Holland, M. J., and Westhead, E. W. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 2627 Abstr.
Jörnvall, H. (1970a), *Eur. J. Biochem. 14*, 521.
Jörnvall, H. (1970b), *Nature (London) 225*, 1133.
Jörnvall, H., and Harris, J. I. (1970), *Eur. J. Biochem. 13*, 565.
Kirschner, K., Eigen, M., Bittman, R., and Voigt, B. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1661.
Kirschner, K., and Voigt, B. (1968), *Z. Phys. Chem. 349*, 632.
Krebs, E. G., Rafter, G. W., and Junge, J. M. (1953), *J. Biol. Chem. 200*, 479.
Lebherz, H. G., and Rutter, W. J. (1967), *Science 157*, 1198.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Smith, E. L., Landon, M., Piszkiewicz, D., Brattin, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.
Stancel, G. M., and Deal, W. C., Jr. (1969), *Biochemistry 8*, 4005.
Sund, H., Ed. (1970), Pyridine Nucleotide-Dependent Dehydrogenases, New York, N. Y., Springer-Verlag.
Surdin, Y. (1967), *Eur. J. Biochem. 2*, 341.
Trentham, D. R. (1968), *Biochem. J. 109*, 603.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Adenosine 5′-Triphosphate Induced Cold Inactivation of Yeast Aspartic β-Semialdehyde Dehydrogenase†

Michael J. Holland‡ and E. W. Westhead*

ABSTRACT: Yeast aspartic β-semialdehyde dehydrogenase undergoes a time-dependent inactivation in the presence of adenosine 5′-triphosphate at 0°. Equilibrium studies of this inactivation at different protein concentrations show that tetrameric aspartic β-semialdehyde dehydrogenase dissociates under these conditions into inactive dimers. Optimum conditions for inactivation are 3.5 mM ATP, 0°, pH 6.5, and 2 mM dithiothreitol. Inactivation can be completely reversed by warming the reaction mixture to 25° in the presence or absence of ATP or by removing ATP at 0°. The reaction can be described as a reversible tetramer–dimer equilibrium with a dissociation constant of 5.2×10^{-8} mol/l. at pH 7.0 in the presence of 20 mM ATP at 0°. Inactivation by ATP under these conditions is cooperative and the data indicate that at least 2.5 mol of ATP/mol of native aspartic β-semialdehyde dehydrogenase is involved in the inactivation. The inactivation of aspartic β-semialdehyde dehydrogenase at 0° is also promoted, but more weakly, by NADPH–Tris-chloride buffer and potassium phosphate. NADH protects against inactivation while $NADP^+$ and NAD^+ appear to have no measurable effect on the enzyme at 0°. These data are discussed and compared with the published results for yeast glyceraldehyde-3-phosphate dehydrogenase.

The phenomenon of cold inactivation is widespread among different classes of enzymes, especially those with multiple subunits. The factors that influence cold inactivation and the structural changes accompanying it vary markedly (see, for example, the discussion in Irias *et al.*, 1969). Glyceraldehyde-3-phosphate dehydrogenase from both rabbit muscle and yeast have been shown by Deal and coworkers to undergo reversible cold inactivation specifically induced by the presence of ATP (Constantinides and Deal, 1969, 1970; Yang and Deal, 1969a,b; Stancel and Deal, 1969). Both of these tetrameric enzymes undergo dissociation during cold inactivation. The rabbit muscle enzyme dissociates into dimers or monomers depending on conditions, while the yeast enzyme was observed to form only monomers.

In our study of aspartic β-semialdehyde dehydrogenase we have been particularly interested in the question of homology between this enzyme and glyceraldehyde-3-phosphate dehydrogenase. The specific cold-induced changes of glyceraldehyde-3-phosphate dehydrogenase, which have been so thoroughly studied, reflect molecular properties of the protein quite separate from details of the catalytic site structure. This paper thus presents a study of the cold inactivation of aspartic β-semialdehyde dehydrogenase showing striking similarity with the behavior of glyceraldehyde-3-phosphate dehydrogenase and adding evidence for close structural homology between the two proteins.

Materials and Methods

Reagents. Adenosine 5′-triphosphate (Sigma grade, disodium salt) was obtained from Sigma Chemical Co., St. Louis, Mo. All other reagents are described in Holland and Westhead, 1973a. The standard assay solution had the following composition: 0.1 M potassium phosphate buffer, pH 7.5, 0.01 M EDTA, 10^{-4} M Clelands reagent, 0.5 mM $NADP^+$, 2 mM aspartic β-semialdehyde, 0.03 M $KHCO_3$, and 0.003 M Tris. The last two components were stored as a concentrated solution of 1 M $KHCO_3$ plus 0.1 M Tris, pH 8.5, and were added to the assay solution in a ratio of 30 μl/ml.

The enzyme used in these studies was either partially puri-

† From the Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received July 11, 1972.* Supported by National Institutes of Health Grant AM11157. A preliminary account of this work has been given (Holland and Westhead, 1969). This work is part of the Ph.D. Thesis (1971) of Michael J. Holland, which is available from University Microfilms, Ann Arbor, Mich. 48106.

‡ Present address: Department of Biochemistry and Biophysics, University of California at San Francisco, San Francisco, Calif. 94122.

fied or fully purified enzyme, with a specific activity of about 13 or 73 μmol of NADPH per minute per milligram at 30°, respectively. The partially pure enzyme had been taken through hydroxylapatite chromatography with a sulfate gradient as previously described (Holland and Westhead, 1973a).

Methods. Routine inactivations of yeast aspartic β-semialdehyde dehydrogenase by adenosine 5′-triphosphate (ATP) were carried out in 0.1 M Tris buffer, pH 7.0, containing 10 mM dithiothreitol and 10 mM EDTA at 0°. Dithiothreitol was required in order to maintain the reversibility of the reaction. Protein concentrations and additions of other effectors are described in Results. Temperatures employed for inactivation studies were maintained at ±0.5° in a refrigerated bath. Reactivations were carried out by warming the reaction mixtures to 30° in a thermostated water bath. The extent of inactivation was determined by measuring the initial velocity of the enzymatic catalysis when an aliquot of the reaction mixture was put into the normal assay solution (minus dithiothreitol) at 30°. Aliquots were transferred in a syringe which had been cooled to the reaction temperature in order to prevent reactivation during the transfer. This procedure specifically measures that fraction of the enzyme which is not very rapidly reactivated under assay conditions. Our assays would detect any reactivation process taking place between 5 and 120 sec after dilution into the assay solution and none was seen. Dilution was generally 30- to 100-fold, which would slow down any second- or higher-order protein association process. Simple inhibition by ATP would be reversed by dilution too rapidly for measurement.

To determine the molecularity for ATP binding to aspartic β-semialdehyde dehydrogenase, inactivations were carried out in 0.01 M phosphate buffer, pH 6.65, containing 10 mM dithiothreitol and 10 mM EDTA at 0°. Under these conditions there was no inactivation in the absence of ATP. The extent of inactivation in the presence of varying concentrations of ATP was measured as described above by diluting aliquots of the reaction mixture into the normal assay mixture.

To test the ability of aspartic β-semialdehyde dehydrogenase to reactivate in the absence of ATP, the enzyme was inactivated in 0.01 M phosphate buffer, pH 6.65, containing 10 mM EDTA, and 20 mM ATP at 0°. Equilibrium was reached after 3 hr at 0°. The protein was then separated from ATP on a Sephadex G-25 column (1 cm × 15 cm) equilibrated at 2° with 0.01 M phosphate buffer, pH 6.65, containing 10 mM dithiothreitol and 10 mM EDTA. This procedure was sufficient to remove all of the free ATP. After removal of ATP, aspartic β-semialdehyde dehydrogenase was allowed to stand at 0° and determination of the extent of reactivation was made by diluting aliquots into the normal assay mixture at 30° and determining activity spectrophotometrically.

Results

Effect of ATP Concentration on Inactivation of Aspartic β-Semialdehyde Dehydrogenase at 0°. Aspartic β-semialdehyde dehydrogenase shows a time-dependent loss of activity in the presence of ATP at 0°. Inactivations were carried out at this temperature for 3 hr at a protein concentration of 0.01 mg/ml as described under Methods. Figure 1 shows the dependence of this inactivation on ATP concentration. These data show that the inactivation is strongly cooperative with respect to ATP. Half-maximal inactivation occurred at an ATP concentration of 2.5 mM. It should be emphasized that the incubation mixture contains 10 mM EDTA which ensures that the ATP

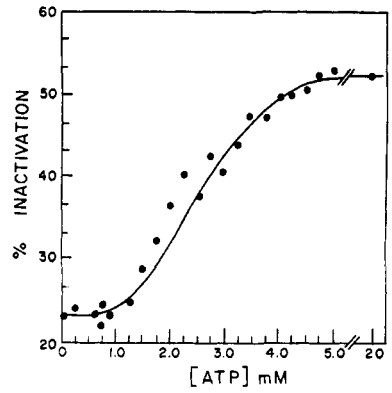

FIGURE 1: Effect of ATP concentration on the inactivation of aspartic β-semialdehyde dehydrogenase at 0°. Inactivations were carried out for 3 hr at a protein concentration of 0.01 mg/ml, using enzyme of specific activity 13.6. Details in Methods.

concentration at half-maximal effect has not been shifted to a higher value due to possible Mg^{2+} contamination in the stock ATP solutions. Levels of inactivation at concentrations of ATP below 0.05 mM are the same as controls without ATP and concentrations of ATP up to 20 mM produce no further inactivation than that observed at 5 mM ATP. These results indicate that active enzyme exists in equilibrium with an inactive form and that both protein species bind ATP, since concentrations of ATP up to 20 mM do not cause complete inactivation of aspartic β-semialdehyde dehydrogenase.

Effect of Protein Concentration on the Inactivation of Aspartic β-Semialdehyde Dehydrogenase by ATP; Evidence for Protein Dissociation. Figure 2 shows the course of ATP inactivation for three different concentrations of enzyme. Inactivations were carried out in 20 mM ATP at 0° as described in Methods. Appropriate dilutions were made of a protein solution containing 0.05 mg/ml for each inactivation. The inactivation is clearly dependent on protein concentration, which would not be the case for a unimolecular reaction going to completion. Maximal inactivation was reached by 150 min for all three protein concentrations and incubation times up to 24 hr caused no further loss of activity. These data indicate that each incubation had come to *equilibrium* by 150 min.

Figure 3 shows the *extent* of inactivation at five different protein concentrations. These data show that the *extent* of inactivation at equilibrium increases with decreasing protein concentration and that protein association stabilizes the active

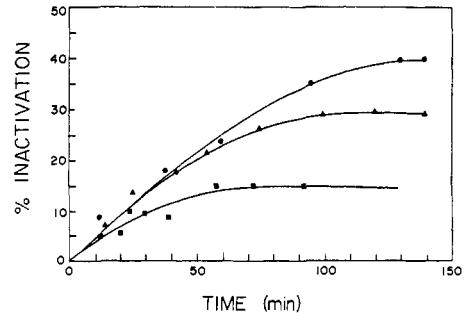

FIGURE 2: Time course of *ATP-induced* inactivation of aspartic β-semialdehyde dehydrogenase at 0°. Inactivations were carried out in 20 mM ATP as described in Methods. Enzyme of specific activity 13.8 was used. Symbols represent protein concentrations: (●) 5 μg/ml; (▲) 10 μg/ml; (■) 50 μg/ml.

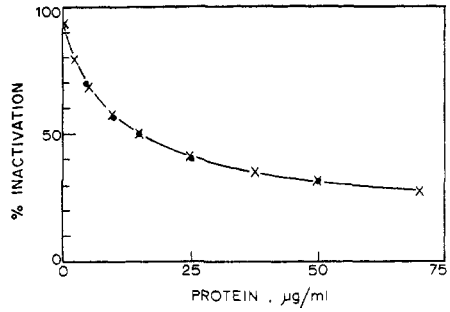

FIGURE 3: Protein concenttrtion dependence of the *ATP-induced* inactivation of aspartic β-semialdehyde dehydrogenase at 0°. Inactivations were carried out in 20 mM ATP for 3 hr, as described in Methods, using protein of specific activity 13.8: (×) points calculated as described in the text; (●) experimental points.

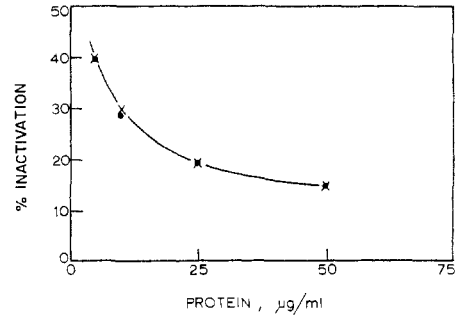

FIGURE 5: Protein concentration dependence of the *phosphate-induced* inactivation of aspartic β-semialdehyde dehydrogenase at 0°. Enzyme with specific activity 13.8 was incubated 3 hr in 0.1 M potassium phosphate, pH 6.0, as described in Methods: (×) points calculated from dimer model (see text for details); (●) experimental points.

form of the enzyme. The order of this association may be deduced from simple curve fitting. The solid line in Figure 3 was generated assuming an equilibrium of the type, E(active) $\rightleftharpoons$ $2E_{1/2}$(inactive), in which association is second order. An equilibrium constant K_D, was calculated with the equation, $K_D = (E_{1/2})^2/E$. This value of K_D was used to calculate the degree of dissociation which would result at any other protein concentration. The very good fit of the data and the calculated line in Figure 3 suggest strongly that the inactive enzyme is half the molecular weight of the active enzyme.

In order to calculate a true molecular dissociation constant a sample of pure enzyme (specific activity = 73) was incubated for 3 hr with 20 mM ATP under standard conditions described in Methods. Concentrations of enzyme based on the molecular weight of the native tetramer (Holland and Westhead, 1973a) were: 6.4×10^{-8}, 1.3×10^{-8}, and 0.6×10^{-8} M. The extents of dissociation observed were 36, 61, and 74%, respectively. Dissociation constants were calculated on the basis of a tetramer to dimer equilibrium and were found to agree closely: 5.2×10^{-8}, 5.0×10^{-8}, and 5.0×10^{-8} M, respectively. At these protein concentrations, dissociation into an inactive form can be either from the native tetramer to the dimer, or alternatively from the dimer to the monomer. Our data do not distinguish between these two cases.

Effect of Protein Concentration on the Inactivation of Aspartic β-Semialdehyde Dehydrogenase by Phosphate. Figure 4 shows the time course for inactivation of aspartic β-semialdehyde dehydrogenase at three concentrations in 0.1 M potassium phosphate. Inactivations were carried out with the same concentrations of protein that were used with ATP as shown in Figure 3. Here, as with ATP, there is a marked dependence of the extent of inactivation on protein concentration. Equilibrium was again reached by 150 min and no further inactivation was observed up to 24 hr. The initial rate of inactivation and the extent of inactivation with 0.1 M phosphate are significantly lower than was observed with ATP, indicating that ATP is more effective an inactivating agent than phosphate.

A theoretical curve for a second-order dependence on protein concentration was generated for the phosphate effect as described for ATP (Figure 5). Again the experimentally determined extents of inactivation at four different protein concentrations superimposed on the theoretical curve for dissociation into halves.

pH Dependence of the ATP- and Phosphate-Induced Inactivations of Aspartic β-Semialdehyde Dehydrogenase at 0°. Figure 6 shows the dependence of the ATP- and phosphate-induced inactivations of aspartic β-semialdehyde dehydrogenase on pH. Inactivations were carried out for 5 hr with 20 mM ATP or 0.1 M potassium phosphate at a protein concentration of 0.05 mg/ml. The pH of each solution was adjusted with KOH or HCl to the desired value, with no further addition of buffer. No drift of pH occurred during the course of any of the incubations. Maximal inactivation by both effectors occurred at

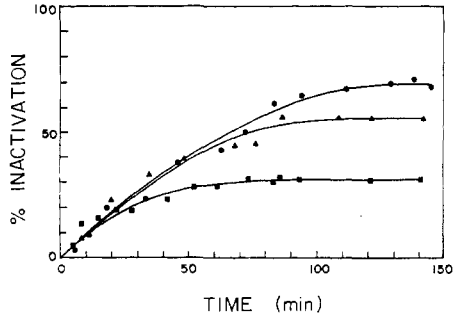

FIGURE 4: Time course of the *phosphate-induced* inactivation of aspartic β-semialdehyde dehydrogenase at 0°. Inactivations were carried out in 0.1 M potassium phosphate, pH 7.0, as described in Methods. The specific activity of the enzyme was 13.8 and the protein concentrations were: (●) 5 μg/ml; (▲) 10 μg/ml; (■) 50 μg/ml.

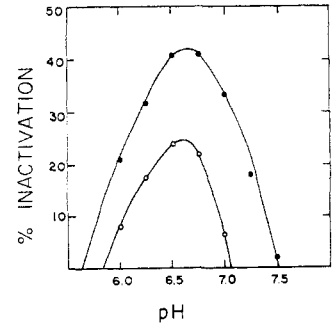

FIGURE 6: Dependence of pH of ATP- and phosphate-induced inactivation of aspartic β-semialdehyde dehydrogenase at 0°. Enzyme with specific activity 13.6 at 50 μg/ml in either 0.1 M potassium phosphate, pH 7.0, or in 0.1 M Tris; 20 mM ATP was titrated to the desired pH with HCl or KOH. No observable drift occurred during the 5-hr incubation at 0°: (●) ATP-induced inactivation; (○) phosphate-induced inactivation.

TABLE I: Reversibility of the ATP-Induced Inactivation of Aspartic β-Semialdehyde Dehydrogenase.[a]

Reaction Condition	Act. (%)
1. 0° for 3 hr − ATP	100
2. 0° for 3 hr + ATP	36
3. 0° for 3 hr + ATP, rewarmed to 30°	100
4. 0° for 3 hr + ATP, ATP removed at 2°	84
5. 0° for 3 hr + ATP, ATP removed at 2°, rewarmed to 30°	84

[a] Inactivations were carried out in phosphate buffer, pH 6.65, containing 20 mM ATP, and 2 mM dithiothreitol at 0° for 3 hr. Aspartic β-semialdehyde dehydrogenase at a concentration of 1.5×10^{-2} μM (specific activity = 73) was used in each incubation. Removal of ATP at 2° after inactivation and subsequent rewarming to 30° are described in Methods.

TABLE II: Effect of Various Ions and Coenzymes on the Inactivation of Aspartic β-Semialdehyde Dehydrogenase.[a]

Effector	% Inactivation with 10 mM ATP	% Inactivation without ATP
0.01 M phosphate	60	0
0.1 M phosphate	61	32
0.1 M Tris	60.5	17
0.1 M phosphate + 0.1 M Tris	59.5	30
0.5 mM $NADP^+$ + 0.1 M Tris	60	18
2 mM NAD^+ + 0.1 M Tris	61	16.5
2 mM NAD^+ + 0.1 M Tris	57	17
0.5 mM NADPH + 0.1 M Tris	62	35
1 mM NADPH + 0.1 M Tris	58	39
2 mM NADPH + 0.1 M Tris	65	35
0.5 mM NADH + 0.1 M Tris	60.5	16
1 mM NADH + 0.1 M Tris	59	13
2 mM NADH + 0.1 M Tris	60.5	8

[a] All inactivations were carried out at 0° for 3 hr at a protein concentration of 0.01 mg/ml (specific activity = 13.6). Measurement of activity and calculation of percentage inactivation are described in Methods.

pH 6.6–6.7. At pH values below 6.8, the enzyme undergoes irreversible inactivation when incubated for periods longer than 5 hr. Incubations carried out at pH 6.6–6.7, the pH optimum for inactivation, underwent 5–10% irreversible inactivation when incubated for 10 hr at 0°. At pH 7.0 no irreversible inactivation was observed up to 24 hr. In order to avoid complications arising from irreversible inactivations, the following experiments were carried out at pH 7.0. The sharpness of the pH dependence at pH 7.0 required careful adjustment of the pH of each incubation to within ±0.05 pH unit.

Reversibility of the Low-Temperature Inactivation Induced by ATP or Phosphate. The inactivation of aspartic β-semialdehyde dehydrogenase by phosphate or ATP can be completely reversed by raising the temperature to 30°. Reactivation occurs within 10 min at 30° and the cycle can be repeated many times with the same protein incubation without loss of enzyme activity. Table I shows that pathway of reversal is not critical. Removal of ATP prior to raising the temperature does not prevent return of most of the activity. Comparison of experiments 4 and 5, Table I, shows that the fraction of activity not recovered is not due to slowness of recovery in the absence of ATP, but may be due to manipulation of the inactivated protein. The results also show that at 30°, ATP is unable to inactivate the enzyme or maintain the inactivated form.

Effects of Buffer Salts and Coenzymes on the ATP-Induced Cold Inactivation of Aspartic β-Semialdehyde Dehydrogenase. The possibility that ATP binds to part of the $NADP^+$–NADPH site of the enzyme was studied in a series of experiments shown in Table II. This table also gives information on the effects of buffer salts and on the effects of combination of effectors. As shown in the first line of Table II, no inactivation of the enzyme was observed at 0° in the presence of 10 mM phosphate, indicating that low temperature alone is not responsible for the inactivation under the conditions used. The first four lines of the table show that both Tris-chloride and potassium phosphate buffer ions do promote inactivation at higher concentrations, but that neither alone nor in combination do they alter the degree of inactivation produced by ATP in saturating concentrations (10 mM ATP). Tris buffer likewise does not alter the degree of inactivation produced by potassium phosphate at 0.1 M.

Looking further down the table, it may be seen that no addition of coenzyme alters the effect of ATP, and that neither oxidized coenzyme (NAD^+ or $NADP^+$) has any effect on the inactivation produced by the Tris buffer alone. The reduced coenzymes are not without effect. NADPH, which is a substrate for aspartic β-semialdehyde dehydrogenase, promotes inactivation, while NADH appears to offer some protection against cold inactivators.

The experimental error in determining the degree of inactivation in these experiments is probably 5–10% of the value shown, so that small differences are not significant. It does seem clear, however, that the ATP site detected in these experiments is not part of the catalytic site. Each dinucleotide coenzyme was used at a concentration at least 50-fold higher than its K_m determined kinetically at 30°. In the case of NADPH, the extent of inactivation in the absence of ATP is constant from 0.5 to 2.0 mM, indicating saturation. Even at these concentrations, NADPH does not alter the ATP effect.

Determination of the Molecularity of ATP Binding. The ATP saturation curve presented in Figure 1 indicated that the binding of ATP to the enzyme at 0° does not fit a simple hyperbolic function. It should be possible, however, to determine the molecularity for ATP binding by suitable analysis of the extent of inactivation at different ATP concentrations. The reaction conditions employed in the experiment presented in Figure 1, however, were such that appreciable inactivation of the enzyme occurred even in the absence of added ATP. Under these conditions description of the equilibrium demands a minimum of four species: active tetramer, active tetramer–ATP complex, inactive dimer, and inactive dimer–ATP complex. The results of the preceding section show that complexes with buffer salts could add another dimension of complexity.

Since this equilibrium situation proves to be too cumber-

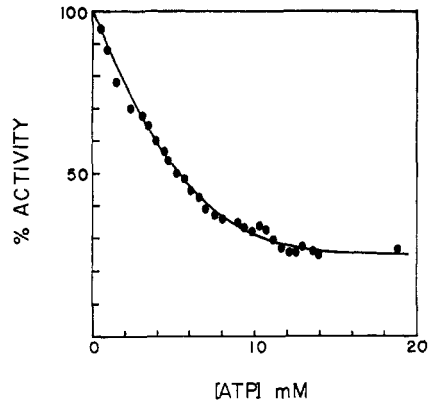

FIGURE 7: Effect of ATP concentration on the inactivation of aspartic β-semialdehyde dehydrogenase at 0°. Inactivations were carried out in phosphate buffer, pH 6.65, containing 2 mM dithiothreitol and appropriate concentrations of ATP at 0° for 3 hr. Aspartic β-semialdehyde dehydrogenase at a concentration of 1.3×10^2 μM (specific activity = 73) was used in each incubation. The extent of inactivation was measured as described in Methods.

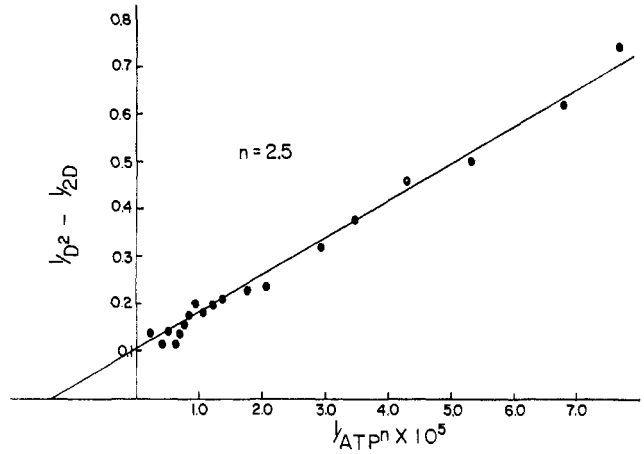

FIGURE 8: Determination of the molecularity of ATP binding to aspartic β-semialdehyde dehydrogenase. Data from Figure 7 are plotted according to eq 10 (see text for details). A value of n equal to 2.5 was used to obtain the best straight line.

some for analysis, a new set of inactivation conditions was employed for the determination of ATP molecularity. Inactivations were carried out in 10 mM phosphate buffer, pH 6.65, instead of 0.1 M Tris, pH 7.0. The ATP saturation curve in Figure 7 shows that under these conditions and at the protein concentration used, there is no inactivation of the enzyme at 0° in the absence of added ATP. Again saturation is not first order in ATP.

The equilibrium state under the new conditions can be written as follows assuming nth-order binding of ATP to the enzyme (eq 1). In this expression, E_2 = native tetrameric

$$E_2 \overset{nA}{\rightleftharpoons} E_2A_n \rightleftharpoons 2EA_{n/2} \tag{1}$$

enzyme, A = ATP, and E = dimer. Letting $E_2A_n = D_2$ and $EA_{n/2} = D$, then eq 1 becomes

$$E_2 \rightleftharpoons D_2 \rightleftharpoons 2D \tag{2}$$

The equilibrium constants for $E_2 \rightleftharpoons D_2$ and $D_2 \rightleftharpoons 2D$ can be written as K_A and K_D, respectively

$$K_A = \frac{[E_2][A]^n}{D_2} \tag{3}$$

$$K_D = \frac{[D]^2}{D_2} \tag{4}$$

Solving for E_2 and D_2

$$E_2 = \frac{K_A D_2}{[A]^n} \tag{5}$$

$$D_2 = \frac{[D]^2}{K_D} \tag{6}$$

Total enzyme, E_0, can be written as:

$$E_0 = E_2 + D_2 + D/2 \tag{7}$$

Substituting eq 5 into eq 7

$$E_0 = D_2[1 + (K_A/[A]^n)] + D/2 \tag{8}$$

Substituting eq 6 into eq 8

$$E_0 = \frac{[D]^2}{K_D}[1 + (K_A/[A]^n)] + D/2 \tag{9}$$

This rearranges to

$$\frac{E_0}{[D]^2} - \frac{1}{2D} = \frac{1}{K_D} + \frac{K_A}{K_D[A]^n} \tag{10}$$

If we let $E_0 = 1$, D becomes the fraction of undissociated enzyme. A and D are then experimentally determined quantities and a plot of $1/[D]^2 - 1/2D$ *vs.* $1/[A]^n$ should give a straight line if the correct value of n is chosen. The slope of this line will be K_A/K_D, and the intercept will be $1/K_D$. Figure 8 shows this type of plot for the data in Figure 7. A value of $n = 2.5$ was used to get a straight line, since values of $n = 2$ or 3 gave lines with marked curvature in opposite directions.

At saturating concentrations of ATP, the concentration of the E_2 species is negligible and K_D can be calculated from eq 2. Since the enzyme is 75% dissociated at high [ATP] (Figure 7), $K_D = 9$ or $1/K_D = 0.11$. The intercept in Figure 8 agrees well with this value.

Discussion

The model we have used for the dissociation reaction (eq 1) is the simplest one that might account for the data. We have equated inactivation with dissociation and justify this assumption by the fact that the data given in Figures 3 and 5 and in the text all fit curves based on that assumption. The same curves indicate that dissociation into dimers is sufficient to account for the data accurately. While we consider that we have shown inactivation to be associated with dissociation into halves, we do not know if we are observing the dissociation of tetramers into dimers or dimers into monomers. The former alternative is assumed in the discussion in the next paragraph. In the cases of both yeast and rabbit muscle glyc-

eraldehyde-3-phosphate dehydrogenase (Stancel and Deal, 1969; Constantinides and Deal, 1969) it has been shown that only the tetramer is an active form.

The equilibrium model of eq 1 requires that removal of ATP at 0° will return the enzyme to its active tetrameric form. The data in the text are in accord with this requirement. The binding of ATP to both dimer and tetramer is required by the fact that appreciable activity is retained at saturating ATP levels (Figures 1 and 7).

It is clear from analysis of Figure 1 and Figure 7 that the ATP effect is cooperative, and we have asked whether one ATP molecule is required per monomer or per dimer to produce inactivation. The objective value of $n = 2.5$, given by the linear plot in Figure 8, cannot have any literal meaning in the model shown even though it indicates that a model with one ATP per dimer fits the data far better than one ATP per monomer. We have fit curves to the data in Figure 7 and found that somewhat more revealing, since n values of 2 and 3 bracket the data. With $n = 2$ the curve fits the data at less than 50% inactivation fairly well. At greater than 50% inactivation the $n = 3$ curve fits the data better. There are probably more than two ATP sites per tetramer and, although one ATP per dimer is enough to promote dissociation into inactive dimers, higher concentrations of ATP may induce further dissociation, pulling the equilibrium farther toward inactivation.

Comparison with data on the behavior of glyceraldehyde-3-phosphate dehydrogenase shows some significant differences between the enzymes. Both rabbit muscle and yeast glyceraldehyde-3-phosphate dehydrogenase appear to be dissociated by ATP in a noncooperative way (Constantinides and Deal, 1969; Stancel and Deal, 1969) and all dissociated forms of those enzymes appear to be inactive. However, only monomers of the yeast enzyme have been observed while dimers and monomers are produced from the rabbit muscle enzyme. The glyceraldehyde-3-phosphate dehydrogenases are much more easily dissociated. ATP concentrations tenfold lower than we use for aspartic β-semialdehyde dehydrogenase are effective, and dissociation takes place at much higher protein concentrations (Constantinides and Deal, 1969; Stancel and Deal, 1969; see also Hoagland and Teller, 1969).

The initial rates of inactivation of all three enzymes are very similar, but the muscle glyceraldehyde-3-phosphate dehydrogenase shows a biphasic inactivation curve. The slow secondary loss of activity (Constantinides and Deal, 1969) may be an irreversible change, in accord with the fact that recovery of that enzyme upon warming is not quantitative—in contrast to aspartic β-semialdehyde dehydrogenase recovery.

Yang and Deal (1969a) postulated that ATP binds at the adenine portion of the NAD^+ site, and reported competition between the two at high ATP concentrations. In contrast our data show almost no competition. At 20 mM, ATP has no effect on the catalytic reaction of the enzyme at 30°, nor does NAD^+ at 50 times its kinetic K_m value alter the ATP effect at 0°. The data of Stancel and Deal (1969) also show that the competition of NAD^+ for the ATP sites is far weaker than predicted from the K_m of NAD^+.

We think it likely that there are separate sites for ATP binding with each of the enzymes, although we have no suggestion about the possible purpose of such sites. The existence of ATP binding sites on the two enzymes, critical for dissociation and inactivation, does seem, however, to add striking evidence in favor of a homologous relationship between the enzymes.

References

Constantinides, S. M., and Deal, W. C., Jr. (1969), *J. Biol. Chem. 244*, 5695.

Constantinides, S. M., and Deal, W. C., Jr. (1970), *J. Biol. Chem. 245*, 246.

Hoagland, V. D., Jr., and Teller, D. C. (1969), *Biochemistry 8*, 594.

Holland, M. J., and Westhead, E. W. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 2627 Abstr.

Holland, M. J., and Westhead, E. W. (1973a), *Biochemistry 12*, 2264.

Holland, M. J., and Westhead, E. W. (1973b), *Biochemistry 12*, 2275.

Irias, J. J., Olmstead, M. R., and Utter, M. F. (1969), *Biochemistry 8*, 5136.

Stancel, G. M., and Deal, W. C., Jr. (1968), *Biochem. Biophys. Res. Commun. 31*, 398.

Stancel, G. M., and Deal, W. C., Jr. (1969), *Biochemistry 8*, 4005.

Yang, S. T., and Deal, W. C., Jr. (1969a), *Biochemistry 8*, 2806.

Yang, S. T., and Deal, W. C., Jr. (1969b), *Biochemistry 8*, 2814.