Concerted Feedback Inhibition

PURIFICATION AND SOME PROPERTIES OF ASPARTOKINASE FROM PSEUDOMONAS FLUORESCENS

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STEPHEN M. DUNGAN* AND PRASANTA DATTA

From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48104

SUMMARY

Aspartokinase of Pseudomonas fluorescens has been purified by heat treatment, ammonium sulfate fractionation, DEAE-cellulose chromatography, and by successive gel filtration steps on Sephadex G-200 in the presence and absence of feedback modifiers. The 700-fold purified enzyme was judged to be greater than 90% pure. Sedimentation velocity centrifugation yielded an $s_{20w}$ value of 6.7 S, a $D_{20w}$ value of $5.3 \times 10^{-7}$ cm$^2$ s$^{-1}$, and a molecular weight of 133,000. The Stokes radius of the native enzyme in 50 mM potassium phosphate buffer, pH 7.5, containing 200 mM KCl was found to be about 43 Å. A diffusion coefficient of $5.2 \times 10^{-7}$ cm$^2$ s$^{-1}$ was calculated from the Stokes-Einstein equation. A molecular weight of 137,000 was also estimated from the gel filtration data. The molecular weight of the subunit was 43,000.

The enzyme had an absolute requirement for ATP and a divalent cation. No other nucleoside phosphates served as the phosphate donor. Addition of 200 mM KCl increased the reaction rate by about 40%; sodium and lithium ions had no effect.

The activity of this enzyme was inhibited by concerted feedback by two pairs of amino acid end products: lysine plus threonine and methionine plus threonine. Individually, lysine and methionine were slightly stimulatory; threonine showed a slight activation at low concentrations, but was weakly inhibitory at concentrations over 10 mM. Concentrated inhibition by low threonine and high lysine combination was compensated by methionine; whereas, methionine did not counteract when enzyme activity was severely decreased by high concentrations of threonine plus lysine. A possible physiological significance of the dual role of methionine is discussed.

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† Predoctoral Trainee of the United States Public Health Service, Grant GM 00187. Present address, Department of Biochemistry, University of Miami School of Medicine, Miami, Fla. 33182.

‡ To whom inquiries should be addressed.

Aspartokinase (ATP:aspartate 4-phosphotransferase EC 2.7.2.4) is the first enzyme of the branched biosynthetic pathway leading to the synthesis of four amino acids of the aspartate family: lysine, methionine, threonine, and isoleucine (1). The enzyme catalyzes the phosphorylation of L-aspartic acid by ATP to produce aspartyl P-phosphate (2). Recent studies have revealed the existence of multiple metabolic feedback patterns of this key enzyme in a variety of microorganisms (3). In Escherichia coli and Salmonella typhimurium, isoenzymes of aspartokinase, each regulated by one metabolite, appear to be the principal mode of control (4, 5); in other organisms such as Rhodopseudomonas capsulata and in several species of Bacillus, concerted action of two metabolites controls the activity of a single enzyme (6-8). In preliminary experiments (3, 9) we found that the enzyme activity in crude extracts from Pseudomonas fluorescens was severely inhibited by the concerted action of low concentrations of L-lysine plus L-threonine; individually, lysine was a mild activator and threonine was weakly inhibitory. Furthermore, the enzyme activity was also inhibited by the combination of L-threonine and L-methionine (3, 9). Although, in metabolic terms, concerted feedback inhibition has proven to be an important physiological device for the control of early enzymes (3, 9-11), relatively little information is available on the mechanism of this phenomenon at the molecular level. In this communication we describe the purification, some general properties, and regulation of the aspartokinase from P. fluorescens. In the accompanying paper (12), we present experimental evidence to support the notion that the mechanism of concerted feedback inhibition may involve modifier-induced formation of inactive enzyme oligomers. A preliminary report of some of these findings has been published (see Ref. 13).

EXPERIMENTAL PROCEDURE

Materials

All ingredients of the bacterial growth medium were obtained from Difco Laboratories. Chromatographically pure amino acids and amino acid analogs were supplied by Sigma Chemical Co., Calbiochem, and Cyclco Chemicals. ATP was supplied by Sigma. Yeast alcohol dehydrogenase, catalase, β-galactosidase, and peroxidase were purchased from Worthington Biochemical Corp.; bovine serum albumin was obtained from Miles Laboratories.
Methods

Aspartokinase Activity Assays—Enzyme activity was routinely assayed by the method of Black (2). The standard reaction mixture, in a final volume of 1.0 ml, contained in micromoles: hydroxylamine hydrochloride, 608; MgCl₂, 10; KCl, 200; ATP, 20; and L-aspartic acid, 50. One unit of enzyme was defined as the amount producing 1 μ mole of aspartohydroxamate in 60 min at 25° as calculated from the molar extinction coefficient of 625 determined with authentic β-aspartohydroxamate. All values reported with this procedure were within the linear range of the assay.

In some instances, a coupled assay using pyruvate kinase and lactate dehydrogenase was described by Wampler and Westcoast (14) and employed. The standard reaction mixture contained 50 mM imidazole-HCl buffer, pH 7.6, 1 mM ATP, 20 mM L-aspartic acid, 200 mM KCl, 10 mM MgCl₂, 2 mM phosphoenolpyruvate, 0.3 mM NADH, 5 μg of lactate dehydrogenase, and 50 μg of pyruvate kinase in a final volume of 1.0 ml. The initial velocities were linear up to 2.5 μg of protein.

Protein Determination—Protein was routinely assayed by the method of Lowry et al. (15).

Polyacrylamide Gel Electrophoresis—Analytical disc gel electrophoresis was carried out in 7.5% gels according to the method described by Brewer and Ashworth (16). Protein bands were located by staining with Coomassie blue. For an in situ activity stain, the gel was incubated in a reaction mixture for the hydroxamate assay; after 15 to 30 min, it was transferred to a tube containing the FeCl₃ color reagent, and a faint brown band could be seen which faded rapidly.

For preparative gel electrophoresis, a 7.5% gel, 2.5 × 7 cm, was pretreated electrophoretically at 5 mA for 1 hour in 0.074 M Tris-0.192 mM glycine buffer, pH 7.4, containing 0.25 mM EDTA and 1 mM each of L-threonine and L-lysine. The gel was eluted with 0.074 M Tris-HCl, pH 7.2, containing 10 mM β-mercaptoethanol and 1 mM each of L-lysine and L-threonine.

The procedure for routine subunit studies and molecular weight calibrations using sodium dodecyl sulfate-polyacrylamide gels was that of Weber and Osborn (17).

Sedimentation Velocity and Diffusion Constant Measurements—Sedimentation velocity experiments were carried out in a Beckman model E analytical ultracentrifuge using the An-D rotor. All sedimentation coefficients were converted to s₂₀,w values after correction for temperature and solvent viscosity. Diffusion coefficients were measured following the standard technique (18).

Stokes Radius Determination—Stokes radius was determined on calibrated Sephadex G-200 columns as described (19, 20). The location of the protein peaks was established by assay for their activities (14, 21).

Strain and Growth Conditions—A wild type strain of P. fluorescens (P-14) was kindly supplied by Dr. Ronald H. Olsen of the Department of Microbiology, the University of Michigan. A stock culture was maintained on nutrient slants. Large scale cultures were grown at 25° in a New Brunswick Farnell fermentor in the minimal salts medium of Vogel and Bonner (22) supplemented with 0.2% glucose.

RESULTS

Enzyme Purification

Preliminary experiments revealed that aspartokinase was stable at 25°. Therefore, except as noted, all purification steps were carried out at room temperature.

1 The abbreviation used is: DTT, dithiothreitol.

Crude Extract—P. fluorescens cells (800 to 900 g, wet weight) were suspended in 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM β-mercaptoethanol, 1 mM L-lysine, and 1 mM L-threonine (Buffer A) at 1 ml of buffer per g of cells. The slurry was passed once through a Manton-Gaulin homogenizer at 9,000 p.s.i. The resulting homogenate was treated with ribonuclease A and deoxyribonuclease I, 2 mg each, stirred for 1 hour, and centrifuged at 48,000 × g for 60 min at 4°. Unbroken cells, resuspended in the same buffer, were sonically disrupted (Branson Sonifier) and centrifuged as above. The two supernatant fluids were combined.

Heat Treatment—The crude extract was divided into 150-ml portions and heated in a shaking water bath for 4 min at 55°. After chilling in ice water the suspension was centrifuged at 39,000 × g for 20 min at 4°.

Ammonium Sulfate Fractionation—The pH of the supernatant fluid following the heat step was adjusted to pH 7.5 by dropwise addition of 1 N KOH. Twenty-one grams of solid ammonium sulfate were added to each 100 ml of heat-treated supernatant fluid over a period of 1 hour. The solution was stirred for 1 hour, centrifuged at 27,000 × g for 40 min, and the precipitate was discarded. For each 100 ml of the supernatant fluid, 0.3 g of ammonium sulfate were added and the suspension was stirred and centrifuged as above; the precipitate was discarded. To precipitate the enzyme, 0.5 g of ammonium sulfate were added to each 100 ml of the supernatant fluid obtained from the previous step. After stirring and centrifugation, the precipitate was resuspended in a minimal volume of Buffer A, and was dialyzed against two changes of 50 volumes of the same buffer.

Ion Exchange Chromatography—A DEAE-cellulose column (4 x 56 cm) was washed with Buffer A. The dialyzed ammonium sulfate fraction was layered on the column at a flow rate of 60 ml per hour and a linear 3000-ml gradient from 0 to 0.4 M KC1 in Buffer A was applied. Flow rate during elution was 160 ml per hour and 15-ml fractions were collected. Elution profiles of enzyme activity and 280-nm absorbing material for this step are shown in Fig. 1. Fractions with high enzyme activity were pooled and concentrated in a Diaflow ultrafiltration apparatus.

![Fig. 1. Chromatography of aspartokinase on DEAE-cellulose. Dialyzed ammonium sulfate fraction (105 ml) was applied on a DEAE-cellulose column (56 x 4 cm) and the enzyme was eluted at 25° with a linear KC1 gradient (from 0 to 0.4 M KC1 in Buffer A). Flow rate during elution was 160 ml per hour and 15-ml fractions were collected. Enzyme activity is expressed as micromoles of aspartohydroxamate per hour per ml. O, Asp, DTT, enzyme activity; o, a magnified profile of the activity eluted between Fractions 30 and 55.](http://www.jbc.org/content/248/2/8535.full.pdf)
Table I

<table>
<thead>
<tr>
<th>Fraction and step</th>
<th>Units</th>
<th>Protein concentration</th>
<th>Specific activity</th>
</tr>
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<tr>
<td></td>
<td>Per ml</td>
<td>Total mg/ml</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>I. Crude extract</td>
<td>63</td>
<td>97,500</td>
<td>32</td>
</tr>
<tr>
<td>II. Heat treatment</td>
<td>64</td>
<td>91,200</td>
<td>24</td>
</tr>
<tr>
<td>III. Ammonium sulfate</td>
<td>720</td>
<td>76,300</td>
<td>68</td>
</tr>
<tr>
<td>IV. DEAE-cellulose (concentrated)</td>
<td>4,330</td>
<td>56,300</td>
<td>63</td>
</tr>
<tr>
<td>V. Sephadex G-200 I (concentrated)</td>
<td>5,520</td>
<td>51,900</td>
<td>8.4</td>
</tr>
<tr>
<td>VI. Sephadex G-200 II (concentrated)</td>
<td>5,780</td>
<td>41,600</td>
<td>4.8</td>
</tr>
<tr>
<td>VII. Sephadex G-200 III Pool A</td>
<td>4,140</td>
<td>25,700</td>
<td>2.9</td>
</tr>
<tr>
<td>VII. Sephadex G-200 III Pool B</td>
<td>1,100</td>
<td>5,400</td>
<td>0.82</td>
</tr>
<tr>
<td>VIII. Preparative gel electrophoresis (concentrated)</td>
<td>300</td>
<td>900</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* Micromoles of aspartohydroxamate per hour at 29°C.

* Estimated by the method of Lowry et al. (15).

Sephadex G-200 I—Sephadex G-200 was allowed to swell in 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM β-mercaptoethanol, 100 mM KCl, and 5 mM each of L-lysine and L-threonine (Buffer B). Two columns (3.2 × 95 cm), poured and equilibrated in the same buffer, were connected in tandem. Concentrated material obtained from the DEAE-cellulose step was applied to the column and eluted with Buffer B at a flow rate of 20 ml per hour; 3-ml fractions were collected. Enzymatically active fractions were pooled and concentrated in the Diaflo apparatus.

Sephadex G-200 II—Two columns (90 × 2.5 cm) were equilibrated and run in tandem with Buffer C (same as Buffer B but without L-lysine and L-threonine). Concentrated material from G-200 I was applied and eluted at 0.5 ml per hour; fraction volume was 2 ml. Peak fractions were concentrated as above.

Sephadex G-800 III The same two columns and buffer system employed for G-200 I were used in this step; flow rates and other conditions were as described above. Fractions with maximum specific activities were pooled and concentrated (Pool A). Several other fractions with slightly lower specific activities were also pooled (Pool B). The results of one such purification procedure are summarized in Table I.

Preparative Gel Electrophoresis—For some experiments concentrated enzyme solution from Sephadex G-200 III step, Pool B, was dialyzed against 10 mM potassium phosphate buffer, pH 7.5, containing DTT, L-lysine, and L-threonine, at 1 ml each. The sample was then subjected to electrophoresis as described under "Methods."

The elution profile given in Fig. 1 shows that a very small fraction (< 4%) of the total activity applied on the DEAE-cellulose column did not adhere to the ion exchanger. The enzyme activity of this early eluting fraction was found to be almost totally insensitive to concerted feedback inhibition by L-threonine plus L-lysine, and to inhibition by L-threonine or L-2,4-diaminopimelic acid. It is not known whether the minor component was a desensitized form of the aspartokinase or an isoenzyme similar to that observed in *Bacillus stearothermophilus* (23) and *Bacillus subtilis* (24).

**Characterization of Enzyme**

**Estimation of Purity**—Enzyme samples were analyzed on analytical polyacrylamide gels in Tris-glycine buffer system, pH 0.5 (16). A densitometric scan of a gel obtained with Pool A from Sephadex G-200 III step and stained with Coomassie blue, as shown in Fig. 2, revealed a major band (Band 2) and a smear of protein-positive material migrating faster than this band. Using the activity stain, the major band was shown to correspond with aspartokinase activity. The minor band (Band 1) and the smeared area did not have enzyme activity. Both Bands 1 and 3, and some of the protein-positive material present in the smeared area were absent from the gels that were obtained with enzyme purified by preparative gel electrophoresis at pH 7.4. Furthermore, when the active enzyme (Band 2) was sliced off, homogenized, and subjected to re-electrophoresis at pH 9.5, several low molecular weight components were visible in addition to the main band. These results suggested extensive dissociation of the enzyme during gel electrophoresis.

Electrophoresis on cellulose acetate paper (25) of the Pool A material from Sephadex G-200 III step revealed only one protein band with no indication of any dissociation product being formed. A photograph of the schlieren pattern during sedimentation velocity centrifugation (see Fig. 3) also did not reveal any small molecular weight contaminants. Based on these results, we conclude that the enzyme was highly purified; from the densitometric scan shown in Fig. 2, and assuming that the low molecular weight material was indeed dissociation product of the native enzyme, we estimated that the enzyme was greater than 90% pure.

**Molecular Weight and Stokes Radius**—The molecular weight of the native enzyme was determined by sedimentation velocity centrifugation. Fig. 3 shows the schlieren pattern of the enzyme at 7 mg per ml. A slight decrease of the sedimentation coefficient on increasing protein concentrations was observed, an increase in the value

1 Several variations in the condition of gel electrophoresis such as the presence or absence of amino acid modifiers and DTT, replacement of Tris with imidazole or sodium phosphate buffers at different pH values, elimination of ammonium persulfate as the catalyst, and prior reduction of the enzyme with 0.1 M DTT did not significantly alter the gel pattern. Addition of 40 mM L-aspartate, or 20 mM ATP plus 10 mM MgCl₂ to both gel and electrophoresis buffer, however, reduced the extent of enzyme breakdown to a large degree.
FIG. 4. Determination of Stokes radius of aspartokinase. Enzyme from Pool A, Table I (420 μg), was mixed with 200 μg of yeast alcohol dehydrogenase, 75 μg of horseradish peroxidase, and 100 μg of rabbit muscle pyruvate kinase in a total volume of 0.15 ml and applied to a Sephadex G-200 column (86 X 1.4 cm) equilibrated at 25° with 50 mM potassium phosphate buffer, pH 7.5, containing 200 mM KCl and 0.02% sodium azide. The proteins were eluted with the same buffer at a flow rate of about 5 ml per hour and 0.75-ml fractions were collected. The location of protein peaks were established by assaying for their activities as given under "Methods." KD values were calculated according to the method given by Siegel and Monty (20). In separate experiments, it was established that 0.02% sodium azide did not influence the elution profile or the enzyme activities of these proteins. AK = aspartokinase; PK = pyruvate kinase; ADH = yeast alcohol dehydrogenase; PEROX = horseradish peroxidase.

TABLE II

Requirements for aspartokinase reaction

The complete reaction mixture contained in a final volume of 1 ml the following: 608 μmoles of hydroxylamine, 10 μmoles of MgCl₂, 200 μmoles of KCl, 20 μmoles of ATP, and 50 μmoles of L-aspartic acid. In Experiment 1, 3 μg of protein from G-200 I step were incubated for 20 min at 25°. In Experiment 2, 8.4 μg of protein from a different enzyme preparation were incubated for 15 min at 25°. Wherever present the concentration of MgCl₂ was 10 mM and 200 mM LiCl or NaCl was used.

<table>
<thead>
<tr>
<th>System</th>
<th>Aspartohydroxamate formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.98</td>
</tr>
<tr>
<td>Omit KCl</td>
<td>0.61</td>
</tr>
<tr>
<td>Omit MgCl₂</td>
<td>0.03</td>
</tr>
<tr>
<td>Omit ATP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Omit KCl and MgCl₂</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Experiment 2

| Complete                    | 1.11                               |
| Omit MgCl₂ but add MgCl₂    | 1.16                               |
| Omit KCl but add LiCl       | 0.61                               |
| Omit KCl but add NaCl       | 0.71                               |

K⁺ from the reaction mixture decreased the activity by about 40%. The requirement for Mg²⁺ could be replaced by Mn²⁺; no other cations tested, including Zn²⁺, Ca²⁺, and Co²⁺, could substitute either Mg²⁺ or Mn²⁺. In the presence of Mg²⁺, Li⁺, or Na⁺ had no effect.

In separate experiments, the enzyme was found to be highly...
specific for both the donor and the acceptor of the phosphate group. Analogs of aspartate such as L-glutamate, dL-α-methyl aspartate, N-acetyl aspartate, and N-aspartate were inactive. ATP was the only known phosphate donor for the phosphorylation reaction; ADP, AMP, UTP, CTP, and GTP were ineffective.

Control of Aspartokinase Activity

Concerted Feedback by Amino Acid Pairs In contrast to the other aspartokinases from a variety of bacterial species the activity of the P. fluorescens aspartokinase was subject to concerted feedback inhibition by two pairs of amino acid end products. The data summarized in Table III show that l-threonine, at low concentration, was a mild activator and exerted a weak inhibitory effect only at concentrations over 10 mM. The other end products of the pathway, l-lysine, l-methionine, and l-isoleucine, showed a small but reproducible stimulatory effect. However, a combination of l-lysine plus l-threonine strongly inhibited the phosphorylation reaction. Concerted feedback inhibition by l-threonine plus l-methionine was not nearly as strong as the effect of the l-lysine plus l-threonine combination.

Control experiments in Table III indicate no concerted feedback inhibition by the other possible pairs of amino acids. That the regulation of enzyme activity by various amino acids was not an artifact of the purification procedure is illustrated in Table IV. The results show that the activation-inhibition patterns seen with crude extracts were virtually unchanged as the enzyme was purified through the Sephadex G-200 step.

Fig. 5 depicts the concentrations of l-lysine and l-threonine required for concerted feedback inhibition. It is clear that a drastic reduction in activity occurred at very low levels of these amino acids; at 0.25 mM each, the enzyme activity was inhibited by approximately 70%.

Structural Specificity of Inhibiting Amino Acids—The results of concerted inhibition studies with structural analogs of l-threonine in the presence and absence of the natural amino acids are summarized in Table V. Only l-threonine amide and l-threonine methyl ester could replace l-threonine for the concerted feedback inhibition when present in combination with either l-lysine or l-methionine. Individually, these analogs were inhibitory, but the effect was less than that observed with equimolar concentration of l-threonine. It is clear, that the α-carboxyl of l-threonine was not required for inhibition; any other alteration of the amino acid structure destroyed the inhibitory potential.

To determine the structural specificity of l-lysine, the following compounds were tested at 5 mM each, either alone or in the presence of 0.25 mM l-threonine: d-lysine, cadaverine, L-arginine, L-2,4-diaminopimelic acid, and S-(β-aminoethyl)-L-cysteine. The only lysine analog that showed concerted inhibition was S-(β-aminoethyl)-L-cysteine indicating that the substitution of a sulfur atom for a methylene group in the side chain reduced but did not eliminate binding on the enzyme molecule.

**Table IV**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>L-Threonine (5 mM)</td>
<td>113</td>
</tr>
<tr>
<td>L-Threonine (10 mM)</td>
<td>106</td>
</tr>
<tr>
<td>L-Threonine (20 mM)</td>
<td>122</td>
</tr>
<tr>
<td>L-Lysine (5 mM)</td>
<td>106</td>
</tr>
<tr>
<td>L-Methionine (5 mM)</td>
<td>124</td>
</tr>
<tr>
<td>L-Isocitrinate (5 mM)</td>
<td>110</td>
</tr>
<tr>
<td>L-Threonine + L-lysine (5 mM each)</td>
<td>20</td>
</tr>
<tr>
<td>L-Threonine + L-methionine (5 mM each)</td>
<td>81</td>
</tr>
<tr>
<td>L-Threonine + L-isoleucine (5 mM each)</td>
<td>121</td>
</tr>
<tr>
<td>L-Methionine + L-lysine (5 mM)</td>
<td>88</td>
</tr>
<tr>
<td>L-Methionine + L-isoleucine (5 mM)</td>
<td>108</td>
</tr>
</tbody>
</table>

**Fig. 5.** Concerted feedback inhibition of aspartokinase by L-lysine plus L-threonine. •, progressive inhibition with increasing L-threonine concentration at a constant level (0.25 mM) of L-lysine. ○, progressive inhibition with increasing L-lysine concentration at a constant level (0.25 mM) of L-threonine. Coupled assay with 2.1 μg of protein (Fraction VI, G-200 II, Table I) were used. One hundred percent activity was equal to 0.015 μmole of aspartohydroxamate formed per min.
amino acid added, 0.034 pmole of aspartohydroxamate per min for ment II, the concentrations were 2 mM. In controls, with no Dex G-200 II was used. For Experiment I, the concentrations of was produced under these conditions.

L-Threonine and the amino acid analogs were 5 mM; for Experiment II, the concentrations of was 100% activity equal to 0.90 pmole of aspartohydroxamate formed. One hundred per cent activity was equal to 0.90 pmole of aspartohydroxamate for 30 min at 25°. Amino acid concentrations were as indicated.

Inhibition by n-lysine plus n-threonine. One hundred and fifty millionths (Table III), inclusion of all three amino acids in the reaction experiment showed that the enzyme isolated from this bacterium has an approximate molecular weight of 135,000; gel electrophoresis in sodium dodecyl sulfate showed a single band corresponding to a subunit molecular weight of 43,000, suggesting that the enzyme is composed of three subunits of equal molecular size. The aspartokinase from Bacillus polymyxa, which is also regulated by concerted feedback inhibition, appears to be a tetramer (molecular weight 116,000) composed of two types of nonidentical subunits of 47,000 and 17,000 (28).

In the context of regulation of enzyme activity, the P. fluorescens aspartokinase shows some properties that are distinguishable from the other aspartokinases subject to concerted feedback inhibition. In Pseudomonas putida (29) the enzyme activity was strongly inhibited by L-lysine or by L-threonine; when added together at low concentrations, L-lysine plus L-threonine brought about drastic inhibition. Similar results were obtained with the B. polymyxa enzyme (30). In contrast, L-threonine inhibited the activity of P. fluorescens aspartokinase only slightly and L-lysine had no inhibitory effect (Table III). When L-lysine and L-threonine were present together the enzyme activity was severely inhibited (cf. Table III and Fig. 5). The important difference between these aspartokinases is, therefore, that in the case of P. fluorescens, only one member of the pair involved in the concerted feedback inhibition shows a weak inhibitory effect, and the second member of the pair is slightly stimulatory. This particular combination is similar to the concerted feedback pattern reported in R. capsulata (6).

In addition to the inhibitory effect of L-methionine in concert with L-threonine, the sulfur-amino acid also exhibited a compensatory role on the concerted feedback inhibition elicited by the L-threonine plus L-lysine pair (Fig. 6). This effect was seen only when the concentration of L-threonine was low (in combination with high L-lysine); under this condition, addition of 10 mM L-methionine almost completely counteracted the concerted inhibition. Although the exact molecular mechanism of the "reversal" by L-methionine of the L-threonine plus L-lysine inhibition is not known, when compared with other over-all control patterns seen in a variety of bacterial species (3), the compensatory phenomenon appears to have some physiological significance. Since L-threonine is an end product by itself as well as a precursor of another end product L-isoleucine (1), it is reasonable to expect that at low L-threonine concentration and at high concentrations of both L-lysine and L-methionine, a severe deprivation of L-threonine (also L-isoleucine) would slow down the cellular growth unless sufficient amounts of the precursors for L-threonine biosynthesis were made available by relieving the concerted feedback of aspartokinase. When L-threonine is present in excess (in addition to excess L-lysine and L-methionine), no limitation of L-threonine is predicted, and the synthesis of all these end products should be efficiently regulated by normal feedback mechanisms of the early enzymes. (Excess L-threonine would be adequate to supply both L-threonine and L-isoleucine.) The
dual role of L-methionine, i.e. its ability to inhibit enzyme activity
in concert with L-threonine as well as its capacity to compensate
the concerted feedback inhibition by L-threonine plus L-lysine,
therefore, appears to be an important physiological device for
the regulation of the activity of the first enzyme of the aspartate
metabolism in P. fluorescens.

Acknowledgments—We thank Mr. John Trojanowski for run-
ing the analytical ultracentrifuge, Mr. Joseph A. Gingras for
constructing the preparative polyacrylamide gel apparatus, and
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    4900-4906
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