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Simian Liver Alcohol Dehydrogenase: Isolation and Characterization of Isozymes from *Macaca nemestrina*†

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Received May 8, 1985

ABSTRACT: Three classes of hepatic alcohol dehydrogenase (ADH), analogous to those of human liver, are present in *Macaca nemestrina*. Their functional, compositional, and structural features have been established with isozymes purified to homogeneity by affinity and conventional ion-exchange chromatography. One unusual molecular form of *M. nemestrina* ADH is electrophoretically indistinguishable as it comigrates with one of the cathodic class I isozymes on starch gel electrophoresis. While its substrate and inhibitor specificity, a high K_m value for ethanol (50 mM at pH 10), and lack of binding to the pyrazole affinity resin are consistent with the kinetics of class II ADH, the physicochemical and compositional properties are virtually identical with all other known mammalian alcohol dehydrogenases. The unexpected presence of this previously unknown ADH variant in livers of *M. nemestrina* demonstrates the need for prudence in assignment of ADH isozymes. Classification based solely on electrophoretic position in starch gels and enzymatic properties of human ADH but without isolation and characterization of individual isozymes may prove insufficient and inadequate. The genetic or phenotypic nature of this isozyme remains to be demonstrated.

The NAD(H)-dependent mammalian alcohol dehydrogenases (ADH)[1] catalyze the interconversion of ethanol and other primary alcohols to their corresponding aldehydes; certain secondary alcohols and steroids can also serve as substrates (Li, 1977). The numerous molecular forms of human liver ADH exhibit a wide range of cathodic and anodic mobilities on starch gel electrophoresis (Smith et al., 1973; Bosron et al., 1977; Parés & Vallee, 1981), and three classes are recognized on the basis of kinetic, physical, chemical, and immunological criteria (Strydom & Vallee, 1982; Vallee & Bazzone, 1983), although they share the same physicochemical and compositional characteristics. Their molecular weight, zinc content, and dimeric structure are virtually identical with those of other known mammalian alcohol dehydrogenases.

Dynamic studies of the structural changes of liver enzymes and their functional consequences cannot be carried out in man. Yet, such information is needed as a basis for and understanding of alcohol-related human enzymological pathology. Primates would seem to offer potential opportunities for the identification of livers with isozymic patterns analogous to those of the human.[2] The hepatic distribution of ADH isozymes of *Macaca nemestrina* was examined for this purpose.

The ADH molecular forms were isolated and purified, and the physicochemical characteristics and kinetics of some of the ADH variants were studied. The liver of this species

† This work was supported by a grant from the Samuel Bronfman Foundation, Inc., with funds provided by Joseph E. Seagram and Sons, Inc.
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[1] Abbreviations: ADH, alcohol:NAD^+ oxidoreductase (EC 1.1.1.1); NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; CapGapp, 4-[3-[(6-aminocaproyl)amino]propyl]pyrazole; DEAE, diethylaminoethyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; DTT, dithiothreitol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; 12-HDDA, 12-hydroxydodecanoic acid; 16-HHDA, 16-hydroxyhexadecanoic acid.

[2] In addition to detailed studies on squirrel monkey, *Saimiri sciureus* (Dafeldecker et al., 1981a), and rhesus monkey, *Macaca mulatta* (Dafeldecker et al., 1981b), multiple isozyme forms have been detected on starch gel in all of the following primate species: tree shrew (*Tupaia glis*), potto (*Peridicticus potto*), owl monkey (*Aotus trivirgatus*), crab-eating macaque (*Macaca fascicularis*), Formosan rock monkey (*Macaca cyclopis*), brown-headed marmoset (*Saguinas fuscicolis*), cotton-top marmoset (*Saguinas oedipus*), African green (*Circopithecus aethiops*), baboon (*Papio papio*), bonnet monkey (*Macaca radiata*), patas (*Erythrocebus patas*), brown capuchin (*Cebus apella*), orangutan (*Pongo pygmaeus*), gorilla (*Gorilla gorilla*), and chimpanzee (*Pan troglodytes*).

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contains the three established classes of ADH and in addition an isozyme of yet unknown identity. This ADH variant was named M for its occurrence so far in *M. nemestrina* only. Electrophoretically it comigrates with one of the class I molecular forms, but its kinetic properties are intermediate between those of class I and those of class II ADH.

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; 4-methylpyrazole and 1,10-phenanthroline were from Aldrich Chemical Co., Milwaukee, WI; DEAE-cellulose (DE-52) and carboxymethylcellulose (CM-52) were from Whatman Inc., Clifton, NJ; agarose–hexane–adenosine 5′-phosphate (AGAMP), type 2, was from P-L Biochemicals, Inc., Milwaukee, WI. Ethanol (100%) was obtained from U.S. Industrial Chemicals Co., New York, NY, other alcohols were from Fisher Scientific, Medford, MA, and all were used without further purification. Deionized, glass-distilled water was used throughout.

Enzymatic Assay. Alcohol dehydrogenase activity was measured in 0.1 M glycine, pH 10.0, at 25 °C by monitoring the production of NADH at 340 nm in the presence of 2.4 mM NAD^+. Through all purification steps activity was determined by means of initial velocities by using 33 mM and 0.5 M ethanol as substrate. Assays were performed on a Cary 219 or Gilford 240 spectrophotometer. Activities (IU) are expressed as micromoles of NADH produced per minute on the basis of an ϵ_{340} of 6.22 mM^{-1} cm^{-1}.

Purification of M. nemestrina Liver Alcohol Dehydrogenase. Livers were obtained from the Primate Research Center, University of Washington, Seattle, WA, and information on age, sex, and principal cause of death was obtained from autopsy records. The isozymes isolated from *M. nemestrina* liver were classified according to the criteria of Vallee & Bazzone (1983). Their electrophoretic mobilities on starch gels are shown in Figure 2.

After sacrifice of the animals, livers were removed immediately and stored at −70 °C. In a typical preparation, two livers (about 40 g), both displaying the starch gel electrophoresis pattern A (Figure 1), were minced and homogenized in 60 mL of water with a Polytron homogenizer (Brinkmann 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 (4.5 × 4.5 cm) prepared on a sintered glass funnel and equilibrated with 10 mM Tris-HCl, pH 8.3. The enzymatically active effluent was concentrated to 67 mL on an Amicon ultrafiltration membrane (PM-10) and dialyzed against 3 × 600 mL of 50 mM NaP_i and 0.1 mM DTT, pH 7.5, for 18 h. The fraction was first adjusted to contain 1.2 mM NAD^+ and 1 mM ascorbic acid and then applied to a 2.5 × 11 cm CapGapp–Sepharose column (Lange & Vallee, 1976), equilibrated with 50 mM NaP_i, 1.2 mM NAD^+, and 1 mM ascorbic acid, pH 7.5. The column was washed with the same buffer at a flow rate of 21 mL/h until the A_{280} of the effluent approached base line. The active fractions, containing the pyrazole-insensitive[3] isozyme M, and class II and III ADH were combined, concentrated to 31 mL by ultrafiltration, and purified further (see below).

The pyrazole-sensitive molecular forms were eluted from the CapGapp–Sepharose affinity column with 0.5 M ethanol in 50 mM NaP_i and 1 mM ascorbic acid, pH 7.5. The active fractions were combined, concentrated by ultrafiltration, and extensively dialyzed against 10 mM Hepes, 1 mM NAD^+, and 0.1 mM DTT, pH 6.8. Application of the dialyzed sample to a CM-52 column (1 × 25 cm), equilibrated with the same buffer, and elution with 200 mL of a linear NaCl gradient, ranging from 0 to 70 mM NaCl at a flow rate of 29 mL/h, yielded three distinct activity peaks, representing the class I isozymes I-1, I-2, and I-3 (Figure 3). No further purification was necessary, and the enzymes were used after extensive dialysis.

Further purification and separation of the pyrazole-insensitive isozymes was achieved by precipitation with 70% saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, 23000*g* for 45 min, and then dissolved in 5 mM Tris-HCl, pH 7.7, containing 1 mM ascorbic acid. Gel filtration through a 1.5 × 30 cm column of Sephadex G-25 (Pharmacia) at a flow rate of 21 mL/h removed $(NH_4)_2SO_4$, NAD^+, and NADH. The active fractions were combined and dialyzed against 4 × 150 mL of 0.1 M Tris-HCl, pH 8.2, containing 1 mM ascorbic acid, and then applied to an AGAMP affinity column (1 × 25 cm) equilibrated at 4 °C with the same buffer. The column was washed with 600 mL of starting buffer, and class III ADH and isozyme M eluted in two distinct activity peaks with 400 mL of a linear NADH gradient, ranging from 0 to 3.2×10^{-5} M NADH at a flow rate of 25 mL/h. Elution with 1 M NaCl in equilibration buffer yielded class II ADH. All three isozyme fractions were concentrated by ultrafiltration and dialyzed against 10 mM Tris-HCl, pH 8.5.

Electrophoretic Procedures. Vertical starch gel electrophoresis of liver homogenates and purified ADH fractions was performed in the presence of NAD^+ according to Li & Magnes (1975). For detection of ADH molecular forms that migrate in either direction, the sample application slit was placed in the center of the Lucite mold (Buchler Instruments, Fort Lee, NJ). Gels were run at pH 7.7 and 220 V for 18 h. Immediately after electrophoresis the gel was sliced longitudinally to yield two slabs of equal thickness. Staining for ADH activity was carried out with 250 mL of either 100 mM ethanol or 1-pentanol, containing 50 mM sodium pyrophosphate, 0.6 mM MTT, 0.26 mM PMS, and 1.2 mM NAD^+, pH 8.6. Isozyme M and class I and II ADHs stain equally well with either alcohol. The gel slices were incubated in the dark at 45 °C for 10–15 min, then washed with water, and photographed immediately.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in analytical slab gels was performed with a Bio-Rad Model 220 instrument. The samples were run at 35 mA in a 10% acrylamide gel by using the system of Laemmli (1970) and stained with Coomassie blue.

Results

Survey of Class I Isozymes by Starch Gel Electrophoresis. The cathodic ADH isozymes in the crude extracts of liver specimens from 9 male and 35 female *M. nemestrina* from 1.2 to 20.9 years of age were resolved by starch gel electrophoresis. Four distinct phenotypic patterns were observed (Figure 1). Seven males and 21 females displayed pattern A (Figure 1). Eleven females and one male contained one additional activity band (Figure 1, lane B). Chromatography of these tissue homogenates on CapGapp–Sepharose reveals that the variant in pattern B binds to the affinity resin and elutes only with 0.5 M ethanol, consistent with the characteristics of class I ADH. These tissue samples also contain the same comigrating pair of ADH isozymes, i.e., the pyra-

[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.

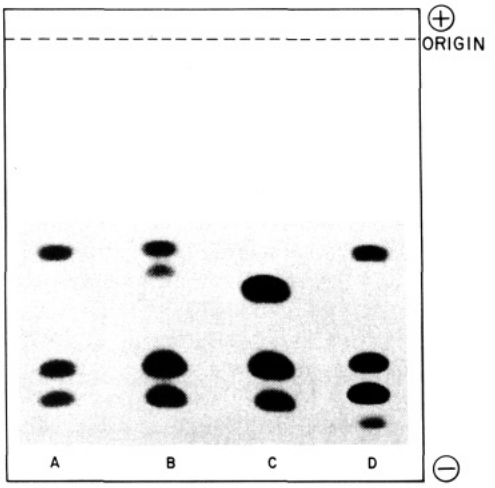

FIGURE 1: Starch gel electrophoresis of *M. nemestrina* liver homogenates. Conditions: pH 7.7; 220 V for 18 h at 4 °C. Enzymatic activity in the homogenate–supernatant of the crude hepatic extract was visualized with ethanol as substrate. Only class I isozymes are clearly detectable in unconcentrated samples. A total of 44 livers were examined, and the phenotypic patterns A–D were observed in 63, 27, 8, and 2% of the homogenates, respectively.

zole-sensitive and -insensitive variants I-2 and M (see below). The remaining two phenotypes (Figure 1, lanes C and D) are expressed in only 7% and 2% of the homogenates examined.

Purification of M. nemestrina Liver Alcohol Dehydrogenase. The nomenclature used for the identification of the individual isozymes is similar to, but not identical with, the classification employed for human liver ADH (Vallee & Bazzone, 1983). First, the molecular forms are grouped according to their pyrazole sensitivity, operationally defined as the isozymes that do or do not bind to the CapGapp–Sepharose affinity resin at pH 7.5. Only class I ADH binds; isozyme M and the molecular forms of class II and III do not. The unbound enzyme fraction was then separated further either by chromatography on DEAE-cellulose or, as described here, by a second affinity resin. Differential substrate specificity and inhibition characteristics of the purified isozymes completed their classificational assignment. In addition, the individual enzyme variants belonging to class I ADH were identified further by their electrophoretic mobilities on starch gel, and the lowest number was assigned to the most cathodal isozyme.

Livers of the major phenotypic pattern (Figure 1, lane A) were used for the isolation of the individual isozymes. The three pyrazole-sensitive class I ADHs, designated I-1, I-2, and I-3, elute from the CapGapp–Sepharose affinity resin with 0.5 M ethanol as an unresolved group (Figure 2). Chromatography on CM-cellulose (Wagner et al., 1983) resolves these three class I isozymes (Figure 3). The pyrazole-insensitive ADHs contained in the void volume of the CapGapp–Sepharose column were purified and separated by using a second affinity resin, agarose–hexane–AMP. A NADH gradient served to separate the class III variant from isozyme M, while the class II molecular form was obtained by eluting the column with 1 M NaCl (Figure 3). Both affinity chromatographic procedures yielded highly purified enzyme fractions, as judged by activity staining on starch gels and by single bands of ~40 000 daltons on $NaDodSO_4$–polyacrylamide electrophoresis gels.

Steady-State Kinetic Parameters. The K_m values for the *M. nemestrina* liver ADH isozymes were determined at pH 10 by the method of Lineweaver and Burk, employing saturating coenzyme concentrations of 2.4 mM NAD^+ (Tables I and II). The kinetic parameters of the three individual class I isozymes I-1, I-2, and I-3 for a series of aliphatic primary alcohols, extending from methanol through 1-octanol, confirmed previous observations (Klyosov et al., 1977) that binding increases with increasing hydrophobicity of the substrate (Table I). K_m values for all class I molecular forms range from 11.5 to 16.1 mM for methanol and from 3 to 8 μM for 1-octanol. All three isozymes oxidize 2-propanol and benzyl alcohol, with Michaelis constants ranging from 0.4 to 8.5 mM for the secondary aliphatic alcohol and from 6 to 48 μM for the aromatic derivative. Moreover, long-chain ω-hydroxy fatty acids are also effectively oxidized by these ADH forms, and relatively constant K_m values averaging 79 and 12 μM were

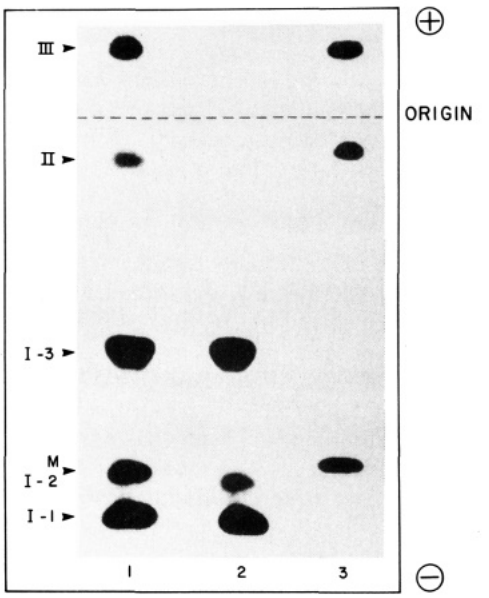

FIGURE 2: Starch gel electrophoresis of pyrazole-sensitive (class I) and pyrazole-insensitive (isozyme M and classes II and III) ADH molecular forms from *M. nemestrina* liver. Conditions: pH 7.7; 220 V for 18 h at 4 °C. Crude liver homogenate (lane 1) was chromatographed on CapGapp–Sepharose and eluted with ethanol, yielding a mixture of three class I variants, designated I-1, I-2, and I-3 (lane 2). The void volume of the affinity resin contained isozyme M, class II ADH, and one class III molecular form (lane 3). All samples were concentrated 10-fold, and the gel was stained for activity with 1-pentanol as the substrate.

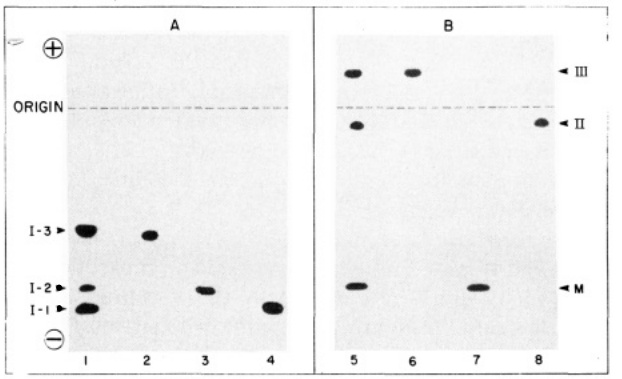

FIGURE 3: Starch gel electrophoresis of *M. nemestrina* liver ADH isozymes. Conditions: pH 7.7; 220 V for 18 h at 4 °C. Panel A shows the pyrazole-sensitive class I isozymes I-1, I-2, and I-3 before (lane 1) and after chromatography on CM-cellulose (lanes 2–4). The gel was stained for ethanol-oxidizing activity. Panel B illustrates the separation of the pyraole-insensitive isozymes (lane 5) by chromatography on AGAMP. A NADH gradient recovered first the class III variant (lane 6) and then the molecular form M (lane 7) from the affinity resin. Further elution with NaCl yielded the class II isozyme (lane 8). The gel was stained for activity with 1-pentanol as the substrate.

Table I: Steady-State Kinetic Parameters for Class I Isozymes of *Macaca nemestrina* Liver Alcohol Dehydrogenase[a]

	I-1		I-2		I-3	
substrate	K (μM)	V_{max}[b]	K_m (μM)	V_{max}[b]	K_m (μM)	V_{max}[b]
methanol	11500	0.04	9800	0.54	16100	0.32
ethanol	1850	1.06	1100	1.27	1600	1.90
1-pentanol	70	1.21	53	1.43	6	0.50
1-octanol	4	0.38	8	1.72	3	1.30
2-propanol	8500	0.97	8000	1.17	440	1.90
benzyl alcohol	48	1.01	40	1.27	6	1.76
12-HDDA	83	1.35	67	1.46	87	1.44
16-HHDA	11	1.12	15	1.46	11	1.69

[a] Oxidations were performed with 2.4 mM NAD^+ at 25 °C in 0.1 M glycine, pH 10. [b] Standard activity of 1 IU/mL was determined with 33 mM ethanol

Table II: Steady-State Kinetic Parameters for Pyrazole-Sensitive Isozymes of *Macaca nemestrina* Liver Alcohol Dehydrogenase[a]

	M		II		III	
substrate	K_m (μM)	V_{max}[b]	K_m (μM)	V_{max}[c]	K_m (μM)	V_{max}[c]
methanol	NA[d]	NA[d]	NA[d]	NA[d]	NA[d]	NA[d]
ethanol	50000	0.44	214000	1.40	NS[e]	NS[e]
1-pentanol	26	1.85	1040	1.07	45000	1.84
1-octanol	6	1.13	76	0.52	700	1.41
2-propanol	256000	0.65	NA[f]	NA[f]	NA[f]	NA[f]
benzyl alcohol	5	1.82	3400	0.51		
12-HDDA[g]	50	0.21				
16-HHDA[g]	7	0.47				

[a] Oxidations were performed with 2.4 mM NAD^+ at 25 °C in 0.1 M glycine, pH 10. [b] Standard activity of 1 IU/mL was determined with 33 mM ethanol. [c] Standard activity of 1 IU/mL was determined with 0.5 M ethanol. [d] Activity up to 0.2 M methanol was <3% of standard activity. [e] Activity was directly proportional to substrate concentration up to 2.5 M ethanol. [f] Activity up to 1 M 2-propanol was <3% of standard activity. [g] 12-Hydroxydodecanoic and 16-hydroxyhexadecanoic acid were dissolved in buffer containing 10% (v/v) methanol.

obtained for 12-hydroxydodecanoic and 16-hydroxyhexadecanoic acid, respectively.

Consistent with the kinetic characteristics of all class II isozymes isolated thus far, methanol does not serve as substrate for the ADH variants M and II (Table II). In addition, the K_m values for ethanol of 50 and 220 mM, respectively, are relatively high when compared to those of the class I molecular forms. However, with increasing chain length of the primary alcohols the K_m values are sharply decreased, i.e., to 6 and 76 μM for 1-octanol. This hydrophobic effect is further expressed in the binding of long-chain ω-hydroxy fatty acids to the isozyme M where the 16-hydroxy derivative binds about 7 times better than the corresponding 12-hydroxy analogue. As judged by their Michaelis constants for benzyl alcohol, there is a pronounced difference between isozyme M and the class II variant of *M. nemestrina* liver. The aromatic alcohol is an excellent substrate for the molecular form M with a K_m value of 5 μM, while the constant is about 700-fold higher for isozyme II.

As shown in Table II, the kinetic characteristics of ethanol oxidation catalyzed by the class III isozyme are substantially different from the other two classes of simian liver ADH. At concentrations up to 2.5 M, ethanol exhibits nonsaturation kinetics with the class III variant, yielding a straight line plot of V vs. [S] as previously observed (Parês & Vallee, 1981). There is no activity with methanol or 2-propanol, but Michaelis–Menten kinetics are observed with long-chain aliphatic primary alcohols. The large decrease of K_m values with these substrates appears to be consistent with a greater affinity of the enzyme for apolar compounds.

Inhibition by Metal-Binding Agents. Suitable metal-binding agents inhibit activity, demonstrating that a metal is essential for enzymatic activity in all ADH isozymes isolated. The concentration-dependent inhibition with 1,10-phenanthroline is both instantaneus and reversible. Further, addition of excess Zn^{2+} or dilution instantaneously restores activity. The two sets of phenomena suggest the formation of a dissociable enzyme–Zn–1,10-phenanthroline complex (Vallee et al., 1959). In contrast, EDTA inhibits the enzymes in a time-dependent manner. Neither dilution nor addition of Zn^{2+} reverses this inhibition, which is consistent with the irreversible removal of catalytically essential metal atoms.

Discussion

The number and diversity of ADH variants detected in and isolated from livers of *M. nemestrina* have turned out greater than initially anticipated. Previous analyses of class I isozymes in liver homogenates of either *Saimiri sciureus* or *Macaca mulatta* consistently showed the same number of ADH variants. However, starch gels of liver homogenates of *M. nemestrina* display four different phenotypes of class I ADHs (Figure 1), although, as judged by position and staining characteristics, all livers contain the same class II and III isozymes (results not shown). A total of 44 livers were examined, and since the electrophoretic pattern observed in lane A of Figure 1 was characteristic of more than 60% of the homogenates, tissues of this phenotype were selected for virtually all preparations here described.

The between-animal variation expressed in distinct class I ADH patterns is provocative since, thus far, it seems to be unique to this simian species. The molecular heterogeneity of human liver ADH appears to be a consequence of genetic control (Smith et al., 1971), and therefore, different class I ADHs are observed. Whether the variability of isozymes in *M. nemestrina* liver can be correlated to the same factors or is a function of age, sex, disease states, or dietary habits cannot be discerned from these results and remains a particularly pertinent question.

The ADH variants of class I were readily purified as a group by affinity chromatography on CapGapp–Sepharose and then separated into the individual molecular forms by ion-exchange chromatography on CM-52. Like all other well-characterized mammalian class I ADHs, the isolated and purified isozymes of *M. nemestrina* exhibit broad substrate specificity. They

all share analogous enzymatic properties and catalyze the oxidation of primary aliphatic and aromatic alcohols, long-chain ω-hydroxy fatty acids, and 2-propanol, a secondary alcohol. In a series of aliphatic primary alcohols the K_m values of all three class I ADHs are highest for methanol, extending from 11.5 to 16.1 mM, and decrease progressively as a function of substrate length to become more than 5000-fold lower for 1-octanol, ranging from 3 to 8 μM. Although steric and other factors may affect the interaction of the substrate with the catalytic domain of the enzyme, hydrophobic forces clearly play a major role in the binding process.

In the past, the utilization of the CapGapp affinity resin has proven the existence of class II ADH in human (Bosron et al., 1977, 1979) and simian livers, i.e., *S. sciureus* (Dafeldecker et al., 1981a) and *M. mulatta* (Dafeldecker et al., 1981b). In all cases the class II molecular forms migrated less cathodically on starch gel electrophoresis between pH 7.7 and pH 8.6 than any one of the class I isozymes. At pH 7.7 the molecular form II from *M. nemestrina* exhibits about the same electrophoretic mobility as the well-characterized human and other simian class II ADHs, while isozyme M comigrates with one of the class I variant forms and can be visualized on starch gel only after separation by affinity chromatography (Figure 2).

The ADH molecular form displaying the least cathodic mobility, II, was further separated and purified from the coeluting isozymes M and III by affinity chromatography on agarose–hexane–AMP (Figure 3). The kinetic properties exhibited by M and II are characteristic of the more limited substrate specificity shown by class II ADHs from other species. Thus, in a series of primary aliphatic alcohols, methanol is not oxidized, the Michaelis constants for ethanol are high, but as previously observed, further elongation of the chain length of the substrate results in a sharp decrease in K_m (Table II). Moreover, analogous to human π-ADH, long-chain ω-hydroxy fatty acids are good substrates, while the secondary alcohol, 2-propanol, is only oxidized by isozyme M. Clearly, a direct comparison between kinetic constants of both isozymes and other mammalian class II ADHs indicates that the Michaelis constants are remarkably similar.

The liver of *M. nemestrina* also contains an anodic molecular form, which closely resembles, though may not be identical with, the class III liver ADH of human (Parês & Vallee, 1981), horse (Dafeldecker & Vallee, 1982), and rhesus monkey (Dafeldecker et al., 1981b) in all respects. Accordingly, the electrophoretic mobility and staining characteristics on starch gel are virtually the same, and a direct comparison of the present data (Table II) with those of previous studies reveals striking similarities of substrate specificity and Michaelis constants. Thus, the class III ADH variant of *M. nemestrina* liver is not inhibited by 4-methylpyrazole and is not active toward methanol or 2-propanol, and ethanol fails to saturate this isozyme when examined at concentrations as high as 2.5 M. However, the catalytic rate of the enzyme increases as a function of the hydrophobicity of the substrate, and 1-pentanol is the shortest primary aliphatic alcohol that is oxidized with a K_m value lower than 100 mM. Clearly, extended studies focusing on additional alcohols and aldehydes of physiological significance are necessary to support the present conclusion that the substrate specificity of this class of *M. nemestrina* ADH is distinct and therefore likely to play an important metabolic role.

While the substrate specificities and kinetic characteristics of all three classes of *M. nemestrina* liver ADH differ markedly from each other, their physicochemical and compositional parameters closely resemble those of all other mammalian alcohol dehydrogenases examined thus far. Gel filtration on Sephadex G-100 using the horse EE isozyme (Pietruszko & Theorell, 1969) as a standard reveals molecular weights of $\sim$80 000, and $NaDodSO_4$ gel electrophoresis indicates the existence of subunits of $\sim$40 000 daltons. Hence, the three classes of ADH and the variant M are composed of two subunits of equal or nearly equal molecular weights. Further, the inhibition of all *M. nemestrina* liver ADHs by chelating agents such as 1,10-phenanthroline and EDTA demonstrates the essentiality of a metal, i.e., zinc, for enzymatic activity. The inhibition with the phenanthroline derivative is instantaneous and reversible on dilution or addition of excess metal ions, indicative of the formation of a mixed enzyme–zinc–chelator complex previously observed 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 a 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 pharmacological and pathological effects as well as the addictive properties of ethanol on humans are extensively documented. However, although postulated (Vallee, 1966), the biological determinants of alcoholism and alcohol abuse have remained elusive and obscure. The reported genetic predisposition in some individuals for the consumption of alcohol (Goodwin et al., 1973; Goodwin et al., 1974) is potentially pertinent to the task of identifying possible biochemical markers. In this context, the delineation of the genetic variability of liver alcohol dehydrogenase, the major enzyme of human ethanol metabolism, has become an important target for alcoholism research. Yet, the scarcity of suitable tissue samples have directed our research efforts toward the identification of an appropriate animal model to elucidate the genesis of alcoholism. Our previous investigations demonstrated the remarkable variability in the expression of simian liver ADH isozymes. While the resemblance to the human pattern is seemingly limited to the orangutan, thus far considerable diversity in anodic and cathodic isozyme migration has been observed in many of the species studied. The present data are sufficiently detailed to recognize the specific characteristics of this new isozyme.

The presence of the M isozyme, so named for its occurrence so far in *M. nemestrina* only and with properties intermediate between those of class I and those of class II, was unexpected and not previously observed in other mammalian species. This circumstance clearly indicates that prudence should be exercised before ADH molecular forms are assigned catagorically on the basis of properties of normal human ADH isozymes. As demonstrated here, classification simply based on electrophoretic position in starch gels but without isolation and kinetic analysis of the individual isozymes may prove to be both inadequate and inaccurate. Polymorphic variation of the class II gene locus in *M. nemestrina* and/or formation of a hybrid heterodimer may give rise to the new ADH variant here described. The study of isozymes of this species, designed to provide a realistic experimental basis for the identification of biochemical links to alcoholism and/or abuse, may not be suitable for these objectives. The investigation of simian liver alcohol dehydrogenases described here and in preceding papers further confirms and illustrates the complexity and metabolic flexibility of this enzyme system.

ACKNOWLEDGMENTS

The excellent technical assistance of B. Z. Tsokanis is gratefully acknowledged.

Registry No. MeOH, 67-56-1; EtOH, 64-17-5; $CH_3(CH_2)_4OH$, 71-41-0; $CH_3(CH_2)_7OH$, 111-87-5; *i*-PrOH, 67-63-0; $PhCH_2OH$, 100-51-6; 12-HDDA, 505-95-3; 16-HHDA, 506-13-8; alcohol dehydrogenase, 9031-72-5.

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Malate Dehydrogenase from *Rhizobium japonicum* 3I1b-143 Bacteroids and *Glycine max* Root-Nodule Mitochondria†

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Received February 19, 1985

ABSTRACT: The enzyme malate dehydrogenase (EC 1.1.1.37) has been purified (85-fold) from mitochondria of *Glycine max* (Williams 79 cultivar) root nodules and from *Rhizobium japonicum* 3I1B-143 bacteroids (400-fold). The mitochondrial malate dehydrogenase (m-MDH) has a native molecular weight of 69 000. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of the protein resulted in two protein bands of equal intensity with molecular weights of 39 000 and 37 000, respectively. The pH optimum of m-MDH is 6.5–7.3 for the conversion of oxaloacetate (OAA) to L-malate and 8.2–8.6 for the conversion of L-malate to OAA. Reduced nicotinamide adenine dinucleotide (NADH), but not OAA, protected m-MDH against heat inactivation at 55 °C. The bacteroid malate dehydrogenase (b-MDH) has a native molecular weight of 139 000 as analyzed by native polyacrylamide gel electrophoresis. SDS–polyacrylamide gel electrophoresis of b-MDH resulted in one protein band with a molecular weight corresponding to 36 000. The pH optimum for conversion of OAA to L-malate is 8.0–8.5, while the pH optimum in the reverse direction is 8.6–9.0. NADH, but not OAA, provided almost complete protection against heat inactivation at 55 °C. Arrhenius plots of the two enzymes gave Q_{10} values of 2.34 for the b-MDH and 1.62 for the m-MDH. The Michaelis constants of both malate dehydrogenases were determined and found to be similar to those of malate dehydrogenases from other sources. Kinetic analysis of b-MDH indicated an ordered bi-bi mechanism with NAD^+ adding first and NADH leaving last.

The metabolism of organic acids fulfills a principal role in the symbiotic nitrogen-fixation process (Bach et al., 1958; Bergersen & Turner, 1967; Finan et al., 1983; Glenn et al., 1980; Reibach & Streeter, 1984; Ronson et al., 1981). In the symbiotic relationship between leguminous plants and the *Rhizobium* sp., for example, soybeans and *Rhizobium japonicum*, the actual site of nitrogen fixation occurs within the bacteroid, the phenotypically distinct state of the bacteria found within the plant root-nodule tissue. The plant provides the energy for the nitrogen-fixation process by supplying the bacteroids with photosynthetically derived carbon compounds.

† Support for this research was provided by U.S. Department of Agriculture Competitive Grant 80-CRCR-1-0503, the Frasch Foundation, and the Missouri Soybean Merchandising Council. This is Contribution No. 9808 of the Agricultural Experiment Station of the Univeristy of Missouri.

0006-2960/85/0424-6479$01.50/0