ISOLATION AND CHARACTERIZATION OF AN ANODIC FORM OF HUMAN LIVER ALCOHOL DEHYDROGENASE

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SUMMARY: A form of human liver alcohol dehydrogenase previously identified on starch gel electrophoresis as the anodic band (Li, T.-K. and Magnes, L.J. Biochem. Biophys. Res. Commun. 63, 202, 1975) has now been separated from the other molecular forms of the enzyme by affinity chromatography on 4-[3-(N-6-aminocaproyl)-aminopropyl]-pyrazole-Sepharose and purified to homogeneity on Agarose-hexane-AMP. Its physical properties are similar to those of other molecular forms already known, suggesting that they may be related. In contrast to other forms, the anodic species is inactive towards methanol, and its K_M for ethanol is as much as 100 times that of the other forms. This anodic form of alcohol dehydrogenase may contribute significantly to alcohol elimination in man, particularly at high alcohol concentrations when the other enzyme species are saturated.

INTRODUCTION: Human liver alcohol dehydrogenase exhibits multiple molecular forms (1-3). A genetic model for their formation as isoenzymes has been proposed which satisfactorily accounts for the multiplicity observed in most autopsy liver specimens (4). However, subsequent studies have demonstrated that both the specific activity and the degree of multiplicity of this enzyme, as identified by starch gel electrophoresis, depend upon the premortem history as well as the subsequent handling of the specimens (5). Thus biopsy and most autopsy material obtained from otherwise healthy individuals within 12 hours after accidental death exhibit high activity and contain a molecular form of the enzyme, previously unrecognized. This form has been designated "the anodic band or species" because of its distinctive electrophoretic behavior on starch gels (5).

Recently, an affinity chromatography method has been developed for

TABLE I. Purification of the Anodic Form.

	Step	Total Activity units	Specific Activity units/mg	Yield %
1	Supernatant	315	0.06	100
2	DEAE-Cellulose	182	0.19	58
3	CapGapp-Sepharose			
	a) Anodic form	30	0.03	10
	b) Other forms	95	2.5	30
4	Agarose-hexane-AMP	20	1.3	6

Alcohol oxidizing activity was determined spectrophotometrically in a 3 ml assay at 25° with 0.1 M glycine, pH 10.0, 33 mM ethanol and 2.4 mM NAD^+.

the specific and rapid purification of alcohol dehydrogenase (6). It utilizes an immobilized, 4-substituted derivative of pyrazole, 4-[3-(N-6-aminocaproyl)-aminopropyl]-pyrazole-Sepharose (CapGapp-Sepharose). We have now employed this technique to separate the anodic species from all other molecular forms, purified it to homogeneity and examined its properties.

METHODS: Alcohol dehydrogenase activity was determined spectrophotometrically (6), expressed as μmoles of NADH produced per min. Protein determinations, starch gel electrophoresis, sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, zinc analyses, analytical ultracentrifugation and amino acid analyses were performed as described previously (5,7).

Liver specimens were obtained at autopsy from apparently healthy individuals who had died suddenly from physical trauma. Liver samples for purification were selected for high activity and the presence of the anodic form as identified by starch gel electrophoresis (5). Samples, 100 g, were homogenized in 250 ml of water at 4° and centrifuged for 30 min at 10^5 x g. The supernatant was subjected to chromatography on DEAE-cellulose and CapGapp-Sepharose according to Lange et al (7). The active effluent fractions were examined by starch gel electrophoresis and those containing the anodic band were precipitated with 70% saturated ammonium sulfate at pH 8.2 and gel filtered on Sephadex G-25 in 5 mM Na phosphate, pH 7.5. The anodic form was then purified to homogeneity by column chromatography on Agarose-hexane-AMP (PL Biochemicals, Type 2). The enzyme was bound to 50 ml of resin equilibrated in 0.1 M Tris-Cl, pH 8.6 at 4°, and eluted with a 300 ml linear gradient of 0 to 7.2 x 10^{-5} M NADH in buffer.

RESULTS AND DISCUSSION: Table I and Figure 1 show the results of a typical purification experiment. The total alcohol dehydrogenase activity in the

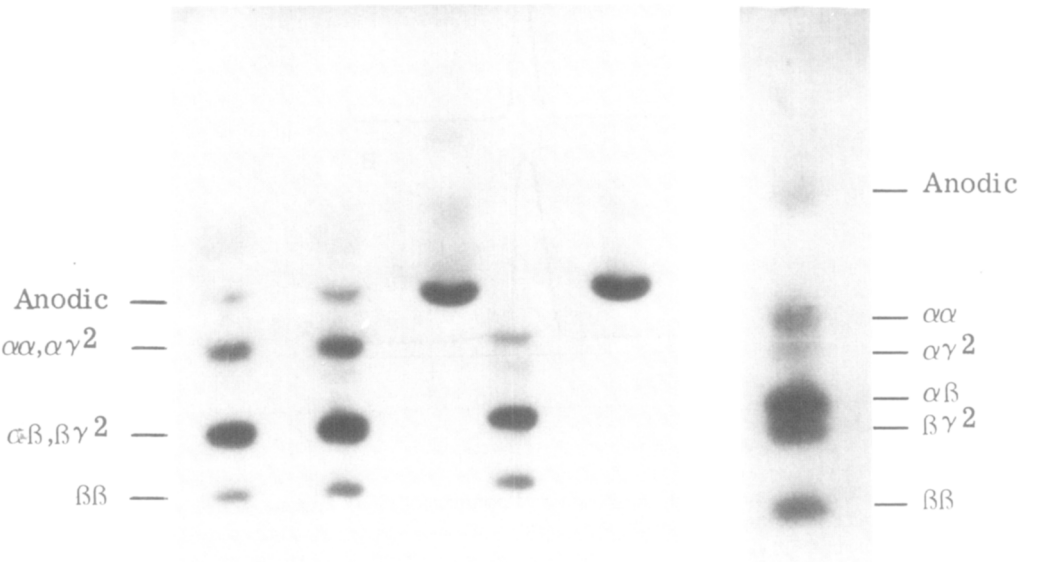

Figure 1 Starch gel electrophoresis of human liver alcohol dehydrogenase. Electrophoresis was performed at pH 7.7 (samples 1-5) and 8.6 (sample 6), and stained for ethanol oxidizing activity (5). Samples 1 and 6 are homogenate supernatants, and samples 2-5 correspond to purification steps 2, 3a, 3b, 4 in Table I. Samples 1, 2, 4 and 6 contain 0.04 units of activity and samples 3 and 5 contain 0.01 units.

homogenate supernatant of 100 g of liver is 315 units. Figure 1 (samples 1 and 6) shows the molecular forms of the enzyme present in this supernatant as examined by starch gel electrophoresis at both pH 7.7 and 8.6. The anodic form is the activity band with the least electrophoretic mobility (5), while the remaining bands are identified as the isoenzymes characteristic of phenotype ADH_3 2 according to the nomenclature of Smith et al (4).

Approximately 58% of the total activity but all of the molecular forms are recovered from the DEAE-cellulose purification step. After separation on CapGapp-Sepharose, the fractions exclusively containing the anodic band, (Figure 1, sample 3) comprise 30 units or 16% of the activity applied to the column. The remaining fractions contain all of the other molecular

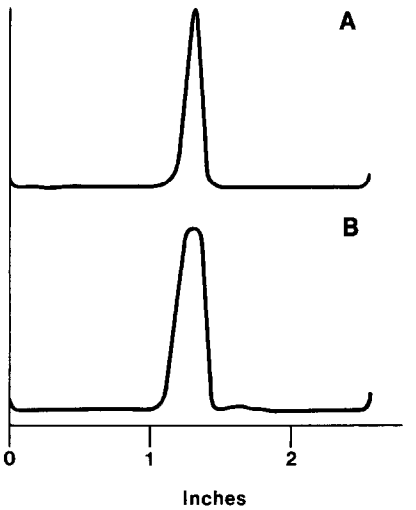

Figure 2 SDS-polyacrylamide gel electrophoresis of human and horse liver alcohol dehydrogenases. The anodic form, 20 μg (panel A) and horse liver alcohol dehydrogenase (Boehringer Mannheim GmbH), 30 μg (panel B), were treated under identical conditions (7). The origin is at the left. Gels were scanned with a Photovolt Densicord at 545 nm.

forms originally present (Figure 1, sample 4) and 95 units of activity. Further chromatography of the anodic species on Agarose-hexane-AMP purifies it to homogeneity, as evidenced by SDS-polyacrylamide gel electrophoresis (Figure 2A). From 100 g of liver, 15 mg of the anodic form is obtained with an overall yield of 6% and a specific activity of 1.3 units/mg. The anodic form is labile, as observed previously (5). In the purified state, it exhibits a half-life of 24 h in 5 mM Na phosphate, pH 7.5, 4°. However, addition of 10^{-2} M ethanol effectively stabilizes it for several weeks.

The purified anodic species has many physical and kinetic properties in common with those previously described for mixtures of human liver alcohol dehydrogenase forms (1,3,5,7-10). Its subunit molecular weight from SDS-polyacrylamide gel electrophoresis (Figure 2A) is approximately 42,000, identical to that of horse (Figure 2B) and human liver alcohol dehydrogenase prepared by CapGapp-Sepharose affinity chromatography (7). It has an amino

TABLE II. Kinetic Constants of the Anodic Form

	pH	K_M (mM)	k_{cat} $(min)^{-1}$
Ethanol	10.0	140	610
Ethanol	7.5	18	42
Butanol	7.5	0.14	42
Pentanol	7.5	0.036	40
3-Pyridylcarbinol	7.5	0.24	41
NAD^+	10.0	0.040	--
NAD^+	7.5	0.026	43

Alcohol oxidizing activity was determined in 0.1 M Na phosphate, pH 7.5, or 0.1 M glycine, pH 10.0, at 25°. When alcohol concentration was varied, NAD^+ was 2.4 mM, and when NAD^+ was varied, ethanol was 100 mM. Kinetic constants were calculated from linear or second order polynomial regression analysis of (A)/v vs. (A) plots and k_{cat} is expressed as moles/min/85,000 daltons.

acid composition similar to that of previous preparations (7), and is a dimer of 78 to 85 x 10^3 daltons, based on ultracentrifugation and amino acid analysis, respectively. It contains approximately 4 atoms of zinc per molecule of protein. Similar to the other molecular forms of the enzyme, this form has a K_M for NAD^+ of 26 μM at pH 7.5 and 40 μM at pH 10.0 (Table II) and is not active with $NADP^+$. It has a pH optimum above 10.0 for ethanol oxidation and exhibits broad substrate specificity (Table II). Increasing the primary alcohol chain length from 2 to 5 carbons decreases K_M, from 18 to 0.036 mM at pH 7.5, while k_{cat} remains relatively constant at 40-42 min^{-1} (Table II). Aromatic alcohols such as 3-pyridylcarbinol are also oxidized efficiently. These similarities suggest that the anodic form is related to the other enzyme forms. However, the current genetic model for their formation as isoenzymes does not predict any molecular form with an electrophoretic mobility less than that of the αα isoenzyme (4). Hence, this model must be reevaluated.

The purified anodic form exhibits certain kinetic properties which are strikingly different from those described for previous preparations of the human enzyme. Thus it does not oxidize methanol and glycerol (100 mM). The K_M for ethanol, which is 18 mM at pH 7.5 and 140 mM at pH 10.0 (Table II), is 20 to 100 times greater than that reported for impure preparations or mixtures of isoenzymes (1,3,5,7-10). Furthermore, the k_{cat} of 610 min^{-1} for ethanol at pH 10.0 is higher than the values of 180 and 240 min^{-1} recently reported for mixtures of isoenzymes (3,7). However, this extrapolated activity of 610 min^{-1} is never actually attained because substrate inhibition occurs at ethanol concentrations of 130 mM and above.

As a result of the lability of the anodic form, it is difficult to quantify the contribution of this species to the total alcohol dehydrogenase activity in liver by starch gel electrophoresis alone. However, separation on CapGapp-Sepharose of a mixture of all of the molecular forms originally present in the homogenate supernatant indicates that the anodic form accounts for not less than 16% of total activity. Consequently, the detection and characterization of this anodic form of alcohol dehydrogenase constitutes an important new finding which is likely to contribute toward the understanding of individual and racial variations in alcohol elimination rates. Its high K_M for ethanol suggests that it may play a significant role in alcohol oxidation at ethanol concentrations which are saturating for the other forms.

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References:

1. Blair, A.H., and Vallee, B.L. (1966) Biochemistry, 5, 2026-2043.
2. Schenker, T.M., Teeple, L.J., and von Wartburg, J.P. (1971) Eur. J. Biochem., 24, 271-279.
3. Pietruszko, R., Theorell, H., and DeZalenski, C. (1972) Arch. Biochem. Biophys., 153, 279-293.

4. Smith, M., Hopkinson, D.A., and Harris, H. (1971) Ann. Hum. Genet., Lond., 34, 251-271.
5. Li, T.-K., and Magnes, L.J. (1975) Biochem. Biophys. Res. Commun., 63, 202-208.
6. Lange, L.G., and Vallee, B.L. Biochemistry (in press).
7. Lange, L.G., Sytkowski, A.J., and Vallee, B.L. Biochemistry (in press).
8. Li, T.-K. and Theorell, H. (1969) Acta. Chem. Scand., 23, 892-902.
9. Pietruszko, R. (1975) Biochem. Pharm. 24, 1603-1607.
10. von Wartburg, J.P., Bethune, J.L., and Vallee, B.L. (1964) Biochemistry, 3, 1775-1782.