Simian Liver Alcohol Dehydrogenase: Isolation and Characterization of Isoenzymes from *Saimiri sciureus*†

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ABSTRACT: The alcohol dehydrogenase (ADH) of squirrel monkey liver can be purified and separated into pyrazole-insensitive and pyrazole-sensitive isoenzymes by affinity chromatography. This is the first demonstration of two functionally distinct classes of ADH in a species other than man. The inhibition of the two enzyme fractions by 4-methylpyrazole is analogous to that observed for the corresponding isoenzymes of human liver. Similarly, the substrate specificity of the pyrazole-insensitive form is more limited and its K_m for ethanol (4 mM at pH 7.5) and acetaldehyde (11 mM at pH 7.0) is larger than that of the pyrazole-sensitive isoenzymes. However, their physicochemical and compositional characteristics, i.e., molecular weight, zinc content, and dimeric structure, are all virtually identical with those of other mammalian alcohol dehydrogenases studied thus far. Zinc is essential for the enzymatic function of both molecular forms as demonstrated by inhibition with chelating agents.

The biochemical features of alcohol dehydrogenase,[1] the principal enzyme responsible for oxidation of ethanol, bear importantly on human alcoholism and alcohol-related diseases. Human liver contains multiple molecular forms of ADH (Blair & Vallee, 1966; Smith et al., 1971; Schenker et al., 1971; Pietruszko et al., 1972); the number and amounts of these forms vary, seemingly dependent on the genetic background and health of the donor (Smith et al., 1971; Azevedo et al., 1974). A newly identified molecular form of human ADH, π-ADH,[2] exhibits kinetic properties that differ strikingly from those of the other isoenzymes (Bosron et al., 1977). Importantly, its K_m value for ethanol exceeds that of the other forms by as much as 100 times and it is remarkably insensitive to inhibition by pyrazole or 4-methylpyrazole, potent inhibitors of all other known mammalian alcohol dehydrogenases. Thus far, π-ADH has been detected only in human livers, and an important role for this enzyme in ethanol metabolism has been postulated (Li et al., 1977).

A genetic predisposition for the capacity to both metabolize and consume ethanol has been proposed, and a genetic basis for some forms of alcoholism has been suggested (Partanen et al., 1966; Goodwin et al., 1974). However, the identification, isolation, and characterization of the postulated biological determinants have remained elusive and obscure so far. The complexity of the problem, compounded by the strict limitations on clinical experiments with human subjects to explore pathogenesis, has generated a search for a suitable animal model. In the course of this pursuit we have concentrated on the livers of nonhuman primates which on an evolutionary basis would be expected to exhibit isoenzyme patterns with pH–rate profiles and pyrazole-insensitive variants similar to those of the human.[3] As shown here, squirrel monkey liver contains such an isoenzyme, the first to be observed in a species other than man. The kinetic and physical properties of the two simian isoenzyme fractions are similar to those of the corresponding forms of human liver ADH. The presence of a pyrazole-insensitive alcohol dehydrogenase, in particular, suggests that the origin and variability of enzymes involved in human ethanol metabolism may be susceptible to experimental delineation in this biochemically related species.

Materials and Methods

NAD^+ (Grade III) and NADH (Grade III) were obtained from Sigma Chemical Co., St. Louis, MO; 4-methylpyrazole was purchased from ICN, K & K Laboratories, Plainview, NY; *o*-phenanthroline was from Aldrich Chemical Co., Milwaukee, WI, and *m*-phenanthroline was from G. F. Smith Chemical Co., Columbus, OH. All other chemicals were reagent grade. Deionized, glass-distilled water was used throughout.

Purification of Squirrel Monkey Liver Alcohol Dehydrogenase. Livers from the common squirrel monkey (*Saimiri sciureus boliviensis*) were obtained either from the New England Regional Primate Research Center or from Pel-Freez Biologicals, Inc. Only livers from apparently healthy adult animals, 2–3 years old, were used. Up to the time of processing, the monkeys were fully fed and the livers were removed immediately after sacrifice and packed in dry ice. All liver homogenates displayed the same isoenzyme patterns, as visualized by starch gel electrophoresis. In a typical preparation, four livers (75 g) cleaned of any nonhepatic tissue were minced and homogenized in 150 mL of water with a Polytron homogenizer (Brinkman Instruments, Inc.). The crude extract was centrifuged for 45 min at 25000*g* and 4 °C. The supernatant was aspirated through a bed of DEAE-cellulose (10 × 7 cm) that had been equilibrated with 0.01 M Tris-HCl, pH 7.9. The enzymatically active effluent was adjusted to 0.05 M NaP_i and 1.2 mM NAD^+, pH 7.5, and applied to a Cap-

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[1] Abbreviations used: ADH, alcohol:NAD^+ oxidoreductase (EC 1.1.1.1); NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; CapGapp, 4-[3-(*N*-6-aminocaproyl)aminopropyl]pyrazole; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; HQSA, 8-hydroxyquinoline-5-sulfonic acid; MCD, magnetic circular dichroism.

[2] π-ADH, initially identified by electrophoresis on starch gels as the "anodic band" (Li & Magnes, 1975) in human liver homogenates, is the isoenzyme with the lowest cathodic electrophoretic mobility, which stains in the presence of 4-methylpyrazole.

[3] Simian liver pyrazole-insensitive ADH is defined operationally as that form of the enzyme that does not bind to the CapGapp–Sepharose affinity resin at pH 7.5.

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Gapp–Sepharose column (1.5 × 23 cm) equilibrated with the same NAD^+ buffer. The column was then washed with equilibrating buffer until the A_{280} of the effluent approached base line. The active fractions, containing pyrazole-insensitive ADH, were combined, concentrated by ultrafiltration (Diaflo PM-10 membrane, Amicon Corp.), and purified further (vide infra). The pyrazole-sensitive ADH was eluted from the affinity resin with 0.5 M ethanol in 0.05 M NaP_i, pH 7.5 (Lange & Vallee, 1976).

Purification of the pyrazole-insensitive ADH was achieved by precipitation with 70% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 5 mM Tris-HCl, pH 7.7, and gel-filtered through a 2.5 × 30 cm column of Sephadex G-25 (Pharmacia) in the same buffer to remove $(NH_4)_2SO_4$ and NAD/NADH. The active fractions were combined, dialyzed against 0.1 M Tris-HCl, pH 8.2, and applied to an agarose–hexane–AMP (P-L Biochemicals, Type 2) affinity column (1.5 × 25 cm) equilibrated at 4 °C with the same buffer. The column was washed with buffer until the A_{280} of the effluent reached 0, and the enzyme was eluted with 400 mL of a linear NADH gradient, ranging from 0 to 7×10^{-5} M NADH. The active fractions were combined, again concentrated by ultrafiltration, and dialyzed against 0.1 M glycine, pH 9.2. The enzyme was then applied to DEAE-cellulose (DE-52, Whatman Biochemicals, Maidstone, United Kingdom) (0.9 × 12.5 cm) equilibrated with the dialysis buffer. Elution of ADH was achieved with 500 mL of a linear gradient of 0–0.1 M NaCl.

The pyrazole-sensitive ADH fractions were combined, concentrated, and dialyzed exhaustively against 0.1 M Tris-HCl, pH 8.2. Application to and elution from agarose–hexane–AMP was carried out as described above. No further purification was necessary, and the enzyme was used after extensive dialysis.

Enzymatic Assays. Alcohol dehydrogenase activity was determined in 0.1 M NaP_i, pH 7.5, or 0.1 M glycine, pH 10.0, by measuring the production of NADH in the presence of alcohol substrates. The reverse reaction, the utilization of NADH with aldehyde substrates, was carried out in 0.1 M NaP_i, pH 7.0. The spectrophotometric assay was performed by use of a Cary 219 or Gilford 240 spectrophotometer, thermostated at 25.0 ± 0.2 °C. The absorbance at 340 nm was recorded after addition of substrate to give 3 mL of reaction mixture, containing enzyme and 2.4 mM NAD^+ or 0.24 mM NADH, respectively. Solutions of the coenzymes were prepared daily and stored at 4 °C. Activities are expressed as IU and represent micromoles of NADH utilized or produced per minute based on an A_{340} of 6.22 mM^{-1} cm^{-1}.

Ultracentrifugation. All centrifugal studies were performed in a Spinco Model E analytical ultracentrifuge (Beckman Instrument Co.). The molecular weight of the pyrazole-sensitive ADH was determined by the low-speed equilibrium method (Van Holde & Baldwin, 1958) at 9341 rpm and 10.5 °C. Enzyme (3.53 mg/mL) was dialyzed against 10 mM Tris-HCl and 0.1 M KCl, pH 7.5, containing 10 mM ethanol. The initial concentration in fringe shifts, C_0, was obtained by a synthetic boundary procedure. The molecular weight was calculated from the slope of log C vs. r^2 by assuming a partial specific volume of 0.743 mL/g (Lange et al., 1976).

The molecular weight of the pyrazole-insensitive ADH was determined by the meniscus depletion sedimentation equilibrium method (Yphantis, 1964) at 23 150 rpm and 21 °C. The enzyme (0.38 mg/mL) was dialyzed against 10 mM Tris-HCl and 0.1 M KCl, pH 7.5. The molecular weight was calculated from the slope of log $(Y_r - Y_0)$ vs. r^2.

Metal Analysis. The Zn content of ADH was determined by atomic absorption spectrometry, employing graphite furnace atomization (Model 5000, Perkin-Elmer Corp.). The enzyme sample to be analyzed was dialyzed extensively against 10 mM Tris-HCl and 0.1 M KCl, pH 7.5, diluted to a final protein concentration of 0.04 μM, and made 50 mM in $NH_4H_2PO_4$ and 10 mM in HNO_3. Each analysis was performed with a 5-μL aliquot of enzyme solution. All reported values, corrected for buffer blanks, constitute the averages of three to five determinations with a coefficient of variation of 1.5%.

Protein Concentration. Enzyme solutions were dialyzed at 4 °C against several changes of 10 mM Tris-HCl and 0.1 M KCl, pH 7.5. The absorbance at 280 nm was measured, and the protein concentration was determined in the analytical ultracentrifuge by counting the interference fringe shift across a synthetic boundary of protein and buffer (Klainer & Kegeles, 1955).

Sodium Dodecyl Sulfate Disc Gel Electrophoresis. Analytical electrophoresis for molecular weight determination and assessment of molecular weight homogeneity was performed as described by Weber & Osborn (1969). Samples were run at 6 mA/gel in the presence and absence of β-mercaptoethanol and stained with Coomassie blue. The results were assessed by absorption spectroscopy using a Gilford Model 240 spectrophotometer equipped with a gel scanner.

Starch Gel Electrophoresis. Crude and purified ADH fractions were subjected to vertical starch gel electrophoresis in the presence of NAD^+ as described by Li & Magnes (1975). ADH activity in the gel was detected with ethanol or 1-pentanol as substrate (Smith et al., 1972).

Control of External Contamination with Metals. All experiments sensitive to metal contamination were carried out in plastic containers or glassware freed of metal by soaking in nitric and sulfuric acids (1:1) followed by rinsing with distilled water. Buffers and reagents were rendered metal free either by dithizone extraction (Thiers, 1957) or by passage over Chelex-100 (Bio-Rad, Richmond, CA).

Amino Acid Analysis. Enzyme (100 μg) was hydrolyzed in 1 mL of 6 N HCl containing 5 μL of redistilled phenol, added to prevent loss of tyrosine, for 24, 48, and 72 h at 110 °C. Each analysis was performed at least in triplicate with a Durrum D-500 amino acid analyzer. The final values for Ser and Thr were extrapolated to zero time, while those for Val and Ile were determined after 72 h of hydrolysis. Cysteine was determined as cysteic acid after performic acid oxidation (Moore, 1963), and tryptophan content was obtained by magnetic circular dichroism (Holmquist & Vallee, 1973). Integral values for amino acids were calculated by assuming 374 residues, based on the primary sequence of horse liver ADH (Jornvall, 1970), per 39 000 subunit molecular weight (Table I).

Results

Purification of Squirrel Monkey Liver Alcohol Dehydrogenase. The results of a typical purification procedure are presented in Table II. There is no loss of activity on passing the homogenate supernatant, from four livers containing 44 IU of total alcohol dehydrogenase activity, over DEAE-cellulose. Chromatography on CapGapp–Sepharose of the active DEAE effluent yields two enzyme fractions: pyrazole-insensitive and pyrazole-sensitive ADH, the molecular forms that do not and do bind to the affinity resin, respectively.

The fractions which solely contain pyrazole-insensitive ADH comprise 7 IU or 16% of the total activity applied to the column. After $(NH_4)_2SO_4$ precipitation and gel filtration over Sephadex G-25, the enzyme is purified further by affinity

Table I: Amino Acid Compositions[a]

residue	residues/subunit: pyrazole-sensitive ADH	pyrazole-insensitive ADH
Asp	30.1 ± 0.2	31.7 ± 0.5
Thr[b]	21.9 ± 0.3	26.3 ± 0.3
Ser[b]	25.0 ± 0.3	21.7 ± 0.2
Glu	29.2 ± 0.1	33.2 ± 0.6
Pro	19.5 ± 0.1	16.1 ± 0.4
Gly	36.6 ± 0.2	36.4 ± 0.3
Ala	30.1 ± 0.2	32.8 ± 0.4
Val[c]	36.1 ± 0.1	24.8 ± 0.3
Met	7.2 ± 0.5	6.3 ± 0.5
Ile[c]	20.8 ± 0.1	24.5 ± 0.4
Leu	29.1 ± 0.5	31.7 ± 0.3
Tyr	5.3 ± 0.2	5.5 ± 0.1
Phe	15.3 ± 0.2	15.2 ± 0.2
His	6.4 ± 0.1	6.5 ± 0.1
Lys	30.7 ± 0.2	29.2 ± 0.6
Arg	10.8 ± 0.1	10.4 ± 0.1
Trp[d]	2.3	2.4
Cys[e]	13.8 ± 0.5	15.6 ± 0.3

[a] Values shown present the mean ± standard deviation of duplicate analyses at 24, 48, and 72 h of hydrolyses, except where otherwise noted. [b] Extrapolated to zero time; average of duplicate determinations. [c] Value after 72 h of hydrolysis; average of duplicate determinations. [d] Determined by MCD. [e] Determined as cysteic acid after performic acid oxidation; average of duplicate analyses. See text for other conditions.

Table II: Purification of Squirrel Monkey Liver Alcohol Dehydrogenase[a]

purification step	vol (mL)	total act. (IU)	act. recovered (%)
crude extract	130	44.2	100
DEAE-cellulose	201	43.7	99
CapGapp–Sepharose			
(1) pyrazole-insensitive ADH[b]	252	7.0	16
agarose–AMP	95	3.8	8.6
DEAE-cellulose	32	1.9	4.3
(2) pyrazole-sensitive ADH[c]	168	21.0	48
agarose–AMP	278	13.1	30

[a] Four squirrel monkey livers (75 g) were homogenized in 150 mL of water and centrifuged. The supernatant was passed through DEAE-cellulose, and the resultant effluent was chromatographed on CapGapp–Sepharose. [b] ADH fraction which did not bind to affinity resin. [c] ADH fraction which bound to affinity resin and was eluted with 0.5 M ethanol. See text for other conditions.

chromatography on agarose–hexane–AMP, yielding 3.8 IU of enzyme. Final chromatography on DEAE-cellulose, followed by exhaustive dialysis, results in 1.9 IU of homogeneous ADH.

Pyrazole-sensitive ADH is eluted from the CapGapp–Sepharose affinity resin with 0.5 M ethanol. The active fractions, comprising 21 IU or 48% of the total activity applied to the column, are concentrated, dialyzed, and further purified by affinity chromatography on agarose–hexane–AMP, yielding 13 IU of homogeneous protein. The CapGapp–Sepharose purification step recovers ~64% of the total activity and all of the molecular forms.

Starch Gel Electrophoresis. The molecular forms of ADH in the supernatant of a squirrel monkey liver homogenate and in the enzyme fractions after separation by CapGapp–Sepharose affinity chromatography are compared in Figure 1. The starch gels after electrophoresis at pH 7.7 or 8.6 were stained for alcohol-oxidizing activity in the presence of NAD^+ either with ethanol or with 1-pentanol as substrate. In the course of the present investigation the crude extracts of 24 liver

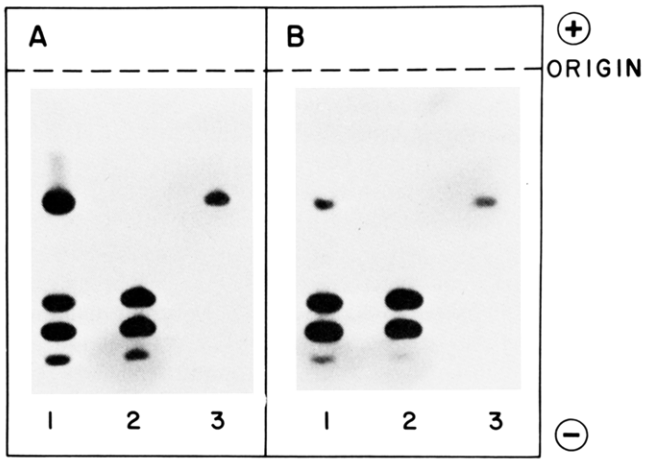

FIGURE 1: Starch gel electrophoresis of squirrel monkey liver alcohol dehydrogenase. Conditions: pH 8.6; 220 V for 18 h at 4 °C. Enzymatic activity of the liver homogenate (sample 1), the pyrazole-sensitive fraction (sample 2), and the pyrazole-insensitive (sample 3) form was detected with either ethanol (panel A) or 1-pentanol (panel B) as substrate.

specimens were analyzed by starch gel electrophoresis, and all of them resulted in virtually the same pattern. Pyrazole-insensitive ADH, the enzyme form which fails to bind to the affinity resin, has the lowest cathodic electrophoretic mobility. The balance of the activity bands correspond to those molecular forms that bind to CapGapp–Sepharose and, hence, collectively represent pyrazole-sensitive ADH.

Purity and Molecular Weight. The two purified enzyme fractions give single bands with identical mobilities on Na-$DodSO_4$ disc gel electrophoresis. Moreover, their mobilities are identical in turn with those of human and horse liver alcohol dehydrogenase, indicative of the same apparent molecular weight, i.e., ~40 000 per subunit. Sedimentation equilibrium experiments employing the Yphantis method (Yphantis, 1964) for pyrazole-insensitive ADH and the conventional, low-speed method (Klainer & Kegeles, 1955) for the pyrazole-sensitive forms yield linear plots of log $(Y_r - Y_0)$ or log C vs. r^2 with slopes of 1.04 and 0.385, respectively, corresponding to molecular weights of 78 430 and 76 230. A molecular weight of 78 000 was used for all calculations. The molar absorptivity at 280 nm, determined by refractive index in the synthetic boundary cell of the ultracentrifuge, was found to be 5.16×10^4 M^{-1} cm^{-1} ($A_{280}^{0.1\%} = 0.66$) and 4.84×10^4 M^{-1} cm^{-1} ($A_{280}^{0.1\%} = 0.62$) for pyrazole-insensitive and pyrazole-sensitive ADH, respectively.

Metal Analysis and Inhibition by Metal-Binding Agents. Zinc in both enzyme fractions was measured by atomic absorption spectrometry on three different preparations of homogeneous enzyme which exhibited comparable specific activities. The analyses revealed 3.9 ± 0.1 and 4.0 ± 0.1 g-atom of zinc/mol of pyrazole-insensitive and pyrazole-sensitive ADH, respectively, and no other metals were detected in significant amounts.

Activity measurements in the presence of suitable metal-binding agents result in inhibition and demonstrate that zinc is essential for enzymatic activity in both enzyme fractions. The concentration-dependent inhibition with *o*-phenanthroline is both instantaneous and reversible. Addition of excess Zn^{2+} or dilution restores activity instantaneously, demonstrating the formation of a dissociable enzyme·*o*-phenanthroline mixed complex (Vallee et al., 1959). In contrast, addition of the nonchelating isomer, *m*-phenanthroline, to the reaction mixture does not result in inhibition. The chelating agents EDTA, HQSA, and dipicolinic acid inhibit both enzyme fractions in

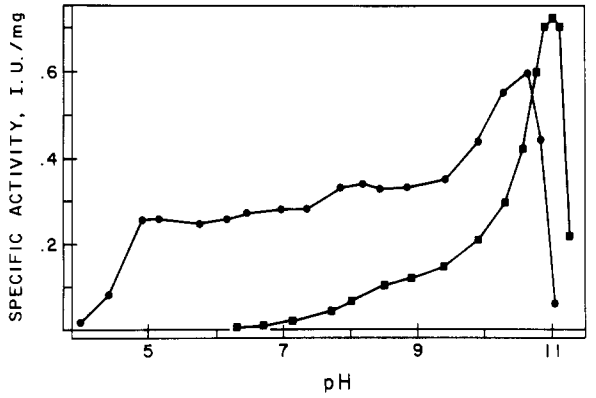

FIGURE 2: pH dependence of ethanol oxidation by pyrazole-sensitive (●) and pyrazole-insensitive (■) squirrel monkey liver alcohol dehydrogenase. Enzymatic activity was assayed with 33 mM ethanol and 2.4 mM NAD^+ in 33 mM NaP_i from pH 4.5–7.5, 33 mM $NaPP_i$ from pH 7.5–9.5, and in 0.1 M glycine–NaOH from pH 9.0–11.5 at 25 °C.

a time-dependent manner. In all three instances, the inhibition is not reversed by either dilution or addition of Zn^{2+}, indicative of the irreversible removal of the catalytically essential metal.

pH Optima of Catalysis. The rates of ethanol oxidation and acetaldehyde reduction were studied as a function of the pH of the assay (Figure 2). For the oxidation of ethanol, the pyrazole-sensitive and pyrazole-insensitive forms exhibit maximal activity at pH 10.6 and 11.0, respectively. Above these pH values the decrease of activity reflects rapid and irreversible inactivation of the enzymes. The pyrazole-sensitive form exhibits a second maximum at pH 8.2 and retains more activity at lower pH values than does the pyrazole-insensitive form. Thus, at pH 5, the pyrazole-sensitive ADH still exhibits 42% of its maximum activity, while the pyrazole-insensitive form is virtually inactive.

Steady-State Kinetic Parameters. The Michaelis and catalytic constants for both forms of squirrel monkey liver ADH were determined at pH 7.5 and pH 10.0 from Lineweaver–Burk plots.

The K_m values of the pyrazole-sensitive ADH fraction for NAD^+, at pH 7.5 and pH 10.0, are 12 μM and 7 μM, respectively, when assayed with ethanol at 10 times its K_m. The kinetic parameters for a homologous series of aliphatic primary alcohols were determined by employing saturating coenzyme concentrations based on these values of K_m (Table III). At both pH values, methanol binds weakest and has the lowest k_{cat} value. K_m values become progressively lower with increasing chain length to reach 0.018 mM for 1-pentanol at pH 10.0 and 0.012 mM for 1-butanol and 1-pentanol at pH 7.5. Similarly, relative to ethanol, the k_{cat} values decrease with increasing chain length to 20 min^{-1} at pH 10.0 and 17 min^{-1} at pH 7.5 for 1-pentanol.

The Michaelis constant of the pyrazole-insensitive ADH fraction for NAD^+ is 5 μM at pH 7.5 and 14 μM at pH 10.0. At saturating coenzyme concentrations and with 100 mM methanol as the substrate there is no activity. An increase in the chain length of the primary alcohol from two to four carbons decreases K_m, from 4 to 0.06 mM at pH 7.5 and from 5 to 0.02 mM at pH 10, while the values for k_{cat} remain constant at 5 min^{-1}, pH 7.5, and 27 min^{-1}, pH 10.

The reduction of acetaldehyde was examined with both enzyme fractions at pH 7. For the pyrazole-insensitive and pyrazole-sensitive ADH forms, the K_m values are 11 and 0.1 mM, while the k_{cat} values are 200 and 1000 min^{-1}, respectively.

Since human π-ADH differs significantly from other molecular forms of ADH with respect to its K_I values for 4-methylpyrazole (Li et al., 1977), the effect of this potent inhibitor on both enzyme fractions of squirrel monkey liver alcohol dehydrogenase was investigated. The K_I values for the pyrazole-insensitive form are 11 and 4 μM at pH 7.5 and pH 10.0, respectively. In contrast, the inhibition constants for the pyrazole-sensitive ADH fraction are 0.2 μM at pH 7.5 and 0.5 μM at pH 10.

Table III: Steady-State Kinetic Parameters for Squirrel Monkey Liver Alcohol Dehydrogenase[a]

		pyrazole-sensitive ADH		pyrazole-insensitive ADH	
substrate	pH	K_m (mM)	k_{cat} (min^{-1})	K_m (mM)	k_{cat} (min^{-1})
methanol	10.0	14	13	no activity	
ethanol		0.8	34	5	27
butanol		0.09	27	0.02	27
pentanol		0.018	20		
NAD^+		0.007	34	0.014	27
methanol	7.5	12	7	no activity	
ethanol		0.18	24	4	5
butanol		0.012	21	0.06	5
pentanol		0.012	17		
NAD^+		0.012	24	0.005	5
acetaldehyde	7.0	0.1	1000	11.4	200

[a] Alcohol oxidations were carried out in 0.1 M glycine, pH 10.0, and 0.1 M NaP_i, pH 7.5. Aldehyde reductions were determined in 0.1 M NaP_i, pH 7.0. Values for K_m and k_{cat} are the averages of three preparations. The estimated standard deviations for the kinetic parameters are consistent with a significant kinetic difference between the two isoenzymes. Other conditions are described under Materials and Methods.

Discussion

CapGapp–Sepharose, an affinity resin that is specific and highly effective for the purification of alcohol dehydrogenases from mammalian livers, has recently served to differentiate two distinct classes of human liver ADH. The first binds to the resin and encompasses a series of electrophoretically distinct variants which are already identified and perhaps yet others which remain to be recognized. As a class, these variants have a low K_m for ethanol (1 mM or less at pH 7.5) and a low K_I for 4-methylpyrazole (<1 μM) and comprise all cathodic molecular forms studied by investigators in the past. The second does not bind to CapGapp–Sepharose and corresponds to the anodic band identified by Li & Magnes (1975) and now referred to as π-ADH. Its identification and isolation was based on the intrinsic relative affinity of the CapGapp ligand, a 4-substituted pyrazole derivative, for the variants of ADH (Lange & Vallee, 1976). The K_I of human liver π-ADH for 4-methylpyrazole is ~500-fold higher than that of the other molecular forms of ADH, and, hence, this inhibitor fails to retain π-ADH on the column (Li et al., 1977). π-ADH has a K_m for ethanol of 34 mM (pH 7.5), and its kinetic properties differ significantly from those of the other class (Bosron et al., 1979). Furthermore, it has recently been shown that human π-ADH is an immunologically distinct variant, and a separate evolutionary origin was postulated for this form (Adinolfi et al., 1978).

This differential affinity chromatography procedure has now been employed to isolate and purify ADH from squirrel monkey liver. Analogous to some human liver homogenates, squirrel monkey liver contains an isoenzyme of ADH, which does not bind to the affinity resin (Figure 1).

The physical properties of both classes of squirrel monkey liver ADH closely resemble those of the horse (Drum et al., 1969) and of human liver ADH (Lange et al., 1976). Ultracentrifugation reveals molecular weights of 78 000, while

$NaDodSO_4$ gel electrophoresis indicates the existence of subunits with identical molecular weights of ~40000. Hence, both classes of squirrel monkey liver ADH are composed of two subunits of equal or nearly equal molecular weights. Atomic absorption spectrometry, utilizing graphite furnace atomization, shows that both classes of squirrel monkey liver ADH contain 4 g-atoms of zinc/mol of protein and no significant concentrations of other metals.

Zinc is essential for enzymatic activity of both classes of squirrel monkey liver ADH, in accord with the characteristics of all yeast and mammalian NAD(H)-dependent alcohol dehydrogenases examined thus far. Further, chelating agents such as 1,10-phenanthroline, EDTA, HQSA, and 2,2′-bipyridine all inhibit the enzyme. The inhibition with 1,10-phenanthroline is instantaneous and reversible on dilution or addition of excess metal ions, indicative of the formation of a mixed enzyme–zinc–chelator complex observed previously with horse (Drum & Vallee, 1970) and human (Von Wartburg et al., 1964; Lange et al., 1976) liver ADH. The results confirm the critical role of the metal in the catalytic action of these liver alcohol dehydrogenases and indicate a close similarity in the chemical properties of their respective catalytic domains.

The kinetic parameters for the oxidation of primary alcohols of pyrazole-sensitive squirrel monkey liver ADH are also similar to those of the pyrazole-sensitive class of human liver ADH's (Lange et al., 1976). Both oxidize methanol, whereas human π-ADH and pyrazole-insensitive squirrel monkey liver ADH do not. Furthermore, both undergo a decrease in K_m values of 1000- and 800-fold at pH 7.5 and 10, respectively, on increasing the chain length of the primary alcohols to five carbons. In contrast, the K_m values for the oxidation of ethanol at pH 7.5 and the reduction of acetaldehyde at pH 7.0 for the pyrazole-insensitive form are 22- and 114-fold higher, respectively, than the corresponding parameters for the pyrazole-sensitive forms (Table III). Though steric and other factors may contribute to the strength of binding of substrate to the active center of the enzyme, hydrophobicity clearly plays an important role in this process (Klyosov et al., 1977). The kinetic similarities between the pyrazole-sensitive forms of human and squirrel monkey liver ADH suggest homology of binding sites in their active centers.

Human π-ADH is much less sensitive to inhibition by 4-methylpyrazole than are the other molecular forms of ADH, and this difference in pyrazole inhibition constitutes the basis for its isolation. The same nomenclature can be applied to the pyrazole-insensitive squirrel monkey liver ADH, separated in like manner from pyrazole-sensitive molecular forms. As might be expected, this differential inhibition is quantitatively expressed by the inhibition constant of the two forms. Thus, the apparent K_I for 4-methylpyrazole at pH 7.5 of the pyrazole-insensitive squirrel monkey liver ADH is 55-fold higher than that of the other forms (vide supra).

Until now, the biological determinants of alcoholism have remained elusive and obscure. However, in recent years increasing evidence has indicated that some individuals have a genetic predisposition for the consumption of alcohol (Partanen et al., 1966) and for alcoholism (Goodwin et al., 1973, 1974). Yet, research directed toward the elucidation of the genesis of alcoholism has suffered from the limitations inherent in direct human experimentation and/or the lack of a suitable animal model. Detailed studies of π-ADH, isolated from some human livers, will likely advance efforts to recognize and understand biochemical mechanisms that may further delineate the genetic variability of the enzymes of ethanol metabolism (Li et al., 1977). Thus far, π-ADH had been detected only in human livers and its potential metabolic role led to the now successful search for its existence in other nonhuman primates.

The π-like isoenzyme of ADH in the liver of *S. sciureus* is similar in all respects to that previously identified in some human livers: its K_m for ethanol and acetaldehyde is high, its cathodic electrophoretic mobility is low, and it is unable to bind to the affinity resin (Lange et al., 1976). The detection of a π-like ADH isoenzyme in *S. sciureus* will permit studies of its genetic properties, functional significance, and inducibility by ethanol or other metabolites. The data provide first evidence of the existence of animal models suitable for the study of human alcoholism when based on biochemical considerations and identities.

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Detection of Ligand-Induced Conformational Changes in Phenylalanyl-tRNA Synthetase of *Escherichia coli* K10 by Laser Light Scattering†

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ABSTRACT: The diffusion constant of phenylalanyl-tRNA synthetase has been measured by laser light scattering under conditions of complex formation with Mg^{2+}, L-phenylalanine, MgATP, $tRNA^{Phe}$, modified $tRNA^{Phe}$, $tRNA^{Phe}$ (yeast), and noncognate tRNA. The diffusion constant (pH 7.5, 20 °C) of the free enzyme is $(2.85 \pm 0.005) \times 10^{-7}$ cm^2 s^{-1}, of the enzyme·Mg^{2+} complex $(2.40 \pm 0.05) \times 10^{-7}$ cm^2 s^{-1}, and of the enzyme·Mg^{2+}·$tRNA^{Phe}$ complex $(2.95 \pm 0.06) \times 10^{-7}$ cm^2 s^{-1}. The effect of $tRNA^{Phe}$ is only seen when the enzyme is saturated with Mg^{2+}. The smaller substrates exhibit no effect besides a small increase of the value of the diffusion constant under conditions where the enzyme·phenylalanyladenylate is synthesized. Of the noncognate $tRNA^{Tyr}$ and $tRNA^{Ile}$, the latter is able to associate with the enzyme, causing the value of the diffusion constant to increase. $tRNA^{Phe}$ (yeast) and $tRNA^{Phe}_{h\nu}$ (photo-cross-linked $tRNA^{Phe}$) exhibit similar effects. The observed variation of the diffusion constant is attributed to conformational changes of the enzyme. The opposite effects of Mg^{2+} and $tRNA^{Phe}$ are interpreted as an expansion and recontraction, respectively, of the enzyme molecule. In several cases, the effects were used to follow a titration of the enzyme with a ligand. Dissociation constants were calculated from the resulting titration curves, yielding values which are in agreement with those obtained by other techniques. It is established by comparison that of the two possible binding sites for each Mg^{2+} and $tRNA^{Phe}$ the diffusion constant reflects occupation of only a single class of sites.

One of the important early steps in protein synthesis is the formation of aminoacyl-tRNA. This process involves two steps, both mediated by a tRNA synthetase specific to a given tRNA–amino acid pair. The first step is the chemical activation of the amino acid, and the second, the attachment of the amino acid to the tRNA.

The second step is commonly believed to be accompanied by one or more changes in the conformation of the enzyme. Indeed, there is a substantial body of literature reporting conformation changes upon binding of tRNA as detected by several techniques. For example, changes in conformation of the enzyme–substrate complex were detected by using kinetic studies of the yeast Phe, *Escherichia coli* Tyr, and yeast Ser systems (Riesner et al., 1976; Krauss et al., 1976; Rigler et al., 1976), circular dichroism measurements of the yeast Tyr system (Ohta et al., 1967), X-ray small-angle scattering of the yeast Phe system (Pilz et al., 1979), and neutron-scattering studies of the *Escherichia coli* Met (Dessen et al., 1978) and yeast Glu (Zaccai et al., 1979) enzymes. Changes in the tRNA conformation upon binding the enzyme have been studied in the *E. coli* Glu (Willick & Kay, 1976) and *E. coli* Phe (Favre et al., 1979) systems by using circular dichroism and in the yeast Phe system by using fluorescence studies of the wybutine base (Krauss et al., 1976).

In spite of this work, a number of questions remain unanswered. Among these are the following: (1) Does the conformation rearrangement of a synthetase·tRNA complex involve changes in both the enzyme and the tRNA or only in the tRNA? (2) It is known that Mg^{2+} has profound effects on reactions catalyzed by phenylalanyl-tRNA synthetase of *E. coli* and on the formation of the enzyme·Phe-$tRNA^{Phe}$ complex (Bartmann et al., 1975a; Holler, 1976). Are these effects caused by or, at least, accompanied by conformation changes? (3) Anticooperative binding of certain substrates has been observed for aminoacyl-tRNA synthetases (Dessen et al., 1978, and references contained therein) and for the Phe-specific enzyme of *E. coli*, L-phenylalanine, and $tRNA^{Phe}$ (Bartmann et al., 1975a). It is of interest to know if the structural changes in the enzyme accompanying the binding of phenylalanine and $tRNA^{Phe}$ are similar. (4) It has been suggested that conformation changes provide a mechanism for the differentiation between cognate and noncognate tRNAs which also bind to the synthetase (Krauss et al., 1976; Rigler et al., 1976). Can the proposed changes be detected by their effect on the hydrodynamic properties of the enzyme as measured by the light-scattering technique?

In the present studies we have employed the laser light-scattering technique to study the diffusion constant of the enzyme *E. coli* phenylalanyl-tRNA synthetase and its complexes with Mg^{2+}, cognate, modified cognate, and noncognate tRNAs and with small substrates L-phenylalanine and MgATP. It provides an ideal tool for studying the questions outlined above. Several factors contribute to this choice of technique in contrast to techniques employed earlier. For example, in

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0006-2960/81/0420-0861$01.00/0 © 1981 American Chemical Society