Kinetic and Molecular Properties of Lysine-sensitive Aspartokinase

QUATERNARY STRUCTURE, CATALYTIC ACTIVITY, AND FEEDBACK CONTROL.*

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SUMMARY

Lysine or Mg++ above at high concentrations brings about partial dimerization of the lysine-sensitive aspartokinase of Escherichia coli B. The native enzyme, which has a molecular weight of 75,300 and consists of 2 subunits, responds to a combination of lysine and Mg++ at much lower concentrations by dimerizing to a tetrameric molecule with a molecular weight approaching 150,000. This dimerization is also observed in the presence of substrates at lysine concentrations known to be inhibitory. The substrates MgATP and aspartate also produce dimerization, and substrate inhibition is observed with MgATP. Aging of aspartokinase results in a loss of cooperativity for lysine inhibition with lysine saturation curves becoming hyperbolic. Aging also results in a loss of lysine-binding capacity and catalytic activity and decreases the extent of dimerization induced by lysine and Mg++. 

Every allosteric enzyme studied to date consists of more than 1 subunit, and, in many cases, the association-dissociation reactions of enzyme subunits have been implicated in the control of catalytic activity. Some examples include: bovine liver glutamate dehydrogenase, whose dissociation is enhanced by, among other things, the regulatory agent, GTP (1); acetyl-CoA carboxylase, which is activated by citrate, a process accompanied by aggregation of the enzyme to a polymer (2); the homoserine dehydrogenase (3) is enhanced by Mg2+. In addition, Mg2+ seems to favor the association of nonidentical subunits in aged preparations of aspartokinase from Bacillus polymyxa as observed electrophoretically (7). The zinc ion associated with the regulatory subunit of aspartate transcarbamylase may be required for normal association of the catalytic and regulatory subunits of this protein (8).

The lysine-sensitive aspartokinase (EC 2.7.2.4) of Escherichia coli is one of three enzymes catalyzing the phosphorylation of the β-carboxyl of aspartate in this organism. It is under feedback control by lysine, and lysine saturation curves, as determined by inhibition of catalytic activity, are sigmoid, giving numerical values of 2.0 for the slope of Hill type plots (9). Although there are several mechanisms which can give rise to this behavior, one possibility is that lysine is involved in a polymerization reaction with enzyme subunits, either by inducing the polymerization or stabilizing spontaneously formed polymers.

With regard to substrates, inhibition of aspartokinase by lysine is noncompetitive with respect to aspartate and ATP (10, 11) but the Vmax is affected much more than is the Ks for these substrates (12). Lysine is a competitive inhibitor with respect to Mg++ (13).

This report presents evidence that Mg++ has a role in lysine-mediated dimerization of aspartokinase, and that the dimerization reaction is related to lysine inhibition.

EXPERIMENTAL PROCEDURE

Growth of E. coli and Enzyme Purification—E. coli B cells were grown in continuous culture at 39°C in a New Brunswick Scientific Company Microferm Fermentor, in a minimal salts and glucose medium previously described (14). They were harvested near the end of the log phase by continuous-flow centrifugation. Lysine-sensitive aspartokinase from 200 liters (300 to 400 g of cells, wet weight) of culture was purified by previously published procedures (15). This method, conducted in 20 mM potassium phosphate, pH 6.75, containing 30 mM β-mercaptoethanol and 0.1 mM MgNa2EDTA, involves sonic disruption, removal of nucleic acid by streptomycin sulfate precipitation, ammonium sul-
fate fractionation, heat denaturation in the presence of lysine (which protects the lysine-sensitive aspartokinase specifically), and chromatography over DEAE-cellulose, Sephadex G-100, and Whatman DE-52. Later preparations were conducted as above, but 0.5 mM lysine was present in all buffers throughout the procedure. These preparations are referred to as type A and the former preparations without lysine as type B preparations. The yield is approximately 20 mg of enzyme for type A preparations and 10 mg for type B. Type A preparations are more stable and retain the allosteric properties of aspartokinase, whereas type B preparations slowly lose the ability to be inhibited by lysine. Both types of preparations have specific activities of around 14 to 16 pmoles of β-aspartyl hydroxamate formed per min per mg, somewhat lower than reported for the enzyme from *E. coli* K-12 (16).

**Assay for Aspartokinase Activity**—Aspartokinase activity was assayed by the hydroxamate procedure as previously described (15), except that the Mg²⁺ concentration was 3.2 mM.

**Determination of Protein**—Purified protein was measured according to the method of Waddell (17).

**Gel Electrophoresis**—Disc electrophoresis of aspartokinase in 7.5% polyacrylamide gel was performed according to the technique of Davis (18), with the glycine-Tris buffer system at a running pH of 7.0. Typical results are shown in Fig. 1.

**Sedimentation Velocity**—Determinations were made at 59,780 rpm in the Beckman Spinco model E ultracentrifuge, using schlieren optics. The protein concentration was 7.5 mg per ml. The solvent was 20 mM potassium phosphate, pH 6.75, 30 mM β-mercaptoethanol. The temperature was 21°C. Lysine (11 mM), or Mg²⁺ (3.2 mM), or both, was present in some determinations.

**Sedimentation Equilibrium**—Sedimentation equilibrium molecular weight determinations were performed in the Beckman Spinco model E ultracentrifuge according to the high speed meniscus depletion method of Yphantis (19) at 11°C. Protein concentrations were 0.1 to 0.4 mg per ml in 20 mM potassium phosphate buffer, pH 6.75, 30 mM β-mercaptoethanol with the indicated additions. Fluorocarbon FC-43 was used for the base fluid in the cell. The partial specific volume of aspartokinase was assumed to be 0.735 ml per g (16).

To determine the number of subunits, the above procedures were carried out in 6 M guanidine hydrochloride and 0.1 M β-mercaptoethanol. Protein was diluted 1:10 in this solution and was dialyzed against it overnight. Under these conditions the partial specific volume was assumed to be 0.72 (20). The protein was sufficiently diluted so as to avoid the decrease in the observed molecular weight which occurs at higher protein concentrations in this solvent (21).

In order to determine the length of time required to attain equilibrium, fringe displacement between the meniscus and a fixed value of r (distance from the center of rotation) was plotted as a function of time, until the displacement became constant.

**Lysine Binding**—Lysine binding to the enzyme was measured at 27°C (room temperature) using the ultrafiltration cell developed by Paulus (22) which was obtained from Metaloglass, Inc., Boston, Mass. The purified enzyme was stored in 20 mM phosphate buffer, pH 6.75, 0.1 mM MgNa₂EDTA, 30 mM β-mercaptoethanol, and 0.5 mM lysine and was passed through a Sephadex G-25 column equilibrated with 20 mM potassium phosphate, pH 6.75, 1 mM Na₄ EDTA, 30 mM β-mercaptoethanol to remove lysine, and Mg²⁺ prior to all binding experiments. In a total volume
of 0.3 ml, the solution for ultrafiltration contained 100 mM Tris buffer (pH 7.0), 10 mM \( \beta \)-mercaptoethanol, 25 to 40 mg of aspartokinase, 0.4 mCi of \(^{14}\)C lysine (specific activity 264 mCi per mmole), and varying amounts of unlabeled lysine. The phosphate concentration arising from the 0.05 ml of enzyme solution routinely used was 3.33 mM, well below the maximum of 10 mM recommended for the Amicon UM-10 filters. The above solution (0.25 ml) was pipetted into the ultrafiltration cell fitted with Amicon UM-10 filter discs. Pressure of 30 p.s.i. was applied, and, after the solution had passed through the membrane, the bottom side of each filter disc was washed with 5 ml of ethylene glycol; the filters were then transferred to 1 ml of distilled water. The radioactivity was determined by liquid scintillation counting in 10 ml of the Cellosolve-dioxane-xylene system of Bruno and Christian (23). Data are expressed as picomoles of lysine bound per microgram of protein. Blank values determined in the absence of protein to compensate for the 2.5 to 3 μl of solution trapped in the UM-10 filters were subtracted from the experimental values for each set of experiments.

**Evaluation of Sigmoidicity of Lysine Saturation Curves**—Velocity determinations made in the presence of various lysine concentrations were plotted as Hill type plots (log \([v/(v_i - v)]\) versus log [lysine]) where \(v_i\) is the velocity in the presence of inhibitor and \(v\) is the velocity in the absence of inhibitor. The lysine concentrations required for 25% and 75% inhibition were determined from the plot by determining the lysine concentrations at the intersection of the experimental plot with log 0.33 and log 3.0. The ratio of the concentration required for 75% inhibition to that required for 25% inhibition was computed and defined as \(R'_s\), according to Koshland et al. (24). A rectangular hyperbola gives a value of 9.0. A value of 3.0 corresponds to a Hill coefficient of 2.0. This \(R'_s\) value and the Hill coefficient (n) are related by the expression \(R'_s^n = 9\). Conformity to a hyperbolic rate law was also tested using Dixon plots, i.e. reciprocal velocity versus inhibitor concentration.

**Aging of Enzyme**—Lysine was removed from the enzyme solution by chromatography on a column (1 × 20 cm) of Sephadex G-25 equilibrated with phosphate buffer, pH 6.75, containing 0.1 mM MgNa₂EDTA and 30 mM \( \beta \)-mercaptoethanol. The enzyme was located in the effluent by its absorbance at 280 nm and was subsequently stored in a glass test tube packed in ice. Aliquots were withdrawn periodically and assayed for catalytic activity, sensitivity to lysine inhibition, and lysine-binding capacity. Type B preparations have already aged significantly, as indicated by their \(R'_s\) value, by the time the preparation procedure is completed.

**RESULTS**

The molecular weight of aspartokinase as determined by sedimentation equilibrium was 75,300 ± 1,600 in the absence of Mg\(^{2+}\) and 74,000 ± 1,100 in 3 mM Mg\(^{2+}\). There was no difference between type A and type B preparations in this respect. In 6 M guanidine hydrochloride plus 0.1 M \( \beta \)-mercaptoethanol, the weight was 39,000 ± 150 with no heterogeneity apparent, indicating 2 polypeptide subunits. Addition of lysine plus Mg\(^{2+}\) causes dimerization to a weight of 150,000 (Figs. 2 and 3). A

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**TABLE I**

**Effect of Mg\(^{2+}\) on lysine binding by aspartokinase**

Data are expressed as picomoles of lysine bound by 1 μg of aspartokinase protein. Binding was measured using L-[\( ^{14}\)C]lysine as described in the text. The Mg\(^{2+}\) concentration was 3.2 mM and 1 mM Na₄ EDTA was included in the absence of Mg\(^{2+}\) and 1 mM MgNa₂EDTA in the presence of Mg\(^{2+}\). Experiments were conducted with type A preparations which were aged in the absence of lysine for various times as follows: Experiment 1, 0 days; Experiment 2, 3 days; Experiment 3, 9 days.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lysine</th>
<th>+Mg(^{2+})</th>
<th>−Mg(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>picomoles/μg</td>
<td>picomoles/μg</td>
</tr>
<tr>
<td>1</td>
<td>0.48</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9.3</td>
<td>10.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10.9</td>
<td>11.2</td>
<td>0.073</td>
</tr>
</tbody>
</table>

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1 Mean ± standard deviation.
plot of molecular weight versus lysine concentration with and without Mg²⁺ and including both fresh (type A) and aged (type B) enzyme preparations is shown in Fig. 2. It is apparent that Mg²⁺ enhances the lysine-mediated dimerization. Furthermore, aging of aspartokinase in the absence of lysine impairs the ability to dimerize.

This monomer-dimer equilibrium is a rapidly reversible phenomenon, since sedimentation at high speed yielded a single peak in the presence of lysine with no Mg²⁺ (S = 6.2) as well as single peaks for the enzyme alone (S = 4.6) and in the presence of lysine plus Mg²⁺ (S = 6.7) (Fig. 4). The height of the peak in Fig. 4B for enzyme plus lysine (no Mg²⁺) is such that even 10% of the 4.6 S component could have been observed had it been present.

The effect of Mg²⁺ is not due to its facilitating the binding of lysine to the enzyme. As shown in Table I, Mg²⁺ is not required for lysine binding.

Since lysine is an inhibitor of this enzyme, one assumes that the dimerization is related to the inhibitory action of lysine. It was considered critical therefore to determine whether this dimerization occurred under assay conditions. This led to the experiments presented in Table II. Lysine (1.0 mM) in the standard assay produces around 95% inhibition. When the molecular weight is determined in this medium, the value obtained approaches that for a dimer of the native protein demonstrating dimerization under conditions in which the enzyme is known to be inactive. All substrates were tested for their effect on quaternary structure at assay concentrations and at 10-fold excess concentrations. Mg²⁺, MgATP, and aspartate produced appreciable dimerization. ATP alone had no apparent effect on molecular weight.

If the tetramer (dimer of the native enzyme) is a consequence or a determinant of inhibition, this dimerization by substrates would predict substrate inhibition. This is observed with MgATP as the variable substrate in Fig. 5. We have not observed substrate inhibition by aspartate, but some inhibition by very high Mg²⁺ has been observed (15) when Mg²⁺ exceeds the ATP concentration.

In Table III we compare the stabilities of type A and type B preparations, both in regard to catalytic activity and lysine inhibition. Type B preparations have aged significantly, as well as the cooperativity of lysine inhibition. Type B preparations lose both catalytic activity and cooperativity, demonstrating that lysine protects both catalytic and control properties of the enzyme. The maximum inhibition attainable by lysine is also increased and is illustrated as percentage of inhibition by 1 mM lysine. These values can be increased somewhat by increasing the lysine concentration drastically. The loss of cooperativity in lysine inhibition is illustrated in Fig. 6. Partially purified type B preparations retain cooperativity and sensitivity to lysine to a greater extent than do the purified preparations. In this respect, they resemble type A preparations stored in the presence of lysine.

Since the type B preparations have aged significantly, as reflected by the Rₖ value, by the time the purification is completed, we have also examined the aging of type A preparations after the removal of lysine. These data are also presented in Table III. Catalytic activity, lysine cooperativity, lysine binding and lysine inhibition all decrease to varying degrees with aging.

**TABLE II**

**Influence of substrates on quaternary structure of aspartokinase**

- Molecular weights were determined as described in the text.
- Type A preparations were used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mol wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartokinase alone</td>
<td>76,000</td>
</tr>
<tr>
<td>ATP (10 mM), Mg²⁺ (3.0 mM)</td>
<td>78,000</td>
</tr>
<tr>
<td>Aspartic acid (10 mM), ATP (10 mM), Mg²⁺ (3.0 mM),</td>
<td>80,500</td>
</tr>
<tr>
<td>lysine (0.5 mM)</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid (10 mM), ATP (10 mM), Mg²⁺ (5.0 mM),</td>
<td>114,200</td>
</tr>
<tr>
<td>lysine (1.0 mM)</td>
<td></td>
</tr>
<tr>
<td>ATP (100 mM)</td>
<td>75,500</td>
</tr>
<tr>
<td>ATP (100 mM), Mg²⁺ (30.2 mM)</td>
<td>130,500</td>
</tr>
<tr>
<td>Aspartic acid (100 mM)</td>
<td>104,000</td>
</tr>
<tr>
<td>Aspartic acid (100 mM), ATP (100 mM), Mg²⁺ (30.2</td>
<td>126,100</td>
</tr>
<tr>
<td>mM)</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺ (40 mM)</td>
<td>108,000</td>
</tr>
</tbody>
</table>

**Fig. 5** (left). Double reciprocal plot of activity versus ATP concentration. The Mg²⁺:ATP ratio was constant at 0.32. Assays were conducted as mentioned in the text. Velocity is expressed as micromoles of β-aspartyl hydroxamate formed per min. Type A preparation was used.

**Fig. 6** (center and right). Dixon plots for fresh and aged preparations of aspartokinase. A, fresh enzyme (type A preparation) showing linear proportionality of 1/v to (Lysine); B, aged enzyme (type B preparation) showing linear proportionality of 1/v to (Lysine). Velocity is expressed as the change in optical density at 560 nm after 30-min incubation.
The change in S values produced by lysine or lysine plus Mg\textsuperscript{2+} are precisely what one would expect for a dimerization reaction assuming other factors remain constant. Since S values were determined at a protein concentration at least 15-fold higher than the molecular weight determinations, the process does not appear to be markedly concentration dependent except possibly when lysine is present alone. In this case, the S values obtained in the presence of lysine or lysine plus Mg\textsuperscript{2+} are very close, suggesting a greater extent of aggregation with lysine alone than is observed at the lower protein concentrations employed for the sedimentation equilibrium determinations. Additional, but less rigorous, support for relative concentration independence in the range of the equilibrium studies is provided by the linearity of most fringe displacement plots for molecular weights intermediate between 75,000 and 150,000. There is, however, a weak tendency\textsuperscript{2} for the observed molecular weight with lysine alone to increase with protein concentration in this range, suggesting that more extensive aggregation would be apparent with lysine alone at higher protein concentrations. This would be consistent with the sedimentation velocity data.

A synergistic action between two or more ligands in altering enzyme activity is not uncommon. Aspartokinase itself displays cooperative, heterotropic interactions between lysine and several other amino acids. Concerted or multivalent inhibition of the B. polymyza aspartokinase by lysine plus threonine is also an example (25). Such interactions are characterized kinetically...
and have usually been ascribed to cooperative binding. This has been demonstrated directly in the case of B. polyoxynus aspartokinase where lysine is required for threonine binding and vice versa (22). Data presented in this paper demonstrate a similar synergism in altering enzyme quaternary structure and suggest the possibility that not all cases of synergism or concerted behavior need necessarily be related to cooperative binding of the two ligands, but some may be manifest at the level of a structural change (quaternary or tertiary) which requires the simultaneous binding of both ligands.

The synergistic relationship of Mg²⁺ and lysine in altering quaternary structure does not provide an explanation for the competitive reversal of lysine inhibition by Mg³⁺ in kinetic experiments. Further exploration will be necessary to relate these two observations at the molecular level.

Two experimental facts indicate that the dimerization reaction is related to inhibition of enzyme activity. Significant dimerization is observed under conditions of substrate and lysine concentrations where the activity is known to be inhibited, and high Mg²⁺ or MgATP concentrations also produce dimerization in the same concentration range which produces substrate inhibition. The failure to demonstrate significant dimerization by ATP alone is consistent with the assumption that MgATP is the true substrate which binds to the enzyme. Whether or not Mg²⁺ may bind at other sites, which may be responsible for the dimerization, cannot be stated but seems unlikely since these sites would have to bind Mg²⁺ with a high affinity to be functional at the low Mg²⁺ concentration available in the presence of MgATP. Such a possibility seems inconsistent with the high concentration of Mg²⁺ required to produce dimerization in the absence of ATP. The failure of aspartate to produce substrate inhibition at high concentrations in the face of its ability to bring about dimerization may indicate that the dimers produced by MgATP differ in some way from those produced by aspartate.

The data in Table III emphasize the complexity of the aging of aspartokinase. Lysine-binding capacity decreases faster than does maximum inhibition but more slowly than catalytic activity. This suggests the formation of enzyme molecules which are inactive but which retain the ability to bind lysine. Such molecules of course contribute to the molecular weight determinations and may or may not dimerize. Enzyme molecules which do not bind lysine presumably do not dimerize and would be measurable in molecular weight determinations as monomers. Only a small fraction of catalytically active molecules fit in this category in view of the slow decline in maximum inhibition. Circumstantial evidence that the dimerization reaction is the basis for the cooperativity of lysine inhibition is provided by the fact that both are decreased in extent in aged enzyme preparations. Inhibition, whether hyperbolic or sigmoid, certainly requires lysine binding, leaving the possibility that dimerization is responsible for the cooperativity observed in fresh preparations. A loss of subunit interactions in the tetrameric state would also explain the loss of cooperativity. In view of their low catalytic activity, lack of cooperativity in lysine inhibition, and decreased dimerization, the bulk of enzyme molecules in aged preparations are probably inactive monomers.

REFERENCES

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