*SUBSTRATE-DEPENDENT ASSOCIATION OF LACTIC DEHYDROGENASE SUBUNITS TO ACTIVE TETRAMER**

By Gary Hathaway and R. S. Criddle

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA (DAVIS)

Communicated by David E. Green, June 22, 1966

Lactate dehydrogenase (LDH) has been reported to be a tetramer of molecular weight near 145,000, with a sedimentation coefficient at infinite dilution of 7.71*S*.[1-3] Millar has shown that appreciable dissociation of the tetramer is observed at concentrations below 0.5 mg per ml and that the dilute enzyme in sodium chloride solution will dissociate into a 5.5*S* dimer with one half the molecular weight of the tetramer.[1]

Reasoning from this data, Reithel has proposed that the enzyme may be present largely as a dimer under the dilute conditions (<1.0 μg/ml) ordinarily employed in an enzymatic assay.[4] This would imply an active dimer form. Kaplan and Chilson *et al.*, on the other hand, feel that the tetramer is the active unit and that the dimer is inactive.[5, 6] This position is strengthened by the report of an apparent decrease in specific activity of the enzyme at concentrations below about 1.0 μg/ml.[7]

The direct measurement of the size of the lactic dehydrogenase molecule, as indicated by its sedimentation coefficient, at concentrations many times more dilute than those previously employed both in the presence and absence of substrate, has been used to resolve some of the controversy concerning the nature of the active particle.

Materials and Methods.—Bovine heart LDH, NADH, and sodium pyruvate were obtained from Sigma Chemical Co. Substrate and coenzyme were used without further purification. The enzyme was either desalted on a Sephadex G-50 column and used as the isoenzyme mixture or separated into the H_4 and H_3M components which are the major isoenzymes in the preparation. Separation was carried out using preparative polyacrylamide gel electrophoresis in a Büchler apparatus using a modification of the procedure of Ornstein and Davis.[8]

Protein was determined by 280-mμ absorbance following the procedure of Pesce *et al.*[9] The isoenzymes were stored in 50% saturated $(NH_4)_2SO_4$ at pH 7.0 in elution buffer and desalted using Sephadex gel filtration immediately prior to use.

Kinetic data was obtained at 25° using an assay procedure similar to that described by Kubowitz and Ott.[10] Initial velocity was recorded by following the decrease in absorbance at 340 mμ using a Cary model 14 recording spectrophotometer for the initial linear portion of the curve.

Sedimentation velocity experiments were performed with a Spinco model E ultracentrifuge using the sedimentation assay technique of Cohen and Hahn.[11] Samples of 0.06 ml of a 7.0 μg/ml solution of enzyme were applied to the layering tube of a 30-mm Vinograd-type synthetic boundary cell,[12] and 1.55 ml of substrate solution containing 0.35 ml of 2.0×10^{-3} *M* NADH, 0.3 ml sodium pyruvate, and 2.29 ml of a NaCl-phosphate buffer identical to that used in the kinetic experiments were added to the cell sector. All buffers were 0.2 *M* in sodium phosphate, pH 6.0, and contained either 1.0 *M* or 0.1 *M* NaCl. The enzyme was layered over the substrate solution at low speed and then spun at 50,740 rpm. A 355-mμ filter was used in the schlieren optical system and the position of the boundary located photographically at various times during sedimentation. A Spinco Analytrol densitometer was used to measure boundary position to $\pm$0.05 cm, and an IBM 7044 digital computer used to determine sedimentation coefficients. Sedimentation was run at 20° and values were corrected for salt concentration.

Sedimentation velocity runs were also carried out using UV optics at an enzyme concentration of 250 μg/ml in 0.1 *M* NaCl-phosphate buffer, pH 6.0.

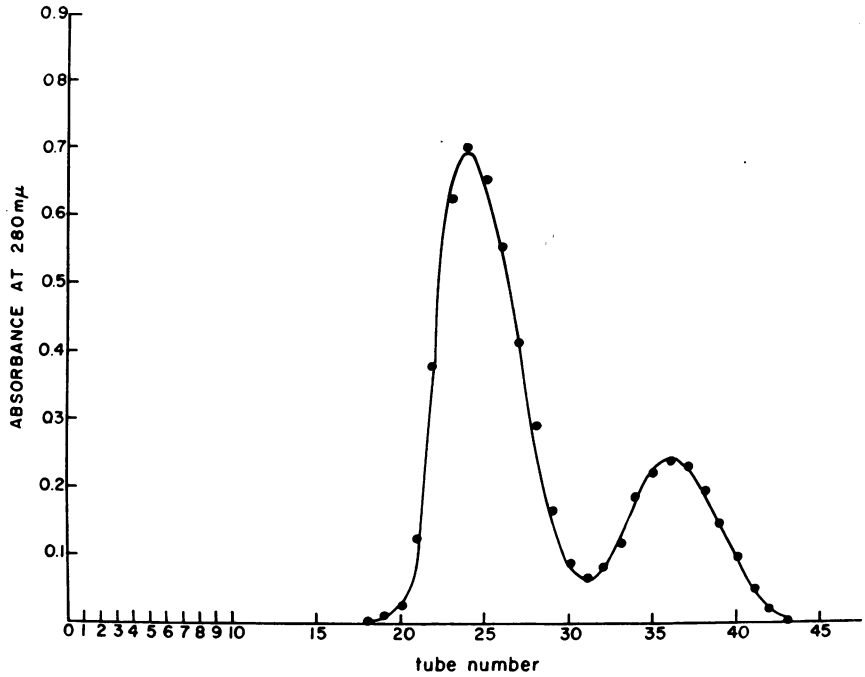

FIG. 1.—Separation of H_4 (large peak) and H_3M isoenzymes. The H_4 enzyme moved as a single band on analytical disk electrophoresis, while the H_3M isoenzyme showed contamination by less than 1% H_4.

Results and Discussion.—Complete separation of the two isoenzymes from beef heart was obtained during 10 hr electrophoresis. Results are shown in Figure 1. A 20 per cent increase in specific activity as measured by change in absorbance at 340 mμ per minute per mg protein accompanied the separation. Disk gel electrophoresis of the isolated isoenzymes and commercial beef heart LDH is shown in Figure 2.

Velocity versus substrate plots were obtained at 0.08 and 0.8 *M* NaCl concentrations and are shown in Figure 3. The shift in the initial velocity curve to higher pyruvate concentrations with increasing salt can be explained if one asssumes an electrostatic interaction between enzyme and substrate as suggested by Winer and Schwert.[13] At low pyruvate concentration in such a system, enzyme-substrate dissociation by salt should be reflected as a decrease in initial velocity, while at high pyruvate in the region of substrate inhibition, salt-induced dissociation should result in an observed increase in velocity. This would then give rise to curves of the type shown in Figure 3.

The kinetics of the H_4 isoenzyme are not of the classical Michaelis-Menten type. R_s (ratio of substrate concentration at 90% maximal activity to that at 10% maximal activity) was found to be 39 instead of the theoretical 81.[14] The parameter $S_{0.5}$ (substrate concentration at one half apparent maximal velocity)[14] increases from 2.0×10^{-5} to 1.6×10^{-4} *M* pyruvate in going to the higher salt concentration. Figure 4 shows a Hill-type plot for the enzyme in 0.8 *M* salt. The slope of the

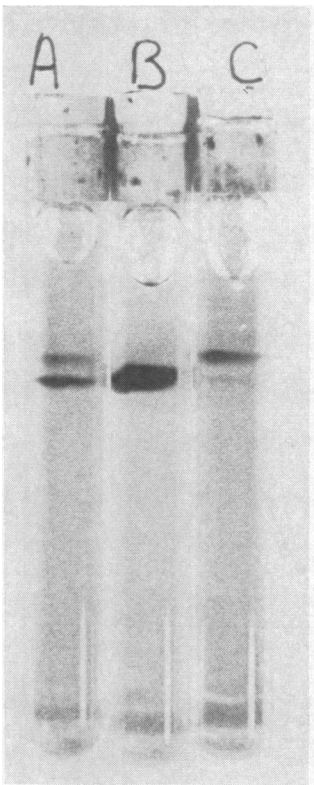

FIG. 2.—(*A*) Disk electrophoresis of crystalline beef heart LDH. (*B*) Enzyme from first peak of Fig. 1. (*C*) Enzyme from second peak of Fig. 1.

straight line was determined by the method of least squares, and n was found to be 1.15.

Sedimentation velocity was followed using UV optics in the absence of substrate and coenzyme at protein concentrations lower than 250 μg/ml, and the enzyme was found to be a 5.6S particle in agreement with Millar's results. In the presence of substrate, however, sedimentation is markedly altered. Figure 3 shows the substrate dependence of the sedimentation coefficient and the correlation with the observed kinetics. Salt concentrations, corresponding to the 0.8 M NaCl curve of enzyme kinetics, were used in the determination of sedimentation values, as the initial portion of the velocity versus substrate curve is shifted to lower pyruvate concentrations at low salt where it becomes technically difficult to observe enzyme sedimentation in the ultracentrifuge. Maximum sedimentation velocity, which corresponds to a 7.7S particle, in agreement with the extrapolated value obtained by Millar for the tetramer, was obtained at 10^{-3} M pyruvate. Note that this also corresponds to the pyruvate concentration at maximum enzyme velocity under these conditions.

A progressively declining value of sedimentation coefficient both in the region of low substrate and in the region of substrate inhibition suggests a mechanism by which pyruvate may shift the reversible dimer-tetramer equilibrium and consequently control enzyme activity. It should be pointed out that while only the fastest-moving active particle is measured by this method, a rapidly reversible dissociation into a smaller particle would be detected as a decrease in the weight average sedimentation coefficient. Although this does not rule out the possibility of an active dimer, the close correlation of the decrease in the Svedberg constant in the region of low substrate concentration and the apparent steepness of the kinetic curve at this point can be explained if one assumes the dimer to be at least less active than the tetramer.

The curves of Figure 5 show the same experiments performed on the commercially obtained heart LDH which has both H_4 and H_3M isoenzymes. Note that the positions of both the maxima for the kinetic curves and the sedimentation curve are shifted and that there is a leveling of the sedimentation velocity curve at high pyruvate. This suggests that LDH molecules containing muscle-type subunits are more stable to dissociation at high pyruvate concentrations. Further study using the M_4 isoenzyme is under way to investigate this.

The fact that LDH is present in high concentrations (2–3 mg/ml as measured in our laboratory in rabbit muscle extracts) makes the control of the heart enzyme by substrate inhibition doubtful unless local high substrate concentrations are invoked.

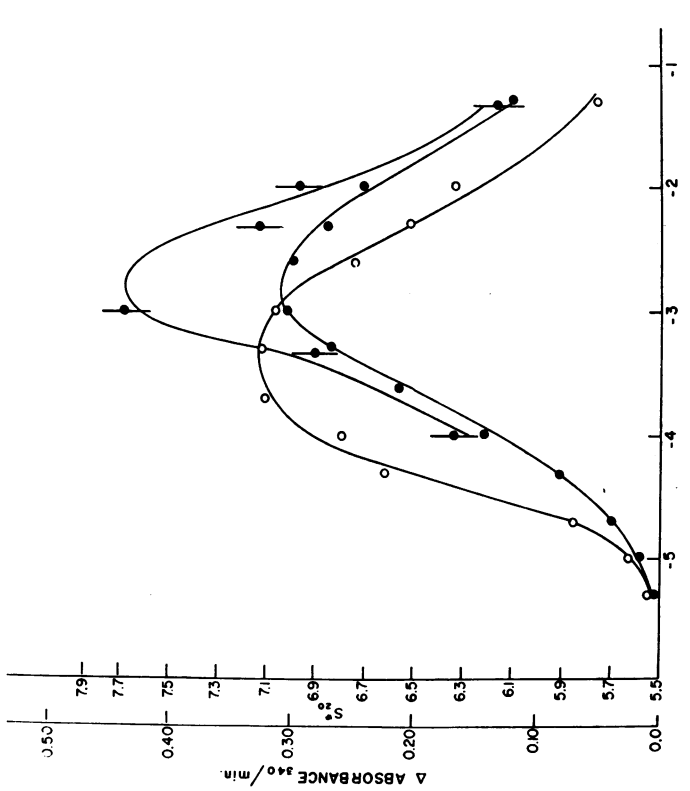

FIG. 3.—Initial velocity and sedimentation assays of H_4 isoenzyme of LDH. Kinetic assay mixtures contained 0.1 ml 10^{-3} *M* NADH, 0.1 ml sodium pyruvate, and 0.8 ml buffer as described in text. NaCl concentrations were 0.08 *M*, —○—○—; and 0.8 *M*, —●—●—. Sedimentation assays, —●—●—, were run at a H_4 isoenzyme concentration of 0.3 μg/ml. Solution conditions were identical to those above in the 0.8 *M* assay, except that NADH was 2.3×10^{-4} *M*. $S^*_{20,w}$ indicates correction only for temperature and for NaCl concentration.

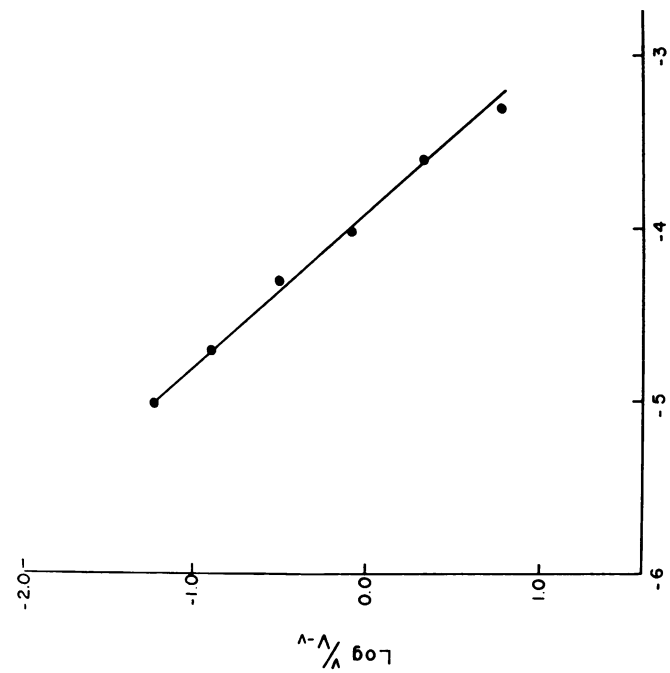

FIG. 4.—Hill plot for H_4 isoenzyme assayed in 0.8 *M* NaCl buffer. Slope was determined by the method of least squares.

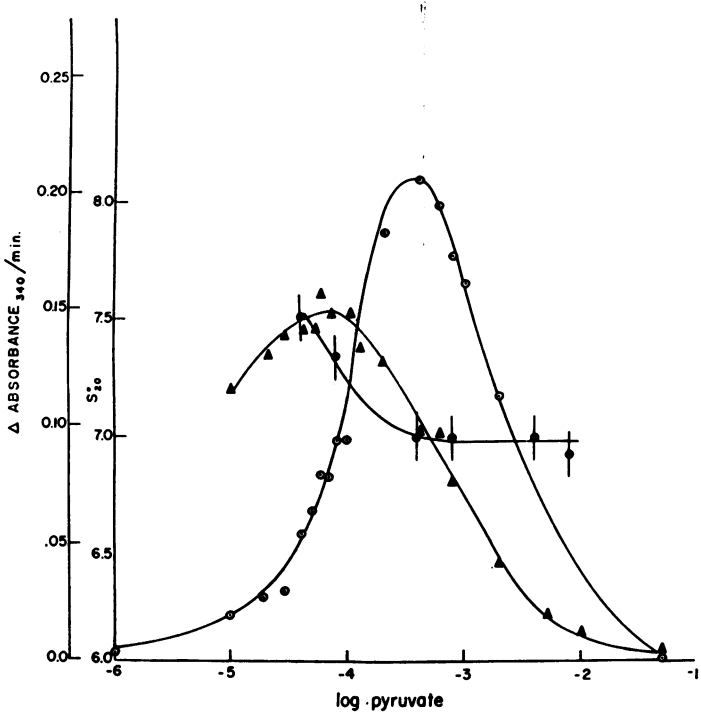

FIG. 5.—Initial velocity and sedimentation assays of beef heart LDH. Kinetic assay mixtures contained 0.1 ml NADH, 2×10^{-3} *M*, 0.1 ml sodium pyruvate, 0.8 ml buffer as described in the text. Final NaCl concentration was 0.08 *M* —▲—. Identical to above, except 0.8 *M* NaCl —⊙—. All points were corrected for enzyme denaturation by running a control plotted against time. —●—, Sedimentation assay of beef heart LDH run at 0.3 μg protein/ml. Conditions were identical to the velocity curve at 0.08 *M* NaCl except that NADH concentration was 2.3×10^{-4} *M*.

Control at low substrate levels by a process of dissociation into an inactive form is not only possible, but most attractive.

Summary. The tetrameric form of lactate dehydrogenase (H_4 isoenzyme) was found to have a sedimentation coefficient of 7.7*S* at a protein concentration of 0.3 μg/ml. This value was very dependent upon pyruvate concentration, however, and showed a marked correlation with kinetic data obtained under nearly identical conditions. These observations are explainable if one assumes a substrate-dependent equilibrium between an inactive dimer and an active tetramer molecule.

We are grateful to Dr. H. Gutfreund for helpful discussions during the course of this investigation.

* Supported in part by USPHS grants GM 10017 and 5T1GM119.

[1] Millar, D. B. S., *J. Biol. Chem.*, **237**, 2135 (1962).
[2] Neilands, J. B., *J. Biol. Chem.*, **208**, 225 (1954).
[3] Appella, E., and C. L. Markert, *Biochem. Biophys. Res. Commun.*, **6**, 171 (1961).
[4] Reithel, F. J., *Advan. Protein Chem.*, **18**, 184 (1963).
[5] Kaplan, N. O., in *Brookhaven Symposia in Biology*, No. 17 (1964), p. 131.
[6] Chilson, O., G. Kitto, J. Pudles, and N. Kaplan, *J. Biol. Chem.*, **241**, 2431 (1966).
[7] Bernfeld, P., B. Berkeley, and R. Bieber, *Arch. Biochem. Biophys.*, **111**, 31 (1965).

[8] Ornstein, L., and B. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 321 (1964).
[9] Pesce, A., R. H. McKay, F. Stolzenbach, R. D. Cahn, and N. O. Kaplan, *J. Biol. Chem.*, **239**, 1753 (1964).
[10] Kubowitz, F., and P. Ott, *Biochem. Z.*, **314**, 94 (1943).
[11] Cohen, R., and C. Hahn, *Compt. Rend.*, **13**, 2077 (1965).
[12] Vinograd, J., R. Bruner, R. Kent, and J. Weigle, these PROCEEDINGS, **49**, 902 (1963).
[13] Winer, A., and G. Schwert, *J. Biol. Chem.*, **231**, 1065 (1958).
[14] Koshland, D., G. Nemethy, and D. Filmer, *Biochemistry*, **5**, 365 (1966).