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Two Aspartokinases from *Escherichia coli*. Nature of the Inhibition and Molecular Changes Accompanying Reversible Inactivation*

D. Eugene Wampler† and Edward W. Westhead

ABSTRACT: Two aspartokinases (adenosine triphosphate: 1-aspartate-4-phosphotransferase, EC 2.7.2.4) have been isolated from *Escherichia coli* K_{12} and some kinetic properties have been studied. Both enzymes are activated by potassium and to a lesser extent by ammonium, but not by sodium or tetramethylammonium ions. Both feedback inhibitors, L-lysine and L-threonine, exhibit cooperative binding to their respective enzymes while aspartate, adenosine triphosphate, and potassium (in the absence of inhibitors) display classical saturation kinetics. In the presence of inhibitor, aspartate binding to the lysine-sensitive enzyme remains hyperbolic, while aspartate binding to the threonine-sensitive enzyme becomes sigmoid. This deviation from classical saturation kinetics is related to the degree of competition between substrate and inhibitor. Aspartate, adenosine triphosphate, and potassium offer little protection to the lysine-sensitive enzyme against lysine inhibition while aspartate and potassium (but not adenosine triphosphate) strongly protect the threonine-sensitive enzyme against threonine inhibition. Both enzymes gradually lose activity when kept in phosphate buffer (pH 7.0), the threonine-sensitive enzyme more rapidly than the lysine-sensitive enzyme. Lost activity can be recovered by adding the feedback inhibitors to the partially inactivated enzyme solution. In the case of the threonine-sensitive enzyme, activity can also be restored by adding potassium chloride. The loss of threonine-sensitive activity is accompanied by a reduction in molecular size as measured by gel filtration.

There are, in *Escherichia coli*, three enzymes which catalyze the conversion of aspartic acid into aspartyl phosphate, the first reaction in the formation of lysine, threonine, methionine, and several other cellular components. For a more detailed discussion of the role of aspartokinase in metabolic control, see Patte *et al.* (1967). Two of these three aspartokinases were originally distinguished by their sensitivity to the feedback inhibitors, lysine and threonine (Stadtmann *et al.*, 1961). The third enzyme is not inhibited by any of the "aspartic acid family" of amino acids but is repressed by methionine (Patte *et al.*, 1967). Both the threonine- and lysine-sensitive aspartokinases are effectively stabilized by

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their feedback inhibitors. Because these two aspartokinases share a common catalytic function but differ in their inhibitor specificity, they offer an attractive opportunity to examine the structural features involved in feedback control mechanisms.

Materials

E. coli K_{12} was the gift of Dr. Martin Freundlich. Catalase, rabbit muscle pyruvate kinase, and rabbit muscle lactic dehydrogenase were obtained as crystalline ammonium sulfate suspensions from commercial sources. Enolase, prepared by the method of Westhead (1966), was a gift of Mr. Thomas Gawronski. Aspartic semialdehyde was prepared by ozonolysis of allylglycine (Black and Wright, 1955). All other reagents were obtained from commercial sources.

Methods

Measurement of Enzyme Activity. A unit of activity is defined as the amount of enzyme required to form 1 μmole of product or remove 1 μmole of substrate per min under the assay conditions. Specific activity is units of activity per milligram of protein. The protein concentration was estimated from absorbancy ratios at 260 and 280 mμ (Warburg and Christian, 1942). Three assay methods were used to measure aspartokinase activity, all at 30°.

Ferric Chloride Assay for Aspartokinase. The hydroxamate–ferric chloride assay was that described by Black (1965). The reaction mixture contained: 10.6 mM aspartic acid, 11.4 mM ATP[1] (disodium salt), 5.0 mM $MgAc_2$, 590 mM KCl, 106 mM Tris-Cl (pH 8.0), 531 mM hydroxylamine, and up to 0.05 unit of aspartokinase in a final volume of 0.7 ml. The reaction was allowed to proceed for 10 min and then stopped by adding 1 ml of the ferric chloride reagent. Commercial aspartic β-hydroxamate (Sigma Chemical Co.) showed a molar absorbancy (540 mμ) under these conditions of 6.3×10^5 cm^2/mole. This value is 5% greater than the absorbance reported by Black (1965).

Aspartic Semialdehyde Dehydrogenase Assay for Aspartokinase. Aspartyl phosphate, formed in the aspartokinase reaction, was reduced to aspartic semialdehyde by aspartic semialdehyde dehydrogenase with the accompanying oxidation of NADPH. The oxidation of NADPH was followed spectrophotometrically as suggested by Black (1965). The reaction mixture contained: 11.6 mM aspartic acid, 7.0 mM ATP, 5.8 mM $MgAc_2$, 0.60 mM NADPH, 588 mM KCl, 140 mM Tris-Cl (pH 8.0), 3–4 units of aspartic semialdehyde dehydrogenase, and up to 0.04 unit of aspartokinase in a final volume of 0.7 ml.

Pyruvate Kinase Assay for Aspartokinase. The third assay used pyruvate kinase and lactic dehydrogenase in a coupled system. ADP formed in the aspartokinase reaction was phosphorylated to ATP at the expense of phosphoenolpyruvate and the resulting pyruvate reduced by NADH. The oxidation of NADH was followed spectrophotometrically at 340 mμ. The reaction mixture contained: 11.4 mM aspartic acid, 3.3 mM ATP, 5.7 mM $MgAc_2$, 0.31 mM NADH, 570 mM KCl, 121 mM Tris-Cl (pH 7.5), 2.8 mM phosphoenolpyruvate, 12 units of pyruvate kinase, 25 units of lactic dehydrogenase, and up to 0.03 unit of aspartokinase in a final volume of 0.7 ml.

Although the oxidation of NADH was normally coupled directly to ADP production, the assay could also be run in the absence of pyruvate kinase and lactic dehydrogenase when the presence of these enzymes or their cofactors was undesirable. In the earlier experiments (potassium binding to the lysine-sensitive enzyme) the reaction was allowed to proceed for 2 or 3 min in the absence of the pyruvate kinase and lactic dehydrogenase mixture. When these two enzymes were added, there was a burst of activity and then a linear decrease in absorbance. The absorbance was extrapolated back to the time at which pyruvate kinase and lactic dehydrogenase were added and the extent of reaction during the initial 2- or 3-min period was measured.

In later experiments (potassium binding to the threonine-sensitive enzyme) the aspartokinase reaction was stopped by adding threonine before adding the auxiliary enzymes. Under these conditions it was much easier to extrapolate back to the time when the auxiliary enzymes were added since the final optical density remained nearly constant.

Comparison of Aspartokinase Assay Methods. The major advantage of the ferric chloride assay was that as many as 20 assays could conveniently be run at the same time, for example, when assaying fractions from a column. The spectrophotometric assays, however, are more sensitive since the molar absorbancy of NADH is ten times that of the iron–hydroxamate complex.

Since the reaction rate decreases rapidly at low aspartate concentrations, the ability to measure the reaction as it is occurring (in the coupled assay systems) was important for determining initial velocities. The pyruvate kinase assay has the added advantages of keeping a constant ATP concentration in the reaction mixture and of being linear over a wide range of optical density changes (2.0–0.5). Finally, the pyruvate kinase assay is convenient because crystalline suspensions of the two auxiliary enzymes are commercially available, usually free from interfering activities.

The principal disadvantage of the aspartic semialdehyde dehydrogenase assay is that threonine-sensitive aspartokinase, which contains homoserine dehydrogenase, can reduce the aspartic semialdehyde formed and thus oxidize a second mole of NADPH. It is hard to be sure that the rate of aspartyl phosphate production is just one-half the rate of NADPH removal.

It should be noted that inhibitor was in the normal working buffer and was therefore usually added to the

[1] Abbreviations are as stated in *Biochemistry 5*, 1445 (1966). Throughout the paper, "buffer P" refers to the normal working buffer which contains: 0.02 M potassium phosphate (pH 6.8), 0.5 mM L-threonine, 0.5 mM L-lysine, 2 mM $MgAc_2$, 2 mM EDTA, and 0.1 mM dithiothreitol. "Buffer T" had the same composition except that the potassium phosphate was replaced by 0.02 M Tris-Cl (pH 7.0).

assay mixture with the enzyme. When 20 μl of enzyme was added to a 0.68-ml reaction mixture, the final inhibitor concentration was 1.5×10^{-5} M. This concentration of inhibitor had no effect on the reaction velocity and kinetic measurements were made using either 10 or 15 μl of enzyme.

Other Assay Methods. Aspartic semialdehyde dehydrogenase was measured by following the reduction of NADP (Black and Wright, 1955), or by using the aspartic semialdehyde dehydrogenase assay for aspartokinase with excess lysine-sensitive aspartokinase. Catalase was measured by following the disappearance of H_2O_2 at 240 mμ (Beers and Sizer, 1952). Enolase was measured by following the appearance of phosphoenolpyruvate at 240 mμ (Warburg and Christian, 1942).

Purification of Enzymes. Aspartic semialdehyde dehydrogenase was purified from commercial yeast following part of the procedure of Black (1965), but substituting chromatography on DEAE-cellulose for ammonium sulfate precipitation.

Our procedures for purifying lysine- and threonine-sensitive aspartokinase from *E. coli* K_{12} were very similar to those described by Patte *et al.* (1966) and Truffa-Bachi and Cohen (1966). Cells were grown in minimal medium (Davis and Mingioli, 1950) containing 0.5% glucose to an absorbance at 650 mμ of 2.0–2.5, then chilled in an ice bath and harvested by continuous-flow contrifugation. The cell paste was suspended in an equal volume of buffer P[1] and sonicated for 10–14 min or until the absorbancy at 650 mμ decreased from about 250 to less than 35. The extract was treated with streptomycin sulfate, precipitated with ammonium sulfate, and then heated as described by Cohen and coworkers (Patte *et al.*, 1966; Truffa-Bachi and Cohen, 1966). The lysine- and threonine-sensitive enzymes were fairly well separated during the first ammonium sulfate precipitation. After each succeeding step, fractions which contained mostly lysine-sensitive enzymes were combined and kept separate from fractions containing mostly threonine-sensitive enzyme.

After heat treatment our purification procedure differed from that of Cohen's group. Heat-treated samples which contained most of the lysine-sensitive aspartokinase were concentrated by precipitating the enzyme with ammonium sulfate (0–0.37 saturation) and applied to a 3.5 $\times$ 97 cm column of Sephadex G-150 which had previously been equilibrated with buffer P. The column was run at room temperature (22°) at a flow rate of 2 ml/min. The most active fractions were combined and precipitated with ammonium sulfate, and the precipitate was redissolved in a small volume of buffer P (protein concentration 15–25 mg/ml). This enzyme solution, stored at 2° in the presence of a small drop of toluene, was stable for several months.

Heat-treated samples containing mostly threonine-sensitive enzyme were concentrated with ammonium sulfate (0.37–0.51 saturation) and passed through a 2.5 $\times$ 70 cm column of Sephadex G-200. The most active fractions were concentrated with ammonium sulfate and stored as described in the preceeding paragraph.

Changing Buffer Conditions. Aspartokinase solutions were exchanged from one buffer into another by passing up to 0.5 ml of the enzyme through a 1 $\times$ 27 cm column of Sephadex G-25. These columns were run at room temperature; 0.8-ml fractions were collected and the four fractions which contained most of the protein were combined. When kept for more than 12 hr, enzyme solutions were protected against bacterial growth by fitting the container with a rubber stopper which held a cotton wad soaked with toluene.

Results

Partial Purification of Aspartokinase. No matter which assay for aspartokinase was used, crude extracts contained some activity which was not inhibited by either lysine or threonine. This unhibited activity was dependent upon the addition of ATP and enzyme, but not aspartate. The amount of uninhibited activity in crude extracts depended to some extent on how the cells were broken. There seemed to be less spurious activity if cells were sonicated in small batches (100 ml or less) at low power (a power setting of 5 or 6 on the Branson Model S-125 Sonifier). In one case, cells were broken by grinding with glass beads in a Waring Blendor. This method did not give significantly better results than sonic oscillation. The French pressure cell gave an extract which contained less uninhibited activity and is probably the best way to extract aspartokinase in small batches, but sonic oscillation was much less work when large quantities of cells were broken.

In any case, the first ammonium sulfate fractionation removed much of the uninhibited activity since this activity remained largely in the final supernatant solution and was discarded. Uninhibited activity was completely separated from threonine-sensitive activity on the Sephadex G-200 column and was slowly separated from lysine-sensitive activity with each ammonium sulfate concentration step.

The specific activities of our preparations of threonine-sensitive aspartokinase were similar to those achieved by Cohen and coworkers at a similar stage of purification (0.99 *vs.* 1.26) but considerably below those achieved for lysine-sensitive aspartokinase (0.99 *vs.* 59) obtained from a derepressed strain.

Potassium Activation. Both aspartokinases, like rabbit muscle pyruvate kinase, require potassium for maximum activity. In order to remove potassium from the reaction mixture completely, aspartokinase was exchanged from buffer P into buffer T by passing 0.2 ml of the enzyme through a 1 $\times$ 27 cm column of Sephadex G-25 (see Methods) which had been equilibrated with buffer T.

Potassium activation was demonstrated by both the aspartic semialdehyde dehydrogenase assay and the pyruvate kinase assay (Figure 1a and Table I). When using the pyruvate kinase assay, the reaction was allowed to proceed in the absence of pyruvate kinase and lactic dehydrogenase as described in Methods. The threonine-sensitive enzyme shows an optimum activity between 0.3 and 0.4 M KCl while the lysine-sensitive enzyme has peak activity between 0.4 and 0.5 M KCl.

Activation by potassium chloride is not simply an ionic strength effect since tetramethylammonium chlo-

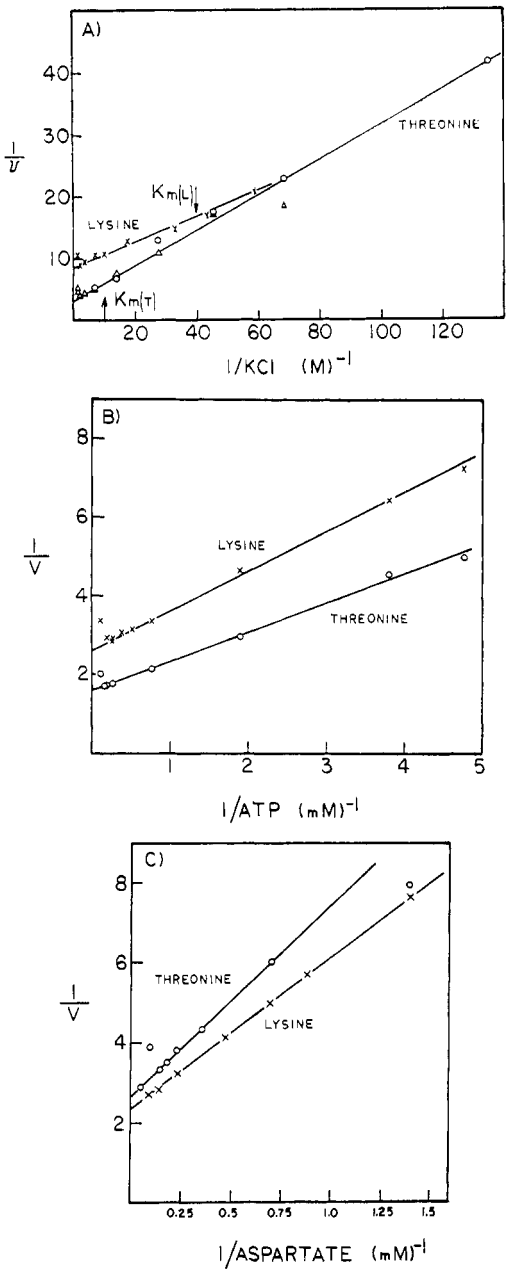

FIGURE 1: Double-reciprocal plot of initial velocity with respect to potassium chloride (A), ATP (B), and L-aspartate (C) concentration for the threonine-sensitive enzyme (△ or ○) and the lysine-sensitive enzyme (×). All data were obtained using the pyruvate kinase assay as described in the text. In part A the assay was modified to measure extent of reaction in the absence of pyruvate kinase and lactic dehydrogenase as described in methods. At potassium chloride concentrations below 0.43 M, tetramethylammonium chloride was added to keep a constant salt concentration of 0.43 M. Aspartate was added as the Tris salt. Arrows indicate the K_m concentration for the lysine-sensitive (K_m(L)) and threonine-sensitive (K_m(T)) enzymes. Data for the threonine-sensitive enzyme were obtained at two concentrations of enzyme, 0.008 unit/assay (△) and 0.012 unit/assay (○).

ride did not activate the enzymes and sodium chloride caused only slight activation (Table I). Ammonium chloride did activate both aspartokinases but the maximum activity was considerably less than the activity that could be obtained in the presence of potassium chloride (Table I).

TABLE I: Activation of Aspartokinase by Monovalent Cations.

Salt	Concn (M)	Aspartokinase Act.[a] Lysine Sensitive	Threonine Sensitive
No additions		0.04	0.00
Me_4NCl	0.43	0.02	0.00
NaCl	0.43	0.04	0.01
NH_4Cl	0.29		0.09
	0.43	0.13	0.08
	0.50		0.07
KCl	0.29		0.25
	0.43	0.21	0.26
	0.70	0.19	0.22

[a] Activity is the change in optical density per minute measured by the pyruvate kinase assay modified to be run in the absence of pyruvate kinase and lactic dehydrogenase.

Apparent K_m Values. The lines drawn in Figure 1a give apparent K_m values for potassium of 91 mM for the threonine-sensitive enzyme and 25 mM for the lysine-sensitive enzyme. Considerable attention was devoted to determining whether potassium activation of the threonine-sensitive enzyme was sigmoid or hyperbolic. The data shown in Figure 1a were obtained at two enzyme concentrations and then corrected to give the same maximum velocity. The resulting Lineweaver–Burk plot gives a straight line even when the potassium concentration is $^1/_{12}$ K_m. No determination was made of the possible influence of aspartate, ATP, or Mg^{2+} concentrations on the K_m values.

The apparent K_m for ATP depended on which assay was used. Using the ferric chloride assay, Patte *et al.* (1966) and Truffa-Bachi and Cohen (1966) have reported K_m values of 2.5 mM for the threonine-sensitive enzyme and 4.5 mM for the lysine-sensitive enzyme. We obtained similar results with the ferric chloride assay. The pyruvate kinase assay, on the other hand, gave K_m values of 0.18 mM for the threonine-sensitive enzyme and 0.38 mM for the lysine-sensitive enzyme (Figure 1b). This large difference probably occurs because the pyruvate kinase assay maintains a stable ATP concentration and prevents the accumulation of ADP while the ferric chloride assay does not. Using the pyruvate kinase assay, both enzymes exhibit maximum activity at an ATP concentration below 10 mM. It should be pointed out that our measurements were made at only one concentration of Mg^{2+}. Since ATP may bind as magnesium–ATP complex, these K_m and V_{max} values apply only to the reaction conditions which have been described. The Mg^{2+} concentration used (5.7 mM) is similar to that used by Patte *et al.* (1966) and by Truffa-Bachi and Cohen (1966).

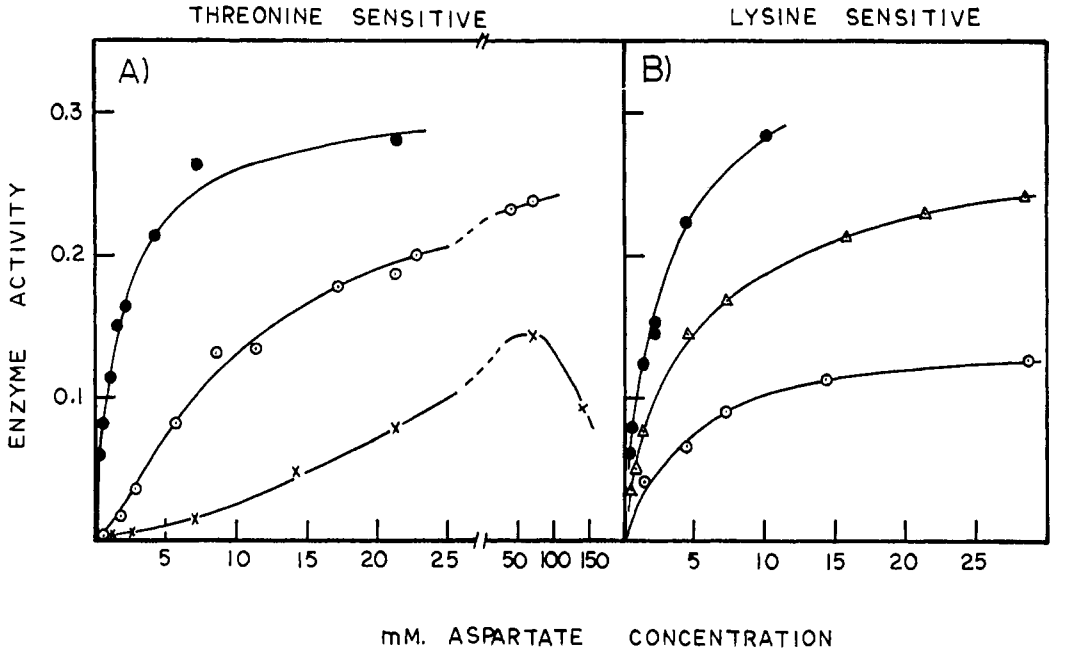

FIGURE 2: Aspartokinase activity as a function of aspartate concentration at low concentrations of the other substrates. Inhibitor concentrations are: 0.007 mM L-threonine or L-lysine (●), 0.14 mM L-lysine (△), 0.2 mM L-threonine or 0.56 mM L-lysine (○), and 0.3 mM L-threonine (×). The concentrations of substrates are: threonine-sensitive aspartokinase: 0.51 mM ATP, 0.57 mM Mg^{2+}, and 57 mM K^+, and lysine-sensitive aspartokinase: 0.49 mM ATP, 0.57 mM Mg^{2+}, and 19 mM K^+. Enzyme activity is the change in optical density per minute as measured in the pyruvate kinase assay.

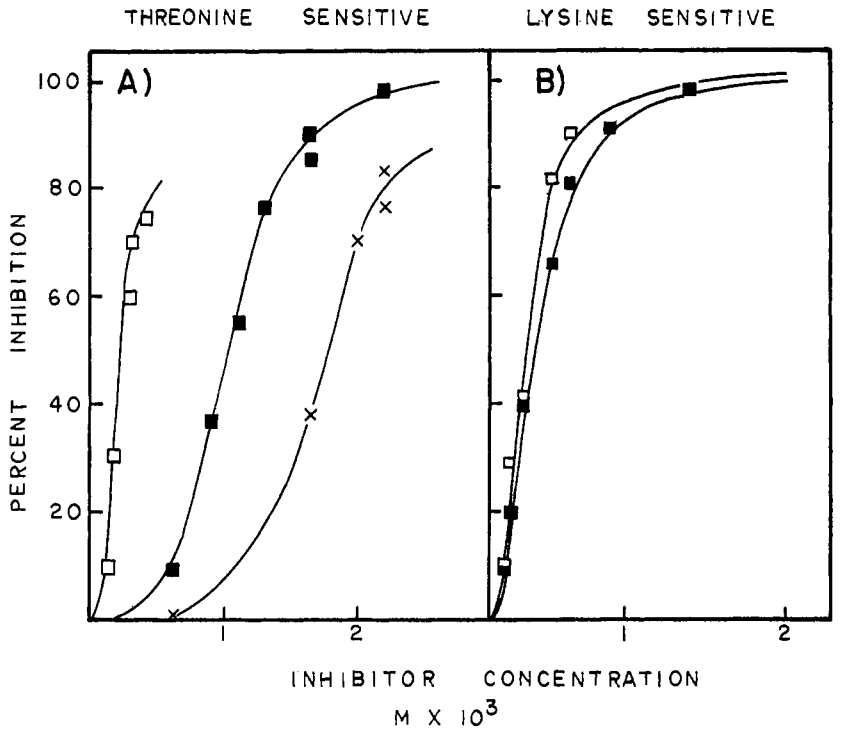

FIGURE 3: Exhibition of threonine-sensitive aspartokinase (left) and lysine-sensitive aspartokinase (right) at 2.5 mM potassium chloride and 0.5 M tetramethylammonium chloride (□), 0.5 M potassium chloride (■) and 1 M potassium chloride (×). Activity was measured by the homoserine dehydrogenase assay.

Apparent K_m values for aspartic acid, using the pyruvate kinase assay, were 1.8 mM for the threonine-sensitive enzyme and 1.6 mM for the lysine-sensitive enzyme (Figure 1c). Under normal reaction conditions, aspartate saturation curves are hyperbolic. Even when the aspartate scale is expanded tenfold, there is no indication of sigmoid character. It might still be possible that aspartate binding is cooperative, however, since saturating concentrations of ATP and potassium might pull the enzyme into the active form even in the absence of

TABLE II: Effect of Substrates and Potassium on the Inhibition of Lysine and Threonine-Sensitive Aspartokinase.

	M/K_m[a]		$I_{1/2}$[b]	
	Threonine	Lysine	Threonine	Lysine
Low K^+	0.33	0.24	0.11	0.25
Normal K^+	6.3	22.8	1.0	0.29
High K^+	11.0		1.61	
Low ATP	1.2	1.4	0.93	0.36
Normal ATP	7.7	8.7	1.11	0.50
Low aspartate	1.1	1.2	0.47	0.36
High aspartate	18.5	20.8	1.03	0.48

[a] M/K_m is the relative affector concentrations which is equal to the molarity divided by K_m. K_m values used are: threonine-sensitive aspartokinase: aspartate = 1.8 mM, ATP = 0.43 mM, potassium = 91 mM, and lysine-sensitive aspartokinase: aspartate = 1.6 mM, ATP = 0.38 mM, and potassium = 25 mM. [b] $I_{1/2}$ is the millimolar inhibitor concentration required for 50% inhibition. The $I_{1/2}$ for different ATP and aspartate concentrations was calculated from Hill plots of the data in Figures 4 and 5. $I_{1/2}$ for different potassium concentrations was calculated from Hill plots of data obtained using the pyruvate kinase assay modified to be run in the absence of pyruvate kinase and lactic dehydrogenase.

aspartate. To increase the sensitivity of the test for cooperative aspartate binding, the concentrations of ATP and potassium were reduced to their K_m values. Even at these low substrate concentrations, the aspartate saturation curves for both enzymes are rectangular hyperbolas.

At low concentrations of magnesium, potassium, and ATP (approximating the K_m's for these species), the K_m for aspartate for the lysine-sensitive enzyme increased only slightly (from 1.6 to 2.8 mM) while the K_m for aspartate for the threonine-sensitive enzyme remained the same (Figure 2).

Nature of the Inhibition. The transition between active and inhibited aspartokinase is rapid. Enzyme in the inhibited form (in the presence of 0.5 mM inhibitor) attained full activity in less than 2 sec (the limit of our observations) when diluted into the assay cuvet. Similarly, full inhibition was achieved in less than 2 sec when inhibitor was added to the reaction mexture (final inhibitor concentration 7.2 mM). Both enzymes produced S-shaped curves when per cent inhibition was measured as a function of inhibitor concentration. The effect of the substrates and activator on these inhibition curves differed markedly for the two enzymes.

Increasing the potassium ion concentration markedly raised the concentration of threonine required for inhibition of threonine-sensitive aspartokinase (Figure 3 and Table II). In contrast, inhibition of the lysine-sensitive enzyme by lysine was affected very little by increased potassium ion concentration.

A sevenfold reduction in the ATP concentration caused a relatively small shift in the lines to lower inhibitor concentrations (Figure 4). The inhibitor concentration required for 50% inhibition decreases 30% for the lysine-sensitive enzyme and 20% for the threonine-sensitive enzyme (Table II).

As shown in Figure 5 and Table II, a sixfold reduction in aspartate concentration causes a 50% reduction in the amount of threonine required to give 50% inhibition but less than a 25% reduction in the lysine concentration required for 50% inhibition.

When the data in Figures 3–5 are put into Hill plots, the resulting lines have slopes of 4 for the threonine-sensitive enzyme and 2 for the lysine-sensitive enzyme, confirming the results of Patte *et al.* (1966) and Truffa-Bachi and Cohen (1966). The inhibitor concentrations which gave 50% inhibition were calculated from these Hill plots.

In order to compare the effect of inhibitors on aspartate saturation curves for the two enzymes at lowered concentrations of potassium and ATP, it was necessary to choose inhibitor concentrations which gave comparable degrees of inhibition. For this purpose, plots were made of per cent inhibition *vs.* inhibitor concentration. ATP and potassium were at their K_m concentrations, magnesium was at the same concentrations as ATP, and aspartate was 10 mM. At these low substrate concentrations, the inhibition curves were again sigmoid; the Hill slope (n_H) for the lysine-sensitive enzyme was unchanged at 2.0, but n_H for the threonine-sensitive enzyme was reduced from 4.0 to 2.9.

From the above data, inhibitor concentrations were chosen which gave approximately 60% inhibition under these conditions (0.2 mM L-threonine and 0.56 mM L-lysine) and aspartate saturation curves were obtained. In the case of the threonine-sensitive enzyme, the curve is distinctly sigmoid (Figure 2a, open circles; $n_H = 1.2$) while the curve for the lysine-sensitive enzyme remains a rectangular hyperbola (Figure 2b).

Although the binding of aspartate to the lysine-sensitive enzyme shows no cooperative character in the presence of lysine, the K_m for aspartate increases slightly to 4.2 mM in the presence of 0.14 mM L-lysine and to 5.0 mM in the presence of 0.56 mM L-lysine.

Enzyme Stability. When aspartokinase solutions were run through a Sephadex G-25 column which had been equilibrated with buffer P lacking the appropriate feedback inhibitor, there was a rapid drop in activity during the first 15 min followed by a slower decay (Figure 6). Throughout this loss of activity, both enzymes remained fully sensitive to their respective inhibitors. When the appropriate inhibitor was added back to the enzyme solutions, most of the lost activity was recovered. Lysine had no effect on the loss or recovery of threonine-sensitive activity nor did threonine have any effect on the loss of lysine-sensitive activity. In the case of the threonine-sensitive enzyme, activity was recovered when potassium chloride was added to

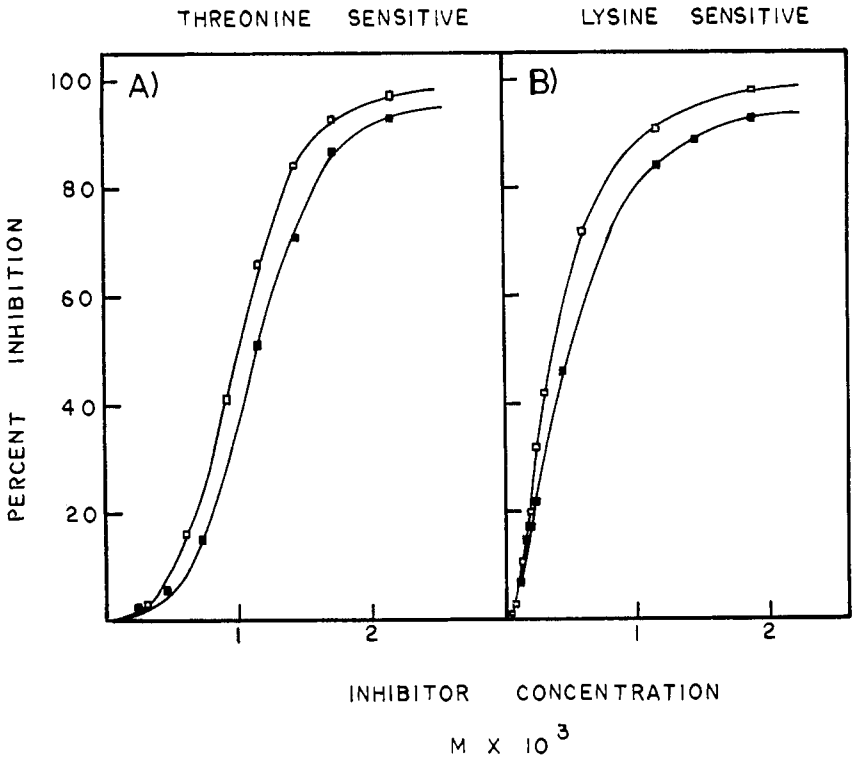

FIGURE 4: Inhibition of threonine-sensitive aspartokinase (left) and lysine-sensitive aspartokinase (right) at 0.52 mM ATP (□) and 3.3 mM ATP (■). Activity was measured by the pyruvate kinase assay as described in the text.

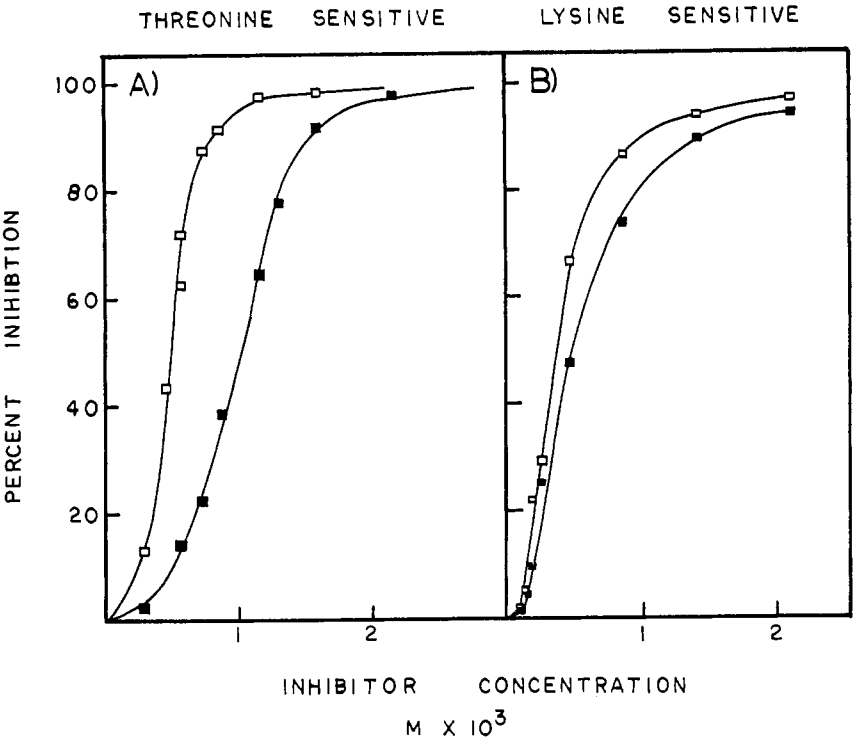

FIGURE 5: Inhibition of threonine-sensitive aspartokinase (left) and lysine-sensitive aspartokinase (right) at 2.0 mM aspartate (□) and 37.5 mM aspartate (■). Activity was measured by the pyruvate kinase assay as described in the text.

the enzyme solution 5 min after it had been exchanged from buffer P (Figure 7). Potassium chloride also increased the extent of reactivation when threonine was added to a solution of threonine-sensitive aspartokinase which had lost 70% of its original activity. In all cases, the aspartokinase activity was fully inhibited by lysine or threonine.

Reduction in Molecular Size. A sample of threonine-

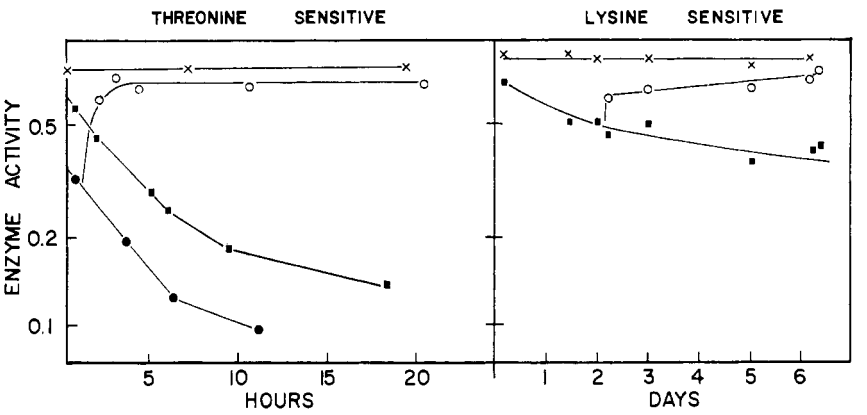

FIGURE 6: Aspartokinase solutions were exchanged into several buffers by passing through Sephadex G-25 as described in Methods. The buffers used were: buffer P (×), 0.02 M potassium phosphate (pH 6.8) containing 1 mM EDTA (■) and 1 mM EDTA brought to pH 7.0 with potassium hydroxide (●). Threonine-sensitive enzyme which had been stored for 1 hr at 0° in the EDTA buffer was made 0.5 mM in L-threonine and 20 mM in potassium phosphate, pH 6.8, and stored at 30° (○). Lysine-sensitive enzyme which had been stored for 54 hr at 0° in phosphate–EDTA buffer was made 5×10^{-4} M in L-lysine and stored at room temperature for 4 hr and stored thereafter at 0° (○). Enzyme activity was measured by the ferric chloride assay.

sensitive aspartokinase was divided into two portions. One portion was mixed with catalase (mol wt 250,000) and enolase (mol wt 68,000) and passed through a 12 × 455 mm column of Sephadex G-200 which had been equilibrated with buffer P. The first 15 ml was discarded and then 3-min, 0.8-ml fractions were collected and the activity of the three enzymes was measured. The results are given in the open symbols in Figure 8.

The second portion was exchanged into 0.02 M phosphate buffer (pH 6.8) containing 10^{-4} M dithiothreitol and allowed to inactivate until it had lost half of the original activity. This enzyme was then mixed with enolase and catalase and passed through the same Sephadex G-200 column which had been equilibrated with the phosphate–dithiothreitol buffer. After the catalase activity had come through the column, concentrated reagents were added to each fraction to give a final solution with the composition of buffer P. The fractions were stored at room temperature for a few hours and then the activity of all three enzymes was measured. The results are given in the solid symbols in Figure 8. While the catalase and enolase peaks are essentially superimposable, the aspartokinase peak has shifted to a region of lower molecular weight.

Discussion

A potassium requirement has been shown for all aspartokinases which have been tested: in *E. coli* by Wampler and Westhead (1966), in *Bacillus polymyxa* by Paulus and Grey (1964), and in *Rhodopseudomonas spheroides* by Datta and Prakash (1966). This relatively unusual requirement is shown by a number of kinases (pyruvate kinase is the best known case) and by at least two dehydrogenases (Dixon and Webb, 1964). It is notable that this requirement is also shown by the threonine-sensitive homoserine dehydrogenase of *E. coli* (Patte *et al.*, 1963) which is associated with the same protein as the threonine-sensitive aspartokinase (Patte *et al.*, 1966).

For both lysine- and threonine-sensitive aspartokinase, a plot of enzyme activity *vs.* aspartate concentration shows Michaelis–Menten kinetics with no evidence of sigmoid character, even when the concentrations of ATP, magnesium, and potassium are reduced to their respective K_m values (Figures 1 and 2). Although the maximum velocities are reduced at low substrate concentrations, the K_m values for aspartate are essentially unchanged. This can only mean that aspartate binds to both enzymes independently of the other substrates or activators. Potassium and magnesium, therefore, do not function as agents for the binding of aspartate.

Both enzymes show cooperative effects when activity is measured as a function of inhibitor concentration. This suggests that more than one molecule of inhibitor binds to each molecule of enzyme and indicates that the enzyme exists in two conformations, one enzymatically active and the other inactive (inhibited). Several models have been proposed which can explain S-shaped binding curves (see the review by Atkinson (1966) as well as recent papers by Kirtley and Koshland (1967) and Rubin and Changeux (1966)). The data in this paper are compatible with more than one of these models and, on the basis of kinetic data alone, we do not intend to prefer any one of them.

Considering first the data for the threonine-sensitive enzyme, it is apparent that the three catalytic ligands differ in the effectiveness with which they counteract threonine inhibition (Figures 3–5). The only parameters which can cause such differences are (a) differences in the concentration of effector in relation to its K_m or (b) differences in the degree to which the binding of effector and inhibitor are mutually exclusive. Complete exclusiveness, of course, results in purely competitive inhibition. The data in Table II show that the different effects illustrated in Figures 3a, 4a, and 5a are not due primarily to the concentration ranges used. Note particularly the differences between the effects of "normal" and "low" ATP on the one hand and the effects of normal and low potassium ion on the other. The differences shown in Figures 3–5 are thus due to differences in the degree of antagonism between threonine and the other affectors.

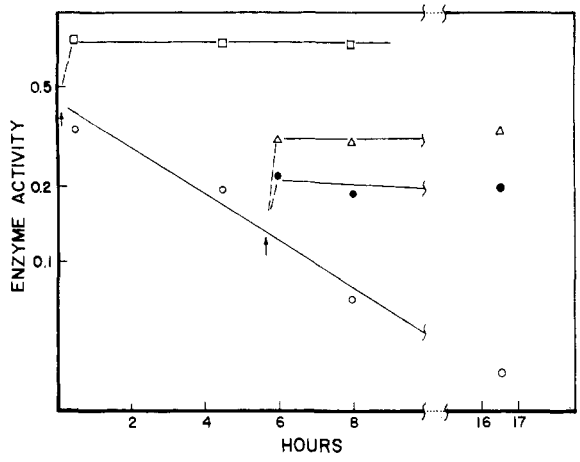

FIGURE 7: Threonine-sensitive aspartokinase was exchanged into 20 mM phosphate buffer (pH 7.8) containing 0.1 mM dithiothreitol as described in Methods and stored at room temperature (○). After 15 min (arrow) 0.5 ml of enzyme was mixed with 1 ml of phosphate buffer containing 0.1 mM dithiothreitol and 0.5 M KCl and stored at room temperature (■). After 5.75 hr 0.3 ml of enzyme was made 0.5 mM in L-threonine and stored at room temperature (●) and another 0.3 ml of enzyme was made 0.5 mM in L-threonine, 0.1 mM in magnesium acetate, 6.1 mM in EDTA, and 0.22 M in potassium chloride and stored at room temperature (△). Enzyme activity was measured by the pyruvate kinase assay.

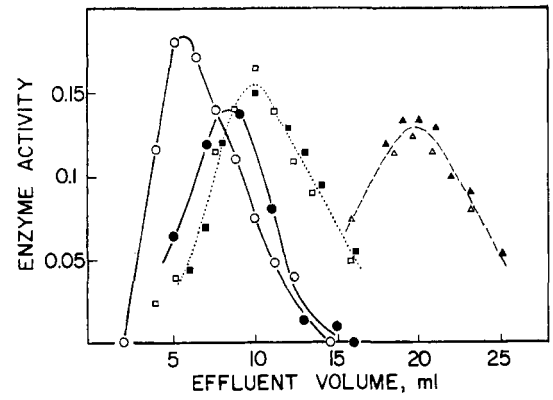

FIGURE 8: A mixture of threonine-sensitive aspartokinase (circles), catalase (squares), and enolase (triangles) was run through Sephadex G-200 in the presence (open figures) and absence (solid figures) of L-threonine and magnesium–EDTA as described in Methods. Aspartokinase was measured by the pyruvate kinase assay and the other two enzymes were measured as described in Methods. Enzyme activity is expressed in arbitrary units. The effluent volume was measured after the void volume (15 ml) had been collected.

Although there is an antagonism between aspartate and potassium binding, on the one hand, and threonine binding, on the other, it is not easy to determine the extent of this antagonism. Lineweaver–Burk plots have been used to show threonine inhibition to be competitive with aspartate (*e.g.*, Stadtmann *et al.*, 1961), but over a longer range of aspartate concentrations, this is evidently unsatisfactory. In the presence of threonine, high concentrations of aspartate inhibit the reaction while low concentrations give less activity than would be predicted by the Michaelis–Menten equation (Figure 2a). Since the resulting Lineweaver–Burke plot is U shaped, an accurate V_{max} cannot be calculated and we cannot be sure whether the inhibition is fully or partially competitive with aspartate. It is, in any case, strongly competitive (Figure 5a).

Turning to the lysine-sensitive enzyme, Figures 3 and 5 show that the antagonism between substrate and inhibitor is very different from that shown by the threonine-sensitive enzyme. Again the data in Table II show that this is not the result of inappropriately chosen substrate concentrations. Apparently none of the ligands binds to the lysine-sensitive enzyme in such a way as to exclude lysine binding. The same conclusion results from a consideration of Figure 2b. Although lysine binding is clearly a cooperative phenomenon the activation by aspartate in the presence of lysine is not cooperative; aspartate saturation curves remain hyperbolic even in the presence of enough lysine to cause 60% inhibition.

As Atkinson has illustrated, one can be misled in such a judgment by an ill-chosen coordinate scale (Atkinson, 1966). In our case, however, expanding the abscissa causes no change in the apparent shape of the curve and all of the data in Figure 2b fit a straight line in a Lineweaver–Burk plot. Although the presence of lysine has dropped the maximum activity to 40% of its uninhibited value, the change in K_m is only from 2.8 to 5.0 mM. It is apparent that the inhibition is, at most, slightly competitive.

Another difference between the two aspartokinases is the apparent order of inhibitor binding. Hill plots of the inhibition data in Figures 3–5 show slopes of two for lysine inhibition and four for threonine inhibition. These slopes give the order of the inhibitor interaction and may indicate the number of cooperating binding sites. It must be pointed out, however, that such an interpretation assumes a very special case in which there is no significant population of molecules with partially filled sites. This condition is met when the ligand has a strong preference for one of two forms and the ligand-free enzyme exists largely in the form to which the ligand is weakly bound. Rubin and Changeux (1966) have made calculations which clearly show that the slope of the Hill plot (n_H) may be far less than the number of binding sites (n) unless both of the above requirements are met. It may be shown, furthermore, that if these special requirements are not met, a significant number of enzyme molecules will have some, but not all, of the sites filled. If the filled sites are active, then the Hill plot must be curved since the ordinate will be the logarithm of a sum of terms containing different powers of the substrate concentration. With real data this curvature may not be seen. Calculations of hypothetical cases which are extreme enough to give greatly reduced values of n_H show very slight curvature.

One of the requirements mentioned above (that the equilibrium between protein confirmations lies far to one side) can be satisfied artificially. If the binding of two ligands (substrate and inhibitor) is mutually exclusive it is possible to shift the protein equilibrium toward one form by adding the ligand which binds more strongly to that form. In such a case the other ligand is in a situation that is thermodynamically indistinguishable from

the case where no ligand is bound and the natural (*i.e.*, ligand free) equilibrium is strongly in favor of one form.

Despite the problems in interpreting Hill plots, some information can be drawn from the data in Figures 3–5. Because cooperative inhibition of the threonine-sensitive enzyme is observed even at low substrate concentrations, and since aspartate and potassium pull the equilibrium toward the active form, at saturating substrate levels the threonine-sensitive enzyme must be almost entirely in the active form. Since threonine has a strong preference for the inactive form, the Hill slope ($n_H = 4$) is probably an accurate measure of the number of threonine binding sites. At lower substrate concentrations, threonine inhibition data give $n_H = 2.9$, showing that the protein equilibrium has shifted toward the inhibited form and the requirements for $n = n_H$ are not met.

Since aspartate has little preference for the active form of the lysine-sensitive enzyme, we have no way of estimating the ratio of molecules in the active and inhibited forms. In view of the discussion above, we cannot estimate the number of lysine binding sites, except that it must be two or more.

Although the binding of aspartate to lysine-sensitive aspartokinase is an example of nonexclusive binding, it differs from the specific case developed by Rubin and Changeux (1966). For the purpose of examining kinetic results they assume that all substrate–enzyme complexes are active (velocity is proportional to the number of substrate sites filled) so that the inhibitor can only affect the K_m of the substrate, not the catalytic constant. Inhibition of lysine-sensitive aspartokinase, on the other hand, changes V_{max} while having little effect on K_m. This means that the substrate–enzyme complex is not active when lysine is also bound.

In the absence of inhibitor, there is a slow transformation of both enzymes to an inactive, unstable form which can be reconverted to the inhibited form by adding lysine or threonine. In the case of the threonine-sensitive enzyme, at least, this transformation to the inactive, unstable form seems to be related to dissociation into subunits, as shown by gel filtration (Figure 8). Other, similar phenomena have been reported. Threonine inhibition of homoserine dehydrogenase from *R. rubrum* is apparently due to aggregation into a catalytically inactive form (Datta *et al.*, 1964), and Scott and Rabinowitz (1967) have shown that potassium causes the association of formyl tetrahydrofolate synthetase into an active tetramer.

The results reported in this paper show significant differences in the kinetic behavior of the two enzymes. Cohen and coworkers have shown striking differences in the structure of the two proteins. The threonine-sensitive complex has a molecular weight of 300,000 while the molecular weight of the lysine-sensitive enzyme is 180,000. Janin *et al.* (1967) have isolated a mutant of *E. coli* which is devoid of homoserine dehydrogenase activity and contains a threonine-sensitive aspartokinase activity with a molecular weight of 180,000. When this aspartokinase activity is desensitized with mercuribenzoate the molecular weight drops to 40,000 (Cohen *et al.*, 1967). If the different functions of these two aspartokinases are associated with separable subunits, as has been shown for aspartic transcarbamylase (Gerhard and Schachman, 1965), it would be interesting to see whether their catalytic subunits are similar or identical and whether the control subunits are greatly different. This system appears to be an ideal one for examining the relationship between structure and control function.

Acknowledgment

We thank Mr. Edward Maclosky and Mr. Ram Mathur for their help with much of this work.

Added in Proof

Recent experiments have shown that the threonine-sensitive enzyme, in 0.02 M phosphate buffer (pH 6.8) containing both 2 mM EDTA and 0.1 mM dithiothreitol, forms subunits without loss of activity. Thus the subunit formation shown in Figure 8 seems to depend simply on the removal of threonine while the reversible loss of activity shown in Figures 6 and 7 is associated with some additional factor.

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The Similarity of the Glyceraldehyde 3-Phosphate Dehydrogenases Isolated from Rabbit Brain and Muscle*

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ABSTRACT: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been isolated from rabbit brain. The crystalline enzyme appears homogeneous by chromatographic, electrophoretic, and ultracentrifugal criteria. The properties of the rabbit brain GAPDH are essentially the same as those of rabbit muscle GAPDH. The following characteristics of the brain enzyme were defined: (a) molecular weight, 147,000; (b) turnover number, 13,750 moles of nicotinamide–adenine dinucleotide (NAD) reduced/mole of enzyme per min; (c) 12.1 sulfhydryl groups/molecule; (d) four "active" sulfhydryl residues per mole; and (e) 2.5 moles of reducible NAD/mole of enzyme. No significant differences were detected in the peptide maps obtained after tryptic digestion or in the immunological properties of the two enzymes. It is concluded that the amino acid sequences of the brain and the muscle GAPDH are remarkably similar or identical.

Since the isolation and studies of D-glyceraldehyde 3-phosphate dehydrogenase from yeast by Warburg and Christian (1939), and from rabbit muscle by Baranowski (1939) and Bailey (1940), the molecular and catalytic properties of GAPDH[1] from these two sources have been studied extensively (Caputto and Dixon, 1945; Cori *et al.*, 1948). In recent years, comparative studies have been performed on GAPDH isolated from the muscle of a number of species including cat, dog, beef, pig, chicken, turkey, halibut, sturgeon, and lobster, as well as from *Escherichia coli* and *Bacillus stereothermophilus* (Elödi and Szörényi, 1956; Elödi, 1958; Allison and Kaplan, 1964; Amelunxen, 1966). From these studies, it seems that these proteins form a series of distinct but closely related and presumably homologous enzymes.

Multiple electrophoretically distinct forms of GAPDH have been found in yeast and, more recently, tissue-specific distribution patterns have been discovered in a number of species (Krebs, 1953; Lebherz and Rutter, 1967); however, no evidence of multiplicity of this enzyme was detected in various mammalian tissues tested (Lebherz and Rutter, 1967). In analogy with the tetrameric molecules, lactic dehydrogenase (Markert and Møller, 1959; Kaplan *et al.*, 1960) and aldolase (Penhoet *et al.*, 1966, 1967), five-membered sets of GAPDH isozymes are usually apparent in tissue extracts which exhibit GAPDH multiplicity (Lebherz and Rutter, 1967). The physiological significance, if any, of these GAPDH isozymes has not been defined. Moreover, the possible absence of multiple forms of this enzyme in various mammalian tissues is curious in view of the known multiplicity of several other glycolytic enzymes in these tissues. GAPDH has been isolated from a number of different tissues of the same species (human) including heart (Allison and Kaplan, 1964), skeletal muscle (Baranowski and Wolny, 1963), and red cells (Oguchi *et al.*, 1966), but no evidence concerning the possible identity of these molecules is available. Recently, Papadopolous and Velick (1967) have reported that crystalline rabbit liver GAPDH resembles the muscle enzyme in many molecular parameters but differs markedly in kinetic characteristics.

In the present investigation, we report the isolation of crystalline GAPDH from rabbit brain and compare its properties with those of rabbit muscle GAPDH.

Experimental Procedure

Substrate. DL-Glyceraldehyde 3-phosphate diethylacetal barium salt was purchased from Sigma Chemical

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: GAPDH, D-glyceraldehyde 3-phosphate dehydrogenase; TPCK, tosyl-2-amidophenylethyl chloromethyl ketone; PMB, *p*-hydroxymercuribenzoate.