JOURNAL OF BACTERIOLOGY, Jan. 1990, p. 218–223
0021-9193/90/010218-06$02.00/0

Vol. 172, No. 1

Aspartokinase III, a New Isozyme in *Bacillus subtilis* 168

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Received 8 August 1989/Accepted 2 October 1989

A previously undetected *Bacillus subtilis* aspartokinase isozyme, which we have called aspartokinase III, has been characterized. The new isozyme was most readily detected in extracts of cells grown with lysine, which repressed aspartokinase II and induced aspartokinase III, or in extracts of strain VS11, a mutant lacking aspartokinase II. Antibodies against aspartokinase II did not cross-react with aspartokinase III. Aspartokinases II and III coeluted on gel filtration chromatography at M_r 120,000, which accounts for the previous inability to detect it. Aspartokinase III was induced by lysine and repressed by threonine. It was synergistically inhibited by lysine and threonine. Aspartokinase III activity, like aspartokinase II activity, declined rapidly in *B. subtilis* cells that were starved for glucose. In contrast, the specific activity of aspartokinase I, the diaminopimelic acid-inhibitable isozyme, was constant under all growth conditions examined.

Aspartokinase (EC 2.7.2.4) catalyzes the first step of a highly branched biosynthetic sequence leading to the amino acids lysine, threonine, methionine, and isoleucine (6, 21). An intermediate of the lysine-biosynthetic branch, *meso*-diaminopimelate, is also a component of the peptidoglycan of the cell wall of *Bacillus* species (20). The appropriate regulation of aspartokinase activity in bacteria is usually achieved by production of multiple aspartokinase isozymes which are subject to differential regulation by end product repression and allosteric inhibition (6, 16). In fact, the discovery and characterization of three aspartokinases in *Escherichia coli* provided the first clear demonstration of the physiological function of isozymes (6, 15, 21).

Bacillus subtilis has been reported to produce two aspartokinases, which are separable by gel filtration chromatography (8, 20). Aspartokinase I is selectively inhibited by diaminopimelate, and aspartokinase II is selectively inhibited by lysine (20). Aspartokinase II has been purified to homogeneity from an overproducing strain (14), and the gene encoding the enzyme has been cloned and sequenced (3). Although partially purified aspartokinase II from *B. subtilis* ATCC 6051 was inhibited by both threonine and lysine (20), the purified enzyme from a derivative of *B. subtilis* 168 was sensitive to lysine only (3, 14). Aspartokinase I activity remained relatively constant throughout the growth and stationary phases in *B. subtilis* cells, but aspartokinase II activity disappeared rapidly during the stationary phase (20). Because of a long-standing interest in the inactivation and degradation of enzymes in *B. subtilis* (22, 24), we undertook a study of aspartokinase II degradation. In the course of these studies, several puzzling observations led us to reexamine the aspartokinases of *B. subtilis*. We report here the discovery of a new aspartokinase, aspartokinase III, which has previously evaded detection because it comigrates with aspartokinase II on gel filtration chromatography. The new isozyme is synergistically inhibited by threonine and lysine and is induced by growth in the presence of lysine.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP was synthesized by the method of Johnson and Walseth (10). $^{32}P_i$ and ^{125}I-protein A were purchased from ICN Radiochemicals. All L-amino acids and DL-α-ε-diaminopimelic acid (a mixture of LL, DD, and *meso* isomers) were obtained from Sigma Chemical Co. Sephacryl S-200 was purchased from Pharmacia. Immobilon P was obtained from Millipore Corp. *N*-Tosyl-L-lysine chloromethylketone was purchased from Research Organics. Phenylmethylsulfonyl fluoride was obtained from Sigma Chemical Co.

Bacterial strains and growth conditions. *B. subtilis* 168 (*trpC*) was obtained from L. Leon Campbell. *B. subtilis* VS11 (*pheA trpC sulR aecA ask::cat*) was a generous gift from Henry Paulus (5) (Boston Biomedical Research Institute). Spores of *B. subtilis* 168 and VS11 in dimethyl sulfoxide stocks were used to inoculate 5-ml starter cultures of supplemented nutrient broth and grown for approximately 6 h at 37°C. A 1-ml portion of cells was transferred from the starter culture and grown overnight in 100 ml of buffered minimal medium (BMM) plus tryptophan (0.05 g/liter) (1). L-Phenylalanine (0.5 g/liter) was included in the growth medium when VS11 was grown. All other amino acids were added to the growth medium at 0.05 g/liter unless stated otherwise. Cells from the overnight cultures were used to inoculate flasks containing fresh BMM and any auxotrophic amino acids. The growth of the cultures was monitored by the use of a Klett-Summerson colorimeter with a no. 66 filter. Cells were harvested by centrifugation and washed twice with buffer A [50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0), 200 mM KCl, 20% ethylene glycol, 0.1 mM EDTA, 0.1 mM *o*-phenanthroline, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium azide].

Assay of aspartokinase. Extracts of cells were prepared for assays by sonicating cells in buffer A plus 10 mM 2-mercaptoethanol with a model W-375 sonicator (Ultrasonics Inc.). Sonication was performed at 0°C with four 15-s pulses with cooling between pulses for a total time of 5 min (setting 5, 50% duty cycle). Extracts were centrifuged at 100,000 × *g* at 4°C for 1 h following sonication. Aspartokinase was assayed by measuring the formation of [^{32}P]aspartyl-β-acylphosphate from [γ-^{32}P]ATP by modifications of the procedure of Paulus and Gray (17). The modifications included the use of a charcoal adsorption step as described by Switzer and Gibson (23) and the use of 50 mM 3-(*N*-morpholino)propane-sulfonic acid buffer, pH 7.0, in place of triethanolamine buffer. All assay mixes were incubated at 25°C for 15 min in the presence of 10 mM L-aspartate. Aspartokinase I activity was determined by assaying ex-

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tracts in the presence of 10 mM L-lysine and 10 mM L-threonine. Aspartokinase II was assayed with the addition of 10 mM diaminopimelic acid and 20 mM L-threonine. Aspartokinase III activity was quantitated by assaying samples in the presence of 10 mM diaminopimelic acid and subtracting the amount of activity due to aspartokinase II. One unit of aspartokinase activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of acylphosphate in 30 min under these conditions. Protein determination was performed by the biuret method (12).

Purification of anti-aspartokinase II IgG. Anti-aspartokinase II antibodies were raised in rabbits by multiple subcutaneous injections of highly purified recombinant aspartokinase II as the antigen (Cocalico Biologicals). Crude serum was obtained from Cocalico Biologicals, where the antiserum was raised in rabbits from highly purified aspartokinase II provided by our laboratory, and was treated with 1 mM phenylmethylsulfonyl fluoride and 1 mM *N*-tosyl-L-lysine chloromethylketone prior to purification of immunoglobulin G (IgG). Anti-aspartokinase IgG was purified by the method described previously (13). Additional purification was performed on DEAE-cellulose (Whatman DE-52) in 15 mM potassium phosphate buffer (pH 7.0) containing 50 mM NaCl. The flowthrough fraction (IgG) was isolated, concentrated to approximately 18 mg/ml, frozen in liquid N_2, and stored at −70°C until use.

Quantitation of immunologically cross-reacting material by immunoblotting. Crude cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels (11). Proteins were transferred to Immobilon P membrane with a Gelman Biotrans model A blotter or a Bio-Rad Trans-Blot SD. Blotting was performed according to the manufacturer's procedure. Blocking of the membrane was achieved by incubation in 2% nonfat milk in TBST (10 mM Tris hydrochloride [pH 8.0]–150 mM NaCl–0.05% Tween 20) with shaking for 15 min. The anti-aspartokinase II antibodies were added at a 1:3,000 dilution in TBST and incubated for 30 min at room temperature. The membrane was then incubated in TBST plus 1 μCi of ^{125}I-protein A per ml for 1 h with shaking at room temperature. Bands corresponding to aspartokinase II (α or β subunit) were visualized by using the Protoblot system (Promega Inc.) or by exposure of the membrane to X-ray film. The aspartokinase bands were cut out and counted in a Beckman model 8000 gamma counter.

RESULTS

Evidence for a third aspartokinase activity in *B. subtilis*. The first clear indication of the existence of a novel aspartokinase was obtained from a comparison of extracts of *B. subtilis* 168 cells which had been grown on BMM with and without lysine (50 μg/ml). The amount of aspartokinase activity, assayed in the presence of 10 mM diaminopimelate to inhibit aspartokinase I, was essentially equal in the two extracts, but quantitative immunoblotting showed that >90% of the aspartokinase II α and β subunits had been repressed in the cells grown with lysine. Unlike purified aspartokinase II, the diaminopimelate-insensitive aspartokinase activity in cells grown with lysine was not inhibited by anti-aspartokinase II antibody and was inhibited by threonine (85% inhibition at 17 mM threonine). These observations led us to postulate the existence of a new aspartokinase which is neither inhibitable by diaminopimelate nor repressible by lysine and which does not cross-react immunochemically with aspartokinase II. We called this new activity aspartokinase III.

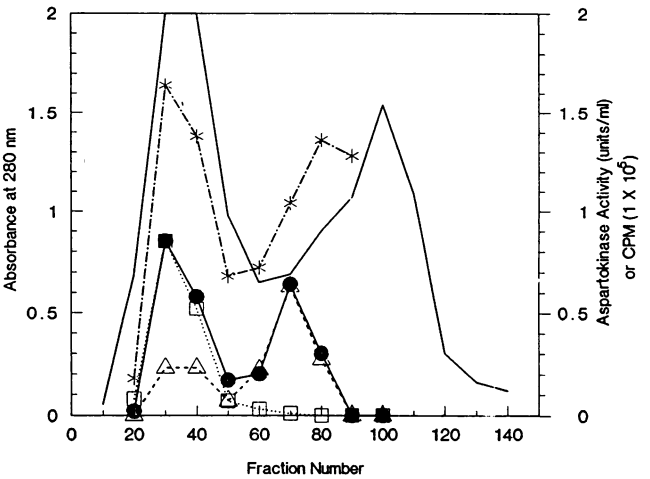

FIG. 1. Separation of aspartokinase I and aspartokinase III. *B. subtilis* VS11 cells were grown on BMM plus tryptophan (0.05 g/liter), phenylalanine (0.5 g/liter), and 0.2% glucose to the end of logarithmic phase. Thawed cells were broken in 1.2 volumes of buffer A and centrifuged at 100,000 × *g* as described in Materials and Methods. A 2.5-ml sample (32 mg/ml) was applied to a column (2.2 by 100 cm) of Sephacryl S-200 (Superfine) equilibrated with buffer B (20 mM potassium phosphate [pH 7.5], 1 mM 2-mercaptoethanol, 1 mM L-threonine, 1 mM L-lysine) plus 20% ethylene glycol. The sample was eluted with the same buffer at a flow rate of 30 ml/h. After 100 ml of buffer had passed through the column, fractions (1.0 ml) were collected and assayed for aspartokinase activity in the absence of added inhibitor (●), in the presence of 10 mM diaminopimelate (△), and in the presence of 10 mM L-lysine plus 10 mM L-threonine (□). Immunoblotting of fractions was performed as described in Materials and Methods. Radioactive bands corresponding to the truncated aspartokinase II α subunit were excised and counted (*). ——, Absorbance of fractions at 280 nm.

Separation of aspartokinases I and III from an aspartokinase II-deficient strain. Characterization of the aspartokinase activity was greatly facilitated by the availability of a strain, VS11 (kindly provided by Henry Paulus of the Boston Biomedical Research Institute), in which the aspartokinase II gene has been disrupted (5). A crude extract of strain VS11 cells which had been grown on BMM plus lysine (50 μg/ml) was analyzed by gel filtration on Sephacryl S-200 (Fig. 1). Two peaks of aspartokinase activity were eluted. The first activity peak eluted at a position corresponding to an M_r of greater than 200,000. The activity in this peak was inhibited by 10 mM diaminopimelate but not by 10 mM lysine plus 10 mM threonine. This activity corresponds to the properties of aspartokinase I as described previously (20). The second peak of aspartokinase activity, which eluted at a position expected for an M_r of 120,000, was insensitive to diaminopimelate but was completely inhibited by lysine plus threonine. Immunoblotting of the Sephacryl fractions demonstrated that the normal α and β subunits of aspartokinase II were absent, as expected. A band corresponding to a truncated form of aspartokinase II (39 kilodaltons [kDa]) was detected and found to be smeared through the eluted fractions; this fragment had the size predicted from the procedure used to construct strain VS11 (5). Because of immunochemical evidence (Table 1, described below) that strain VS11 produces no aspartokinase II activity, we concluded that the peak eluting in a position corresponding to M_r 120,000 contained aspartokinase III.

Separation of aspartokinase I from aspartokinases II and III

TABLE 1. Differentiation between aspartokinases II and III by inhibition with antibodies and threonine

Source of extract[a]	Addition	% Inhibition	Aspartokinase isozyme(s) present
168	Anti-aspartokinase II antibody[b]	63	II + III
VS11	Anti-aspartokinase II antibody	0	III
168	Threonine (20 mM)	28	II + III
VS11	Threonine (20 mM)	84	III
168	Anti-aspartokinase II antibody + threonine (20 mM)	93	II + III
VS11	Anti-aspartokinase II antibody + threonine (30 mM)	86	III

[a] Strain 168 was grown on BMM with only tryptophan added; strain VS11 was grown on BMM plus 50 μg of lysine per ml. Crude extracts from each were chromatographed on Sephacryl S-200 as described for Fig. 1 and 2, and fractions corresponding to the aspartokinase activity peak of M_r 120,000 (i.e., excluding aspartokinase I) were pooled and assayed under standard conditions.

[b] An amount of anti-aspartokinase II antibody (2.7 mg) sufficient to inhibit 0.8 U of purified enzyme by 95% was added, and the samples were incubated for 1 h at 25°C prior to assay.

TABLE 2. Inhibition of aspartokinase III by amino acids[a]

Amino acid added	Concn (mM)	% Inhibition
Threonine	0.2	5
	1.0	14
	2.5	26
	5.0	59
	10.0	86
	20.0	95
Lysine	0.2	9
	1.0	23
	2.5	30
	5.0	47
	10.0	64
	20.0	73
Threonine plus lysine	0.2 (each)	76
	1.0 (each)	100
	2.5 (each)	100

[a] The pooled M_r 120,000 peak from Sephacryl S-200 analysis of a crude extract of strain VS11 cells, grown and analyzed as described for Fig. 1, was dialyzed against buffer A and assayed under standard conditions with the indicated concentrations of amino acids.

from *B. subtilis* 168. An extract of *B. subtilis* 168 cells which had been grown on BMM without amino acids (except tryptophan) was also analyzed by chromatography on Sephacryl S-200 (Fig. 2). Aspartokinase I eluted first, as with extracts of VS11 cells; this activity was completely inhibited by diaminopimelate and insensitive to lysine plus threonine. A second peak of aspartokinase activity eluted at a position equivalent to M_r 120,000; this activity was inhibited by lysine plus threonine and insensitive to diaminopimelate. Immunoblot analysis showed a close correspondence between the abundance of aspartokinase II α and β subunits and the second aspartokinase activity peak in the Sephacryl fractions. These results led us to suspect that aspartokinases II and III coeluted from the Sephacryl column.

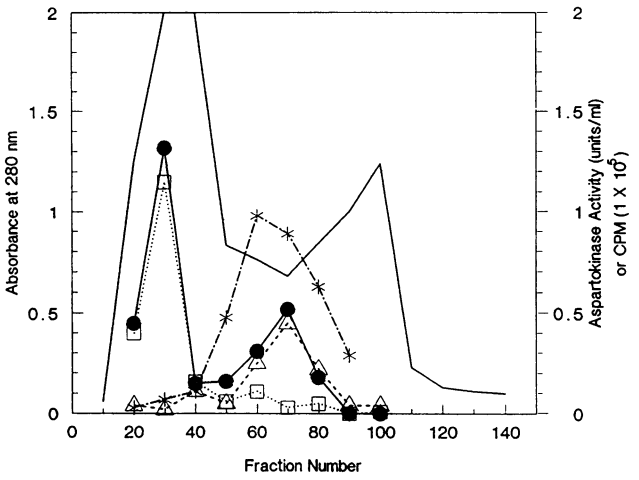

FIG. 2. Separation of aspartokinase I from aspartokinases II and III. *B. subtilis* LC168 cells were grown on BMM plus tryptophan (0.05 g/liter) and 0.2% glucose to the end of logarithmic phase. Thawed cells of *B. subtilis* LC168 were broken and centrifuged as described in Materials and Methods. A 2.5-ml sample (25 mg/ml) was applied and chromatographed as described in the legend to Fig. 1. Fractions (1.0 ml) were collected and assayed for aspartokinase activity in the absence of added inhibitor (●), in the presence of 10 mM diaminopimelate (△), and in the presence of 10 mM L-lysine plus 10 mM L-threonine (□). Fractions were immunoblotted as described in Materials and Methods, and the radioactivity corresponding to the aspartokinase II α subunit was plotted (*). ——, Absorbance of fractions at 280 nm.

This conclusion was confirmed by analysis of the properties of the pooled M_r 120,000 aspartokinase peaks from Sephacryl analysis of extracts of strains 168 and VS11 (Table 1). This fraction from strain VS11 was insensitive to inhibition by anti-aspartokinase II antibodies. In contrast, an extract of strain 168 grown without amino acids was inhibited 63% by the anti-aspartokinase II antibody, suggesting that aspartokinase II and aspartokinase III were present in a 2-to-1 ratio in these cells. Further experiments (Table 1) indicated that aspartokinase III was inhibited by at least 85% by 20 mM threonine. Aspartokinase II was insensitive to threonine inhibition, as previously shown for the purified enzyme from strain VB217 (14). The previous conclusion that aspartokinase II was subject to multivalent inhibition by lysine and threonine (20) was probably the result of having studied a mixture of aspartokinases II and III.

The ability to separate aspartokinase III from aspartokinase I by Sephacryl S-200 chromatography and the use of extracts of strain VS11 allowed us to examine the sensitivity of the new isozyme to end product inhibitors (Table 2). Aspartokinase III was sensitive to inhibition by lysine or threonine alone or, much more significantly, in combinations. Individually, the amino acids threonine and lysine inhibited the isozyme by approximately 50% at concentrations of 4 and 5 mM, respectively. Synergistic inhibition was observed at much lower concentrations of lysine and threonine. A concentration of 0.2 mM lysine plus 0.2 mM threonine inhibited aspartokinase III by approximately 76%, and higher concentrations (1.0 mM each) completely inhibited the enzyme.

Amino acid induction and repression of *B. subtilis* aspartokinases. *B. subtilis* 168 was grown to late exponential phase in BMM containing various amino acids at 50 μg/ml each. The specific activities of aspartokinases I, II, and III were determined by the specific assay procedures described in Materials and Methods, which are based on the preceding observations of their specific inhibitory metabolites. Aspartokinase II was repressed by lysine, whereas aspartokinase

TABLE 3. Effects of amino acids in the growth medium on specific activities of *B. subtilis* aspartokinase isozymes[a]

Addition	Sp act (U/mg of protein) of aspartokinase isozyme:		
	I	II	III
None	0.40	0.26	0.09
Lysine	0.37	0.11	0.34
Methionine	0.45	0.78	0.14
Threonine	0.41	0.29	0.06
Isoleucine	0.41	0.27	0.09
Diaminopimelate	0.39	0.23	0.14
20 amino acids[b]	0.37	0.07	0.03

[a] *B. subtilis* strain 168 was grown to late exponential phase on BMM plus tryptophan (50 μg/ml). Additional amino acids were added at 50 μg/ml each.
[b] A mixture of the amino acids commonly found in proteins was added at 50 μg/ml each.

III was induced under the same growth conditions (Table 3). Methionine caused a three-fold induction of aspartokinase II and had little effect on aspartokinase III levels. Both aspartokinases II and III were repressed by growth on 20 amino acids. Aspartokinase III levels were found to be significantly lower than aspartokinase II levels except under inducing conditions (growth with lysine). Aspartokinase I levels were essentially invariant under all growth conditions tested. The conclusions based on specific activities of aspartokinase II were confirmed by immunoblot analysis, which demonstrated that aspartokinase II activity levels reflect the amount of enzyme protein rather than activation or inhibition of a constant amount of enzyme.

TABLE 4. Specific activity of *B. subtilis* aspartokinase isozymes during growth and stationary phases[a]

Addition to medium	Time of growth[b]	Sp act (U/mg of protein) of aspartokinase isozyme:		
		I	II	III
None	−2.0	0.33	0.22	0.02
	−1.0	0.39	0.28	0.08
	0.0	0.44	0.37	0.09
	+1.0	0.40	0.24	0.10
	+2.0	0.38	0.20	0.02
	+3.0	0.38	0.13	0.04
Lysine	−2.0	0.34	0.08	0.24
	−1.0	0.37	0.10	0.28
	0.0	0.44	0.12	0.32
	+1.0	0.35	0.08	0.15
	+2.0	0.36	0.06	0.14
	+3.0	0.39	0.07	0.04

[a] *B. subtilis* strain 168 was grown on BMM plus tryptophan (50 μg/ml) with or without lysine (50 μg/ml). Samples were harvested at the indicated times and assayed for the aspartokinase isozymes as described in Materials and Methods.
[b] Time (in hours) before (−) or after (+) the end of exponential growth.

Levels of aspartokinase isozymes at various stages of growth. Table 4 summarizes observation of the specific activities of aspartokinase isozymes in wild-type *B. subtilis* grown on BMM. The experiments were conducted with cells grown in the absence of amino acids that affect aspartokinase expression and in cells grown with lysine, which represses aspartokinase II and induces aspartokinase III, to increase the sensitivity of the determination of the activity of the new isozyme. In both cases, aspartokinase II levels rose to a maximum at the end of exponential growth and declined significantly during the stationary phase (the cells were limited for glucose), as has been reported by others (20). We have shown that the decline in aspartokinase II activity is accompanied by parallel decreases in the amount of α and β subunits, as measured immunochemically (unpublished experiments). Interestingly, aspartokinase III levels showed a similar pattern of development. In contrast, the specific activity of aspartokinase I was nearly constant in all phases of growth. This result agrees with the observations of others (20) for aspartokinase I.

DISCUSSION

The properties of the three aspartokinase isozymes found in *B. subtilis* are summarized in Table 5. The regulatory properties of the newly discovered aspartokinase III, together with those described previously (and confirmed by us) for aspartokinases I and II, allow a rational picture of the overall regulation of the complex aspartyl-β-phosphate pathway to be drawn. Feedback inhibition and repression of aspartokinase II by lysine indicates the importance of this isozyme in control of lysine biosynthesis. Repression of aspartokinase III by threonine and induction of the isozyme by lysine suggest to us that it functions primarily to regulate threonine biosynthesis. It may seem paradoxical that aspartokinase III is induced by lysine and also inhibited by it. However, the concentrations of lysine alone needed to inhibit aspartokinase III (K_i, ≈5 mM) are much higher than needed to inhibit aspartokinase II (K_i, ≈0.1 mM [14]). Only in the presence of threonine does aspartokinase III become inhibitable by physiological levels of lysine. The induction of aspartokinase II by methionine (not previously described by others) may serve to prevent starvation for this amino acid when lysine and threonine are present in excess. Assuming that aspartokinase I is unable to supply precursors to amino acid biosynthesis other than diaminopimelate, excess lysine plus threonine would fully repress aspartokinases II and III and prevent methionine formation unless methionine could antagonize this repression in some manner. Little regulation of aspartokinases by isoleucine has been detected. Perhaps the major regulation of this pathway is at the level of the

TABLE 5. Comparison of *B. subtilis* aspartokinases I, II, and III

Aspartokinase	Mol wt	Inhibitor(s)	Inducer	Repressor	Sp act[a]		Cross-reaction with anti-aspartokinase II antibody
					Exponential phase	Stationary phase	
I	>200,000	*meso*-Diaminopimelate	None known	None known	Constant	Constant	No
II	120,000	Lysine	Methionine	Lysine	Increases	Declines	Yes
III	120,000	Lysine and threonine	Lysine	Threonine and 20 amino acids[b]	Increases	Declines	No

[a] During growth on minimal medium and entering stationary phase because of glucose exhaustion.
[b] See text.

threonine-to-isoleucine branch only with the presence of the "constitutive" aspartokinase I sufficient to ensure flow of aspartyl-β-phosphate to that pathway.

The rapid rise in activity of aspartokinases II and III in *B. subtilis* cells growing on minimal medium and their rapid decline during glucose starvation also suggest the participation of these enzymes in the provision of aspartyl-β-phosphate for amino acid biosynthesis during rapid growth. The disappearance of aspartokinase II is known to result from degradation of this enzyme (L. M. Graves and R. L. Switzer, J. Cell. Biol. vol. 107, abstr. 3465, p. 612a, 1989). Probably the same is true for aspartokinase III. Both aspartokinases II and III are fully repressed by growth of *B. subtilis* cells in medium containing a full complement of amino acids.

Aspartokinase I activity is invariant during the exponential and stationary phases of growth and is not altered by addition of amino acids to the growth medium. The role of this isozyme is most likely to provide a constant level of aspartyl-β-phosphate for the biosynthesis of diaminopimelate for peptidoglycan synthesis, as was proposed earlier (20). It would be interesting to learn by mutational inactivation of aspartokinases II and III whether aspartokinase I alone is able to supply sufficient precursor for amino acid biosynthesis during rapid growth.

Aspartokinase III apparently escaped detection in earlier studies because it comigrates closely with aspartokinase II on gel filtration chromatography and its levels are quite low in cells grown under many conditions. A key to its discovery was our observation that aspartokinase III is induced by addition of lysine to the growth medium and the repression of aspartokinase II under the same conditions. The availability of a mutant strain, VS11, lacking aspartokinase II activity was also an important tool in demonstrating the existence of the new isozyme. In previous studies, Rosner and Paulus (20) described two aspartokinase isozymes in *B. subtilis*, which were distinguishable by their molecular weights and sensitivity to inhibitors. One of these was aspartokinase I. Comparison of our findings with the properties of a partially purified fraction called aspartokinase II by Rosner and Paulus indicates that this fraction was largely aspartokinase III, because of its synergistic inhibition by lysine and threonine. Recently it has been found that the *B. subtilis* strain (ATCC 6051) used by Rosner and Paulus contains very little aspartokinase II and elevated levels of aspartokinase III (H. Paulus, personal communication). Subsequently, Moir and Paulus (14) purified aspartokinase II to homogeneity from an overproducing mutant of *B. subtilis*, VB217. The properties of the purified enzyme are those which we have ascribed to aspartokinase II in this work.

Lysine-sensitive and lysine-insensitive aspartokinase activities from *B. subtilis* cells were also described by Hampton et al. (8). They separated two peaks of activity on a gel filtration column, a result which was similar to the observations of Rosner and Paulus. The larger, lysine-insensitive aspartokinase species was probably aspartokinase I, but was not tested for inhibition by *meso*-diaminopimelate. The smaller aspartokinase species was inhibited by lysine but contained substantial lysine-insensitive activity, which might have been due to aspartokinase III. However, no inhibition by threonine was observed, as we would have expected. Most of the experiments conducted by Hampton et al. were done with cells grown in the presence of methionine, so the dominant lower-molecular-weight isozyme in the extracts would be expected to be aspartokinase II.

The properties of aspartokinase activities from *B. polymyxa* (2, 17–19), *B. brevis* (9), and *B. licheniformis* (7) have all been described. In each case the observations are suggestive of the presence of two or three isozymes, although they have not always been so interpreted by the authors. In each case it seems to us that a reinvestigation, guided by the collective findings on *B. subtilis* aspartokinases, might lead to the discovery of one or more new isozymes.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI11121 from the National Institute of Allergy and Infectious Diseases.

We gratefully acknowledge Henry Paulus of the Boston Biomedical Research Institute for providing strain VS11, for providing clones bearing recombinant DNA encoding aspartokinase II, and for many helpful discussions.

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