Biochimica et Biophysica Acta, 445 (1976) 350—363

BBA 67895

LYSINE- AND LYSINE-PLUS-THREONINE-INHIBITABLE ASPARTOKINASES IN *BACILLUS BREVIS* *

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(Received March 11th, 1976)

Summary

Two aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) enzyme activities have been identified and partially purified from *Bacillus brevis*. Aspartokinase I is subject to both inhibition and repression by lysine, and has a molecular weight in the region of 110 000. Aspartokinase II is a lysine-stabilised enzyme, inhibited multivalently by lysine plus threonine and has a molecular weight in the region of 95 000. This pattern of aspartokinase activity has not been described previously and is unusual in that one end product (lysine) regulates two isoenzymes catalysing the first reaction of a branched biosynthetic pathway. In the absence of lysine, aspartokinase II changes to a more unstable non-inhibitable enzyme. Both enzymes are stabilised by sulphydryl reducing agents and have similar affinities for ATP, aspartate and lysine. However, there is no evidence for a view that they are products of a common gene. Problems concerned with the regulation of aspartokinase activities in *Bacillus* species are discussed.

Introduction

Aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) catalyses the first step in the branched biosynthetic pathway leading to the production of the aspartate family of amino acids, L-lysine, L-threonine, L-methionine and L-isoleucine. Most bacteria studied regulate the production of these four amino acids and *meso*-diaminopimelate (a cell-wall component) by mechanisms which include effects on the aspartokinase activity. These vary from the controls by single end-products on separate isofunctional enzymes such as found in *Escherichia coli* K-12 [1] and other members of the family Enterobacteriaceae [2] to

* A preliminary report of this work was presented at the 1975 Meeting of the Australian Biochemical Society 8, 57.

controls exerted by various combinations of any or all four amino acid end products on the single aspartokinases of *Rhodospirillum tenue* [3] various members of the family Pseudomonadaceae [2] and other marine eubacteria [4].

In the Bacillaceae the problems involved in providing a balanced synthesis of the four end-products and diaminopimelate during vegetative growth will be similar to those in non-sporing bacteria. During sporulation balanced quantities of lysine, threonine, methionine and isoleucine are mostly provided for protein synthesis by the protein turnover reactions. This turnover occurring at a rate of about 20% of the cell protein per hour is activated during sporulation [5,6]. However, the carbon flow along the lysine branch of the aspartate family pathways must continue in order to supply dipicolinic acid, a unique constituent of bacterial spores, and the extra *meso*-diaminopimelate required for the spore cortex [7].

In *Bacillus polymyxa* [8], *Bacillus subtilis* [9], *Bacillus licheniformis* [10], *Bacillus colistinus* [11], *Bacillus stearothermophilus* [12], and *Bacillus cereus* [13] aspartokinase activity during vegetative growth appears to be regulated in the main by either L-lysine or by multivalent feedback inhibition by L-lysine and L-threonine. During sporulation, the regulation of aspartokinase activity by lysine can be lost as in *B. cereus* [13] or carbon flow can be maintained by a meso-diaminopimelate sensitive enzyme as in *B. subtilis* [9], and *B. stearothermophilus* [14]. In *Clostridium perfringens* [15] where because of the complex nature of the growth medium the aspartate family of amino acids might be expected to be available at all times, only a *meso*-diaminopimelate-sensitive aspartokinase activity has been detected at all stages of growth.

Additional complexities in the control mechanisms of aspartokinase activity in Bacillus species have been indicated by a number of observations. Stahly and Bernlohr [10] demonstrated that when growth of *B. lichenformis* ceased there was a rapid decrease in aspartokinase activity which was much greater than would be expected by degradation due to turnover. Gray and Bernlohr [16] claimed that a lysine sensitive aspartokinase activity of *B. licheniformis* was changed to a lysine plus threonine sensitive activity during either growth in the presence of lysine or following the cessation of growth. Paulus and Gray [17] observed that L-methionine reversed the inhibition of activity produced by L-lysine plus L-threonine with the one detected aspartokinase activity in *B. polymyxa*.

In the Bacillaceae there are thus a variety of mechanisms which control the activities of aspartokinase, and these together with the mechanisms involved in the control of aspartokinase synthesis presumably ensure that the various branches of the aspartate family pathways are efficiently supplied with the appropriate quantities of substrates under different physiological conditions.

In this paper we report on the properties of the aspartokinase activities of *Bacillus brevis* ATCC 10068.

Materials and Methods

Materials. Unless otherwise stated all chemicals were analytical reagent grade commercial preparations. All L-amino acids, DL-α-ϵ-diaminopimelate, dithiothreitol, disodium ATP, L-aspartyl-β-hydroxamate, rabbit muscle aldolase and

xanthine oxidase were purchased from Sigma Chemical Co. Crystalline trypsin, egg albumin, human immunoglobulin came from Calbiochem. Bovine serum albumin and crystalline serum albumin Cohn Fraction V were products from C.S.L., Melbourne, Victoria. Streptomycin sulphate came from Glaxo. Sephadex G-25, G-200 and DEAE A-50 were products of Pharmacia.

Culturing of the micro-organism. The origins of *B. brevis* ATCC 10068 and the culture conditions used have been mostly described previously [18]. The asparagine, glycerol minimal medium [19] was modified to contain 1.67 g/l glycerol and 0.83 g/l L-asparagine. 500-ml cultures in 1.5-l flasks were shaken rapidly at 280 cycles/min in an orbital incubator and 20-l cultures were stirred and sparged with air (5 l/min) in stainless steel fermentors. Growth was determined using a Klett-Summerson photoelectric colorimeter with filter No. 54.

Protein determination. Protein concentration was determined by the method of Lowry et al. [20] with predried bovine serum albumin as standard, or by the method of Warburg and Christian [21].

Assay of aspartokinase activity. The aspartokinase assay mixtures contained Tris, 200 μmol; magnesium sulphate, 6 μmol; L-aspartate, 20 μmol; ATP, 10 μmol; hydroxylamine, 800 μmol; KCl, approximately 800 μmol and enzyme solution at a final pH of 8.0. The total volume was 1.0 ml. Incubation was at either 25° C or 37° C for 30 min. Reactions were stopped with 1.5 ml of ferric chloride solution (100 g, $FeCl_3 \cdot 6H_2O$; 33 g, trichloroacetic acid per l of 0.7 M HCl, [22]). Solutions were mixed and centrifuged at 2000 × *g* for 10 min. Amounts of aspartylhydroxamate formed were determined by measuring absorbance at 540 nm with a Unicam SP 500 and comparing the values obtained with a calibration curve constructed with authentic aspartyl hydroxamate as standard. One unit of activity forms 1 μmol of aspartyl hydroxamate per min. Specific activities are given in units/mg of protein.

Preparation of cell extracts. Cells were harvested in the logarithmic phase of growth by centrifuging for 5 min at 11 500 × *g* from volumes of media up to 1.5 l and for larger volumes by continuous centrifugation at 19 500 × *g* in a Sharples Super centrifuge, Sedimented bacteria were resuspended in buffer (50 mM Tris · HCl, pH 8.0 at 20° C) at 4° C, resedimented (11 500 × *g*, 10 min), and resuspended in buffer (4 ml/g wet weight). Cells were broken by one passage through on Aminco-French pressure cell (10 000 lb/in^2) and unbroken cells and debris sedimented for 15 min at 27 000 × *g* to form a pellet. The supernatant solution served as the source of crude enzyme.

Removal of amino acids from cell extracts. 2.0 ml aliquots of cell extracts were washed through a 10 ml column of Sephadex G-25 equilibrated with 50 mM Tris · HCl buffer pH 8.0 containing 1 mM dithiothreitol and the void volume fractions used to measure aspartokinase activity of the crude enzyme fraction.

Fractionation procedures. The buffer used throughout contained 50 mM Tris · HCl (pH 8.0 at 20° C) and all steps were performed at 0—4° C.

Step 1, streptomycin precipitation: 0.05 vol. of 20% w/v aqueous streptomycin sulphate was slowly added to 1 vol. cell extract. After 1 h this was centrifuged for 15 min at 27 000 × *g* and the supernatant solution (fraction II) was decanted.

Step 2, ammonium sulphate fractionation: Finely ground solid ammonium

sulphate was added slowly with stirring to fraction II to 35% saturation. After stirring for 1 h and centrifuging for 15 min at 27 000 × *g*, more solid ammonium sulphate was slowly added to the supernatant solution to 55% saturation, stirred for 1 h and again centrifuged for 15 min at 27 000 × *g*. Sedimented material was in each case dispersed in the minimal quantity of the buffer which effected solution. Following assay of all fractions for aspartokinase activity, in all cases the bulk of the aspartokinase activity was located in the material sedimenting from the 55% saturated ammonium sulphate solution (fraction III).

Step 3, DEAE-Sephadex chromatography: A DEAE-Sephadex A-50 column (25.0 × 1.5 cm) was developed with a linear gradient from 0.15 or 0.25 to 0.5 M KCl in a total volume of 200 or 400 ml of buffer containing 10 mM 2-mercaptoethanol or 1 mM dithiothreitol and in some cases 1 mM L-lysine. A sample was in each case dialysed overnight against the starting buffer before being applied to the column. Fractions of 5 ml were collected.

Step 4, Sephadex G-200 chromatography: Active fractions from Step 3 (fraction IV) were concentrated by diaflo (Amicon, PM10 filter) and applied to a Sephadex G-200 column (2.64 × 93 cm) pre-equilibrated with buffer containing 1 mM dithiothreitol, 1 mM L-lysine and 0.15 M KCl. Fractions of 5.12 ml were collected.

Determination of molecular weights. Molecular weights for the aspartokinase enzymes were estimated by the method of Andrews [23] using a 2.64 × 93-cm column of Sephadex G-200. Xanthine oxidase, human immunoglobulin, aldolase, bovine serum albumin, ovalbumin and trypsin were used as standards. 5-ml fractions were collected and the elution volume (V_e) for each standard was determined.

Results

Inhibition of aspartokinase activity

Crude extracts from cells grown with minimal medium and minimal medium plus 2 mM L-lysine were treated to remove endogenous amino acids and assayed for aspartokinase activity in the presence or absence of various amino acids (Table I). The activity measured in the absence of additions was almost

TABLE I

INHIBITION OF ASPARTOKINASE ACTIVITY

Cell extracts after passage through a Sephadex G-25 column equilibrated with Tris · HCl buffer containing 1 mM dithiothreitol were assayed for aspartokinase activity at 25° C.

Enzyme from cells grown with	Specific activity (units/mg protein × 10^3)	Activity (%) in the presence of		
		2.5 mM L-lysine	2.5 mM L-threonine	2.5 mM L-lysine and 2.5 mM L-threonine
Minimal medium	14.2	59	98	32
Minimal medium plus 2 mM L-lysine	13.9	122	116	47

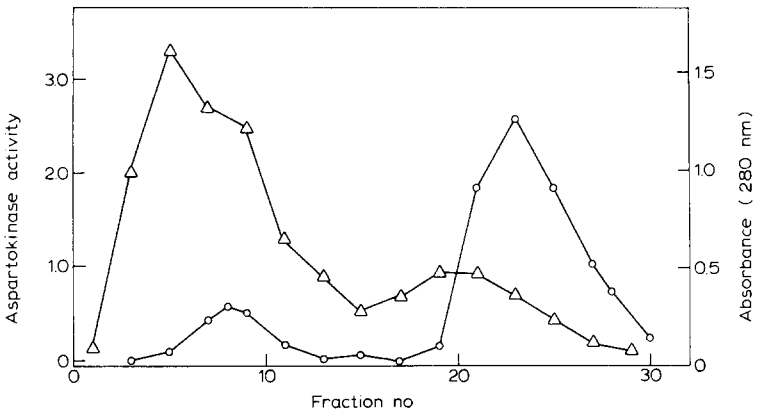

Fig. 1. DEAE-Sephadex chromatography of extracts from cells grown with minimal medium. Fraction III (Table II) was absorbed onto a DEAE-Sephadex column equilibrated with Tris · HCl buffer containing 10 mM 2-mercaptoethanol and 0.25 M KCl. Protein was eluted with a 200-ml linear gradient of 0.25—0.5 M KCl in the equilibration buffer. 5.0-ml fractions were collected ○———○, aspartokinase activity in μmol of aspartylhydroxamate produced/30 min determined in the standard assay at 37°C for a 0.20-ml aliquot of each fraction. △———△, absorbance at 280 nm.

identical for both extracts, and the presence of 2.5 mM L-lysine plus 2.5 mM L-threonine caused a significant inhibition in both cases (68% and 53% respectively). Increasing the concentrations of L-threonine and L-lysine to 5.0 mM did not further reduce the activities. L-lysine caused inhibition (41%) only in extracts from cells grown with minimal medium, but L-threonine itself had negligible effect. L-methionine and L-isoleucine at 2.5 mM and DL-α,ε-diaminopimelate at 5.0 mM did not affect activity and did not modify the inhibition caused by lysine and threonine.

Fractionation of an extract from cells grown with minimal medium

To test for the presence of separable enzymes, an extract was fractionated (Table II, Fig. 1). Following treatment with ammonium sulphate, 88% of the

TABLE II

FRACTIONATION OF AN EXTRACT FROM CELLS GROWN WITH MINIMAL MEDIUM

For details concerning experimental procedures See Fig. 1. Aspartokinase activity was assayed at 37°C. n.d. = not detectable.

Fraction		Total protein (g)	Total activity (units)	Specific activity (units/mg)	Sensitivity to 2.5 mM L-lysine (% inhibition)
I	Cell extract	6.08	255	0.042	66
II	Streptomycin sulphate-treated supernatant	5.50	268	0.049	64
	0—35% $(NH_4)_2SO_4$		37		
III	35—55% $(NH_4)_2SO_4$	2.14	297	0.139	68
	>55% $(NH_4)_2SO_4$		n.d.		
IV	DEAE-Sephadex pooled fractions 19—30	0.102	49.1	0.483	96

recovered activity was present in the fraction precipitating between 35 and 55% saturation. Two peaks of activity were detected in the fractions eluted from the DEAE-Sephadex column; the first (fractions 5—11) had 17% of the recovered activity and showed no inhibition by lysine plus threonine, the second (fractions 19—30) with 83% of the recovered activity showing 96% inhibition by 2.5 mM L-lysine. The activity which was inhibited by L-lysine plus L-threonine was not detected. Total activity recovered from the column accounted for only 20% of the activity of the original cell extract. The presence of a reducing agent in the elution buffer for the DEAE-Sephadex column was essential, or very little activity was recovered. In later experiments 1 mM dithiothreitol was found to be a more effective stabilising agent than was 10 mM 2-mercaptoethanol. With 1 mM dithiothreitol in the elution buffer more than 65% of the total activity applied to a DEAE-Sephadex column was recovered and 95% of the recovered activity was present in the second peak. The enzyme associated with the activity which is sensitive to inhibition by lysine alone will be referred to as aspartokinase I.

Fractionation of an extract from cells grown with minimal medium plus 2 mM L-lysine

Using a procedure similar to that described in Table II it was found that although most of the aspartokinase activity was recovered in the fraction precipitating between 35 and 55% saturation with ammonium sulphate, no activity was detected in fractions eluted from a DEAE-Sephadex column. When L-lysine was added to extracts from cells grown with L-lysine the measured aspartokinase activity was greater than when there was no added lysine (Table I). On other occasions, a stimulation of up to 1.4-fold has been observed. A check on the effect of L-lysine on the time course of the aspartokinase reaction showed

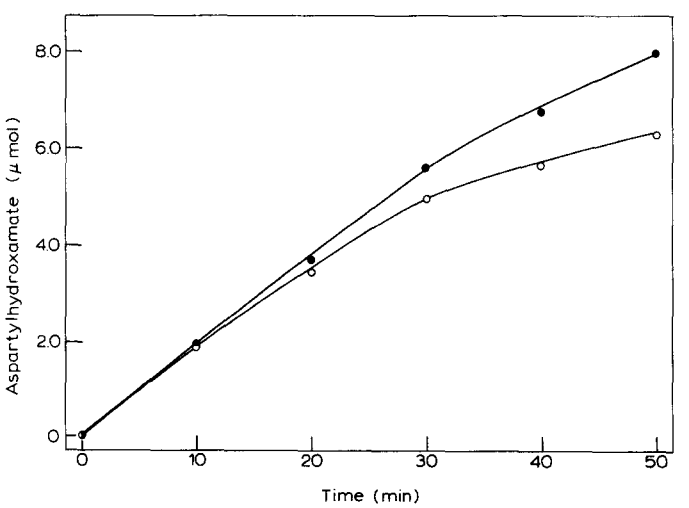

Fig. 2. Stabilisation by L-lysine of aspartokinase activity sensitive to lysine-plus-threonine inhibition. 0.1-ml aliquots (containing approx. 2 mg protein) of fraction III (Table III) of an extract from cells grown with minimal medium plus lysine were assayed at 37°C in the presence (●———●) or absence (○———○) of 2.5 mM L-lysine.

TABLE III

FRACTIONATION OF AN EXTRACT FROM CELLS GROWN WITH MINIMAL MEDIUM PLUS 2 mM L-LYSINE

For details concerning experimental procedures see Fig. 3. Aspartokinase activity was assayed at 37°C.

Fraction		Total protein (g)	Total activity (units)	Specific activity (units/mg)
I	Cell extract	3.48	240	0.069
II	Streptomycin sulphate-treated supernatant	3.34	290	0.087
	0—35% $(NH_4)_2SO_4$		12.5	
III	35—55% $(NH_4)_2SO_4$	1.82	266	0.146
	>55% $(NH_4)_2SO_4$		8.9	
IV	DEAE-Sephadex pooled fractions 35—55	0.025	68	2.730

that its effect was to prevent loss of activity during incubation rather than to increase the initial rate of reaction (Fig. 2). When 1.0 mM L-lysine was added to all the buffers used in the purification steps, activity was again recovered in two peaks from the DEAE-Sephadex column (Table III, Fig. 3). 4% of the recovered activity (fractions 4—11) was insensitive to inhibition by lysine plus threonine and the remaining activity (fractions 35—55) was inhibited 93% by 2.5 mM L-lysine plus 2.5 mM L-threonine. In this case 28% of the initial activity was recovered following purification and again activity was stabilised by 1 mM dithiothreitol. The enzyme associated with the activity which is inhibited by L-lysine plus L-threonine, but not by these amino acids independently, will be referred to as aspartokinase II.

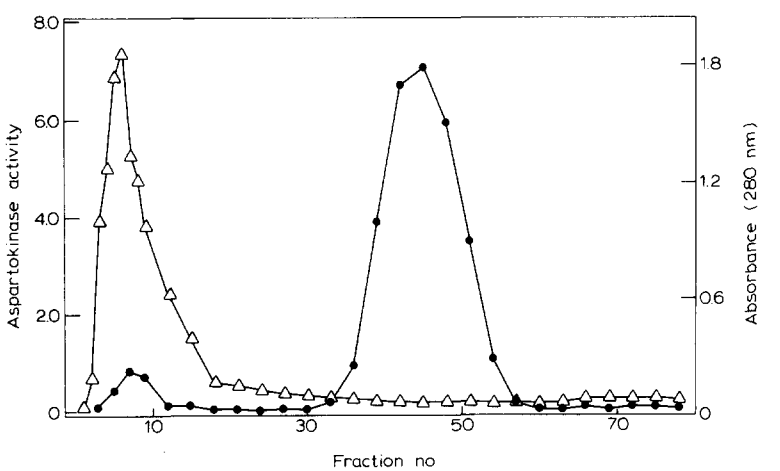

Fig. 3. DEAE-Sephadex chromatography of extracts from cells grown with minimal medium plus 2 mM L-lysine. Fraction III (Table III) was absorbed onto a DEAE-Sephadex column equilibrated with Tris · HCl buffer containing 10 mM 2-mercaptoethanol, 1 mM L-lysine and 0.25 M KCl. Protein was eluted with a 400-ml gradient of 0.25—0.5 M KCl in the equilibration buffer and 5.0-ml fractions were collected. Aspartokinase activities: ●———●; and protein: △———△ as for Fig. 1.

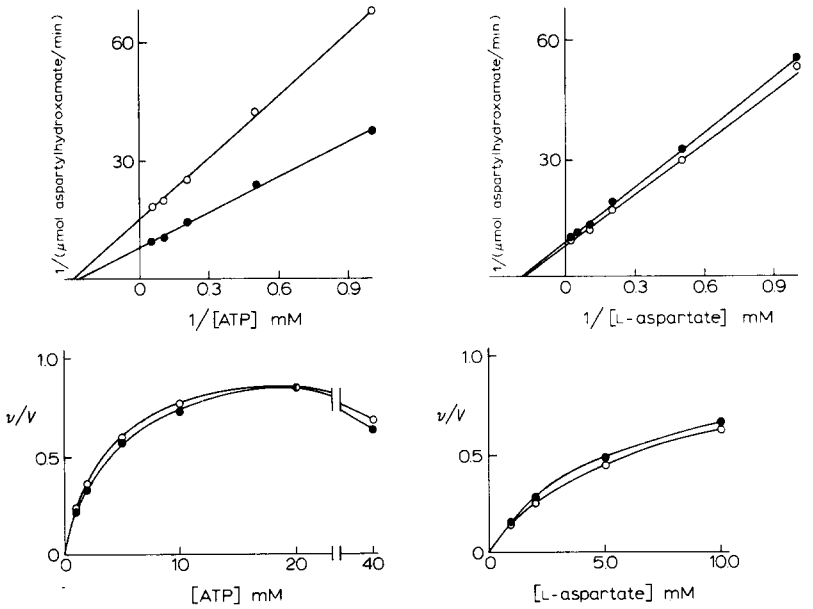

Fig. 4. Effect of variation of substrate concentrations on activities of aspartokinases I and II. DEAE-Sephadex-purified aspartokinases I and II were assayed at 37° C as described in the methods except the concentrations of ATP and L-aspartate were varied. Effect of ATP concentration was determined in the presence of 20 mM L-aspartate and effect of L-aspartate concentration was determined in the presence of 20 mM ATP. All assays for aspartokinase II were performed at 2.5 mM L-lysine to stabilise activity. ○———○, aspartokinase I; ●———●, aspartokinase II.

Properties of aspartokinases I and II

(a) Variation of substrate concentration. Apparent kinetic constants for aspartate and ATP were determined using double reciprocal plots of activity against concentration (Fig. 4). As the curves showed no sigmoidal effects, there is probably no homotrophic co-operation of substrate binding. Although the nucleotide substrate for the enzymes is probably $Mg \cdot ATP^{2-}$ [24] in these experiments it is not possible to determine the actual $Mg \cdot ATP^{2-}$ concentration because of the high salt concentration present in the reaction mixtures. The kinetic constants calculated are therefore not absolute, but as the same conditions were used with both enzymes the values obtained can be compared. Thus $[Mg^{2+}]$ was kept constant at 6 mM while [ATP] was varied between 1.0 and 40 mM. For aspartokinase II determinations, 2.5 mM L-lysine was added to all reactions to stabilise the enzyme. Apparent K_m values for aspartate and ATP were 6.3 mM and 3.5 mM for aspartokinase I and 5.3 mM and 3.8 mM for aspartokinase II respectively.

(b) Variation of inhibitor concentrations. The effects of increasing inhibitor concentration on activity are shown in Fig. 5. Since aspartokinase II activity is not inhibited unless both L-lysine and L-threonine are present together, inhibition curves for this enzyme were constructed using a saturating concentration of one inhibitor and a varying concentration of the other. Again the curves showed no sigmoidal effects. The concentration of lysine required to give 50% inhibition of either enzyme was similar (90 ± 10 μM), and the concentration of

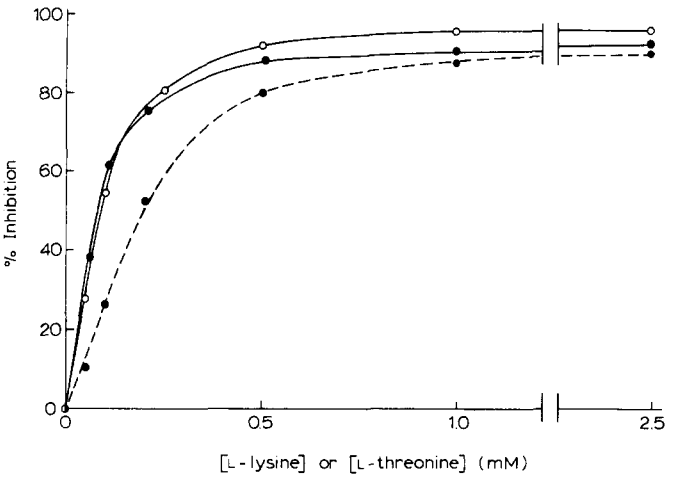

Fig. 5. Effect of variation of lysine and threonine concentration on the inhibition of aspartokinases I and II. DEAE-Sephadex-purified aspartokinases I and II were assayed at 37° C as described in Methods, except inhibitors were added at varying concentrations. Inhibition of aspartokinase II by lysine was determined at a saturating (2.5 mM) concentration of threonine and vice versa. ○———○, effect of lysine on aspartokinase I; ●———●, effect of lysine on aspartokinase II; ●- - - - -●, effect of threonine on aspartokinase II.

threonine causing 50% inhibition of aspartokinase II in the presence of L-lysine was 200 μM.

Separation of the aspartokinase I and II activities

(a) Fractionation. The results of the fractionation experiments on extracts from cells grown with L-lysine showed that aspartokinase II was lost during elution from a DEAE-Sephadex column unless lysine was added to the elution

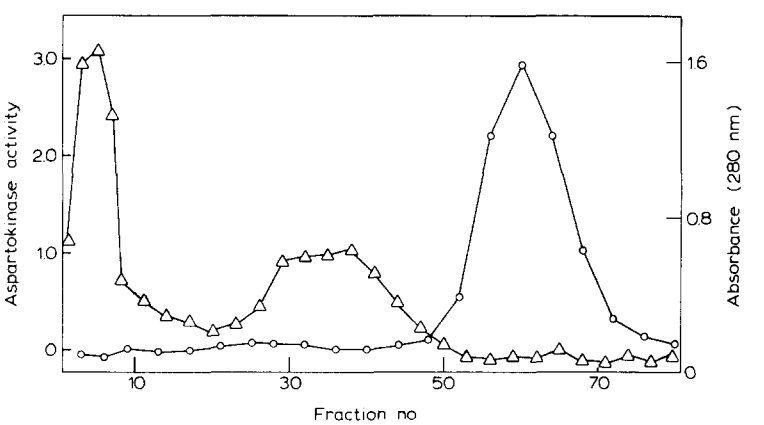

Fig. 6. DEAE-Sephadex chromatography in the presence of lysine of extracts from cells grown with minimal medium. Conditions were the same as for Fig. 1 except 1 mM L-lysine was present in all buffers used throughout the purification, 1 mM dithiothreitol replaced 10 mM 2-mercaptoethanol and protein was eluted with a 400-ml linear gradient of 0.15—0.50 M KCl in the equilibration buffer. ○———○ aspartokinase activity in μmol of aspartylhydroxymate produced/30 min determined for a 0.10-ml aliquot in the standard assay at 37° C. △———△ absorbance at 280 nm.

buffer. Since the results in Table I indicated that both aspartokinase I and II activities were present in extracts from cells grown with minimal medium, the fractionation procedure described in Table II was repeated with 1 mM L-lysine added to all buffers and 1 mM dithiothreitol replacing the 10 mM 2-mercaptoethanol. Only one peak of activity was eluted from the DEAE-Sephadex column (Fig. 6) and in each fraction activity was inhibited 48% ± 8% by the addition of 2.5 mM L-lysine and 94% ± 2% by the addition of 2.5 mM L-lysine plus 2.5 mM L-threonine. The pooled active fractions (53—72) were concentrated and washed through a precalibrated Sephadex G-200 column. Only one peak of activity (fractions 55—69) was recovered (Fig. 7). When each fraction was re-assayed in the presence of 2.5 mM L-lysine and the residual activity replotted (Fig. 7), the peak of residual activity was displaced towards the right when compared with the peak of activity measured in the absence of lysine.

(b) Effect of temperature on activities of partially purified aspartokinases I and II at a low lysine concentration. The aspartokinase I activity present in the pooled peak fractions from the DEAE-Sephadex column (Fig. 6) behaves differently from the aspartokinase II activity present in the same fraction (Table IV). When the preparation is diluted to reduce the lysine concentration the aspartokinase II activity is quickly lost even when the preparation is maintained at 0°C and after 15 min at 37°C no aspartokinase II activity remains. The activity of aspartokinase I however remains virtually unaffected by these treatments.

Aspartokinase activity insensitive to lysine and threonine inhibition.

Although 2.5 mM L-lysine plus 2.5 mM L-threonine caused greater than 90% inhibition of partially purified aspartokinases I and II, (Fig. 5), the activity of crude extracts was inhibited less than 70% (Table I). It was therefore possible that a third enzyme was present. This was named aspartokinase III. This activity was separable from aspartokinases I and II by DEAE-Sephadex chromatog-

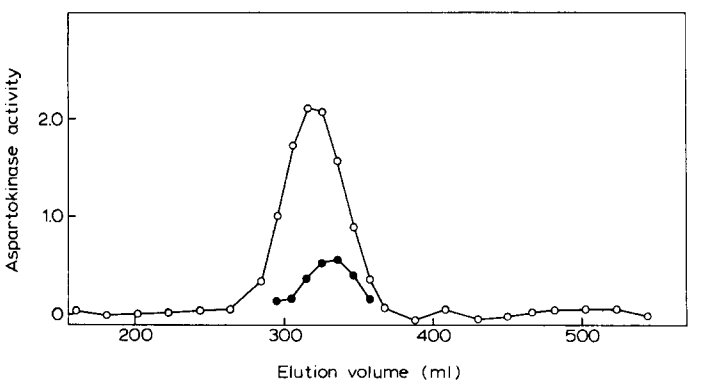

Fig. 7. Sephadex G-200 chromatography of partially purified aspartokinase from cells grown with minimal medium. Pooled active fractions from the DEAE-Sephadex column (Fig. 6) were concentrated and chromatographed on Sephadex G-200 with Tris · HCl buffer containing 1 mM L-lysine, 1 mM dithiothreitol and 0.15 M KCl. Fractions (5.12 ml) were collected and 0.1-ml aliquots assayed for aspartokinase at 37°C either in the absence (○———○) or presence (●———●) of added L-lysine at 2.5 mM. Aspartokinase activity is expressed as μmol of aspartohydroxamate produced/30 min.

TABLE IV

STABILITY OF ASPARTOKINASES IN THE ABSENCE OF L-LYSINE

An aliquot of enzyme from pooled fractions from the DEAE-Sephadex column (Fig. 6) was diluted 50-fold with Tris · HCl buffer to reduce the lysine concentration to 20 μM. Immediately following dilution, 0.25-ml aliquots were assayed at 37°C for aspartokinase activity and then after 15 min incubation at either 0°C or 37°C. Aspartokinase I activity is the difference between the activity in the presence and absence of lysine, and aspartokinase II activity is the difference between the activity in the presence of lysine and in the presence of lysine plus threonine. Inhibitors were added at 2.5 mM. N.D. = not detectable.

	Aspartokinase activity (units/ml)	
	I	II
Preincubation	10.9	4.2
Enzyme incubated at		
0°C	12.0	1.7
37°C	11.0	N.D.

raphy (Figs. 1 and 3) and was found to bc insensitive to L-lysine, L-threonine, L-methionine, L-isoleucine and DL-diaminopimelate either singly or in combination. Attempts to further purify this activity were unsuccessful as additional treatments following DEAE-Sephadex chromatography led to loss of activity.

As there was no peak corresponding to aspartokinase III in the experiment described in Fig. 6 it was possible that aspartokinase III was an artifact of preparation, produced by modification of one of the other enzymes in the absence of lysine. To test this possibility two aliquots of a crude extract from cells grown with minimal medium were washed through a Sephadex G-25 column, one with L-lysine present and the other with no added lysine. The activities of aspartokinases I, II and III in the void fraction were estimated (Table V). Although the given values for aspartokinase II and III activities are likely to be accurate those for aspartokinase I are likely to be underestimated. In preparation A the activity measured in the absence of added lysine will be the sum of activities of the three aspartokinase activities but as aspartokinase II is unstable in the absence of added lysine (Fig. 2) the contribution of the enzyme to the total

TABLE V

EFFECT OF LYSINE DURING ELUTION OF AN EXTRACT FROM A SEPHADEX G-25 COLUMN

1.5-ml aliquots of crude extract from cells grown with minimal medium were washed through a 7.5-ml G-25 Sephadex column pre-equilibrated with Tris · HCl buffer containing 1 mM dithiothreitol and L-lysine at either zero (Prep. A) or 1.0 mM (Prep. B). Void fractions were pooled and assayed for aspartokinase (0.15 ml aliquots) at 25°C and protein content. Aspartokinase I activity is the difference between activities in the presence and absence of added 2.5 mM lysine, aspartokinase II activity the difference between activities in the presence of 2.5 mM L-lysine and the presence of 2.5 mM L-lysine plus 2.5 mM L-threonine, and aspartokinase III activity is the residual uninhibited reaction. All activies are expressed as units/mg × 10^3.

	Preparation A	Preparation B
Concentration of L-lysine in elution buffer (mM)	0	1.0
Aspartokinase I (units/mg × 10^3)	7.5	4.6
Aspartokinase II (units/mg × 10^3)	8.8	12.1
Aspartokinase III (units/mg × 10^3)	6.4	2.8

will be less than it should be. In preparation B the elution buffer contained lysine so in this case the contribution of enzyme I to the total will be less than it should be due to inhibition by the presence of the 0.15 μmol of lysine added to the assay with the enzyme. Taking these factors into consideration it was calculated that the correct value for aspartokinase I activity was in each case approximately 10^{-2} units/mg.

The aspartokinase III activity is lowest and the aspartokinase II activity greatest when lysine is used in the elution buffer. The total activity of II and III however remains approximately constant.

Discussion

We have detected three aspartokinase activities in extracts from *B. brevis* ATCC 10068 growing with a glycerol asparagine mineral salts medium. One, (aspartokinase I) is inhibited by lysine alone and another (aspartokinase II) is inhibited only by combinations of lysine and threonine. The third activity (aspartokinase III) is not inhibited by any of the known effectors of aspartokinase activities and we consider this activity to be produced by a modification of aspartokinase II. This system is different from that reported for any other Bacillus species.

The properties of partially purified aspartokinases I and II are very similar in several respects. They have similar affinities for their substrates ATP and aspartate and the effector lysine. Both are stabilised by a reducing agent such as 2-mercaptoethanol, and, more effectively, dithiothreitol. Under the conditions used in this paper they elute together from a DEAE-Sephadex column and they are both precipitated by a 35—55% saturated ammonium sulphate solution. They are different only in the response of their activities to lysine and or threonine, their sensitivity to the absence of lysine and their elution position from a Sephadex G-200 column (Fig. 7). Upon elution from a Sephadex G-200 column aspartokinase II activity is displaced towards a greater elution volume compared to the peak of aspartokinase I activity. Since in the experiment described aspartokinase II activity is low compared with aspartokinase I and aspartokinase I is almost completely inhibited in the presence of lysine we have assumed that the two peak positions (Fig. 7) approximated the peaks of aspartokinases I and II. We have calculated that proteins eluted in these positions should have molecular weights of 110 000 and 95 000 respectively.

Although the total aspartokinase activity is similar in extracts from cells growing with or without added lysine, with lysine, aspartokinase I activity cannot be detected. This phenomenon is similar to the one described by Gray and Bernlohr [16] for *B. licheniformis*. However they found that the lysine sensitive activity was unstable and lost during purification and they claimed that this enzyme was converted both in vivo and in vitro to the activity which was sensitive to lysine plus threonine. In *B. brevis* it is the aspartokinase II activity which is unstable and there is no evidence that either activity can be converted into the other.

In spite of the findings that the properties of two enzymes catalysing the same reaction in the one organism are similar there is no need to assume that a common gene must be involved for in *E. coli* the aspartokinase I (threonine-

sensitive) and aspartokinase III (lysine-sensitive) enzymes are indistinguishable with respect to kinetic constants yet they are indisputably products of separate genes [1]. This similarity is more likely to reflect a common evolutionary origin for the two enzymes [25].

We favour the view that aspartokinase I and II activities of *B. brevis* are the products of separate genes. Aspartokinase I is subject to repression by lysine. There is also evidence that repression controls do operate on aspartokinase II, for extracts from cells grown with peptone, yeast extract medium or minimal medium plus threonine and isoleucine contain very low levels of aspartokinase II. The nature of these repression controls has not been investigated. However, they may be complicated for in the presence of both lysine and threonine the two detected aspartokinase activities will be minimal yet the organism will still require carbon to flow into diaminopimelate, methionine and probably dipicolinate. We have not been able to detect any aspartokinase whose activity is modified by either diaminopimelate or methionine.

The postulate that aspartokinase II activity can be converted into aspartokinase III activity is based on the following observations. Aspartokinase III activity is at a lower level when lysine is not removed from a crude extract and aspartokinase II activity is higher (Table V). When lysine is absent during purification procedures aspartokinase II activity is lost (Fig. 1 cf. Table I), but when lysine is present aspartokinase III activity cannot be detected (Fig. 6). The separation of aspartokinase II and III activities on DEAE-Sephadex (Fig. 3) might be explained by a ligand dependant subunit dissociation phenomenon similar to that found for other aspartokinase enzymes [26—28] resulting in an altered ionic charge distribution. We have not been able to find conditions to reverse this modification for in this form activity is readily lost.

This finding that aspartokinase II activity is rapidly lost in extracts which do not contain added lysine may not be of physiological significance, for under normal conditions the level of lysine within the cell would not be expected to fall to sufficiently low levels. However if this property is a more general property of aspartokinase enzymes in vitro then there are a number of important consequences. In the case of *B. licheniformis* should the stability of the lysine sensitive aspartokinase activity depend on the presence of either lysine or some other such compound, then a different interpretation might be given to the results of Gray and Bernlohr [16]. Theirs has been the only example of an apparent in vivo conversion of two aspartokinase activities. In situations where only one aspartokinase activity has been reported for certain Bacillus species [8,11,29] this might have resulted from a loss of other activities caused by the absence of the appropriate stabilising compounds in the buffers.

The detection of other aspartokinase activities in these species might enable us to understand how a flow of carbon could be maintained into the aspartate family of amino acids and particularly diaminopimelate and dipicolinate under conditions when according to the published properties, the specific activity and enzyme activity of the identifiable aspartokinase would be expected to be minimal. In *B. brevis* we do not yet know how the level of diaminopimelate synthesis is maintained during vegetative growth in the presence of both lysine and threonine nor how the organism makes available diaminopimelate and dipicolinate during sporulation. Obviously these situations in various Bacillus species now need to be re-examined more carefully.

Acknowledgment

M.J.M.H. was supported by a University of Melbourne Research Scholarship.

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