the o-phenanthroline concentration producing 50% inhibition, 1.8×10^{-5} M (Figure 2), is similar to that resulting in an analogous response of other known zinc metalloenzymes (Auld et al., 1975), with 1 mole of inhibitor binding/active site. The inhibition is instantaneous and reversible on dilution or on addition of excess metal ions, in agreement with earlier studies of partially purified enzyme showing inhibition as a function of azide, sulfide, EDTA, α,α'-bipyridine, and o-phenanthroline concentration (von Wartburg et al., 1964). Further, the data confirm that human liver alcohol dehydrogenase forms a mixed enzyme–chelator complex, analogous to the interaction of o-phenanthroline (OP) with horse liver alcohol dehydrogenase (LADH):

$$[(LADH)Zn_2Zn_2] + 2OP \rightleftharpoons [(LADH)Zn_2(Zn \cdot OP)_2]$$

Here, o-phenanthroline interacts with, but does not remove,

the active site zinc atoms of the enzyme (Drum and Vallee, 1970). In marked contrast, the interaction of *o*-phenanthroline with other metalloenzymes results in time-dependent inhibition due to removal of the metal atom (Me) from the enzyme (E) (Vallee and Wacker, 1970):

$$E \cdot Me + nOP \rightleftharpoons E + Me(OP)_n$$

Such differential behavior constitutes a probe of the nature of the metal atoms in such enzymes and their coordinating ligands (Sytkowski and Vallee, 1976). Since *o*-phenanthroline interacts with both native human and horse liver alcohol dehydrogenase in an analogous manner, a similarity in the chemical properties of their respective catalytic metal binding sites is likely.

The mode of substrate binding to alcohol dehydrogenase has been the subject of considerable interest. Although in the horse liver enzyme the hydrophobicity of the substrate side chain has been thought to play a role (Dalziel and Dickinson, 1966), systematic, quantitative studies relating binding to substrate hydrophobicity have not been performed.

The overall change in chemical structure of a series of substrates, such as pairs of primary aliphatic alcohol and the corresponding aldehydes, is minimal, but hydrophobicity increases linearly as a function of chain length (Leo et al., 1971). The strength of binding to human liver alcohol dehydrogenase is lowest for the one carbon substrates and increases progressively with substrate length to become 88- and 1090-fold stronger for the four carbon alcohol and aldehyde, respectively (Table V). However, further elongation does not additionally increase binding. Though steric and other factors may contribute to the stability of the interaction of substrate with enzyme, hydrophobicity clearly is important in this process.

The temperature dependence of ethanol binding to human liver alcohol dehydrogenase provides further evidence that hydrophobic forces effect substrate binding, at least in part. As temperature increases from 290 to 317 °K, K_M decreases 3.3-fold, a response characteristic of hydrophobic interactions (Jencks, 1969). The linear relationship of temperature vs. $-\ln K_M$ results in a $\Delta H = +8.8$ kcal/mol and $\Delta S = +41.6$ eu for substrate binding. The large positive entropy change is consistent with a significant hydrophobic contribution to the overall binding process, and could be the result of substrate binding, displacing water from the enzyme.

The present study documents preparation of human liver alcohol dehydrogenase with well-defined properties. In a larger sense, the species of origin lends importance to the characterization of this particular alcohol dehydrogenase. Although most organisms oxidize ethanol (Brändén et al., 1975), only humans manifest the complex collection of psychological, sociological, and pathological maladaptations labeled alcoholism. In the United States alone, chronic alcohol abuse afflicts five to ten million individuals with intense personal suffering and huge losses to the national economy. Surprisingly, the study of possible biochemical features that might underlie aspects of these afflictions has in some way been disregarded, and certainly underemphasized, either owing to the overwhelmingly societal nature of the consequences or, alternatively, for want of appropriate biochemical approaches.

Importantly, since these afflictions are unique to man, suitable animal systems which could serve as models for the study of alcoholism are not readily defined. As a consequence, knowledge of the molecular events underlying normal alcohol metabolism is fragmentary; that of related disease processes is almost nonexistent. Of necessity, relationships of human liver alcohol dehydrogenase to normal and abnormal ethanol metabolism and, hence, to human pathological states have remained unexplored.

The existence of isozymes of human alcohol dehydrogenase has been noted (Blair and Vallee, 1966; Jörnvall and Pietruszko, 1972) and they may gain importance in relation to such considerations, since there is evidence that some of them may differ in specific activities (von Wartburg and Schürch, 1968). Based on starch gel electrophoresis of crude tissue extracts, Smith et al. (1973) have proposed the existence of three separate structural gene loci coding for monomers of human alcohol dehydrogenase. At least two of the loci are thought to code for two different polypeptide chains, and the existence of all homo- and heterodimeric combinations has been suggested. This genetic model seems to account for the multiplicity of enzymatically active bands separated by electrophoresis, and the differences in electrophoretic mobility may well correlate with changes in the primary structure of the enzyme yet to be identified. The presence of multiple, enzymatically active species could also denote posttranslational modifications, of course, in accord with such findings in crude extracts of *Drosophila* (Jacobson et al., 1970).

In this regard, although human liver alcohol dehydrogenase isolated by affinity chromatography is homogeneous by molecular weight criteria, preliminary studies employing isoelectric focusing and starch gel electrophoresis, indeed, indicate heterogeneity with respect to charge, in accord with earlier observations (Smith et al., 1973; Li and Magnes, 1975).

Potentially, differences in the relative activities and distribution of isozymes of human liver alcohol dehydrogenase could affect the rate of human ethanol metabolism which, in turn, might assign to them a role in the genesis of human alcoholism. Observations that American Eskimos metabolize ingested ethanol at a rate 50% slower than that observed in a matched population of American Caucasians (Fenna et al., 1971) are consistent with such a viewpoint. The present procedure enables the isolation and characterization of human alcohol dehydrogenases to examine these and related hypotheses (Vallee, 1966).

References

Auld, D. S., Kawaguchi, H., Livingston, D. M., and Vallee, B. L. (1975), *Biochem. Biophys. Res. Commun. 62,* 296.

Blair, A. H., and Vallee, B. L. (1966), *Biochemistry 5,* 2026.

Bonnichsen, R. K., and Brink, N. G. (1955), *Methods Enzymol. 1,* 495.

Brändén, C. I., Jörnvall, H., Eklund, H., and Furugren, B. (1975), *Enzymes, 3rd Ed. 9A,* 104.

Cohn, E. J., and Edsall, J. T. (1943) Proteins, Amino Acids and Peptides as Ions and Dipoles Ions, New York, N.Y., Hafner Publishing Co.

Dalziel, K., and Dickinson, F. M. (1966), *Biochem. J. 100,* 34.

Drum, D. E., and Vallee, B. L. (1970), *Biochemistry 9,* 4078.

Fenna, D., Mix, O., Schaefer, A., and Gilbert, J. A. (1971), *Can. Med. Assoc. J. 105,* 472.

Fuwa, K., Pulido, P. A., McKay, R. H., and Vallee, B. L. (1964), *Anal. Chem. 36,* 2407.

Hoch, F. L., and Vallee, B. L. (1953), *Anal. Chem. 25,* 317.

Holmquist, B., and Vallee, B. L. (1973), *Biochemistry 12,* 4409.

Jacobson, K. B., Murphy, J. B., Hartman, F. C. (1970), *J. Biol. Chem. 245,* 1075.

Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology,

New York, N.Y., McGraw-Hill, p 393.
Jörnvall, H. (1970), *Eur. J. Biochem. 16,* 25.
Jörnvall, H., and Pietruszko, R. (1972), *Eur. J. Biochem. 25,* 283.
Kawaguchi, H., and Vallee, B. L. (1975), *Anal. Chem. 47,* 1029.
Kistiakowsky, G. B., and Shaw, W. H. R. (1953), *J. Am. Chem. Soc. 75,* 2751.
Klainer, S. M., and Kegeles, G. (1955), *J. Phys. Chem. 59,* 592.
Lange, L. G., and Vallee, B. L. (1976), *Biochemistry 15* (preceeding paper in this issue).
Leo, A., Hansch, C., and Elkins, D. (1971), *Chem. Rev. 71,* 525.
Li, T.-K., and Magnes, L. J. (1975), *Biochem. Biophys. Res. Commun. 63,* 202.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56,* 658.
Moore, S. (1963), *J. Biol. Chem. 238,* 235.
Mourad, N., and Woronick, C. L. (1967), *Arch. Biochem. Biophys. 121,* 431.
Racker, E. (1955), *Methods Enzymol. 1,* 500.
Smith, M., Hopkinson, D. A., and Harris, H. (1973), *Ann. Hum. Genet. 37,* 4.
Sytkowski, A. J., and Vallee, B. L. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 344.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5,* 273.
Vallee, B. L. (1955), *Adv. Protein Chem. 10,* 317.
Vallee, B. L. (1966), *Ther. Notes 73,* 71.
Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 2nd Ed., 5.*
Vallee, B. L., Williams, R. J. P., and Hoch, F. L. (1959), *J. Biol. Chem. 234,* 2621.
von Wartburg, J. P., Bethune, J. L., and Vallee, B. L. (1964), *Biochemistry 3,* 1775.
von Wartburg, J. P., and Schürch, P. M. (1968), *Ann. N.Y. Acad. Sci. 151,* 936.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

Regulatory Properties of the Pyridine Nucleotide Transhydrogenase from *Pseudomonas aeruginosa.* Kinetic Studies and Fluorescence Titration†

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ABSTRACT: Mechanisms involved in the action of the pyridine nucleotide transhydrogenase from *Pseudomonas aeruginosa* (EC 1.6.1.1) have been investigated by means of kinetic studies and fluorescence titration. Our results, as well as those from previous investigations, suggest that the allosteric MWC model (Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88–118) may be used as a first step for the explanation of the properties of the transhydrogenase. The basic reaction of the enzyme is the oxidation of reduced triphosphopyridine nucleotide (TPNH) by diphosphopyridine nucleotide (DPN^+). In terms of the model, the functional R state is favored by TPNH, whereas the product triphosphopyridine nucleotide (TPN^+) behaves as an allosteric inhibitor, and is therefore assumed to favor the nonfunctional T state. To a slight extent, the T state is also favored by inorganic phosphate. On the other hand, adenosine 2′-monophosphate and several other 2′-phosphate nucleotides function as activators, and hence are presumed to shift the allosteric equilibrium toward the R state. The studies in this paper suggest a specific regulatory site for the transhydrogenase.

In 1952 an enzyme possessing pyridine nucleotide transhydrogenase activity was discovered in a strain of *Pseudomonas* (Colowick et al., 1952). This enzyme has recently been crystallized from extracts of *Pseudomonas aeruginosa,* and it has been possible to carry out extensive studies on its kinetic and physical properties (Cohen, 1967; Cohen and Kaplan, 1970a,b; Louie and Kaplan, 1970; Louie et al., 1972).

It has been demonstrated that PATH[1] catalyzes the following reaction:

$$TPNH + DPN^+ \rightarrow TPN^+ + DPNH \qquad (1)$$

The reverse reaction occurs at a negligible rate, although from thermodynamic parameters the reaction should be freely reversible. The apparent irreversibility is due to the fact that TPN^+ is a very efficient inhibitor of the enzyme. This also explains why the forward reaction does not reach equilibrium in a reasonable time.

PATH is not highly specific with regard to the pyridine nucleotides and, therefore, several coenzyme analogues can be used as substrates (Kaplan et al., 1952, 1953; Cohen, 1967;

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[1] Abbreviations used are: PATH, *Pseudomonas aeruginosa* pyridine nucleotide transhydrogenase; DPN^+, TPN^+, di- and oxidized triphosphopyridine nucleotide; DPNH, TPNH, reduced DPN and TPN; FAD, flavin adenine dinucleotide; IMP, inosine 5′-monophosphate; AMP, ADP, adenosine mono- and diphosphates; $(AcPy)TPN^+$, 3-acetylpyridine analogue of TPN^+; $deaDPN^+$, and $deaTPN^+$, hypoxanthine analogues of DPN^+ and TPN^+; $(TN)DPN^+$ and $(TN)TPN^+$, thionicotinamide analogues of DPN^+ and TPN^+; ϵ-2′-AMP and ϵ-TPN^+, 1,N^6-ethenoadenine derivatives of 2′-AMP and TPN^+; Tris, tris(hydroxymethyl)aminomethane.