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Simian Liver Alcohol Dehydrogenase: Isolation and Characterization of Isoenzymes from *Macaca mulatta*†

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ABSTRACT: Like human liver alcohol dehydrogenase, that of *Macaca mulatta* can be purified and separated into anodic and cathodic pyrazole-insensitive and cathodic pyrazole-sensitive enzyme forms. Their inhibition by 4-methylpyrazole and their substrate specificities are analogous to those observed for the corresponding isoenzymes of human liver. However, on the basis of data available so far, the physicochemical and compositional characteristics, i.e., molecular weight, zinc content, and dimeric structure, of all simian alcohol dehydrogenase forms are virtually identical with those of other mammalian alcohol dehydrogenases studied up to now. Zinc is essential for their enzymatic function, as demonstrated by inhibition with chelating agents.

Knowledge about the biochemical features of alcohol dehydrogenase (ADH),[1] the principle enzyme catalyzing the oxidation of ethanol, bears importantly on an understanding of alcohol metabolism and alcohol-related pathology. The presence of multiple molecular forms of the enzyme in human liver was recognized early (Blair & Vallee, 1966), and their identities have been the subject of intensive studies which have now identified isoenzymes π-ADH (Bosron et al., 1979), χ-ADH (Parés & Vallee, 1981), and $ADH_{Indianapolis}$ (Bosron et al., 1980), whose electrophoretic and kinetic properties differ strikingly from those of the other isoenzymes. 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. In contrast, χ-ADH, a temporary designation of the new forms of human ADH, migrates toward the anode between pH 7.7 and 8.6. These isoenzymes are not inhibited by 4-methylpyrazole and stain only with long-chain monohydric alcohols, i.e., 1-pentanol or 1-octanol, as substrates. Until now, π-ADH and χ-ADH forms have been detected only in human livers, and they have been postulated to have an important role in ethanol metabolism (Li et al., 1977; Parés & Vallee, 1981). The recent isolation and characterization of pyrazole-sensitive and pyrazole-insensitive[2] ADH isoenzymes from squirrel monkey liver provided the first evidence of the existence of similar molecular forms in another species (Dafeldecker et al., 1981).

Excessive alcohol ingestion is a problem peculiar to the human, and studies of its pertinent biochemical consequences would ideally be performed in that species, though this is clearly not feasible. Hence, it would seem prudent to identify another species whose cellular biochemistry resembles that of the human as closely as possible. Toward this end, we are examining a number of primates by using the hepatic distribution of ADH isoenzymes as the criterion of selection. Among the species examined by starch gel electrophoresis displaying isoenzyme patterns similar to that of the human, the rhesus monkey was chosen for further investigation. The liver of *Macaca mulatta* contains the same three classes of ADH isoenzymes, i.e., anodic and cathodic pyrazole-insensitive and cathodic pyrazole-sensitive forms, as those found in human liver. The kinetic and physical properties of these three simian isoenzyme fractions are similar to the corresponding human liver ADH variants, suggesting that studies of the liver ADH of this species may serve to advance understanding of human alcohol metabolism and its pathological consequences.

Materials and Methods

NAD^+ (grade III), NADH (grade III), 12-hydroxydodecanoic acid, and 16-hydroxyhexadecanoic acid were obtained from Sigma Chemical Co., St. Louis, MO; alcohols, aldehydes, 4-methylpyrazole, EDTA, and 1,10-phenanthroline were from Aldrich Chemical Co., Milwaukee, WI; 4-bromopyrazole was from Research Plus Laboratories, Denville, NJ; 2,2′-bipyridine was from G. Frederic Smith Chemical Co., Columbus, OH; DEAE-cellulose (DE-52) was from Whatman Inc., Clifton, NJ; and agarose–hexane–AMP, type 2, was from P-L Biochemicals, Inc., Milwaukee, WI.

Enzymatic Assay. Alcohol dehydrogenase activity was determined in 0.1 M glycine, pH 10.0, at 25 °C by measuring the production of NADH in the presence of alcohol. The initial velocity at pH 10 in the presence of 2.4 mM NAD^+ and 33 mM ethanol for the pyrazole-sensitive fraction or 0.5 M ethanol for both pyrazole-insensitive isoenzyme fractions was

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[1] Abbreviations used: ADH, alcohol:NAD^+ oxidoreductase (EC 1.1.1.1); CapGapp, 4-[3-[(6-aminocaproyl)amino]propyl]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; CD, circular dichroism.

[2] 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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considered as the standard activity through all the purification steps. The reverse reaction, the utilization of NADH, was carried out in 0.1 M NaP_i (pH 7.5). The spectrophotometric assay was performed by use of a Cary 219 or Gilford 240 spectrophotometer. Activities are expressed as micromoles of NADH utilized or produced per minute based on an ϵ_{340} of 6.22 mM^{-1} cm^{-1}.

Purification of Rhesus Monkey Liver Alcohol Dehydrogenase. All rhesus monkey livers were commercially obtained from the Primate Imports Corp., Port Washington, NY. Up to the time of processing, the animals were fully fed, and the livers were removed immediately after sacrifice and packed in dry ice. All liver homogenates displayed the same isoenzyme pattern, as visualized by starch gel electrophoresis. In a typical preparation, 75 g of liver, cleaned of any nonhepatic tissue, was minced and homogenized in 150 mL of water. The crude extract was centrifuged and the supernatant was dialyzed against 10 mM Tris-HCl, pH 7.9 (3 × 1 L), for 6 h. The cathodic pyrazole-sensitive and pyrazole-insensitive ADH fractions were separated from the anodic pyrazole-insensitive isoenzymes by DEAE-cellulose chromatography (Parês & Vallee, 1981).

CapGapp–Sepharose Chromatography. The fractions containing the cathodic ADH variants were combined, concentrated by ultrafiltration (Diaflo PM-10 membrane, Amicon Corp.), adjusted to 50 mM NaP_i and 1.2 mM NAD^+, pH 7.5, and applied to a CapGapp–Sepharose column (2.6 × 23 cm), equilibrated with the same NAD^+ buffer. The pyrazole-insensitive isoenzyme was separated from the pyrazole-sensitive forms by following the conditions of Dafeldecker et al. (1981).

Agarose–Hexane–AMP Chromatography. The fractions containing the anodic pyrazole-insensitive isoenzymes were combined, concentrated, and dialyzed against 10 mM NaP_i, pH 7.3. The sample was then applied to an agarose–hexane–AMP column (1.5 × 25 cm), equilibrated with the same buffer, and the enzyme was eluted as previously described (Parês & Vallee, 1981).

Purification of the cathodic pyrazole-insensitive and pyrazole-sensitive forms was achieved as described (Dafeldecker et al., 1981).

DEAE-cellulose Chromatography. The AMP column fractions, containing anodic pyrazole-insensitive ADH, were combined, concentrated, and extensively dialyzed against 10 mM Tris-HCl, pH 7.9. The enzyme was then applied to a DEAE-cellulose column (1.5 × 30 cm), equilibrated with the dialysis buffer. Elution and separation of the two anodic ADH isoenzymes were achieved with 400 mL of a gradient of 0–0.1 M NaCl.

Starch Gel Electrophoresis. Liver homogenates and purified ADH fractions were subjected to vertical starch gel electrophoresis at pH 7.7 in the presence of NAD^+ (Figure 1) as described by Li & Magnes (1975). For detection of ADH isoenzymes that migrate in either direction, the sample application slit of the electrophoresis apparatus (Buchler Instruments, Fort Lee, NJ) was placed in the center of the Lucite mold. ADH activity in the gel was detected with ethanol or 1-pentanol as substrate (Smith et al., 1972).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Analytical slab gel electrophoresis was performed with a Bio-Rad Model 220 instrument. The samples were run at 35 mA in a 10% acrylamide gel, using the system of Laemmli (1970). Coomassie blue was employed to stain the gels.

Ultracentrifugation. All centrifugal studies were performed in a Spinco Model E analytical ultracentrifuge (Beckman Instrument Co.). The molecular weights of the pyrazole-sensitive isoenzymes, and one of the anodic pyrazole-insensitive forms (A_1), were determined at concentrations of 0.28 and 0.32 mg/mL, respectively, by the meniscus depletion sedimentation equilibrium method (Yphantis, 1964). The enzymes were dialyzed against 10 mM Tris-HCl and 0.1 M KCl, pH 7.5, and operating speeds were 20 410 and 24 630 rpm at 22 °C for the pyrazole-sensitive and 21 740 rpm at 26 °C for the pyrazole-insensitive isoenzymes. The molecular weight was calculated from the slope of log $(Y_r - Y_0)$ vs. r^2, assuming a partial specific volume of 0.743 mL/g (Lange et al., 1976).

Sedimentation velocity experiments were carried out with the pyrazole-sensitive forms, 7.6 mg/mL, in the same buffer at 56 100 rpm and 21 °C.

Protein Concentration. Enzyme solutions containing pyrazole-sensitive rhesus monkey liver ADH 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).

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).

Metal Analysis. The Zn content of the pyrazole-sensitive and anodic pyrazole-insensitive ADH fractions 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, and the analyses were carried out as described (Dafeldecker et al., 1981).

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 100 °C. Each analysis of pyrazole-sensitive rhesus monkey liver ADH was performed at least in triplicate, using 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

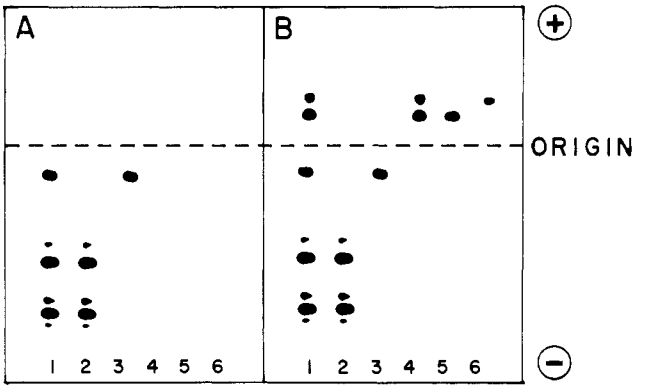

FIGURE 1: Starch gel electrophoresis of rhesus monkey liver alcohol dehydrogenase. Conditions: pH 7.7, 220 V for 18 h at 4 °C. Sample 1, whole liver homogenate; sample 2, cathodic pyrazole-sensitive fraction; sample 3, cathodic pyrazole-insensitive form; sample 4, mixture of anodic pyrazole-insensitive isoenzymes; sample 5, anodic pyrazole-insensitive isoenzyme A_1; sample 6, anodic pyrazole-insensitive isoenzyme A_2. The gels were stained for enzymatic activity by using ethanol (A) or 1-pentanol (B) as substrate. Samples 1–3 stain with both ethanol and 1-pentanol in the cathodic region of the gel, while samples 4–6 stain only in the presence of 1-pentanol.

Table I: Amino Acid Composition of Cathodic Pyrazole-Sensitive Rhesus Liver Alcohol Dehydrogenase[a]

residue	residues/subunit	
	rhesus liver ADH	horse liver ADH[f]
Asp	28.9 ± 0.4	25
Thr[b]	22.8 ± 0.4	24
Ser[b]	23.8 ± 0.3	26
Glu	27.2 ± 0.6	29
Pro	19.9 ± 0.2	20
Gly	38.3 ± 0.6	38
Ala	30.9 ± 0.4	28
Val[c]	36.2 ± 0.4	29
Met	6.3 ± 0.2	9
Ile[c]	21.8 ± 0.3	24
Leu	27.6 ± 0.3	25
Tyr	6.4 ± 0.2	4
Phe	15.8 ± 0.5	18
His	5.6 ± 0.2	7
Lys	31.3 ± 0.3	30
Arg	11.7 ± 0.4	12
Trp[d]	3.2	2
Cys[e]	14.0 ± 0.4	14

[a] Values shown represent the mean ± the standard deviation of duplicate analyses at 24-, 48-, and 72-h hydrolyses, except where otherwise noted. [b] Extrapolated to zero time; average of duplicate determinations. [c] Value after 72-h hydrolysis; average of duplicate determinations. [d] Determined by MCD. [e] Determined as cysteic acid after performic acid oxidation; average of duplicate analyses. [f] Calculated from the primary sequence (Jörnvall, 1970). See text for other conditions.

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 (Jörnvall, 1970), per 39 000 subunit molecular weight (Table I).

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 or passage over Chelex-100.

Circular Dichroic Spectra. Circular dichroism (CD) of pyrazole-sensitive rhesus monkey liver ADH was determined with a Cary 61 spectropolarimeter using a quartz sample cell of 2-mm path length. Units of molar ellipticity, $[\theta]$, are degrees per squared centimeter per decimole.

Results

Extracts of livers of *M. mulatta* which were devoid of pathology exhibit two distinct activity bands which migrate toward the anode and only stain when a long-chain primary alcohol is the substrate. No activity is apparent in the presence of ethanol.

Molecular Weight and Sedimentation Coefficient. Sedimentation velocity experiments of pyrazole-sensitive rhesus liver alcohol dehydrogenase reveal one symmetrical boundary, suggesting molecules of but one size, with s_{obsd} = 4.85 at 21 °C in 0.01 M Tris–0.1 M KCl, pH 7.5. Sedimentation equilibrium experiments performed by the Yphantis method (1964) at two different speeds, 20 410 and 24 630 rpm, yield linear plots of log $(Y_r - Y_0)$ vs. r^2, with slopes of 0.813 and 1.208, respectively, equivalent to molecular weights of 76 000 and 77 000.

A linear slope of 0.950, corresponding to a molecular weight of 82 800, was obtained for one of the anodic pyrazole-insensitive isoenzymes (A_1) by employing the same method at 21 740 rpm.

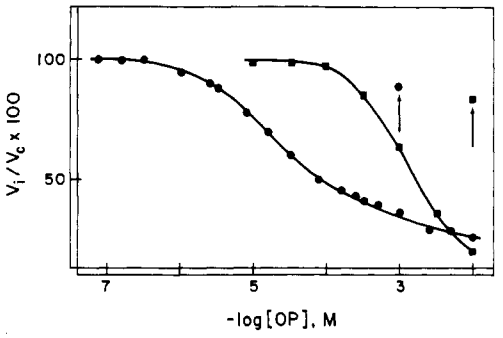

FIGURE 2: Instantaneous inhibition of rhesus monkey liver alcohol dehydrogenase by 1,10-phenanthroline at 25 °C, pH 10. The assay mixture of the pyrazole-sensitive isoenzymes (●) contained 100 μmol of ethanol, 7.6 μmol of NAD^+, and 283 μmol of glycine. The activity of the anodic pyrazole-insensitive form (■) was determined in the presence of 0.4 mM 1-octanol as substrate. Reversal of inhibition, the arrows, was obtained by addition of zinc, 6.6×10^{-2} M.

Metal Analysis. The Zn content of the pyrazole-sensitive ADH form was measured on two different preparations of homogeneous enzyme with comparable specific activities. Atomic absorption spectrometry detects 2560–2960 μg of zinc/g of protein, corresponding to a molar ratio of metal to protein varying from 3.7 to 4.2 mol/mol, based on a molecular weight of 77 000.

The analysis of the purified anodic pyrazole-insensitive ADH form (A_1) revealed 3.6 ± 0.1 mol of zinc per mol of enzyme, based on a molecular weight of 82 800. This isoenzyme was also analyzed for the presence of Cu, Fe, Mn, and Mo. Only detectable amounts of Cu (0.5 mol/mol) were found.

Inhibition by Metal-Binding Agents. Activity measurements in the presence of suitable metal-binding agents result in inhibition and demonstrate the importance of zinc to the enzymatic activity of all three classes of rhesus monkey liver ADH. The chelators, 1,10-phenanthroline, 2,2′-bipyridine, and EDTA, reduce the activity of the pyrazole-sensitive ADH fraction. The concentration-dependent inhibition with 1,10-phenanthroline in the presence of 33 mM ethanol and 2.4 mM NAD^+ in 0.1 M glycine, pH 10.0, is instantaneous with a pK_I of 4.1 (Figure 2). Dilution restores activity to the level expected for the 1,10-phenanthroline concentration in the final assay mixture. Moreover, addition of zinc restores activity from 25% to 92% of native activity. The number of moles of inhibitor bound per active site, n, is 1.00 as calculated by the method of Kistiakowsky & Shaw (1953). These results demonstrate that the enzyme and 1,10-phenanthroline form a dissociable mixed complex, accounting for the reversibility of the inhibition (Drum & Vallee, 1970).

Inhibition studies were performed with the pyrazole-insensitive ADH fractions. The standard activity of the cathodic isoenzyme, determined with 0.5 M ethanol in 0.1 M glycine, pH 10.0, is reduced instantaneously by 72% on addition of 10 mM 1,10-phenanthroline. Moreover, the concentration-dependent inhibition of the anodic forms by the same chelator in the presence of 0.4 mM 1-octanol and 2.4 mM NAD^+ in 0.1 M glycine, pH 10.0, is similar, and a pK_I of 2.75 was obtained (Figure 2).

pH Optima of Catalysis. The rhesus liver ADH catalyzed rate of alcohol oxidation was studied as a function of the pH of the assay. The oxidation of ethanol by the pyrazole-sensitive form (Figure 3A) has a pH optimum at about 11.2, exhibits 15% of this rate at pH 7, and demonstrates little or no activity toward ethanol below pH 6, although the enzyme is still physically stable at pH 5.5. In contrast, the decrease of activity above pH 11.2 reflects rapid, irreversible inactivation of the enzyme. The pH optimum of catalysis for the anodic forms

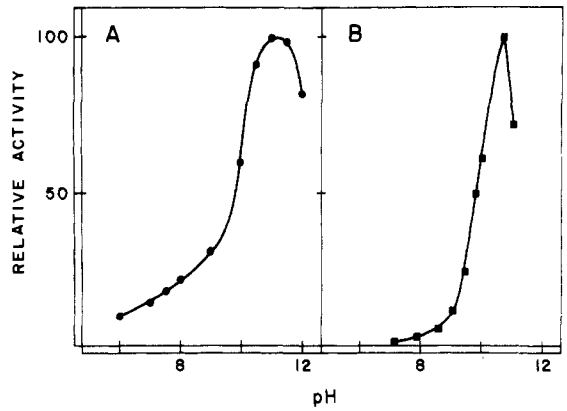

FIGURE 3: pH dependence of alcohol oxidation by rhesus liver alcohol dehydrogenase. Enzymatic activity of the pyrazole-sensitive isoenzymes (A) was assayed with 33 mM ethanol and 2.4 mM NAD^+ in 33 mM NaP_i from pH 4.5 to 7.5, in 33 mM $NaPP_i$ from pH 7.5 to 9.5, and in 0.1 M glycine–NaOH from pH 9.0 to 11.5 at 25 °C. Enzymatic activity of the cathodic pyrazole-insensitive ADH (B) was assayed with 0.2 mM 1-octanol and 2.4 mM NAD^+ in the same buffers.

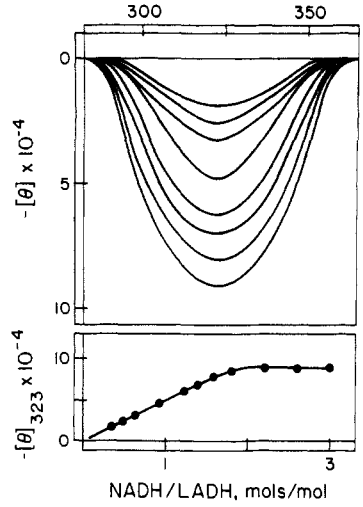

FIGURE 4: Circular dichroism titration of pyrazole-sensitive rhesus liver alcohol dehydrogenase with NADH. Upper panel: the magnitude of the negative circular dichroic extremum at 323 nm increased as NADH, 5–50 μL, was added sequentially. Lower panel: extrapolation of titration data indicates binding stoichiometry of 1.8 mol of NADH/mol of rhesus liver ADH; 7.7 μM enzyme and 2.8 mM NADH in 0.05 M NaP_i, pH 7.5. Units of molar ellipticity, $[\theta]$, are degrees per squared centimeter per decimole.

(Figure 3B) was determined with 1-octanol as the substrate. The enzyme exhibits virtually the same profile as that of the pyrazole-sensitive form with an optimum at about pH 10.6.

Stoichiometry of Coenzyme Binding. Binding of NADH to rhesus liver alcohol dehydrogenase was examined by absorption and circular dichroic spectral titrations. Incremental amounts, 5–50 μL, of 2.8 mM NADH added to 7.7 μM of the pyrazole-sensitive form in 50 mM NaP_i, pH 7.5, reveal a negative extrinsic Cotton effect centered at 323 nm, with a molar ellipticity of $[\theta]_{323} = -9.10 \times 10^4$ deg cm^2/dmol, identifying the formation of an enzyme–NADH complex (Brändén et al., 1975). A plot of the change of ellipticity at 323 nm vs. moles of NADH added indicates a stoichiometry of 1.8 mol of NADH/mol of enzyme (Figure 4).

Steady-State Kinetic Parameters. The Michaelis and catalytic constants for the rhesus liver ADH catalyzed oxidation of alcohols, examined at pH 10.0, and reduction of acetaldehyde, studied at pH 7.0, were determined by the method of Lineweaver and Burk.

The K_m value of the pyrazole-sensitive ADH fraction for NAD^+ is 52 μM, when assayed with ethanol at 10 times its Michaelis constant. The kinetic parameters for a homologous series of aliphatic primary alcohols were determined by employing saturating coenzyme concentrations based on this constant (Table II). While methanol binds weakest and has the lowest value of k_{cat}, those of K_m become progressively lower with increasing chain length of the substrate to reach 0.006 mM for 1-octanol. In contrast to both pyrazole-insensitive variants, this ADH form utilizes cyclohexanol as a substrate with a K_m value of 0.02 mM. In the reverse reaction, the K_m for NADH was assumed to be 18 μM (Lange et al., 1976), and a K_m of 0.91 mM for acetaldehyde was obtained, when assayed at 10 times the K_m of the cofactor.

Table II: Steady-State Kinetic Parameters for Rhesus Monkey Liver Alcohol Dehydrogenase[a]

	cathodic pyrazole-sensitive ADH		cathodic pyrazole-insensitive ADH K_m (mM)	anodic pyrazole-insensitive ADH	
substrate	K_m (mM)	k_{cat} (min^{-1})		K_m (mM)	k_{cat} (min^{-1})
methanol	12	40	no activity	no activity	
ethanol	2.2	177	400	no saturation[b]	
pentanol	0.012	200	1.5	19	240
octanol	0.006	225	0.087	0.5	340
cyclohexanol	0.020	155	no activity	no activity	
NAD^+	0.052	177		0.059[c]	340[c]
acetaldehyde	0.91	13700		no saturation	

[a] Alcohol oxidations were carried out in 0.1 M glycine, pH 10.0. Aldehyde reductions were determined in 0.1 M NaP_i, pH 7.0. [b] The activity is directly proportional to the ethanol concentration up to 2 M as observed for X-ADH (Parés & Vallee, 1981). [c] Determined with 0.4 mM 1-octanol. Values for K_m and k_{cat} are the averages for three preparations. The estimated standard deviations for the kinetic parameters are consistent with a significant kinetic difference between the three isoenzyme fractions. Other conditions are described under Materials and Methods.

Methanol and cyclohexanol do not serve as substrates for the cathodic pyrazole-insensitive isoenzyme, and the K_m for ethanol, 400 mM, is relatively high. However, increasing the chain length of the primary alcohols sharply decreases the K_m to 1.5 mM for 1-pentanol and 0.09 mM for 1-octanol, respectively, while V_{max} remains constant at 0.16 unit/mL (Table II).

The Michaelis constant of the anodic pyrazole-insensitive ADH fraction for NAD^+, 0.06 mM, was determined with 1-octanol as the substrate, since the oxidation of ethanol and the reduction of acetaldehyde showed nonsaturating kinetics; i.e., a straight line plot of V vs. s was obtained. Also, the reciprocal plot of $1/V$ vs. $1/s$ intersects at the origin, and therefore, K_m and k_{cat} values cannot be calculated. At saturating coenzyme concentrations and up to 2 M methanol or 0.3 M cyclohexanol, there is no activity. However, the enzyme shows Michaelis–Menten kinetics with a series of aliphatic primary alcohols ranging from 1-pentanol to 1-octanol. Again, an increase in the chain length of these substrates results in a sharp decrease in K_m values from 19 mM for 1-pentanol to 0.5 mM for 1-octanol, while k_{cat} remains relatively constant. Moreover, long-chain ω-hydroxy fatty acids are also good substrates for this enzyme, and K_m values of 0.24 and 0.06 mM were obtained for 12-hydroxydodecanoic and 16-hydroxyhexadecanoic acid, respectively.

Discussion

The recent isolation of a physicochemically homogeneous form of human liver alcohol dehydrogenase by double ternary complex affinity chromatography (Lange et al., 1976) has made the major ethanol-oxidizing enzyme available for detailed

investigations. Moreover, the application of this technique led to the isolation of π-ADH (Bosron et al., 1979) and χ-ADH (Parês & Vallee, 1981), hitherto unidentified isoenzymes of human liver ADH with unique electrophoretic and kinetic properties. The recent characterization of an ADH form from squirrel monkey liver (Dafeldecker et al., 1981), whose properties are similar to those of human π-ADH, generated the present survey of simian liver alcohol dehydrogenases.

Liver homogenates of *Macaca mulatta* contain two isoenzymes which migrate toward the anode of the gel and stain only with long-chain primary alcohols, i.e., 1-pentanol or 1-octanol, consistent with the characteristics of human liver χ-ADH (Figure 1). These variants are apparently also similar to isoenzymes present in cultured human skin fibroblasts, detected by isoelectrofocusing (Petersen et al., 1979). Moreover, no enzymatic activity in the anodic region of the gel was observed with ethanol as the substrate.

Identification of human liver ADH isoenzymes in the cathodic region of the starch gel has proven to be a useful and sensitive tool in probing the heterogeneity of alcohol dehydrogenase. On the basis of the electrophoretic mobility of ADH variants, a genetic model was proposed (Smith et al., 1971) that satisfactorily accounted for the molecular forms observed up to the time it was suggested. It obviously could encompass neither π-ADH nor χ-ADH, which were discovered subsequently. The present data indicate that human π-ADH as well as the simian pyrazole-insensitive isoenzymes of *Saimiri sciureus* (Dafeldecker et al., 1981) and those of *M. mulatta* share similar electrophoretic and kinetic properties. They all exhibit the least electrophoretic mobility, are inactive toward methanol, display a higher K_m for ethanol, and are less susceptible to inhibition by 4-methylpyrazole than all other cathodic forms.

The purified pyrazole-sensitive and pyrazole-insensitive forms of alcohol dehydrogenase isolated from livers of reportedly healthy *Macaca mulatta* share many physicochemical properties with human (Lange et al., 1976), horse (Drum et al., 1969), and squirrel monkey liver alcohol dehydrogenase (Dafeldecker et al., 1981). Ultracentrifugation reveals molecular weights of 77 000 and 83 000 for the pyrazole-sensitive and anodic pyrazole-insensitive forms, respectively, while $NaDodSO_4$ gel electrophoresis indicates the existence of subunits with identical molecular weights of approximately 40 000. Hence, all three classes of rhesus monkey liver ADH are composed of two subunits of equal or nearly equal molecular weights. Atomic absorption spectrometry, utilizing graphite furnace atomization, shows that the pyrazole-sensitive and the anodic pyrazole-insensitive forms contain 4 mol of zinc per mol of protein and no significant concentration of other metals.

Consistent with the characteristics of all yeast and mammalian NAD(H)-dependent alcohol dehydrogenases examined thus far, rhesus liver ADH requires zinc for enzymatic activity. All three enzyme forms are inhibited by 1,10-phenanthroline, a known chelator of this metal ion. The inhibtion is instantaneous and reversible on dilution or addition of excess metal ions (Figure 2). Thus, the results demonstrate the formation of a mixed enzyme–zinc chelator complex (Drum & Vallee, 1970) and confirm the critical role of the metal in the catalytic action of these alcohol dehydrogenases.

A total of 2 mol of NADH bind to 1 mol of pyrazole-sensitive rhesus alcohol dehydrogenase, identical with the stoichiometry reported for the horse (Sytkowski & Vallee, 1978) and human (Lange et al., 1976) enzymes. The ADH–NADH complex is optically active, displaying a negative circular dichroic extremum at 323 nm and a maximum molar ellipticity, $[\theta]_{323}$, of -9.1×10^4 deg cm^2/dmol (Figure 4). These values are in close agreement with previously reported data of $[\theta]_{330} = -9.42 \times 10^4$ deg cm^2/dmol for the horse and human enzymes, respectively, suggesting similar modes of coenzyme binding in these alcohol dehydrogenases.

Kinetic characteristics and inhibition by 4-methylpyrazole serve to differentiate the three classes of rhesus liver alcohol dehydrogenase and reveal striking similarities to the corresponding classes of human liver ADH. Methanol serves as a substrate for the pyrazole-sensitive variants of human and rhesus liver ADH, and both enzymes show a low K_m for other primary alcohols and cyclohexanol and are markedly inhibited by 4-methylpyrazole. Analogous to human π-ADH, the cathodic pyrazole-insensitive form does not oxidize methanol and has a high K_m for ethanol and other primary alcohols and a high K_I for 4-methylpyrazole. The anodic pyrazole-insensitive enzyme presents features virtually identical with those of human χ-ADH (Parês & Vallee, 1981). It is not active toward methanol and cyclohexanol, short-chain primary alcohols and aldehydes do not saturate the enzyme, and inhibition with 4-methylpyrazole occurs only at high concentrations, possibly in a nonspecific manner. Accordingly, this isoenzyme form does not bind to the pyrazole affinity resin and is visualized on starch gel electrophoresis only by activity staining with long-chain primary alcohols, i.e., 1-pentanol or 1-octanol. The isolation and characterization of anodic pyrazole-insensitive ADH from rhesus monkey liver may indicate that this class of alcohol dehydrogenases is widely distributed in this order of mammals.

The catalytic specificity of human and simian ADH isoenzymes toward long-chain aliphatic alcohols and ω-hydroxy fatty acids is particularly intriguing. Previous studies on the substrate specificity of alcohol dehydrogenase have been limited largely to a small number of specific alcohols and sterols or their analogues, and a number of these compounds have been proposed to serve as physiological substrates. Arguments for activities toward metabolically important substrates have been based generally on the fact that the K_m values for many of these alcohols are lower than those for ethanol. The behavior of straight chain, primary alcohols, substrates for the human and simian isoenzymes, illustrates this point: binding increases with increasing chain length for all isoenzymes studied thus far. Overall, the very number of potentially important substrates for ADH suggests that the enzyme's metabolic role is not limited to ethanol, and it may perform a more general function in metabolism and detoxification, acting on a variety of alcohols and aldehydes. Further studies of ADH isoenzyme composition in nonhuman primates, either inherited or induced, may reveal hitherto unknown physiological functions of the enzyme.

Previous efforts to discern the role of ADH in regulating alcohol metabolism and alcoholism were based extensively on studies with experimental animals, particularly the rat (Li, 1977; Hawkins & Kalant, 1972). Although these studies have yielded fundamental information, it has become increasingly apparent that degrees of complexity and genetic heterogeneity exist in the human that have no counterpart in lower animal species. Since the limitations inherent in direct human experimentation preclude the formulation of critical questions to be answered by decisive experiments in man, the present studies on nonhuman, but genetically closely related, primates give the first indication that a suitable simian model might be at hand. Extension of the present studies promises to open the way to novel approaches designed to solve the urgent

problem of ethanol abuse and its pathologic consequences.

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Resonance Raman Spectra for Flavin Derivatives Modified in the 8 Position†

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ABSTRACT: Spontaneous resonance Raman or resonance-enhanced AC-coupled inverse Raman spectra were obtained for 8-mercaptoriboflavin, 8-(dimethylamino)riboflavin, 8-hydroxyriboflavin, and 8-aminoriboflavin all free in solution. These Raman spectra were all similar to one another and markedly different from that of riboflavin. In addition, the Raman spectra of 8-mercaptoflavin bound to the apoproteins of old yellow enzyme, glucose oxidase, and L-lactate oxidase were determined. The Raman spectra of these protein-bound flavins were distinctly different from both those of the above 8-substituted riboflavins and that of riboflavin. An argument is presented in favor of assigning a quinonoid electronic structure to these protein-bound flavins.

On the basis of Raman spectroscopic studies, several investigators have recently proposed that the π-electronic structures of 8-methoxyriboflavin (Nishina et al., 1980), 8-aminoriboflavin (Nishina et al., 1980), 8-hydroxyriboflavin (Dutta et al., 1980), and 8-(methylamino)riboflavin (Dutta et al., 1980) all contain substantial contributions from a quinonoid form (see Figure 1). This conclusion was based primarily on the observation that the bands which occur at 1582 and 1547 cm^{-1} for riboflavin (bands II and III, see Table I) were markedly shifted in the Raman spectra of these modified riboflavins (Nishina et al., 1980; Dutta et al., 1980). These bands were previously assigned to carbon–nitrogen double-bond stretching modes in ring II, primarily based on isotopic substitution studies (Kitagawa et al., 1979) and normal mode analysis (Bowman & Spiro, 1981). Thus, a shift from a benzenoid to a quinonoid structure was expected to perturb these bands. Further support for this position comes from earlier work on 8-hydroxyflavins, which indicated that at neutral pH 8-hydroxyriboflavin was predominantly in the quinonoid form (Ghisla & Mayhew, 1976).

Recent studies on flavoproteins have employed modified flavins as probes of both structure and mechanism [see Massey & Hemmerich (1980) for a review]. A significant feature of this work has been the observation that the flavoprotein oxidases (as well as some other enzymes) stabilize the quinonoid form of the flavin (Massey et al., 1979). These findings provide important support for the thesis that the native flavin in flavoprotein oxidases stabilizes a negative charge in the N(1)–C(2α) locus during the course of normal reduction (Massey & Hemmerich, 1980; Massey et al., 1979; Ghisla & Massey, 1980).

The enzymological studies involving modified flavins were performed in large part with 8-mercaptoflavins. Therefore, we have sought to extend the Raman studies to include this

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