CRYSTALLIZATION OF A SEPARATE STEROID-ACTIVE LIVER ALCOHOL DEHYDROGENASE

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Dalziel (1958) reported the presence of an ethanol-active subfraction, more acidic than the main horse liver alcohol dehydrogenase (LADH), in his crude LADH sample crystallized from phosphate buffer containing 6% ethanol. In 1963 (unpublished observation), we carried out recrystallizations of LADH both from ordinary and ethanol-containing phosphate buffer. In the mother liquor from these crystallizations we found, on free moving boundary electrophoresis, a second ethanol-active subfraction of more basic character than the main LADH component. After subsequent removal of crystals from the original material containing a few percent of the basic fraction, the relative concentration of the basic subfraction increased to 25% of the total soluble enzyme in the supernatant. A typical electrophoresis pattern of such a supernatant is shown in Fig. 1.

The basic subfraction isolated by electrophoresis was found to have two coenzyme-binding sites and four zinc atoms per molecule, just as the main LADH component (Åkeson, 1964), but only half the activity when tested with NAD^+ and ethanol. Amino acid analyses revealed a remarkable similarity between the subfraction and the main component. We therefore concluded that the subfraction was a slightly modified LADH[xx]. The steroid activity found and studied in LADH preparations by Ungar (1960), Ungar et al. (1965), Waller et al. (1965) and Graves et al. (1965 a;b) was not ascribed by these

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[xx] It may be of interest, however, to report that the well-known property of earlier LADH preparations to stubbornly retain alcohol against all attempts at removal by dialysis was found to be entirely due to the basic subfraction. The main fraction of LADH can easily be dialyzed free from alcohol.

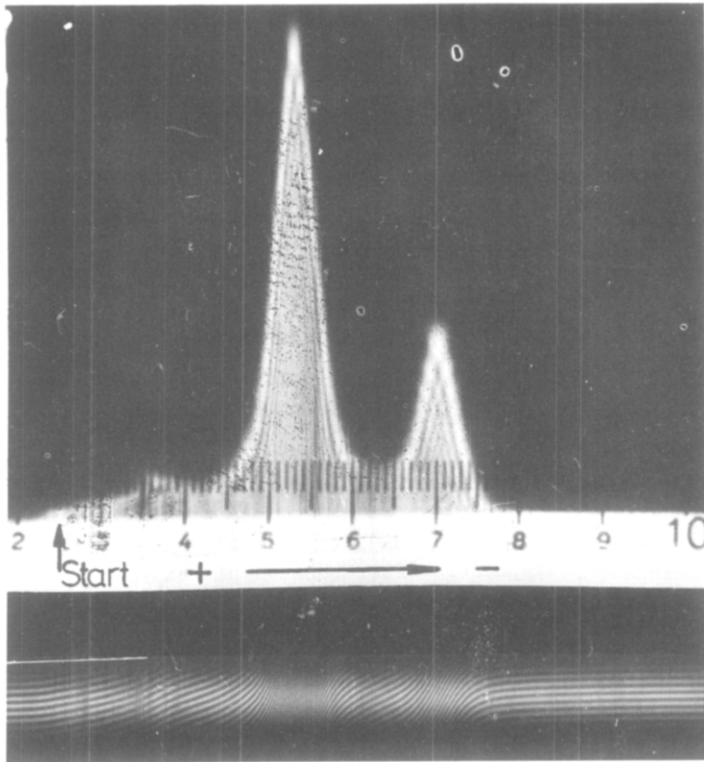

Fig. 1. Electrophoresis pattern of supernatant from crystallization of unresolved LADH. Phosphate buffer, pH 6.0, I = 0.1, 0°.

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workers to this basic subfraction. H.J. Ringold in a letter of Dec. 3, 1965 kindly drew our attention to the fact that commercial LADH preparations contain various amounts of steroid-active fractions that could be separated from LADH by gel electrophoresis. These results were recently published (Pietruszko et al., 1966). In the meantime we have carried out a systematic reinvestigation of our isolation methods. This led to the conclusion that our formerly isolated basic subfraction ($LADH_S$) is solely responsible for the steroid activity, whereas the main LADH ($LADH_E$) is free from such activity. $LADH_S$ was now isolated, crystallized and found nearly electrophoretically homogeneous (see Fig. 2).

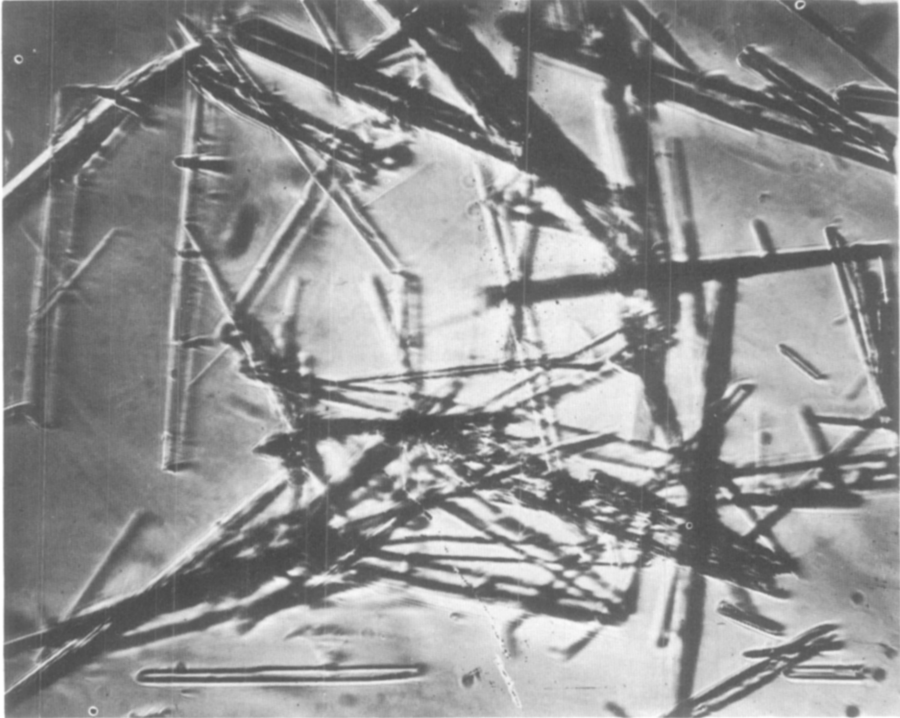

Fig. 2. Crystals of $LADH_S$ from 25% ethanol in phosphate buffer, pH 8.0, I = 0.02 (120 x magnification).

The amino acid composition of $LADH_E$ and $LADH_S$ are indistinguishable within the limits of error, except for possibly threonine, as is also the sedimentation constant. The difference in electrophoretic properties is presumably due to a difference in the content of amide nitrogen. This could result in conformational changes and hence a variation in substrate specificity.

Experimental and Results

The hydroxysteroid dehydrogenase activity was assayed by the rate determination of the reduction of NAD^+ (200 μM) by 3β-hydroxy-5β-cholanic acid (3β-OHCA) (10 μM) as a standard substrate in 0.1 M glycine buffer,

pH 9.5 at 23.5° by means of a sensitive fluorometer (Theorell _et al._, 1955). The reaction rate thus determined was converted to enzyme concentration by a factor determined by crystalline $LADH_S$ sample. The activity towards ethanol was assayed according to the routine procedure (Dalziel, 1957).

In order to avoid possible modification of the enzyme during the usual purification procedures, we devised a milder preparation method. Our attempts to prepare hydroxysteroid dehydrogenase were made with water extracts (pH adjusted to 7.0 with ammonia) from fresh horse liver. These extracts were immediately precipitated with 3.4 M ammonium sulfate at pH 7.0 and 5°. The ammonium sulfate precipitate, dissolved in phosphate buffer, pH 7.0, I = 0.1, was fractionated by the addition of solid ammonium sulfate. Ninety per cent of the total hydroxysteroid dehydrogenase activity was recovered in the fractions obtained between 2.0-3.2 M ammonium sulfate together with the majority of ethanol dehydrogenase activity. The combined active fractions were dialyzed against phosphate buffer, pH 6.0, I = 0.05, and adsorbed on a carboxymethyl-cellulose (CMC) column equilibrated with the same buffer. From this column were at least three distinct LADH fractions eluted with phosphate buffer of I = 0.05 and stepwise increment of pH from 6.0 to 7.3.

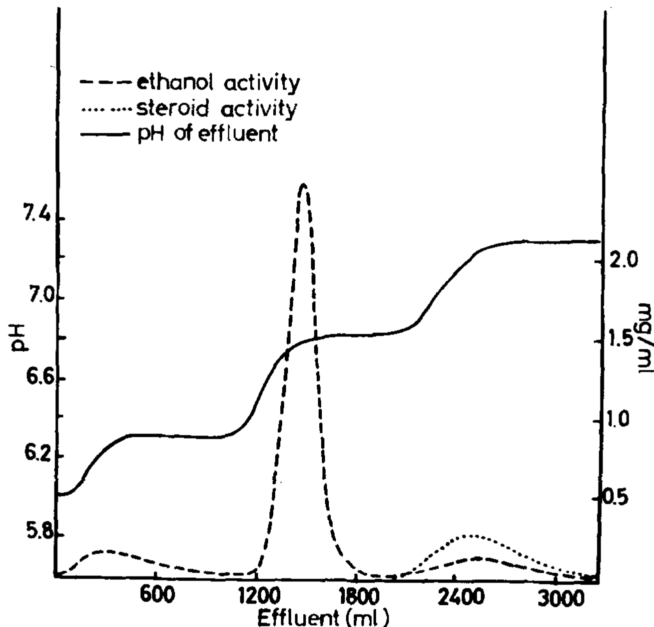

Fig. 3. CMC column elution diagram of LADH from the fraction obtained between 2.0 and 3.2 M ammonium sulfate. Approx. 500 mg LADH, calculated from $LADH_E$ activity was subjected to the chromatography.

As recorded in Fig. 3, the first active fraction was eluted at pH 6.0-6.3. Whether this fraction is active also on other substrates than ethanol is now being investigated. The second, main LADH fraction came out as a sharp peak at pH 6.3-6.8. This was followed by a third active fraction which was eluted at pH 6.8-7.3. As indicated in the same figure, steroid activity was located only in the third fraction, whereas ethanol activity was common to all three fractions. The activity of the last peak assayed with ethanol is accounted for by the activity of $LADH_S$ towards ethanol, which was shown to be about half of that of $LADH_E$. The concentration of the first active zone does not reflect the real one in the original liver extracts, since we found that the fraction was enriched in the precipitate produced by lower than 2.0 M ammonium sulfate concentration. Crystalline $LADH_S$ of a high degree of purity was prepared from the chromatographically isolated $LADH_S$ fractions by subsequent precipitation with 35% (v/v) ethanol at pH 8.0 and -15°, passage through a diethylaminoethyl-cellulose column equilibrated with phosphate buffer, pH 7.8, I = 0.006, to remove hemoglobin by adsorption, and triple crystallization from phosphate buffer, pH 8.0, I = 0.02, containing 25% ethanol. The average yield of final $LADH_S$ crystals from 5 kg of liver was 100-300 mg, approximately 1/10 of the yield of $LADH_E$. $LADH_E$ crystals were prepared from the main $LADH_E$ fraction by ethanol precipitation and rechromatography on CMC. The crystals of $LADH_S$ were more fragile than $LADH_E$ crystals, and showed higher solubility both in ordinary and ethanolic phosphate buffer, pH 7.0[xxx]. The activity towards ethanol, as previously observed with the electrophoretically isolated $LADH_S$ samples, was again about half compared with $LADH_E$. $LADH_S$ does not seem to be an artifact produced during the isolation procedure, but is most probably one of the physiologically functioning forms of LADH.

The crystallized $LADH_S$ showed a single and monodisperse boundary during analytical sedimentation in a Spinco Model E apparatus in phosphate buffer, pH 7.0, I = 0.1 containing 1% NaCl, but two small acidic components of less than 10% of the total protein were revealed on electrophoretic analysis at pH 7.0-9.0. The isoelectric point of $LADH_S$ was estimated from the electrophoretic mobility of the main boundary associated with hydroxy-steroid dehydrogenase activity to be around 10 (in borate or glycine buffer, I = 0.1) compared with 6.8 (in phosphate buffer, I = 0.1) reported for $LADH_E$

[xxx]The fact that $LADH_S$ is more soluble than $LADH_E$ could explain why the $LADH_S$ fraction was not detected on electrophoretic analysis of Dalziel's crude LADH crystallized from alcohol concentrations as low as 6% (Dalziel, 1958).

(Dalziel, 1958). Judging from our S_{20}^{o} value (4.9 S) of $LADH_S$ extrapolated to infinite dilution determined from our preliminary sedimentation experiments, it is suggested that the molecular weight of $LADH_S$ is nearly the same as $LADH_E$ (83300) determined under essentially the same conditions, although the concentration dependence of the sedimentation constant of the $LADH_S$ seems to be less than that reported for $LADH_E$ (Ehrenberg et al., 1958). $LADH_S$ was found to have nearly the same absorption at 280 mμ as was found with $LADH_E$ ($\varepsilon_{cm}^{1\%}$ = 4.60, unpublished data). Our amino acid analyses of crystalline $LADH_S$ samples revealed it to be probably identical with that of $LADH_E$, except for differences in amide nitrogen (higher in $LADH_S$) which may explain the difference in electrophoretic behaviour, and possibly threonine (-6% in $LADH_S$), as indicated in Table 1.

Table I

Amino acid composition of $LADH_S$ and $LADH_E$.

Amino acid	Residues/Molecule[1] $LADH_S$	$LADH_E$
Aspartic acid	54	54
Threonine	47	50
Serine	53	53
Glutamic acid	65	66
Proline	45	46
Glycine	83	83
Alanine	62	61
Valine	84	82
Methionine	19	19
Isoleucine	49	49
Leucine	55	54
Tyrosine	8	8
Phenylalanine	37	38
Tryptophan[2]	4	4
Lysine	63	62
Histidine	15	14
Arginine	24	25

[1]Based on a molecular weight of 83300.

[2]Calculated from absorption at 280 mμ and tyrosine content.

Discussion

Differences in the number of amide groups might be enough to cause difference in the secondary and tertiary structure even if the amino acid sequence were unchanged. The fact that $LADH_S$ appears to contain more glutamine and/or asparagine residues is particularly reminiscent of the recent observations made in our laboratory on multiple components of lacto-peroxidase (Carlström, 1966) and beef heart cytochrome c (Flatmark, 1966)

where hydrolysis of amide groups is strongly suggested as the primary cause of the observed microheterogeneity. In agreement with our previous observations it was confirmed that $LADH_S$, just as $LADH_E$, contains two coenzyme-binding sites per molecule, as indicated by titration with NAD^+ in the presence of excess 4-bromo-pyrazole[xxxx], assuming the same molecular weight as that determined for $LADH_E$.

Table II

Summary of turnover number, K_M and K_I values of $LADH_S$ and $LADH_E$. All values are averages of several experiments carried out at pH 9.5, 23.5° with 200 μM NAD^+.

LADH	$LADH_S$		$LADH_E$
substrate	3β-OHCA	ethanol	ethanol
Turnover number (sec^{-1}, per coenzyme-binding site) [substrate] → ∞	0.26	2.6	5.0
K_M (mM)	0.002	0.60	0.60
K_I (μM) for pyrazole	1.7	0.07	0.06
3α-OHCA	2.4	(540)	(1340)

Table II summarizes some of the kinetic properties of $LADH_S$ and $LADH_E$ determined at pH 9.5. It was also shown that methanol, isopropanol and tertiary butanol were extremely poor substrates, as was the case with $LADH_E$. On the other hand, our previous observation that $LADH_S$ was about half as active as $LADH_E$ towards ethanol per coenzyme-binding site was not only confirmed but shown to be valid also for C_3-C_5 aliphatic normal alcohols and cyclohexanol. It is interesting to notice from Table I that 3α-OHCA is strongly competitive with 3β-OHCA in $LADH_S$, but practically inactive

[xxxx] In a survey of different halogen substituted pyrazole derivatives, the 4-bromo-pyrazole was found to be a potent competitive inhibitor towards alcohol with K_I = 1/10 of the K_I for pyrazole. This derivative is consequently more suitable for use in titrating the coenzyme-binding site in view of the apparent greater dissociation of $LADH_S$•DPN•pyrazole complex as compared with $LADH_E$ (Theorell and Yonetani, 1963).

as an inhibitor when ethanol is used as substrate for either $LADH_S$ or $LADH_E$. The binding sites for ethanol and for 3β-OHCA in $LADH_S$ therefore must be different and independent of each other, as suggested by Pietruszko et al., (1966). Furthermore, it should be noticed that the binding sites for ethanol in $LADH_E$ and $LADH_S$ must be similar as judged from the similarity of the K_I for pyrazole (0.06-0.07 μM) in both, whereas the K_I is much higher for $LADH_S$ (1.7 μM) when 3β-OHCA is used as substrate.

Since our attempts to separate the hydroxysteroid activity of $LADH_S$ from its ethanol activity have hitherto been unsuccessful, it seems reasonable to assume the occurrence of two different binding sites for the two substrates in the same molecule. Our preliminary determinations of the dissociation constants between $LADH_S$ and coenzymes gave quite different values from those determined with $LADH_E$. Kinetic studies of the mechanism of the $LADH_S$ reaction with hydroxysteroids as well as ethanol are in progress, particularly with the view of testing their conformity with the Theorell-Chance mechanism (1951) that is valid for the $LADH_E$ system.

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