DISSOCIATION OF LACTATE DEHYDROGENASE INTO SUBUNITS WITH GUANIDINE HYDROCHLORIDE [1]

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Analysis of proteins commonly reveals that the functional molecule is a polymer or aggregate of smaller polypeptide chains. Furthermore, it is increasingly evident that many proteins with enzymatic activity exist in multiple molecular forms, or as isozymes, even within the cells of a single organism. Among these is lactate dehydrogenase (LDH), which has been extensively investigated and shown to be resolvable into numerous isozymes (Markert and Møller, 1959). In searching for an explanation of this molecular heterogeneity we have subjected crystalline LDH to a variety of physico-chemical analyses. This report presents the results of treating the enzyme with guanidine hydrochloride -- a hydrogen bonding reagent which effectively ruptures the hydrogen bonds responsible for the secondary structure of many proteins. With the destruction of their secondary structure the polypeptide chains unfold and aggregates of such polypeptide chains then commonly dissociate. LDH behaves in this way and appears to be dissociated into four inactive subunits by treatment with guanidine hydrochloride.

Materials and Methods

Crystalline lactate dehydrogenase was prepared from beef heart by Straub's procedure (Straub, 1940). Two of the isozymes (Markert and Appella, 1961) contained in such crystalline preparations were then separated on a cellulose column according to Porath's method (Porath, 1956). The most negatively charged isozyme at pH 8.6, that is LDH-1, was used in these

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experiments. Assays of enzyme activity were performed as previously described (Markert and Appella, 1961) using DPN and with lactate as a substrate at pH 9. Protein concentration was estimated by microkjeldhal analysis and by spectrophotometry using an $E^{1\%}_{1\ cm}$ of 14.55 at 280 mμ in 5 M guanidine-HCl. Fluorescence measurements were made with the Aminco-Bowman spectrophotofluorimeter (Bowman et al., 1955) at room temperature in a quartz cell having a 1 cm. light path. The intensities reported are expressed in terms of the deflection of the automatic recorder which is calibrated in inches.

Optical rotation studies were done with a Rudolph Model 80 spectropolarimeter equipped with an oscillating polarizer prism. The temperature was maintained at 20°C. Sedimentation was observed in a Spinco model E ultracentrifuge at 56,100 rpm with the temperature held at 20°C with the RTIC unit. Considering the viscosity and density of the guanidine-HCl solvent the sedimentation coefficients were corrected to the standard state of water at 20°C. Diffusion measurements were made with a synthetic boundary cell (Pickels et al., 1952). Our reported value represents the average of duplicate determinations. The partial specific volume of the protein was determined with a two ml pycnometer equilibrated in a constant temperature bath at 20.2°C. Molecular weight measurements of the native enzyme and of the dissociated subunits after guanidine treatment were determined by the Archibald approach to sedimentation equilibrium using a multichannel cell as described by Yphantis (1960) and employing the schlieren optical system.

The guanidine-HCl used in these experiments was twice recrystallized from methanol. This crystalline salt was added directly to samples of LDH in 0.1 M phosphate buffer at pH 7.2 to give the desired concentrations of guanidine-HCl. Then the mixture was dialyzed against guanidine-HCl solutions of the same molarity and pH in the presence of 0.1 M mercaptoethanol in order to prevent disulfide bridging among the dissociated chains. Guanidine-

HCl binding to the enzyme was calculated by the equilibrium dialysis method as outlined by Kielley and Harrington (1960).

Results and Discussion

Fig. 1 shows the effect of exposing the enzyme for five minutes to various concentrations of guanidine-HCl. Slight inactivation is apparent at 0.5 M and complete inactivation is produced by 1.25 M. However prolonged exposure of the enzyme even to 0.5 M guanidine gradually reduces enzyme activity until complete inactivation is reached after several hours. Removal of the guanidine by dialysis against buffer did not restore enzymatic activity.

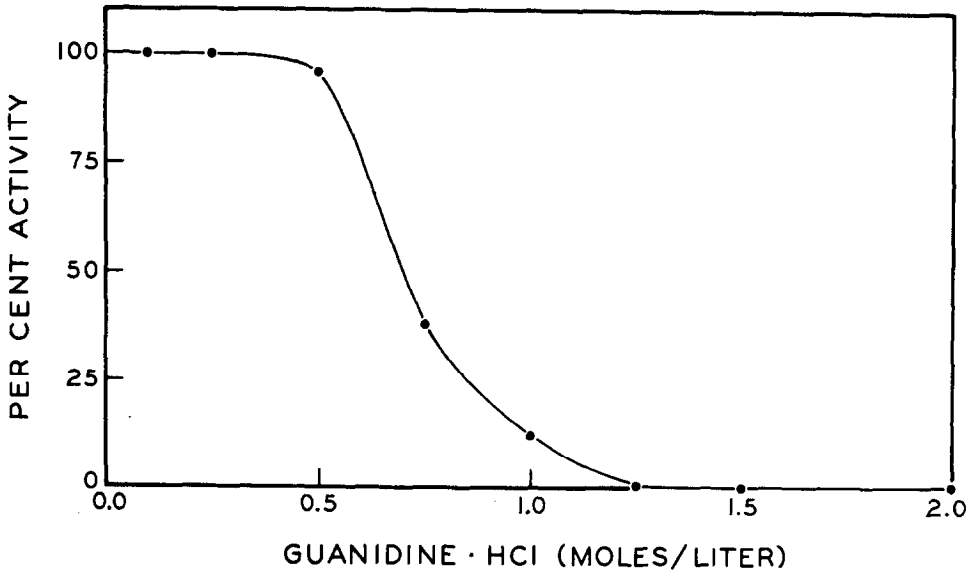

Fig. 1 Inhibition of lactate dehydrogenase by urea. Assays were conducted after 5 minutes incubation of the enzyme with urea at the indicated concentrations.

In Fig. 2 is shown the fluorescence intensity of LDH-1 at 340 mμ as a function of guanidine concentration. Between 0.5 M and 2.0 M a sharp decrease was observed in the fluorescence but no further change was apparent at higher concentrations. The coincidence of loss in enzymatic activity with decreased fluorescence intensity in the same range of guanidine-HCl concentration suggests that the ruptured hydrogen bonds of the

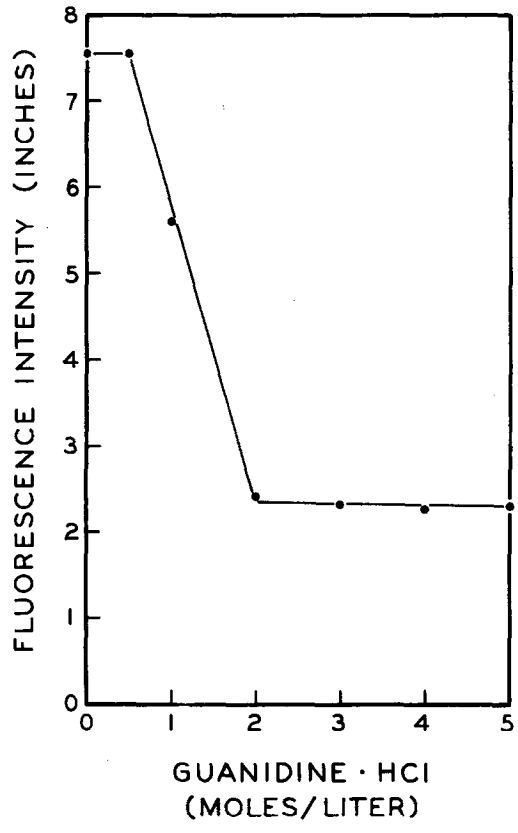

Fig. 2 Changes in the fluorescence intensities at 340 mμ of lactate dehydrogenase upon addition of urea. The excitation wave length was 288 mμ.

secondary structure of the enzyme were essential for stabilizing the active site of the enzyme. The disorienting effect of rupturing hydrogen bonds was confirmed by measuring changes in optical rotation during treatment with guanidine-HCl. As judged by the increasing levorotation at higher concentrations of guanidine a considerable unfolding of the molecule occurred. This unfolding paralled the loss of enzymatic activity. No effect was found below 0.5 M but a gradual increase occurred thereafter until the maximum levorotation was measured at a concentration of 5 M. The change in optical rotation measured at 589 mμ was from a specific rotation of -30° in buffer to -72° in 5 M guanidine-HCl. The optical rotation in guanidine was corrected for the index of refraction of the solvent.

The velocity sedimentation properties of LDH have been investigated in different concentrations of guanidine-HCl. At concentrations below 5 M a complex pattern was observed indicating the presence of more than one component. In 5 M only a single symmetric peak was apparent at all of the protein concentrations examined with the use of the synthetic boundary cell. The extrapolated value of the sedimentation constant for this single peak was calculated as 1.75 S. The diffusion coefficient was determined from the boundary spreading in a synthetic boundary cell. At a concentration of 1% a value of 6.74×10^{-7} cm^2 sec^{-1} was obtained. With the use of a pycnometer the partial specific volume was calculated to be 0.740 g/ml. The molecular weight of lactate dehydrogenase from beef has been reported as 135,000 (Neilands, 1954) and 134,000 (Markert and Appella, 1961). The latter value was again obtained by our equilibrium sedimentation experiments. The reproducible appearance of a homogeneous peak with a much lower sedimentation constant in guanidine-HCl suggests that the enzyme dissociates into discrete subunits of equal molecular weight. The molecular weight of the polypeptide subunits is 34,000 $\pm$ 2,000 as determined by calculation from the average molecular weight extrapolated to zero time and corrected for the 4% calculated binding of the guanidine-HCl to the protein. This result is consistent with the results of the sedimentation, diffusion, and partial specific volume measurements from which we calculate a molecular weight of 35,000. We believe the equilibrium sedimentation measurements of the dissociated subunits provide the most accurate estimate of their molecular weight. Considering the molecular weights of the intact enzyme molecule and of the dissociated subunits we may conclude that the LDH molecule is composed of four dissociable polypeptide chains.

In addition to these investigations a chemical analysis of the separated isozymes and the dissociated subunits is in progress and will be reported in detail elsewhere. Determinations of N and C terminal residues, total amino acid composition, and the pattern of peptides resulting from complete trypsin digestion have all been made. The number of peptides found

corresponds to about one fourth the number of arginine plus lysine residues found on total amino acid analysis. This peptide analysis therefore tends to confirm the conclusion that LDH is made up of four very similar polypeptide chains. That LDH may be dissociated into polypeptide subunits has also been confirmed by the use of 12 M urea. The response of LDH to urea treatment closely parallels the behavior of LDH in solutions of guanidine-HCl. It is interesting to note that the polypeptide subunits can be separated into at least two classes on the basis of charge. Assorting these two kinds of subunits into all possible groups of four would yield five isozymes, all distinguishable by charge. Indeed, five isozymes of LDH is the number commonly found in several mammals.

References

Bowman, R. L., Caulfield, P. A., and Udenfriend, S., Science 122: 32 (1955).

Kielley, W. W., and Harrington, W. F., Biochim. et Biophys. Acta 41: 401 (1960).

Markert, C. L., and Appella, E., Ann. N.Y. Acad. of Science, in press (1961).

Markert, C. L., and Møller, F., Proc. Natl. Acad. Sci. U.S. 45: 753 (1959).

Neilands, J. B., J. Biol. Chem. 208: 225 (1954).

Pickels, E. G., Harrington, W. F., and Schachman, H. K., Proc. Natl. Acad. Sci. U.S., 38: 943 (1952).

Porath, J., Biochim. et Biophys. Acta 22: 151 (1956).

Straub, F. B., Biochem. J. 34: 483 (1940).

Yphantis, D. A., Ann. N.Y. Acad. of Science 88: 586 (1960).