THE EFFECT OF 2-MERCAPTOETHANOL (ME) AND EDTA ON THE SUB-SPECIES STRUCTURE OF HORSE LIVER ALCOHOL DEHYDROGENASE (LADH) IN 8 M UREA*

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While incubation of LADH in 8 M urea gives rise primarily to sub-units of molecular weight 40,000, addition of ME and EDTA yields sub-species of molecular weights between 36,000 and 20,000 as determined by time-lapse ultracentrifugation and sodium dodecyl sulphate (SDS)-electrophoresis.

Horse liver alcohol dehydrogenase is a metalloenzyme containing 3.6-4 atoms of zinc per mole of protein (1,2). These may be differentiated into two distinct classes based on their physiochemical properties (3): two of the zinc atoms are catalytically active and the others apparently involved in structural stabilization (4).

LADH, of molecular weight 80,000 (4), is a two subunit enzyme, on the basis of enzymatically active metal content (3), binding of coenzyme (5), isolation of active cysteinyl peptides (6), sequence analysis (7,8), crystallographic studies (9) as well as dissociation into two subunits in 0.05 M SDS (10), in 5 or 6 M guanidine hydrochloride in the presence of reducing agents (11,12) or in 7 or 8 M urea (13,4).

However, upon removal of a fraction or of all of the zinc by addition of 0.1 M ME and/or 0.01 M EDTA, dissociation in 8 M urea apparently proceeds further to smaller species of a weight average molecular weight of approximately 20,000,

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as determined by low speed sedimentation equilibrium studies (4), or light scattering measurements (14). Moreover, electrophoresis and chromatography both identify up to 9 or even 12 isoenzymes (15,16).

To further investigate the problem, we have employed ultracentrifuge time lapse photography, a technique particularly suitable for comparative studies of protein structure in different solvents. This, as well as high speed sedimentation equilibrium studies, show that treatment of LADH with 8 M urea, 0.1 M ME and 0.01 M EDTA, results in sub-species of LADH of molecular weight below 40,000.

In confirmation of these findings, electrophoresis on polyacrylamide gels in the presence of SDS (18), visualizes the separation of the different components of this system and allows determination of their approximate molecular weights.

MATERIALS AND METHODS: Ultrapure urea and guanidine hydrochloride were obtained from Mann Research Laboratories, Inc. Other reagents of the highest purity available were used without further purification.

Crystalline LADH obtained from Boehringer Mannheim Corporation was dialyzed for three days against 0.1 M sodium phosphate buffer, pH 7.5, 4°. Protein concentration was determined from the absorbance at 280 mμ based on an absorptivity of 0.455 mg^{-1} cm^2 (19). Dissociation of LADH was achieved through dilution of the enzyme solution with the appropriate solvents. To satisfy the conditions of Casassa and Eisenberg (20), the material was dialyzed against four changes of 100 volumes of the relevant solvent for at least four days at 23°. These solvents contained either 8 M urea, 0.1 M phosphate pH 7.5, with or without 0.1 M ME and/or 0.01 M EDTA or 5 M guanidine hydrochloride, 0.1 M ME, 0.05 M Tris pH 7.5 with or without 0.1 M KCl and 0.01 M EDTA. For ultracentrifuge time-lapse photography (17), protein concentrations of 0.7 to 5 mg/ml were employed. Some high speed sedimentation equilibrium runs were also performed (21).

For SDS-electrophoresis, after dialysis against the relevant dissociating solvent (above), the LADH solution (5 mg/ml) was re-dialyzed against 0.01 M

sodium phosphate buffer, pH 7.0, 0.1% in SDS and ME, usually against 4 changes of 100 volumes for 30 to 72 hours. About 0.1 mg of protein was applied to 10% polyacrylamide gels and electrophoresis was carried out according to Weber and Osborn (22), either in 0.1 M sodium phosphate buffer, or to decrease the time of electrophoresis at half this molarity or in 0.1 M H_3PO_4, adjusted to pH 7.0 with Tris, for the gel buffer, being diluted 1/1 with water to yield the electrophoresis buffer. After electrophoresis, the gels were fixed in 12% trichloroacetic acid for 1-2 hours, and stained at least three hours.

RESULTS AND DISCUSSION: Time-lapse photography is the method of choice for ultracentrifugation in solvents containing high concentrations of guanidine hydrochloride or urea (17). It allows the unambiguous determination of the reduced refractometric molecular weights of macromolecules at low speed sedimentation equilibrium, and neither assumptions nor ancillary experiments.

When higher concentrations (2 ~ 5 mg/ml) of LADH were dialyzed against ME, EDTA and 8 M urea, a weight average molecular weight of 29,600 ± 2,500 was obtained (Figure 1A). However, at lower concentrations of enzyme, 0.7 to 1.5 mg/ml, two slopes were found corresponding to molecular weights of 23,000 ± 1,000

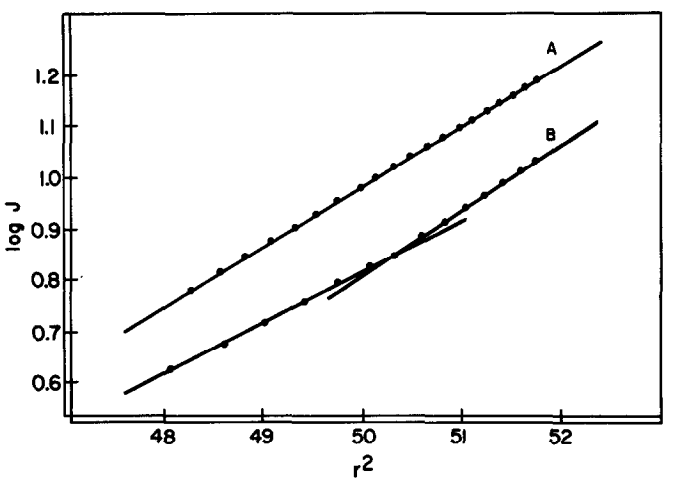

Figure 1. Molecular weight determination by ultracentrifuge time-lapse photography. LADH in 8M urea, 0.1M ME, 0.01M EDTA, 0.1M KCl, 0.1M phosphate, pH 7.5. Meniscus depletion at 42,040 rpm. Equilibrium at 14,290 rpm. Temperature: 20°. A: 2.8 mg/ml in a 12 mm cell. B: 0.7 mg/ml in a 30 mm cell.

and 29,000 ± 1,500, respectively (Figure 1B). High speed sedimentation equilibrium studies also gave correspondent molecular weights of 25,600 ± 800 and 30,700 ± 700, depending on the protein concentration used.

However, the enzyme dialyzed against 5 M guanidine hydrochloride, 0.1 M ME, 0.05 M Tris pH 7.5, with or without 0.1 M EDTA, yielded a single slope corresponding to a molecular weight of 44,300 ± 700 in agreement with results, in the same solvent of Green and McKay (11) or in urea (4,13).

SDS-electrophoresis of LADH dialyzed against 8 M urea alone, and then made 0.1% in SDS, resulted in a species of 40,000 daltons and two others of higher molecular weight.[1] Control LADH, at the same concentration, treated only with SDS and ME gave the 40,000 species, as expected. In contrast, addition of ME and EDTA to the incubation mixture yields sub-species of lower molecular weights. Thus, lower molecular weight species have been detected, so far, only after incubation in ME, EDTA and 8 M urea.

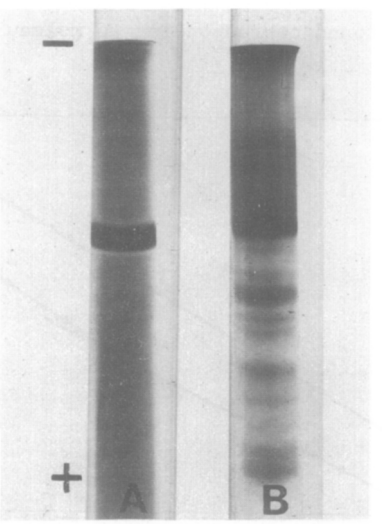

Figure 2. SDS-electrophoresis of LADH. A: Control enzyme treated only with SDS and ME. B: enzyme treated with 8M urea, ME and then dialyzed against SDS and ME. For details, see "Materials and Methods."

[1] Probably formed by aggregation in the absence of ME.

Figure 2 compares results of SDS-electrophoresis of LADH, after dialysis against 8 M urea, 0.1 M ME and/or 0.01 M EDTA (Figure 2B) with those of the control enzyme (Figure 2A). After exposure to urea-ME several components are visualized with approximate molecular weights of 40,000, 27,000, 22,000, 15,000 and 10,000 (Figure 3). Some weaker bands correspond to 36,000, 30,000, 20,000, 17,000 and 10,000 daltons.[2]

These results demonstrate that under the conditions described treatment of LADH by 8 M urea, 0.1 M ME and 0.01 M EDTA generate the multicomponent system invariably detected by these methods, being consistent with a disjuction of the 40,000 subunits into several smaller species.

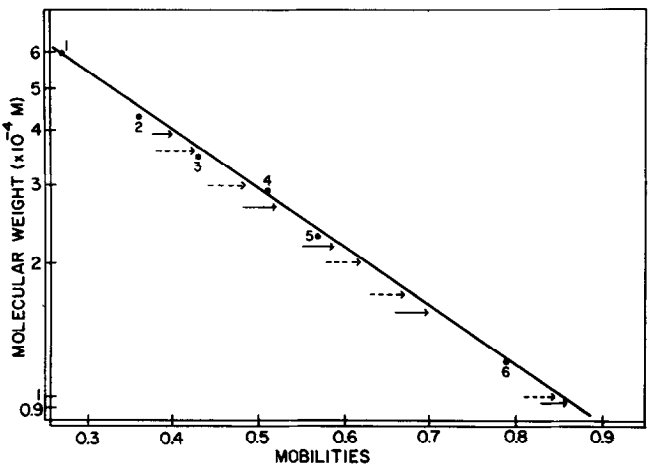

Figure 3. Typical semi-log plot of molecular weights versus mobilities for the determination of the molecular weights of dissociation products of LADH. The mobilities of standard proteins ( ) are averages obtained from duplicate gels. Those of LADH dissociation products (arrows, or dotted arrows for weak bands) are averages of values from four gels. The following standard proteins, listed with their polypeptide chain molecular weights, were used: 1) catalase MW 60,000 (Calbiochem.); 2) crystalline ovalbumin, MW 43,000; 3) pepsin, twice crystallized, MW 35,000; 4) carbonic anhydrase, MW 29,000; 5) trypsin, twice crystallized, MW 23,300 (all from Worthington); 6) horse heart cytochrome c, twice crystallized, MW 12,000, was a gift of Dr. David D. Ulmer.

[2]While the reliability of this technique when used in the determination of molecular weights of 10,000 or less remains to be ascertained, the different results in electrophoresis after treatment with urea alone and with the addition of ME and EDTA rules out the presence of electrophoretic artifacts (23).

Such data give new insight regarding the structure of the enzyme as detected by sedimentation data. Whereas 8 M urea dissociates LADH into two subunits of 40,000 daltons, ME and/or EDTA results in further dissociation into several smaller species, accounting for the lower weight-average molecular weights cited here and by Drum et al. (4).

In a substantial number of instances details of primary or quaternary protein structure are revealed in response to proteolytic or chemical cleavage which were not apparent either from amino acid sequence or X-ray diffraction data of the native material either for single chain proteins, such as ribonuclease (24), DNA-polymerase (25,26), or ovalbumin (21) or for multichain proteins, such as myosin (28).

Chemical cleavage of LADH, of yet unknown nature, may account for the data observed which in turn may or may not explain the results of light scattering measurements (19) or account for the great numbers of isoenzymes found (20,21). The elucidation of the mechanism underlying these phenomena are the objective of current studies.

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