Characterization of *Coturnix* Quail Liver Alcohol Dehydrogenase Enzymes

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Received October 11, 1988; Revised Manuscript Received May 10, 1989

ABSTRACT: Livers from male or female *Coturnix* quail possess up to four electrophoretically distinct bands of alcohol dehydrogenase (ADH) activity. Three pyrazole-sensitive bands of enzymatic activity, designated ADH-1, ADH-2, and ADH-3, are cathodic at pH 8.2, and the fourth, ADH-An, is neutral to slightly anodic and insensitive to pyrazole. ADH-2 and ADH-3, and occasionally ADH-1, are present in livers from immature females. The predominant enzyme in immature male livers is ADH-3. At sexual maturity all three pyrazole-sensitive enzymes are present in livers from male birds, and livers from females possess predominantly ADH-3. ADH-2 and ADH-3, purified from female livers, are dimers of 80 000 daltons possessing 4 mol of Zn^{2+}/mol of native protein. Both ADH-2 and ADH-3 were inhibited by 4-methylpyrazole with K_I values of 430 and 335 nM, respectively. These values are similar to those of human class I isoenzymes. Neither enzyme oxidized methanol or ethylene glycol, which distinguished them from mammalian pyrazole-sensitive ADH isoenzymes. Both ADH-2 and ADH-3 showed specificity toward hydrophobic primary alcohols and were most active toward benzyl alcohol and *n*-octanol.

Liver alcohol dehydrogenases (ADH,[1] EC 1.1.1.1) have been purified and characterized from many mammalian species, including human (Wagner et al., 1983, 1984; Ditlow et al., 1984), horse (Lutstorf et al., 1970), rat (Markovič et al., 1971), and monkey (Dafeldecker et al., 1981). All of these sources have multiple isoenzymes of ADH, which cumulatively act on numerous physiologically important compounds such as precursors of norepinephrine, digitoxigenin, digoxigen, gitoxigenin, and other steroids (Frey & Vallee, 1980; Mårdh et al., 1986), and polyglycols (Wagner et al., 1983). While these studies have implicated metabolic roles for ADH isoenzymes other than ethanol detoxication, evidence does not exist to correlate isoenzyme composition or specificity with any metabolic or physiological state of the specimen.

Avian alcohol dehydrogenases have not been as extensively studied as the mammalian enzymes. Previous work with *Coturnix* liver postulated the existence of one gene locus with detection of three different alleles (Castro-Sierra & Ohno, 1968). ADH has also been isolated and partially characterized from chicken livers (von Bahr-Lindström et al., 1978); however, little information is available concerning the properties or substrate specificity of avian ADH isoenzymes. We have undertaken to characterize the alcohol dehydrogenases of quail liver with respect to composition and occurrence as a function of sex, age, and maturity of donors. We have also examined the physical and enzymatic properties of two of the principal forms of the enzymes.

Experimental Procedures

Materials. NAD^+ (grade III) and DTT were purchased from Sigma Chemical Co., St. Louis, MO. Ethanol (100%) was purchased from Aldrich Chemical Co., Milwaukee, WI. Other alcohols and aldehydes were obtained from Fischer Scientific Co. and used without further purification. All other chemicals used were of reagent grade. Ion-exchange resins (CM 52 and DEAE 52) were obtained from Whatman Chemical Separation, Inc., Clifton, NJ. AMP–hexane–agarose and CapGapp–Sepharose were generous gifts from Dr. Bert L. Vallee, Center for Biochemical and Biophysical Sciences and Medicine, 250 Longwood Ave, Harvard Medical School, Boston, MA 02115.

Purification of Quail Liver ADH. Livers from Japanese quail (*Coturnix coturnix japonica*) were used for the purification and characterization of ADH. Birds used in these experiments were from two different sources. For earlier work, quail were hatched from eggs of the Pharoah D-1 strain purchased from Fatima Quail Farms, Artesia, CA (source 1), while for later work the quail were hatched from eggs obtained from Quail Genetic Stock Center, University of British Columbia, Vancouver, BC, Canada (source 2).

Birds were anesthetized by exposure to a CO_2 atmosphere, decapitated, and bled. The livers were removed by dissection, weighed, and immediately frozen on dry ice. All livers were stored at −70 °C until use.

Livers were thawed prior to use, rinsed to remove blood, and homogenized at 4 °C, by using a Sorvall Omni-Mixer, in 10 mM Tris-HCl buffer (1:2 w/v) containing 0.5 mM DTT, pH 8.0. Unless otherwise noted, subsequent steps were performed at 4 °C. The pH of the homogenate was then adjusted to 8.0 with 2.0 M Tris base and stirred with a magnetic stirrer for 2 h. The homogenate was filtered through cheesecloth to remove debris and lipid and then centrifuged at 27000*g* for 45 min.

The supernatant fluid was applied to DEAE-cellulose (100 mL of extract from 50 g of liver per 2.5 × 32 cm column), equilibrated with 10 mM Tris-HCl buffer containing 0.5 mM DTT, pH 8.0. After the sample was applied, the ADH activity

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[1] Abbreviations: ADH, alcohol dehydrogenase; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, oxidized nicotinamide adenine dinucleotide phosphate; DTT, dithiothreitol; CapGapp, 4-[3-[*N*-(6-aminocaproyl)-amino]propyl]pyrazole; AMP–hexane–agarose, agarose–hexane–adenosine 5′-phosphate, type 2; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMS, phenazine methosulfate; $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl; DEAE, diethylaminoethyl; 4MPz, 4-methylpyrazole; K_m, Michaelis constant; k_{cat}, maximum velocity (micromoles per minute) divided by total enzyme (micromoles).

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was eluted with approximately 100 mL of the equilibrating buffer, pooled, and concentrated to about 50 mL in an Amicon concentrator equipped with a UM-50 membrane and operated at 40 psi. The concentrated extract was dialyzed against 50 mM phosphate buffer, pH 7.5, containing 0.5 mM DTT.

After dialysis, the ADH sample was applied to an AMP–hexane–agarose column preequilibrated with 50 mM sodium phosphate and 0.5 mM DTT, pH 7.5. After the unbound fraction eluted, which contained the majority of the protein and no ADH activity, the column was washed with 50 mM sodium phosphate containing 0.5 mM DTT, 0.2 M NaCl, and 20 mM galactose, pH 7.5, and then with the same buffer containing 0.2 mM NAD^+ (1 column volume). ADH activity was eluted with 50 mM sodium phosphate containing 0.5 mM DTT and 3.0 mM NAD^+, pH 7.5, as an unresolved mixture of two or three isoenzymes.

The mixture of ADH activities recovered from the AMP–hexane–agarose column was further purified and resolved according to a modified procedure of Crabb et al. (1983). The mixture was dialyzed extensively against 5 mM Hepes–NaOH buffer, pH 7.5, containing 0.5 mM DTT and 10 μM zinc sulfate and then applied to CM-cellulose (2 × 7.5 cm column), equilibrated with the same buffer. The conductivities of the equilibrating buffer, the enzyme-containing solution, and the effluent from the CM-cellulose column were measured prior to chromatography to verify that they all were of the same conductance. After contaminating protein was removed with equilibrating buffer, the enzymes were resolved with a linear gradient of 5–500 mM Hepes buffer, pH 7.5, containing 0.5 mM DTT and 10 μM zinc sulfate.

Enzymatic Assay. Alcohol dehydrogenase activity toward alcohols and aldehydes was measured spectrophotometrically, by using a Cary 219 recording spectrophotometer, either as the increase in absorbance at 340 nm due to the reduction of NAD^+ or as the decrease in absorbance at 340 nm due to the oxidation of NADH. When alcohols served as the substrate, assays were performed at 25 °C in 0.1 M glycine–NaOH buffer, pH 10, and 2.6 mM NAD^+. Reactions were initiated by the addition of enzyme. Assays performed to monitor elution from columns and to assess enzymatic activity were performed with 33 mM ethanol. For all steps in the enzyme preparation prior to chromatography on CM-cellulose, a blank without ethanol was monitored for NADH formation as a control. Enzymatic activity was calculated as the difference between the velocities in the presence and absence of ethanol. When octanal served as the substrate, assays were performed at 25 °C in 0.1 M Mes–NaOH buffer, pH 6.5, 0.26 mM NADH, and 0.5 mM octanal. One unit of ADH activity is defined as the amount of enzyme required to catalyze the reduction of 1 μmol of NAD^+ or the oxidation of 1 μmol of NADH/min at 25 °C, using a molar absorptivity of 6220 M^{-1} cm^{-1} for NADH.

Kinetic measurements were performed in a Cary 219 spectrophotometer interfaced with an Apple II computer. Data were collected and stored with software supplied by Varian Associates and analyzed by a least squares fit to the Michaelis velocity equation.

Protein Determination. Protein concentration was determined colorimetrically by the method of either Lowry et al. (1951) or Bradford (1976). In both cases bovine serum albumin was used as a standard. Appropriate blanks were used to correct for interference by buffers and DTT.

Electrophoretic Procedures. Starch gel electrophoresis was performed according to a modified method of Bosron et al. (1979). The gels (11 × 24.5 × 0.5 cm) contained 13% starch, 1.0 mM NAD^+, and 5.0 mM Tris-HCl buffer, pH 8.2. Samples were applied to paper wicks (0.5 × 1.0 cm) and inserted into the gel at the center of the slab. Gels were run at 720 V for 6 h at 4 °C. Immediately after electrophoresis, gels were sliced longitudinally into three slabs. The top slab (ca. 1 mm) was discarded. The center and bottom slabs (1.5–2 mm in thickness) were stained for ADH activity. The staining solution (200 mL) contained 50 mM sodium pyrophosphate, pH 8.5, either 100 mM ethanol or 100 mM pentanol, 0.6 mM MTT,[2] and 0.26 mM PMS.[2] Gel slabs were incubated in the dark at 40 °C for 15–20 min by submersion in the staining solution. After being stained, the gels were washed with water and photographed.

Electrophoresis in $NaDodSO_4$–polyacrylamide gels was performed by the procedure of Laemmli (1970), using a 5% stacking gel and a 12% running gel. Gels were stained with Coomassie blue and destained with methanol–glacial acetic acid–water (4.5:1:4.5) or were silver stained by using a slightly modified technique (Morrissey, 1981).

Metal Analysis. Before the zinc content of purified quail ADH was determined, the enzyme preparation was dialyzed against 3000 volumes of 10 mM Hepes buffer and 0.5 mM DTT, pH 7.5, over a period of 24 h. All glassware was washed with 20% nitric acid and then rinsed with glass-distilled water. The zinc content was measured by using a graphite furnace atomic absorption spectrophotometer. The analysis was generously performed by S. H. Wilkes, Institute for Occupational Medicine, Texas A&M University, College Station, TX 77843.

Molecular Weight and Molar Absorptivity Determinations. The molecular weights of purified quail liver ADH enzymes were determined separately for each enzyme by the sedimentation equilibrium method of Yphantis (1964). Quail ADH enzymes (0.25 mg/mL) were dialyzed for 36 h against 50 mM phosphate buffer, pH 7.5, containing 0.2 M NaCl and then loaded into a double-sector cell. Centrifuge measurements were performed in a Spinco Model E analytical ultracentrifugation equipped with Rayleigh interference optics. Centrifugation was performed at 22 000 rpm at 15 °C for 24 h, when equilibrium had been achieved.

Molar absorptivities were calculated after determination of total nitrogen, using a modified micro-Kjeldahl procedure of Ballentine (1957). The enzyme samples were desalted by using a prepacked G-25 column (PD-10, Pharmacia, Inc., Piscataway, NJ) equilibrated with glass-distilled water. Bovine serum albumin was used for the standard curve. All samples and standards were run in triplicate. Prior to digestion, absorbances at 280 nm were determined by using a Cary 219 spectrophotometer.

Amino Acid Analysis. Purified enzyme was desalted as previously described and then lyophilized. Approximately 5–10 μL of sample, diluted with water to a concentration of 1 mg/mL, or 5 μL of amino acid standards (standard H, Pierce, Rockford, IL) was placed in 6 × 50 mm Pyrex tubes. Tubes containing samples and standards (performed in duplicate) were placed in a vial fitted with a Teflon valve, and then 350 μL of constant boiling HCl was added to the bottom of each vial. The vial was sealed under vacuum and subjected to vapor-phase hydrolysis for 24, 48, and 72 h at 110 °C according to the procedure of Moore and Stein (1963). After hydrolysis, residual HCl was removed under vacuum at room temperature. Samples and standards were derivatized with phenyl isothiocyanate, to form phenylthiocarbamyl amino

[2] MTT and PMS are carcinogens and should be handled with extreme caution.

Table I: Purification of Quail Liver ADH[a]

step	protein (mg)	total act. (units)	sp act. (units/mg)	purification (x-fold)	yield (%)
crude extract	5809	55	0.009	1	100
DEAE-cellulose	960	36	0.038	4.2	68
AMP-agarose	28	12	0.41	46	22
CM-cellulose					
ADH-2	1.2	3	2.5	278	5.5
ADH-3	3.2	11	3.3	367	20

[a] Purification was performed with 100 g of tissue (about 20 livers). Activity was measured at pH 10.0, with 33 mM ethanol and 2.6 mM NAD^+.

acids, and analyzed according to the Pico-Tag methodology of Waters Associates Inc., Milford, MA, by using a Waters HPLC system. An Apple II computer was used with CHROMATOCHART (Interactive Microware Inc., State College, PA) to collect and process data. Cysteine was determined as cysteic acid by treating the enzyme with performic acid prior to hydrolysis as described by Hirs (1967). Tryptophan was estimated spectrophotometrically according to the method of Edelhoch (1967).

Results

Occurrence of ADH Enzymes in Quail Liver. Starch gel electrophoresis of liver homogenates (source 1) followed by staining for ADH activity revealed the existence of three distinct cathodic bands of activity (Figure 1). ADH-3, the most cathodic of the bands, was present in the highest relative activity in liver tissue; ADH-2, which was slightly less cathodic than ADH-3, possessed a less intense activity. A third band of activity, ADH-1, was present in lower concentrations depending on the age and sex of the bird. The kinetic behavior of this enzyme form was not investigated due to its low activity. Purification of ADH-2 and ADH-3 (Table I) confirmed observations from gel electrophoresis and demonstrated ADH-3 to be the enzyme form present in greatest concentrations in livers from mature (16-week-old) females. Livers from both males and females contained a neutral to slightly anodic band of enzymatic activity, designated ADH-An, which was similar to human χ-ADH. It was detected with ethanol (Figure 1) and preferentially with pentanol. It was not inhibited by 4MPz.

Distinct differences between the male and female ADH electrophoretic patterns correlated with age (Figure 1). At 2 weeks of age, the liver of male birds exhibited only one band on starch gels, corresponding to ADH-3, while the liver of female birds contained two electrophoretic bands, ADH-2 and ADH-3. Occasionally, trace amounts of ADH-1 could be detected in livers from young female birds. As the male birds matured, the number of ADH forms present in the liver increased to three distinct forms. In contrast, as the female birds aged, the intensity of ADH-2 decreased relative to ADH-3. Livers from some female birds more than 1 year old possessed only ADH-3 (data not shown). One form of the enzyme, ADH-3, predominated and could be detected when the female birds reached sexual maturity and were able to lay eggs.

Purification of Quail Liver ADH. The results of a typical purification procedure for quail liver ADH are presented in Table I. Livers were obtained from 16-week-old birds (source 1). It was critical that all buffers used contained DTT, to prevent inactivation of ADH, presumably due to oxidation. Passage of the crude extract over DEAE-cellulose (equilibrated in 50 mM Tris-HCl and 0.5 mM DTT, pH 8.0) resulted in the removal of about 40% of the total protein in the crude extract, while ADH-2 and ADH-3 appeared in the unbound fraction. Most of the loss in activity was accounted for by the

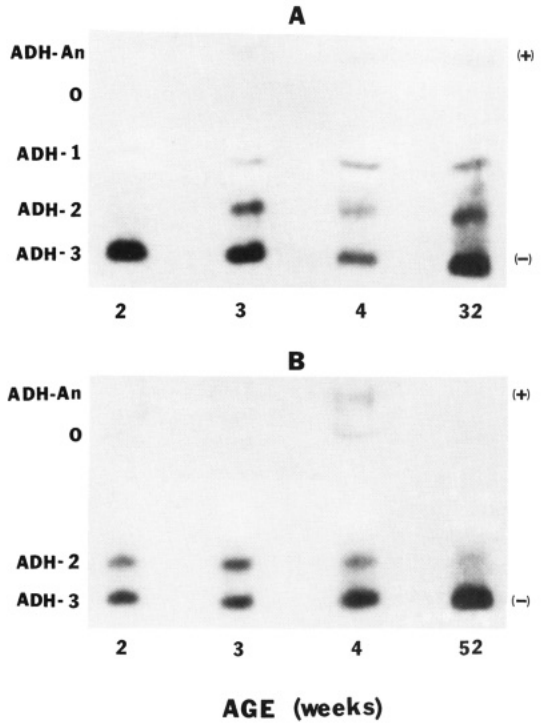

FIGURE 1: Starch gel electrophoresis (pH 8.2) of quail liver homogenates correlated with age and sex. (A) Supernatant fluid from liver extracts of male birds stained by using ethanol as the substrate. (Lane 1) 2 weeks; (lane 2) 3 weeks; (lane 3) 4 weeks; (lane 4) 8 months. (B) Supernatant fluid from liver extracts of female birds stained by using ethanol as the substrate. (Lane 1) 2 weeks; (lane 2) 3 weeks; (lane 3) 4 weeks; (lane 4) 1 year. O indicates the origin.

binding of the anodic ADH to the resin.

All ADH activity isolated from the DEAE step adhered to AMP–hexane–agarose. Prior to the adsorbed enzyme eluting, the column was extensively washed to remove any unbound or weakly bound protein. After the unbound protein was eluted, the column was washed with 50 mM sodium phosphate containing 0.2 M NaCl and 20 mM galactose, pH 7.5, to disrupt nonspecific binding of proteins and glycoproteins to the resin. The enzyme was eluted by complex formation with NAD^+. A low concentration of the cofactor (0.2 mM NAD^+) was first used to remove other weakly bound pyridine nucleotide-dependent proteins. A small amount of ADH coeluted in this step, accounting for some decrease in the yield. The bulk of ADH activity was eluted with the addition of 3 mM NAD^+, along with some other proteins. Starch gel electrophoresis revealed this fraction to possess ADH-2 and ADH-3 and ADH-1 when it was present in the crude material.

The three enzyme forms were separated and purified to homogeneity on a CM-cellulose column (Figure 2). These enzymes were adsorbed at pH 7.5, while the contaminating protein eluted in the unbound fraction. Subsequently, the enzymes were separated by using a linear gradient of Hepes buffer (5–100 mM). ADH-2 eluted prior to ADH-3, and the specific activities of the isolated enzymes were 2.5 and 3.3 units/mg, respectively. When liver samples contained detectable levels of ADH-1, it eluted prior to ADH-2, at approximately 25 mM Hepes. The purified enzymes gave single bands on SDS–polyacrylamide gel electrophoresis when stained with Coomassie blue (Figure 3). Starch gel electrophoresis revealed a single band for each enzyme form when stained with ethanol for activity (Figure 4). Notably, the numbers and quantities of ADH activities isolated by this procedure correlated well with electrophoresis results obtained by using liver extracts.

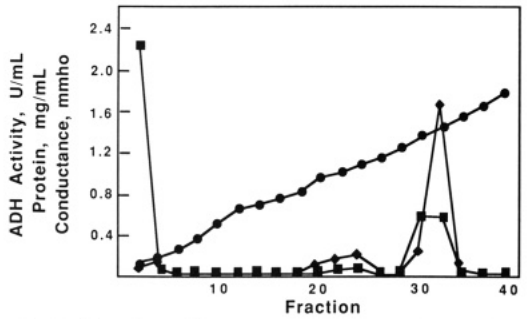

FIGURE 2: Purification and separation of ADH activities from quail liver extracts by chromatography on CM-cellulose. Fractions (3 mL) 22–27 contained ADH-2, and fractions 31–36 contained ADH-3. (◆) Activity with 33 mM ethanol (units/mL; (■) protein concentration (mg/mL); (●) conductance.

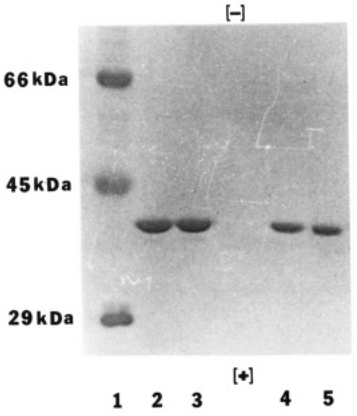

FIGURE 3: SDS–polyacrylamide gel electrophoresis of quail liver ADH isoenzymes, Coomassie blue stained gel. (Lane 1) Molecular weight standards containing bovine serum albumin (66 000), egg albumin (45 000), and carbonic anhydrase (29 000); (lane 2) ADH-2; (lane 3) ADH-3; (lane 4) horse liver ADH; (lane 5) mixture of ADH-2 and ADH-3.

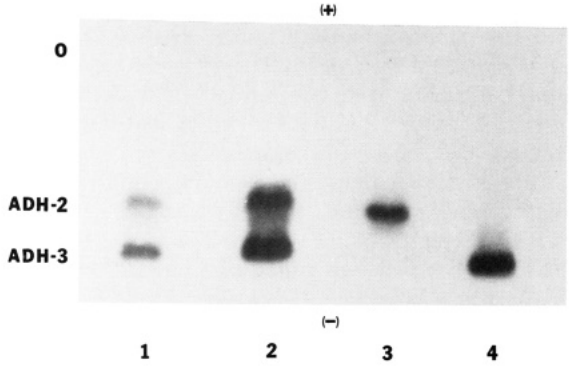

FIGURE 4: Starch gel electrophoresis of the purification of quail liver ADH activities. (Lane 1) Crude extract; (lane 2) DEAE-cellulose fraction; (lane 3) ADH-2; (lane 4) ADH-3. Enzymes in lanes 3 and 4 were obtained after CM-cellulose chromatography.

Molecular Properties. $NaDodSO_4$–polyacrylamide gel electrophoresis revealed the mobility of ADH-2 and ADH-3 to be nearly identical with that of horse liver ADH (Figure 3), with apparent subunit masses of 42 000 daltons for both. ADH-1 had an identical mobility on $NaDodSO_4$–polyacrylamide gel electrophoresis when compared to ADH-2 and ADH-3 (data not shown). Native molecular weights were calculated for each purified form of the enzyme from sedimentation equilibrium data. Data for ADH-3 were collected in duplicate and resulted in molecular weights of 82 700 and 82 800, very similar to the molecular weights of well-characterized mammalian ADH isoenzymes. These data indicate that quail liver ADH-3 is also a dimeric protein, composed

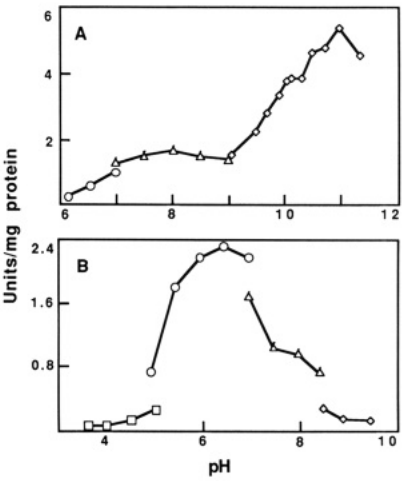

FIGURE 5: pH activity profile of ADH-3. Activity was measured by using either 33 mM ethanol and 2.6 mM NAD^+ (A) or 0.5 mM octanal and 0.26 mM NADH (B). The buffers used were (□) 0.1 M sodium acetate, (○) 0.1 M Mes, (△) 0.1 M Tris, and (◇) M glycine.

Table II: Kinetic Constant of ADH-2[a]

substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
ethylene glycol	NA[b]		
methanol	NA[b]		
ethanol	4.1	295	71
cyclohexanol	2.8	45	16
NAD^+[c]	0.19	280	1470

[a] Assay conditions are described under Experimental Procedures. [b] NA, no activity detected up to 5 M. [c] Determined at pH 10.0 by using 33 mM ethanol.

of two subunits of similar molecular weights. The sedimentation equilibrium of ADH-2 and ADH-1 resulted in data that indicated these enzymes had become partially dissociated, presumably by the high salt concentration (0.2 M) used in the buffer. A plot of the fringe displacement versus r^2 was biphasic, one portion with a slope corresponding to the subunit molecular weight and the other corresponding to the native molecular weight.

Samples of ADH-2 and ADH-3 were dialyzed against metal-free buffers (Holmquist, 1988) and then analyzed for zinc by atomic absorption. Metal analysis of ADH-2 and ADH-3 showed a zinc content of 1.7–1.9 and 1.6–1.9 atoms/subunit, respectively, on the basis of a subunit molecular weight of 42 000. Molar absorptivities were determined by micro-Kjeldahl estimations of protein concentration. Paucity of pure ADH-2 and ADH-3 did not allow for multiple analysis either of Zn or of the molar absorptivities. Thus, these results only provide for a first approximation of the metal content, which is in agreement with the well-established stoichiometry for mammalian enzymes.

pH Optima of Catalysis. The rate of ethanol oxidation for both ADH-2 and ADH-3 exhibited a pH maximum at 11.0 (Figure 5A). These enzymes were only about 30% as active at pH 7.5. Below pH 7.0 there was a rapid reversible decrease in activity down to pH 4.5. In contrast, the rapid decrease above pH 11.0 reflected irreversible inactivation of the enzyme.

The rate of octanal reduction by ADH-3 was also studied as a function of pH (Figure 5B). This enzyme exhibited a sharp pH maximum at pH 6.5. Below pH 5.0 and above pH 8.0 the activity declined to zero.

Kinetic Analysis. Kinetic results for the oxidation of some alcohols by ADH-2 and ADH-3 are presented in Tables II and III, along with the kinetic values for NAD^+ and NADH. Both

Table III: Kinetic Constants of ADH-3[a]

substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
ethylene glycol	NA[b]		
methanol	NA[b]		
ethanol	8.1	398	49
1-butanol	2.0	433	216
1-pentanol	0.2	175	648
1-octanol	0.011	111	9740
12-HDA	0.04	87	2180
cyclohexanol	5.2	213	41
benzyl alcohol	0.013	151	11700
octanal[c]	0.043	1020	23700
NAD^+[d]	0.23	371	1610
NADH[e]	0.0051	533	105000

[a] Assay conditions are described under Experimental Procedures. [b] NA, no activity detected up to 5 M. [c] Determined at pH 6.5 by using 0.26 mM NADH. [d] Determined at pH 10.0 by using 33 mM ethanol. [e] Determined at pH 6.5 by using 0.5 mM octanal.

forms proved to be specific for NAD^+ as the cofactor in the oxidation reaction inasmuch as neither ADH-2 nor ADH-3 oxidized ethanol in the presence of $NADP^+$, up to a concentration of 15 mM $NADP^+$. A pH value of 10.0 was selected for these assays rather than pH 11.0, where optimal activity occurs, to compare kinetic parameters with those obtained by using mammalian ADH isoenzymes.

The K_m values for NAD^+ measured with 33 mM ethanol as the substrate, at pH 10.0, were determined to be 0.226 and 0.192 mM for ADH-3 and ADH-2, respectively (Tables II and III). The K_m for NADH, measured with 0.5 mM octanal as the substrate, at pH 6.5, was found to be 5.13 μM for ADH-3.

Kinetic constants for a series of aliphatic alcohols were determined for both enzymes by using saturating concentrations of coenzymes. Because of the limited amount of ADH-2 available, the investigation of its properties was not as extensive as that of ADH-3. Neither enzyme oxidized ethylene glycol or methanol at concentrations up to 5 M, and of the alcohols examined, both forms of the enzyme have the highest K_m values for ethanol. As can be seen from Tables II and III, binding of alcohols to ADH-3 increases (evidenced by decreased K_m values) with their hydrophobicity. Values of k_{cat} were relatively constant and varied no more than 2-fold from the mean of 224 min^{-1}, a value similar to those for human liver isoenzymes (Wagner et al., 1983).

With ethanol as the variable substrate, NAD^+ as the fixed, saturating substrate, and 4MPz as an inhibitor, K_I values of 430 and 335 nM were obtained for ADH-2 and ADH-3, respectively.

Amino Acid Analysis. The results of the amino acid analysis are presented in Table IV. These data were calculated on the basis of a subunit molecular weight of 42000. The overall composition was very similar to that of the residue composition of human ADH enzymes (Hempel et al., 1984; Höög et al. 1986, 1987; Ikuta et al., 1986; von Bahr-Lindström et al., 1986), with the exception of AsX and methionine, the latter of which was somewhat lower in the quail enzymes. Both forms of the quail enzyme are rich in glycine, alanine, valine, and lysine residues, typical of all mammalian ADH enzymes. ADH-2 contains 8 residues of half-cystine, while the half-cystine content for ADH-3 was found to be 12 residues. The low half-cystine value for ADH-2 was consistent in three separate preparations of these isoenzymes.

Values for Asx for both ADH-2 and ADH-3 were almost half those for class I or class II human isoenzymes (Table IV). Glx values for human and quail liver enzymes were similar, however.

Table IV: Amino Acid Composition of Quail Liver ADH-2 and ADH-3 Compared to the Residue Composition Human Class I and Class II ADH[a]

			residue composition	
residue	ADH-2 (mol/subunit)	ADH-3 (mol/subunit)	class I[d] (α, β, γ)	class II[e] (π)
Cys[b]	8.4 ± 1.9	11.7 ± 1.6	16, 15, 15	16
Asx	17.0 ± 1.8	18.8 ± 2.4	28, 29, 28	33
Glx	30.1 ± 2.0	29.4 ± 2.5	26, 24, 26	23
Ser	27.6 ± 2.0	28.2 ± 2.2	23, 21, 24	24
Gly	45.3 ± 2.0	44.0 ± 1.8	36, 38, 37	39
His	8.1 ± 0.4	8.3 ± 0.7	8, 7, 6	6
Arg	12.9 ± 1.9	12.1 ± 1.5	9, 11, 11	8
Thr	28.5 ± 2.0	28.7 ± 2.4	22, 24, 22	26
Ala	36.4 ± 2.0	36.3 ± 2.0	31, 31, 30	35
Pro	22.5 ± 3.0	20.8 ± 2.4	20, 20, 20	17
Tyr	5.6 ± 1.2	5.9 ± 1.3	4, 6, 4	5
Val	37.5 ± 2.4	36.1 ± 3.0	36, 39, 38	26
Met	4.3 ± 0.4	5.0 ± 0.8	10, 7, 7	4
Ile	21.4 ± 3.6	20.9 ± 2.8	26, 22, 24	32
Leu	28.3 ± 3.9	24.9 ± 2.3	29, 29, 29	30
Phe	17.9 ± 2.0	16.9 ± 1.1	15, 16, 17	19
Lys	31.2 ± 2.4	33.8 ± 2.2	32, 32, 33	33
Trp[c]	2.2	3.0	2, 2, 2	3

[a] Assay conditions are described under Experimental Procedures. Amino acid residues are averages from results of six data sets. [b] Cysteine was determined as cysteic acid. [c] Tryptophan was determined spectrophotometrically. [d] From sequence data of Ikuta et al. (1986), Hempel et al. (1984), Höög et al. (1986), and von Bahr-Lindström et al. (1986). [e] From Höög et al. (1987).

Discussion

Electrophoretically, three cathodic bands of ADH activity constitute the majority of the detectable ethanol oxidizing activity in *Coturnix* quail liver. Additionally, slightly anodic ADH activity is easily detected when pentanol is used as the test substrate and is barely perceptible when ethanol is the substrate. This enzymatic form, which was not further characterized, correlates with χ-ADH from human liver (Wagner et al., 1984).

Quail liver ADH enzymes have been reported to be derived from three autosomally inherited alleles, designated A, B, and C (Castro-Sierra & Ohno, 1968). Thus, a liver could possess a single homozygous phenotype or three phenotypes derived from heterozygous gene, namely, A/B, B/C, or A/C. In the present study, livers from a common population were pooled for ADH isolation. Under these circumstances, one might expect to observe a very complex electrophoretic pattern of up to six bands. We consistently observed just three bands of activity in preparations from two different sources. This could mean that one allele was missing from these populations or that these alleles are behaving as homodimeric electromorphs of this genetic locus (Batzer et al., 1988). Since we could not directly relate our observations with the allelic phenotypes reported by Castro-Sierra and Ohno (1968), we have used the designations ADH-1, ADH-2, and ADH-3, which indicates the order of electrophoretic separation of *Coturnix* quail liver ADH activity but does not imply any genetic relation between them. However, we do not yet know whether these forms are all homodimeric or if one represents a heterodimer. Preliminary evidence indicates that ADH-3 is homodimeric.[3]

The most cationic form, ADH-3, appeared to be predominant in all pooled liver extracts subjected to electrophoresis regardless of sex or age; however, the relative amounts of the

[3] Personal communication from Dr. Hans Jörnvall, Department of Physiological Chemistry, Karolinska Institutet, Box 60400, S-104-01 Stockholm, Sweden.

three cathodic forms of ADH varied with age and sex. Extracts of pooled livers from young female birds from ages 2 weeks to 8 weeks possessed all three of the ADH activities, but as females matured and began laying, first ADH-1 and later ADH-2 disappeared, leaving ADH-3 as the predominant and often the only detectable ADH enzyme form. In pooled liver extracts from males the reverse was true: young males had predominantly ADH-3 and developed ADH-1 and ADH-2 as they matured. In both males and females the changing ADH pattern correlated well with the onset of sexual maturity and, in females, with egg production.

The cathodic ADH activities from female quail liver, like those from mammalian livers (Pietruszko, 1975), were inhibited by 4MPz. Thus, procedures developed for the purification of ADH isoenzymes from mammalian tissues (Wagner et al., 1983) were adapted for use. Unfortunately, affinity chromatography on CapGapp (employed for the purification of human liver cathodic isoenzymes) (Lange & Vallee, 1976) could not be employed. Quail ADH enzymes had to be kept in millimolar concentrations of DTT to maintain activity. Under these conditions the enzymes did not adhere to CapGapp. Others have shown that DTT interferes with CapGapp affinity chromatography of ADH isoenzymes (Hoshino et al., 1985). Affinity chromatography on AMP–hexane–agarose provided the purification of ADH enzymes from other proteins as an unresolved mixture. Chromatography on CM-cellulose afforded subsequent separation of the three ADH activities. On the basis of specific activity measurements (Table I) ADH constitutes 0.75% of liver weight in 16-week-old female birds, about one-third that for the human isoenzymes (Ditlow et al., 1984).

Physical properties of quail ADH enzymes are markedly similar to those of mammalian enzymes (Wagner et al., 1983, 1984; Ditlow et al., 1984); they are dimers of 80 000 daltons composed of subunits of 40 000 daltons and possess 4 mol of Zn/mol of native protein. The amino acid compositions of ADH-2 and ADH-3 were markedly similar to those of mammalian cathodic ADH isoenzymes (Jörnvall & Markovic, 1972; Hempel et al., 1984, Höög et al., 1986, 1987; Ikuta et al., 1986; von Bahr-Lindström et al., 1986), with the exception of their methionine content, which was half that of the human isoenzymes. Further, ADH-2 had two-thirds the half-cystine content (8 residues) of ADH-3 (12 residues), the latter of which compared favorably with that of human isoenzymes.

Oxidation of simple alcohols occurred optimally at pH 11, and reduction of simple aldehydes occurred optimally at pH 6.5. These optima are characteristic of virtually all mammalian alcohol dehydrogenases.

Specificity of the quail ADH enzymes for the oxidation of alcohols revealed apparent differences between these enzymes and those isolated from mammalian sources. Human liver ADH isoenzymes are subdividied into classes on the basis of their sensitivity to inhibition of ethanol oxidation by 4MPz, electrophoretic mobility, and substrate specificity (Strydom & Vallee, 1982). Thus, class I isoenzymes (a) are the most cathodic, (b) are strongly inhibited by 4MPz, and (c) oxidize methanol, ethanol, and a host of other primary aliphatic and aromatic alcohols. Class II ADH isoenzymes are less cathodic than class I isoenzymes, are not effectively inhibited by 4MPz, and oxidize ethanol, but neither methanol nor ethylene glycol. Class II human ADH isoenzymes oxidize other primary alcohols with a specificity similar to that of class I. Class III isoenzymes are anodic, are not inhibited by 4MPz, and oxidize neither methanol nor ethylene glycol. They do oxidize ethanol, but cannot be saturated by this substrate at concentrations as high as 2.5 M (Wagner et al., 1984). Livers from other mammalian sources, including horse (Lutstorf et al., 1970), mouse (Holmes et al., 1981), and monkey (Dafeldecker et al., 1981), all possess cathodic, pyrazole-sensitive enzymes that oxidize methanol and ethylene glycol as well as ethanol and other aromatic and aliphatic primary alcohols. Livers from monkeys have been shown to possess pyrazole-insensitive ADH isoenzymes that oxidize neither methanol nor ethylene glycol, but are active toward ethanol (Dafeldecker et al., 1981), and therefore resemble human class II ADH isoenzymes. Thus, it appears that activity toward methanol and ethylene glycol is an enzymatic characteristic of mammalian pyrazole-sensitive ADH isoenzymes.

The enzymes isolated in this study, ADH-2 and ADH-3, have characteristics consistent with class I human isoenzymes. First, like class I human isoenzymes, both ADH-2 and ADH-3 are cathodic and inhibited by 4MPz (K_I = 430 nM for ADH-2 and 335 nM for ADH-3, at pH 10). The human isoenzyme β_1 has a K_I for 4MPz 290 nM at pH 10; the K_I decreases to a minimum of 44 nM at pH 8.5.[4] Thus, the 4MPz inhibition of ADH-2 and ADH-3 is of the same level of magnitude as that of human β_1. The K_m values for ADH-2 and ADH-3 toward ethanol are more similar to those of class I human isoenzymes (1–2 mM; Wagner et al., 1983) than to class II (K_m = 20 mM for class II; Ditlow et al., 1984). Neither ADH-2 nor ADH-3 is capable of oxidizing methanol or ethylene glycol, but their pronounced inhibition by 4MPz clearly characterizes them as class I "like" alcohol dehydrogenase enzymes. ADH-3 has highest specificity for benzyl alcohol and 1-octanol, demonstrating a selectivity for hydrophobic alcohols. It has been previously reported (Levine & Haley, 1975) that embryonic quail liver ADH was active toward aliphatic alcohols up to eight carbons. Methanol and 1-propanol were reported to be poor substrates. A determination of K_m for ethanol was also made for an extract of embryonic chicken liver containing two ADH isoenzymes (Wilson et al., 1984). These workers found the K_m to vary from 1 to 5 mM, depending on the age of embryonic development. This variation may be a reflection of isoenzyme composition in their preparation.

Like mammalian liver ADH isoenzymes, k_{cat} values are relatively constant at pH 10, consistent with the mechanism established for horse liver ADH in which ethanol oxidation is limited by dissociation of NADH from the ADH·NADH complex (Brooks & Shore, 1971).

While the methodology used in this investigation did yield two highly purified forms of ADH from quail liver, we have concentrated our efforts on the ADH-3 enzyme from female livers. We do not know if the three cathodic ADH enzymes in male livers are the same as ADH-1, ADH-2, and ADH-3. Our interest in ADH-3 stems from the fact that it was the only form of the enzyme present in fully mature female livers and predominant in livers from younger laying females. Since it was the most predominant, it was obtained in greatest quantity. The correlation of the change in patterns of enzyme activity with the onset of maturity and egg production suggests the possibility that ADH-3 may be involved in the metabolism of metabolically functional complex alcohols. Whether this changing pattern is unique to birds is not certain. However, corresponding changes have not been reported for mammalian species. If correlation of the state of liver metabolism to the identity of ADH present is significant, then clearly, ADH-3

[4] Personal communication from Dr. Barton Holmquist, Center for Biochemical and Biophysical Sciences and Medicine, 250 Longwood Ave Harvard Medical School, Boston, MA 02115.

is the most significant form of ADH in adult female quail livers.

ACKNOWLEDGMENTS

We thank Dr. Mary Beck for her cooperation in providing quail used in this study.

Registry No. ADH, 9031-72-5; NAD, 53-84-9; NADH, 58-68-4; 4MPz, 7554-65-6; ethylene glycol, 107-21-1; methanol, 67-56-1; ethanol, 64-17-5; cyclohexanol, 108-93-0; 1-butanol, 71-36-3; 1-pentanol, 71-41-0; 1-octanol, 111-87-5; benzyl alcohol, 100-51-6; octanal, 124-13-0.

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