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NEW HUMAN LIVER ALCOHOL DEHYDROGENASE FORMS WITH UNIQUE KINETIC CHARACTERISTICS

Xavier Parés and Bert L. Vallee*

Center for Biochemical and Biophysical Sciences and Medicine
Harvard Medical School, Brigham and Women's Hospital,
Boston, MA 02115.

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SUMMARY: All adult and infant human liver homogenates studied thus far show two previously unreported forms of alcohol dehydrogenase on starch gel electrophoresis. Under the conditions employed, these forms migrate toward the anode and readily stain for pentanol but virtually not for ethanol oxidizing activity. In contrast, all human ADH isoenzymes identified previously are cathodic and react equally well with either substrate. These new ADH forms have been separated from the other known ones by DEAE-cellulose chromatography and are then purified on Agarose-hexane-AMP. Although the physical characteristics of the new anodic ADH forms are similar to those of the known human ADH isoenzymes, the former are not inhibited by 12 mM 4-methyl pyrazole, oxidize ethanol very poorly and appear to prefer longer chain alcohols as substrates.

INTRODUCTION: Alcohol dehydrogenase (Alcohol: NAD^+ oxidoreductase, EC 1.1.1.1) from adult human liver[1] exhibits multiple molecular forms that can be identified on starch gel electrophoresis (1,2). The electrophoretic patterns of isoenzymes change from liver to liver, seemingly dependent upon the genetic endowment and the health of the donors (3). The genetic model proposed by Smith et al. (2) is satisfactory for variations in LADH isoenzymes observed most frequently, but it

*To whom correspondence should be addressed.

[1]Abbreviations: LADH or ADH, human liver alcohol dehydrogenase; χ-ADH, temporary designation of the new forms of human ADH described here, migrating toward the cathode between pH 7.7 and 8.6.

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does not encompass either π-ADH or $ADH_{Indianapolis}$ (4,5) which have been described since it was first advanced. The present data underscore the complexity and diversity of this enzyme. They further suggest that existent knowledge is incomplete and insufficient to account for either the origin or physiological roles of all the various ADH forms.

Past investigations of LADH isoenzymes have been limited to those exhibiting cathodic mobility on starch gel electrophoresis at pH 7.7. Even the "anodic band" of Li and Magnes (6), now known as π-ADH (7), moves toward the cathode, although it has the least mobility of all the cathodic forms. Under conditions generally employed (pH 7.7 to 8.6), no isoenzymes had been described that actually move toward the anode.

We now report the detection, purification and initial characterization of two ADH forms that do migrate towards the anode on starch gel electrophoresis and that stain with pentanol but not with ethanol. So far these two anodic bands have been found in every human liver homogenate that we have examined. The prefix "χ" is here employed as a temporary designation until the nature of the relationship of these anodic to the known forms of human ADH is established. While their physical properties are remarkably similar, their substrate specificities and kinetic characteristics differ significantly.

METHODS: LADH activity was determined spectrophotometrically (8); units of activity are expressed as μmoles of NADH produced per min. Determinations of protein concentration, analytical ultracentrifugation, metal analysis and starch gel electrophoresis were performed as described previously (9,6).

Human liver samples were obtained within 12 h postmortem from adults who had succumbed to sudden, traumatic death and from 1 day to 4 months old children whose demise was the result of prematurity or cardiac disease. In a typical experiment a 180 g

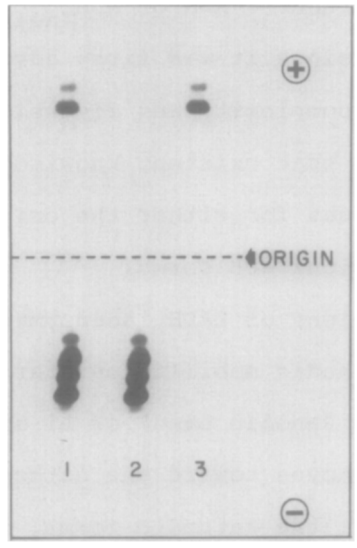

Figure 1 Starch gel electrophoresis of human liver alcohol dehydrogenase at pH 7.7 according to the method of Li and Magnes (6). The gel was stained for activity to oxidize pentanol: 1, homogenate supernatant of an adult human liver; 2, breakthrough of the DEAE-cellulose chromatography; 3, salt gradient eluted χ-ADH.

sample was homogenized in 250 ml of water at 4° and centrifuged for 2 h at 25,400 g and 4°. The supernatant was dialyzed against 10 mM Tris-Cl pH 7.9 and applied to a DEAE-cellulose column (DE-52, 5 x 40 cm) equilibrated with the same buffer. The column was washed successively with 1,700 ml 10 mM Tris-Cl, 1400 ml 10 mM Tris 25 mM NaCl, and 2000 ml of a linear gradient containing from 25 to 100 mM NaCl, all at pH 7.9. Fractions exhibiting ADH activity and containing the anodic forms as assessed by starch gel electrophoresis were combined, concentrated and applied to an Agarose-hexane-AMP column. The purified enzyme was eluted using an NADH gradient (0 - 7 x 10^{-5} M), in 0.1 M Tris-Cl, pH 8.6, 4°.

RESULTS AND DISCUSSION: Starch gel electrophoresis of the crude homogenates obtained from five adult and five infant livers all revealed the two previously unreported LADH forms, χ_1-ADH and χ_2-ADH. Both migrate toward the anode, in contrast to all other ADH isoenzymes reported and studied thus far (Figure 1). Their electrophoretic mobilities are consistent with a much lower pI for χ_1-ADH and χ_2-ADH than for all other isoenzymes. Moreover, they exhibit an important feature that differentiates them from

TABLE I

Purification of χ-ADH isoenzymes

	Total Activity[a]	Specific Activity	Yield
	Units	U/mg protein	%
Crude Homogenate	390	0.04	100
DEAE-Cellulose			
Cathodic ADHs[b]	270	0.15	69
χ-ADH[c]	10	0.03	2.5
Agarose-hexane-AMP	9	0.65	2.3

[a]The rate of ethanol oxidation was determined spectrophotometrically in a 3 ml assay with 0.5 M ethanol and 2.4 mM NAD in 0.1 M glycine, pH 10.0, at 25°.

[b]Breakthrough fraction.

[c]Gradient elution fraction.

the other forms: pentanol is effective in staining them for activity but ethanol is not.

Table I summarizes a typical purification. The χ-ADH isoenzymes are separated from the cathodic LADH forms by DEAE-cellulose chromatography. The latter enzymes are in the breakthrough fraction, while χ_1-ADH and χ_2-ADH elute in the sodium chloride gradient. Chromatography on Agarose-hexane-AMP further separates the χ-ADHs from other, extraneous proteins. The final enzyme preparation is exclusively χ-ADH as evaluated by starch gel electrophoresis (Figure 1), and by SDS-gel electrophoresis. From 180 g of adult liver a final amount of 13.8 mg (9 activity units in 0.5 M ethanol, pH 10) of the χ-ADH forms were obtained. A 20 μM solution of the purified χ-ADH forms has a half-life of 7 days when stored in 0.1M Tris-Cl pH 8.6 at 4°.

The purified mixture of both χ_1-ADH and χ_2-ADH (Figure 1), designated χ-ADH, was used throughout to determine the enzymatic characteristics. It has many of the physical properties of the non-π cathodic human ADH forms (9), of human π-ADH (8) and of horse liver ADH (10). Thus, its subunit molecular weight is approximately 40,000 by SDS-polyacrylamide gel electrophoresis, and based on ultracentrifuge analysis it is a dimer of 79 to 84 x 10^3 daltons, containing from 3.6 to 4.2 g-atoms of zinc per mol by atomic absorption spectrometry. It is inhibited by chelating agents such as 1,10-phenanthroline, dipicolinic acid and 8-hydroxyquinoline-5-sulfonic acid, demonstrating that the metal is essential for the catalytic activity (Table II).

Like all other molecular forms of the human enzyme, χ-ADH requires NAD^+ for the oxidation and NADH for the reduction reactions; neither $NADP^+$ nor NADPH is effective (Table II). Further, the catalytic rate of the enzyme increases as a function of increasing hydrophobic chain length of the substrate.

However, the kinetic properties of χ-ADH differ significantly from those of any other mammalian alcohol dehydrogenases known so far. Short chain (2 to 4 carbons) primary alcohols and aldehydes do not saturate this enzyme even when tested at concentrations as high as 1 M, either at pH 10.0 or at pH 7.5. The activity is directly proportional to ethanol concentration up to 2 M (Figure 2) at both pH values. Thus - up to this concentration of ethanol - saturation of the enzyme is not observed, though the binding constants for NAD(H) are quite analogous to those for the conventional ADH forms. At higher ethanol concentrations substrate activation becomes apparent. In contrast, the cathodic forms are saturated by 30 mM ethanol, and substrate inhibition appears at 100 mM (Figure 2). However,

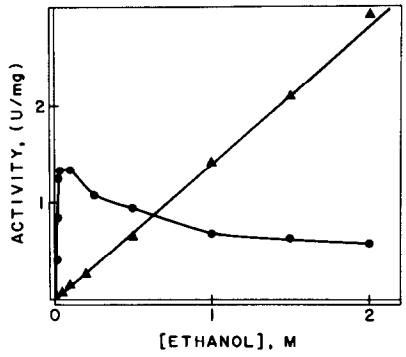

Figure 2 Variation of ethanol oxidizing activity as a function of substrate concentration. Activity was measured with 2.4 mM NAD^+ in 0.1 M glycine, pH 10.0, (▲—▲), χ-ADH; (●—●), electrophoretically unresolved ADH was obtained from a type 1-2 liver by the method of Lange and Vallee (9). This material contained all cathodic forms except π-ADH.

primary aliphatic alcohols containing more than 4 carbon atoms do saturate χ-ADH and K_m values can be calculated. These constants, e.g. 17 mM for hexanol and 0.8 mM for octanol at pH 7.5, are higher than the corresponding ones measured for the cathodic forms, 12 μM and 5 μM, respectively. When methanol is the substrate of χ-ADH, no activity is detected.

Another remarkable characteristic of the χ-ADHs is their complete failure to be inhibited by 12 mM 4-methyl-pyrazole (Table II). At this concentration, all the other ADH forms are inhibited, including even π-ADH which up to now has been the form thought to be least sensitive to 4-methyl-pyrazole (K_I = 2 mM) (8).

The data available so far indicate that all variants of ADH and the χ-ADH forms are related closely both in terms of their physical properties and their capacity to oxidize alcohols, identifying them as isoenzymes of LADH. However, their functional behavior is sufficiently distinctive to generate questions regarding appropriate terminology which would describe

TABLE II

Activity of χ-ADH in the Presence of Various Inhibitors or Coenzymes[a]

	Concentration (mM)	Relative[b] Activity (%)
[c]$NADP^+$	2.4	0
4-methyl-pyrazole	12	100
1,10-phenanthroline	10	16
[d]dipicolinic acid	10	13
[d]8-hydroxyquinoline-5-sulfonic acid	1	44

[a]Initial velocity was determined with 0.4 mM octanol and 2.4 mM NAD^+ in 0.1 M NaPi, pH 7.5.

[b]100% activity is the initial velocity, measured in the absence of any agent.

[c]No NAD^+ was added to the assay.

[d]Activity was measured by addition of NAD^+ to the reaction mixture after 120 minutes incubation.

their differences, at least when assuming ethanol to be the "standard" substrate of this group of enzymes. Thus, the χ-ADH forms are the first mammalian ADH's which oxidize ethanol very poorly, even at concentrations which can be considered toxic i.e. 20 mM. They would play a role in the oxidation of ethanol only when this substrate would be present at extraordinarily high concentrations, so that - save for π-ADH - all other known ADH forms would not only be saturated but even inhibited. Further, the catalytic efficiency of χ-ADH increases as the hydrophobic chain of the alcohol is elongated. While all LADH isoenzymes studied previously also prefer hydrophobic alcohols, short chain primary alcohols are also relatively good substrates for them

(11). χ-ADH forms are much more specific in this regard, since hexanol is the shortest primary alcohol with a K_m lower than 100 mM. The substrate specificity and kinetics of these forms are sufficiently distinctive to suggest that they may reflect different and characteristic biological function(s). However, the data available at this juncture do not as yet provide a structural basis which might underly the unique specificity of these new forms of alcohol dehydrogenases.

Over and above these remarkable kinetic properties, three additional, important features characterize the χ-ADH forms: they have been found in all of the human livers examined so far, in livers of premature infants, i.e. in early stages of development, and their concentration is high i.e. about 1 mg/10 g of tissue. Jointly, all of these properties suggest that the χ-ADH forms play an important and likely hitherto unknown role in the metabolism of long chain alcohols and aldehydes.

The identification and characterization of the χ-ADHs may well constitute an important advance in the understanding of human alcohol dehydrogenases and their potential enzymatic and metabolic roles. The model of Smith et al. (2) must now be reconsidered, since, at the time it was proposed, the very existence as well as the relative proportions of these new forms were unknown and could not be envisioned. It is apparent that the functional and structural features of the human alcohol dehydrogenase forms are much more complex than had been anticipated. Their detection may call for revisions of current concepts regarding the genetics, biochemistry and physiology governing human alcohol metabolism.

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