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Control of Lysine Biosynthesis in *Bacillus subtilis*: Inhibition of Diaminopimelate Decarboxylase by Lysine

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Diaminopimelate decarboxylase has been characterized in extracts of *Bacillus subtilis* and resolved from aspartokinases I and II. Under certain conditions, the enzyme is specifically inhibited by physiological concentrations of L-lysine, but less specificity and altered kinetics of inhibition are observed if lower ionic strengths are employed in the assay procedure. Diaminopimelate decarboxylase can be desensitized to lysine inhibition by either lowering the pH or diluting the enzyme in Tris buffer in the absence of pyridoxal phosphate. Evidence is presented to indicate that, under proper conditions, lysine inhibition involves an interaction of the amino acid with the enzyme rather than competition for available pyridoxal phosphate in the assay. Lysine, by affecting the level of *meso*-diaminopimelate, may thus regulate its biosynthesis through sequential feedback inhibition. Analysis of the diaminopimelate decarboxylase of 15 revertants of mutants that had originally lacked diaminopimelate decarboxylase activity indicates that as little as 5% of the specific activity of enzyme observed in the wild-type strain is sufficient to permit normal growth rates. In the growing cell, diaminopimelate decarboxylase may therefore exist largely in an inhibited state.

Since its discovery in *Escherichia coli* (5), diaminopimelate decarboxylase (*meso*-2,6-diaminopimelate carboxylyase; EC 4.1.1.20) has been shown to be repressed but not inhibited by L-lysine in the enteric bacteria (24, 35). Subsequent studies have indicated that the enzyme from several species of nonenteric bacteria can be slightly inhibited by L-lysine (11, 12, 21, 32), but only at relatively high concentrations (20 mM).

This paper describes the characterization of diaminopimelate decarboxylase from the Marburg strain of *Bacillus subtilis*. Assay conditions were developed under which the enzyme is specifically inhibited by low concentrations of L-lysine, suggesting that L-lysine can regulate its production from *meso*-diaminopimelate.

The finding is of special significance in *B. subtilis*, since it was recently shown in that organism that *meso*-diaminopimelate is capable of regulating its own biosynthesis (27). Lysine inhibition of diaminopimelate decarboxylase in *B. subtilis*, therefore, would indicate that L-lysine ultimately regulates its own biosynthesis through at least two units of sequential feedback inhibition in the aspartate pathway.

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MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]lysine and Omnifluor were obtained from New England Nuclear; α, ϵ-[1,7-^{14}C]diaminopimelate (mixed isomers), amino acids, putrescine, and cadaverine were from Calbiochem; L-lysine methylester, *N*-ethylmaleimide, and ammonium sulfate were from Schwarz-Mann; *p*-hydroxymercuriphenylsulfonate was from Sigma; *N*-ϵ-acetyl lysine was from Cyclo; 2-mercaptoethanol and phenethylamine were from Eastman; and pancreatic deoxyribonuclease (DNase) was from Worthington. Sephadex G-200 (40-120 μ) was treated by the method of Kawata and Chase (16) to remove fines.

Enzyme assays. Diaminopimelate decarboxylase was assayed by the conversion of α, ϵ-[1,7-^{14}C]diaminopimelate to radioactive CO_2. The following components were added to 25-ml Erlenmeyer flasks at 0 C, in a final volume of 2 ml: potassium phosphate, pH 8.3 (200 mM); triethanolamine hydrochloride, pH 8.3 (30 mM); potassium bicarbonate, pH 7.5 (10 mM); ethylenediaminetetraacetic acid (EDTA) (1 mM); pyridoxal phsophate (20 μM); ethylene glycol (15%); [^{14}C]diaminopimelate (0.1 mM; 65,000 counts per min per μmol of the mixed isomers); and appropriate amounts of enzyme. The flasks were then sealed with serum stoppers that supported plastic cups containing 0.2 ml of phenethylamine and a folded strip of glass fiber filter paper. After incubation at 25 C for 30 min, the flasks were cooled to 0 C and acidified by the injection of 1 ml of 2 N H_2SO_4. The flasks were allowed to equilibrate at 25 C for 30 min, whereupon the strips of filter paper

were transferred to vials containing 5 ml of a toluene-based scintillation fluid (4 g of Omnifluor per liter of toluene) for determination of radioactivity in a Packard liquid scintillation spectrometer. The amount of enzyme used was adjusted so that not more than 5% of the reactants were converted to products, thereby assuring linearity of the assay with time and enzyme concentration. Under these conditions, no lysine decarboxylase activity (assayed in a similar manner, except that L-[U-^{14}C]lysine [0.5 mM; 400,000 counts per min per μmol] replaced [^{14}C]diaminopimelate in the reaction) could be detected in extracts of *B. subtilis*.

Aspartokinase activity was measured by the formation of [^{32}P]acyl phosphate from γ-[^{32}P]adenosine 5'-triphosphate, and L-aspartate, as described previously (26).

Protein determination. Protein was estimated by the biuret method (10).

Bacterial strains and growth conditions. *B. subtilis* ATCC 6051, the Marburg strain, was grown and harvested as described previously (27). Mutants lacking diaminopimelate decarboxylase were derived from the Marburg strain and kindly provided by E. Freese: A and B (*lys*$^-$); C (*lys*$^-$*ileu*$^-$); D, E, and F (*lys*$^-$*trp*$^-$); and G, H, and I (*lys*$^-$*trp*$^-$*met*$^-$). Revertants were isolated from these strains at frequencies of 1×10^{-5} to 2×10^{-7} as separate colonies that grew on solid minimal salts media in the absence of lysine but in the presence of any other amino acid originally required by the auxotroph.

After overnight preincubation on tryptose blood agar plates at 37 C, each mutant and revertant was grown in a 500-ml portion of the minimal salts medium of Spizizen (29), with L-tryptophan (25 μg/ml) and L-methionine (10 μg/ml) included. Aeration was accomplished in a 2-liter culture flask with shaking until the mid-exponential phase of growth (A_{600} = 0.6; 65 Klett units with a no. 42 filter) was attained. Bacteria were harvested by centrifugation, washed in chilled PM medium (0.07 M potassium phosphate, pH 7.0, containing 0.8 mM $MgSO_4 \cdot 7H_2O$), and stored at −20 C.

Preparation of extracts. All operations were carried out at 2 C. Thawed cells of *B. subtilis* (10 g) were suspended in 15 ml of buffer A (0.1 M potassium phosphate, pH 7.1; 1 mM 2-mercaptoethanol; 0.02 mM pyridoxal phosphate; and 20% ethylene glycol) with 2 mg of pancreatic DNase and were disrupted by passage through an Aminco French pressure cell at 10,000 lb/in^2. The extract was centrifuged at 100,000 $\times$ *g* for 1 h, and the supernatant solution (crude extract) was fractionated by the addition of solid ammonium sulfate. The material precipitating between 3 and 4 g of ammonium sulfate per 10 ml of crude extract was redissolved in buffer A (modified to contain 40% ethylene glycol) to give a protein concentration of 35 mg/ml. Recovery of diaminopimelate decarboxylase in the twofold purification was 70% and the enzyme could be stored at −15 C for over 2 months without loss of activity.

RESULTS

Gel filtration. When crude extracts were subjected to gel filtration on Sephadex G-200, a single symmetrical peak of diaminopimelate decarboxylase activity eluted slightly after aspartokinase II (27), in a position indicative of a molecular weight of 105,000 (Fig. 1).

Effect of salts. Optimal decarboxylation rates were observed at 150 mM potassium phosphate, with half-maximal activity at 30 mM and no activity at less than 15 mM of the salt (Fig. 2). This effect was relatively nonspecific, for sodium phosphate, sodium citrate, and sodium acetate stimulated the enzyme in a similar manner. Sodium chloride, potassium chloride, and potassium tartrate were considerably less effective. As discussed below, ionic strength also had a significant effect on the specificity of inhibition of the enzyme.

The addition of EDTA stimulated diaminopimelate decarboxylase. Maximal activity was observed at 0.1 mM EDTA, and higher concentrations (up to 5 mM) were not inhibitory.

Substrate kinetics. Under standard assay conditions, the concentration of diaminopimelate required for half-maximal activity of the enzyme was 1 mM (Fig. 3B), whereas the apparent K_m with respect to pyridoxal phosphate was 2 μM (Fig. 3C).

Inhibition by L-lysine. At high ionic strengths, diaminopimelate decarboxylase was specifically inhibited by L-lysine, other diamino acids (D-lysine, L-aminoethylcysteine, L-ornithine, L-2,4-diaminobutyrate), as well as *N*-ϵ-acetyl L-lysine, L-lysine methyl ester, putrescine, and cadaverine being less effective (Table

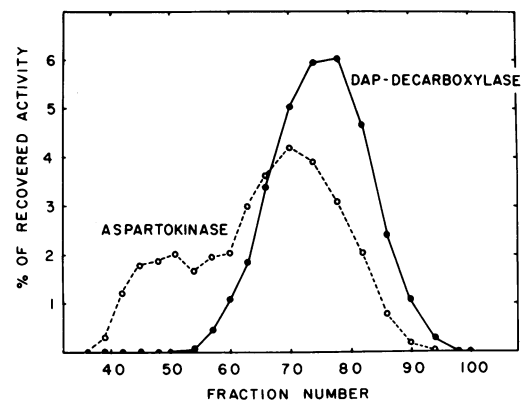

FIG. 1. *Separation of diaminopimelate decarboxylase and aspartokinases I and II. Crude extract was diluted with buffer A to give a final protein concentration of 25 mg/ml. A sample (5 ml) of this diluted extract was applied to a column (1 by 143 cm) of Sephadex G-200 and eluted with the same buffer. Fractions (1 ml) were collected and assayed for either aspartokinase* (○) *or diaminopimelate decarboxylase* (●) *activity. Recovery of both activities was nearly quantitative.*

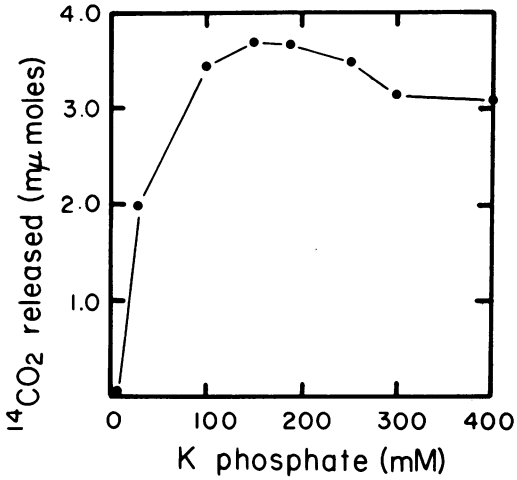

FIG. 2. *Effect of potassium phosphate on diaminopimelate decarboxylase activity. The partially purified diaminopimelate decarboxylase (2×10^{-4} units of ammonium sulfate fraction) was assayed for activity under the conditions described in Materials and Methods, except that potassium phosphate was added at the concentrations indicated. The reaction velocities are expressed as the amount of $^{14}CO_2$ liberated in 30 min.*

1). In contrast, at low ionic strength (30 mM potassium phosphate), all diamino compounds inhibited the enzyme. Compounds with only a single amino group were not inhibitory under any condition.

TABLE 1. *Effect of potassium phosphate upon the specificity of inhibition of diaminopimelate decarboxylase by* L-*lysine*[a]

Additions (2 mM)	Inhibition (%) at	
	30 mM potassium phosphate	200 mM potassium phosphate
L-Lysine	80	45
D-Lysine	71	19
L-*S*-aminoethylcysteine	64	17
L-Ornithine	58	16
L-2,4-Diaminobutyrate	40	18
4-Aminobutyrate	0	0
2-Aminobutyrate	2	4
n-Butylamine	8	4

[a] Diaminopimelate decarboxylase (2×10^{-4} units of ammonium sulfate fraction) was assayed as described under Materials and Methods, except that potassium phosphate was added at the concentrations indicated.

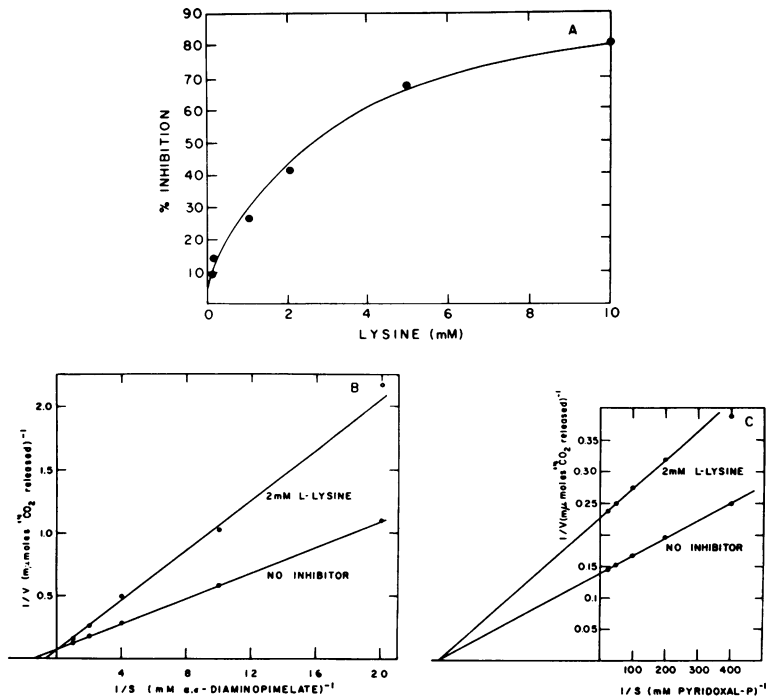

FIG. 3. *Inhibition of diaminopimelate decarboxylase by* L-*lysine. (A) Diaminopimelate decarboxylase (2×10^{-4} units of ammonium sulfate fraction) was assayed at various concentrations of* L-*lysine. (B) Diaminopimelate decarboxylase was assayed at various diaminopimelate concentrations at 20 μM pyridoxal phosphate under standard conditions in the absence of inhibitor (●) and in the presence of 2 mM* L-*lysine (○). (C) Diaminopimelate decarboxylase was assayed at various pyridoxal phosphate concentrations at 0.1 mM diaminopimelate in the absence of inhibitor (●) or in the presence of 2 mM* L-*lysine (○).*

The inhibition of diaminopimelate decarboxylase by L-lysine was a hyperbolic function of inhibitor concentration (Fig. 3A). L-lysine had no effect on the linearity of double reciprocal plots with either substrate, and behaved as a competitive inhibitor with respect to diaminopimelate (Fig. 3B) and as a noncompetitive inhibitor with respect to pyridoxal phosphate (Fig. 3C) with a K_i of 1.5 mM. Under conditions where the specificity of inhibition was less (such as at 30 mM potassium phosphate), the inhibition with respect to pyridoxal phosphate became partially competitive.

Effect of pH. Diaminopimelate decarboxylase exhibited more than half-maximal activity between pH 7.5 and 9.0, with an optimum at pH 8.5 (Fig. 4A). Inhibition by L-lysine was also dependent on pH, being very low at pH 7.0 and reaching a maximum at pH 8.5 (Fig. 4B). It must be noted that nonspecific inhibition by diamino compounds other than L-lysine also became more pronounced at higher pH values. The results (Fig. 4B) were corrected for this nonspecific effect by subtracting the inhibition caused by an equilvalent amount of D-lysine.

Effect of dilution in Tris buffer. When crude extracts of *B. subtilis* were diluted with tris (hydroxymethyl)aminomethane (Tris) buffer in the absence of pyridoxal phosphate, a large loss of diaminopimelate decarboxylase activity resulted, but the residual enzyme had become virtually insensitive to inhibition by L-lysine. These changes were less pronounced if the dilution was carried out in the presence of pyridoxal phosphate (Table 2). In contrast, other attempts to desensitize the enzyme towards inhibition by L-lysine, such as treatment with *N*-ethylmaleimide, *p*-hydroxymercuriphenylsulfonate, or acetic anhydride, resulted in substantial loss of diaminopimelate decarboxylase activity with no concomitant decrease in sensitivity towards the inhibitor.

Effect of growth medium on diaminopimelate decarboxylase. In minimal medium, the level of diaminopimelate decarboxylase declined about twofold as growth progressed from exponential to stationary phase, and the addition of L-lysine (5 mM) to exponentially growing cultures had a similar effect. Much lower levels (5 to 10%) of diaminopimelate decarboxylase were observed in a rich medium (30). The response of diaminopimelate decarboxylase to various growth conditions was different from that of aspartokinase I and aspartokinase II, indicating that the synthesis of the three enzymes is not coordinately controlled (Table 3).

Effect of ionic strength on Schiff base formation. The formation of a Schiff base between pyridoxal phosphate and L-lysine was measured by its absorbance at 414 nm (19). The stability of the complex was found to depend on ionic strength, its dissociation constant being 0.3 mM and 1.4 mM at 30 mM and 200 mM potassium phosphate, respectively (Fig. 5).

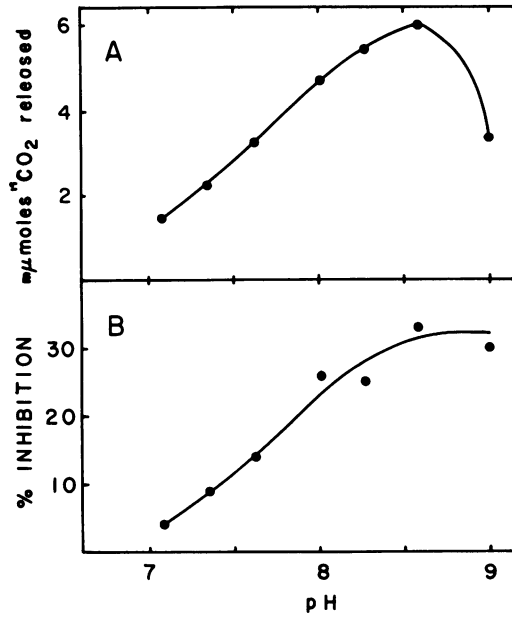

FIG. 4. *Effect of pH on diaminopimelate decarboxylase. (A) Diaminopimelate decarboxylase (2 × 10^{-4} units of ammonium sulfate fraction) was assayed at the pH indicated. (B) The inhibition of diaminopimelate decarboxylase by 2 mM L-lysine was determined at the pH shown. Minor nonspecific inhibitions by 2 mM D-lysine were also observed at various pH and subtracted to yield the final specific L-lysine inhibitions indicated. The reaction velocities are expressed as the amount of $^{14}CO_2$ liberated in 30 min.*

TABLE 2. *Effect of dilution in Tris buffer upon the activity and inhibition of diaminopimelate decarboxylase*[a]

Addition during dilution	$^{14}CO_2$ released (nmol)	Inhibition by 1 mM L-lysine (%)
Expt 1		
None	4.00	13
Pyridoxal phosphate	10.60	46
Expt 2		
None	1.44	0
Pyridoxal phosphate	6.32	35

[a] Two ten-thousandths unit of fresh extract (25 mg protein/ml) was diluted with 1.5 volumes of 0.1 M Tris-acetate, pH 7.2, in the presence or absence of pyridoxal phosphate (50 μg/ml) and incubated at 0 C for 20 min. Samples (0.1 ml) of the diluted extracts were then assayed for diaminopimelate decarboxylase activity.

TABLE 3. *Relative enzyme levels in Bacillus subtilis ATCC 6051 grown under various conditions*[a]

Conditions	Relative enzyme levels[b]		
	Aspartokinase I	Aspartokinase II	DAP decarboxylase
Minimal medium: midlog growth	[100]	[100]	[100]
Minimal medium: stationary growth	135	15	43
Rich medium: midlog growth	161	10	7

[a] Fresh extracts from *B. subtilis* grown as indicated were assayed for aspartokinase and diaminopimelate (DAP) decarboxylase activity, as described in Materials and Methods. The amount of aspartokinase I was estimated as the residual activity in the presence of L-threonine plus L-lysine (2 mM each), whereas the activity that was subject to inhibition by these amino acids was taken as a measure of aspartokinase II.

[b] Expressed as percent of the corresponding activities in extracts from cells grown to the mid-exponential phase in minimal salts medium.

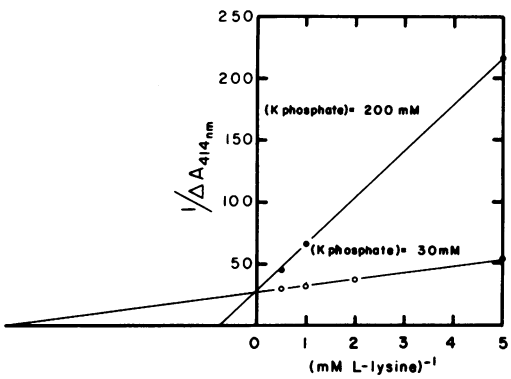

FIG. 5. *Determination of dissociation constant of Schiff base between pyridoxal phosphate and L-lysine at different concentrations of potassium phosphate. The mixtures contained in a final volume of 1.90 ml: potassium phosphate, pH 8.3 (30 mM); triethanolamine hydrochloride, pH 8.3 (30 mM); potassium bicarbonate, pH 7.5 (10 mM); pyridoxal phosphate (20 μM); EDTA (1 mM); and ethylene glycol (15%). Samples (0.1 ml) of L-lysine at different concentrations were then added. The absorbance was measured at 414 nm after 40 min at 25 C to give an indication of the extent of Schiff base formation between pyridoxal phosphate and lysine in the presence of either 30 mM (○) or 200 mM (●) potassium phosphate.*

Diaminopimelate decarboxylase activity in spontaneous revertant of lysine-requiring strains. Specific activities of the diaminopimelate decarboxylase of the revertants varied to as little as 5% of the value observed for the wild-type Marburg strain and were sometimes only three times the value observed in strains which required lysine for growth (Table 4). The reduced growth rates of certain strains, shown by their prolonged doubling times, did not necessarily correspond to low specific activities of diaminopimelate decarboxylase. All revertant activities were virtually as sensitive to inhibition by L-lysine as the diaminopimelate decarboxylase extracted from the Marburg strain.

DISCUSSION

Meso-diaminopimelate occupies a unique position in the lysine branch of the aspartate pathway (see Fig. 6). The amino acid occurs both as an intermediate in the biosynthesis of L-lysine and as a structural component (end-product) which is an essential constitutent of the vegetative and spore cell wall peptidoglycan

TABLE 4. *Diaminopimelate decarboxylase activity in revertants of lysine-requiring strains of Bacillus subtilis*[a]

Strain	Doubling time (h)	Sp act (units/mg protein)	Inhibition by 2 mM L-lysine (%)
Marburg	0.8	16.5	54
I	0.9[b]	0.2	
Ir1[c]	1.1	9.6	52
Ir2	1.0	8.0	52
Hr1	1.1	2.6	45
Hr2	1.0	3.9	47
Hr3	0.9	2.5	44
Gr1	0.9	14.6	44
Gr2	1.1	0.7	40
Fr1	1.4	17.0	48
Er1	2.4	1.1	35
Dr1	1.0	16.2	52
Cr1	1.0[d]	14.1	48
Br1	1.5	3.0	39
Br2	1.5	0.7	32
Br3	1.0	11.5	65
Ar1	1.0	15.9	49

[a] Thawed cells (0.4 g) were suspended in 3 ml of buffer A for the preparation of crude extracts. Cell disruption, centrifugation, analysis of protein, and assay of diaminopimelate decarboxylase were performed as described in Materials and Methods. All extracts were dialyzed for 1 h against two changes of 100-fold excess of buffer A at 0 C before use. The final concentration of pyridoxal phosphate in the assay was 0.2 mM. (Under these conditions, 2 mM D-lysine produced no inhibition.)

[b] L-Lysine (3 mM) was included in growth medium.

[c] Independently isolated revertants of their respective lysine-requiring parent strains are shown as r1, r2, . . . rn.

[d] L-Isoleucine (5 mM) was included in growth medium.

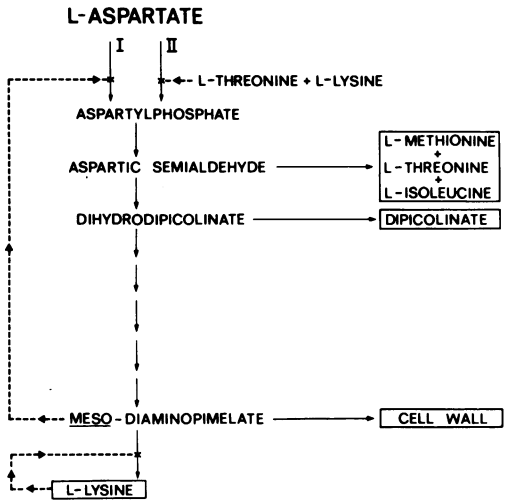

FIG. 6. *Biosynthesis of L-lysine by the aspartate pathway. Inhibitions of participating enzymes by amino acids in the pathway in B. subtilis are indicated by the dashed lines.*

in *B. subtilis* and several other bacteria (28). Thus, one might postulate that diaminopimelate regulates its own biosynthesis, a proposal supported by the observation that *meso*-diaminopimelate is capable of inhibiting an aspartokinase in *B. subtilis* (27) and other bacterial species (17). Because diaminopimelate decarboxylase plays a role in regulating the level of intracellular diaminopimelate, the decarboxylase might therefore be assumed to ultimately determine the degree of activity of *meso*-diaminopimelate-sensitive aspartokinase in the cell. The data in this paper demonstrate that physiological concentrations of L-lysine are capable of inhibiting the diaminopimelate decarboxylase extracted from *B. subtilis*. In this manner, lysine may indirectly regulate its own biosynthesis, with the lysine branch of the aspartate pathway comprised of two units of feedback inhibition acting sequentially.

Sequential feedback inhibition is observed also at other points in the aspartate pathway. The first enzyme in the threonine branch, homoserine dehydrogenase, is susceptible to L-threonine inhibition in *Escherichia coli* (23), *Salmonella typhimurium* (8), *Bacillus polymyxa* (M. Brenner and H. Paulus, unpublished observations), *B. subtilis* (A. Rosner and H. Paulus, unpublished observations), and *Rhodopseudomonas spheroides* (9). In the latter species (4) but not in *B. subtilis* (27), the substrate of the enzyme, aspartic β-semialdehyde, has been shown to be inhibitory to aspartokinase activity. Thus, an accumulation of L-threonine, leading to the inhibition of homoserine dehydrogenase and a subsequent accumulation of aspartic β-semialdehyde, is capable of inhibiting the aspartate pathway in two sequential units of feedback inhibition.

Because it has been demonstrated that several species of homoserine dehydrogenase and aspartokinase exist as multienzyme complexes in *E. coli* (22, 25), the question arises whether the sequentially inhibited enzymes in *B. subtilis*, diaminopimelate decarboxylase and *meso*-diaminopimelate-sensitive aspartokinase (aspartokinase I), exist as a complex in *B. subtilis*. This possibility is eliminated by my observation that the aspartokinase and diaminopimelate decarboxylase have different molecular weights (Fig. 1) and are independently regulated during growth (Table 3). Furthermore, the activity of aspartokinase I is unaffected by variations of potassium phosphate concentrations from 16 to 200 mM, whereas diaminopimelate decarboxylase is inactive if the salt concentration is less than 30 mM (Fig. 2). Finally, diaminopimelate decarboxylase and aspartokinase I activities can be partially separated by ammonium sulfate precipitation.

Because the literature concerning diaminopimelate decarboxylase is sparse, relatively few comparisons of the properties of the diaminopimelate decarboxylase of *B. subtilis* ATCC 6051 can be made with the corresponding enzyme in other bacterial species. As with the *B. subtilis* diaminopimelate decarboxylase, stimulation of amino acid decarboxylases by phosphates and other inorganic salts has been observed in *E. coli* (7). In solutions of higher ionic strength, this phenomenon could be due to the reduction of electrostatic interactions between the phosphoryl group of pyridoxal phosphate, metal cations, and the carboxyl group of the amino acid in the Schiff base intermediate (14). Such interactions would have induced the carboxyl group of the amino acid about to be decarboxylated into a coplanar configuration with the phosphoryl group of pyridoxal phosphate (14), an orientation that would have prohibited the carboxyl group's labilization and removal (6). By this mechanism, EDTA would facilitate decarboxylation by chelating those metals which would otherwise have been involved in the electrostatic interactions unfavorable to decarboxylation.

In contrast to the recent observation that two diaminopimelate decarboxylases may be present in a lysine-accumulating strain of *Brevibacterium* (15), the diaminopimelate decarboxylase activity from the Marburg strain of *B. subtilis* appears to be a single enzyme. At different stages of the growth cycle, the properties of the diaminopimelate decarboxylase ac-

tivity from *B. subtilis* are invariant. Furthermore, the linear double reciprocal plots depicting the kinetics of the diaminopimelate decarboxylase under study suggest that only a single component of activity has been isolated. Finally, similar values of inhibition by L-lysine of many revertant diaminopimelate decarboxylase activities (Table 4) suggest that only a single enzyme exists in *B. subtilis*.

The phenomenon of lysine inhibition of diaminopimelate decarboxylase reported in this paper agrees with the observations of others (11, 12, 21, 34); however, the earlier studies employed 10 to 40 mM lysine, considerably greater than the inhibitor concentrations utilized in this research and an order of magnitude higher than physiological levels (31). Because the inhibition by L-lysine is competitive with respect to *meso*-diaminopimelate (Fig. 3B), the concentration of diaminopimelate in the assay determines the amount of inhibitor required to reduce the activity of the enzyme. The sensitive assay method described here permitted the use of 0.1 mM diaminopimelate for assays, a concentration two orders of magnitude lower than that used in previous determinations of diaminopimelate decarboxylase (11, 12, 21, 34, 36) and more closely approximating physiological concentrations of the amino acid (13).

Several inferences concerning the mechanism of lysine inhibition can be drawn. Because decarboxylation is irreversible, the inhibition could not be due to mass action. Another possibility is allosteric inhibition, in which the inhibitor (L-lysine) and substrate bind to separate sites on the enzyme molecule. This interpretation is supported by the fact that diaminopimelate decarboxylase from *B. subtilis* can be rendered insensitive to L-lysine inhibition by either lowering the pH to 7.0 or by diluting the enzyme in Tris in the absence of pyridoxal phosphate. The molecular weight of diaminopimelate decarboxylase from *B. subtilis* is 105,000, sufficiently large to suggest the presence of subunits. However, no cooperative interactions of substrate or inhibitor molecules with the enzyme have been detected. Furthermore, of the 15 revertants that regained decarboxylase activity (Table 4), none differed significantly from the Marburg strain in their sensitivity to inhibition by 2 mM L-lysine. In the reversion studies, therefore, it was impossible to define a genetic locus specifically governing the sensitivity of the enzyme to inhibition by L-lysine, as would have been expected with an allosteric enzyme. Nevertheless, a definitive answer as to whether the enzyme is allosteric must await the study of the binding of substrate and effector molecules to highly purified preparations of the enzyme.

Another possibility is that the inhibition by L-lysine is a case of product inhibition. Assuming that lysine and diaminopimelate combine only with the enzyme-pyridoxal phosphate complex, one would predict product inhibition of the enzyme by lysine to be competitive with respect to diaminopimelate and uncompetitive with respect to pyridoxal phosphate (3). Because lysine inhibition with respect to pyridoxal phosphate was actually observed to be noncompetitive (Fig. 3C), the conditions for product inhibition have been contradicted for the *B. subtilis* enzyme. Product inhibition as a possible mechanism of lysine inhibition of diaminopimelate decarboxylase can therefore be disputed. (This argument, however, is not applicable if both L-lysine and diaminopimelate are capable of binding to the apoenzyme.)

All previous investigations that cite the inhibition of diaminopimelate decarboxylase by L-lysine (11, 12, 21), except one (34), do not consider the possibility that this could have been an indirect consequence of competition for pyridoxal phosphate by primary amines (such as lysine). Indeed, Schiff base formations between pyridoxal phosphate and primary amines have been studied spectrophotometrically for some time (18, 19). Enzyme inhibition by this mechanism does not involve an interaction of the inhibitor with the enzyme molecule and would hardly lend itself to the specific control by L-lysine of its own biosynthesis in the aspartate pathway. The data presented in this report eliminate this artifact by demonstrating that, under the reaction conditions used for diaminopimelate decarboxylase, L-lysine was the only significant inhibitor among several diamino compounds. Under such assay conditions, where the inhibition by L-lysine was specific, the competitive element in the mixed competitive-noncompetitive kinetics of lysine inhibition with respect to pyridoxal phosphate was absent (Fig. 3C). Similarly, under the same conditions, the extent of Schiff base formations between L-lysine and pyridoxal phosphate in solution decreased fivefold (Fig. 5). It thus appears that the nonspecific inhibition of diaminopimelate decarboxylase (Table 1) and the competitive element of inhibition kinetics with respect to pyridoxal phosphate were due to the formation of a Schiff base between the diamino compound and pyridoxal phosphate, which occurred to a significant extent only if the concentration of potassium phosphate was appreciably lower than the 200 mM routinely used in the enzyme assays in this research. (This suggestion is

supported elsewhere by the observation that nonspecific inhibition of the diaminopimelate decarboxylase from *E. coli* by primary amines was completely abolished by increasing the concentration of pyridoxal phosphate in the assay, whereas L-lysine alone remained an effective inhibitor [34]. The reason that the nonspecific effect appears to be confined to diamino compounds is most likely due to the fact that amines of this type have abnormally low pK values due to electrostatic interactions, and that it is the un-ionized amine that reacts with pyridoxal phosphate. This would also explain why the nonspecific inhibition is reduced in solutions of high ionic strength where intramolecular electrostatic interactions are minimized, such that pK values of diamino compounds will increase.) At 200 mM potassium phosphate, the reduction of diaminopimelate decarboxylase activity by L-lysine was specific, strongly implying that the inhibition was achieved by the direct attachment of the effector to the enzyme molecule. This, rather than the depletion of pyridoxal phosphate by diamino compounds, is the more attractive model by which L-lysine could specifically regulate diaminopimelate decarboxylase in the living bacterium.

The lysine inhibition of diaminopimelate decarboxylase demonstrated in this research could be especially critical during the later stages of growth of *B. subtilis*. It was previously reported that the activity of the lysine- and threonine-sensitive aspartokinase (aspartokinase II) of *B. subtilis* declines sharply toward the end of the growth cycle (27). In addition, in *B. subtilis*, unlike *E. coli* (37), the first enzyme of the lysine branch of the aspartate pathway, dihydrodipicolinate synthetase, is not inhibited by L-lysine. Instead, the activity of the enzyme increases two- to threefold prior to sporulation (1,2). The level of lysine might thus be expected to rise late in the growth cycle. Indeed, in *B. subtilis*, lysine has been shown to be one of only three abundant intracellular amino acids at the time of sporulation (20). The higher concentrations of L-lysine thus present would then be capable of inhibiting diaminopimelate decarboxylase, permitting the increased levels of intracellular diaminopimelate thus produced to be diverted into the cell wall peptidoglycan (Fig. 6) at precisely the time a greater degree of cell wall biosynthesis is necessary. Only when biosynthesis of spore cortex has progressed to the stage when large amounts of diaminopimelate are no longer required, will this amino acid accumulate to sufficiently high concentrations to shut off aspartokinase I and completely inactivate the lysine branch of the aspartate pathway. From these considerations, the significant role of diaminopimelate decarboxylase in the regulation of lysine biosynthesis in *B. subtilis* can be appreciated.

Evidence in support of this model has been presented elsewhere. In *Bacillus cereus*, a burst of [^{14}C]diaminopimelate incorporation into the cell wall mucopeptide was observed shortly before dipicolinic acid production. The conversion of [^{14}C]diaminopimelate to L-[^{14}C]lysine, which had persisted through most of the growth cycle, suddenly ceased during the onset of dipicolinate synthesis (33). More recently it has been found in the same species that the actual pool of intracellular diaminopimelate increased threefold, concomitant with both its incorporation into the cell wall peptidoglycan and the curtailment of its conversion to L-lysine (13). The enzyme responsible for this conversion, diaminopimelate decarboxylase, is apparently inactivated at the time of sporulation by a mechanism which is not yet understood.

That lysine independence in growth can be achieved by a strain of *B. subtilis* possessing only three or four times the specific activity of the diaminopimelate decarboxylase of a lysine auxotroph (whose auxotrophy is apparently due only to an insufficient level of activity of that enzyme) suggests that very low levels of diaminopimelate decarboxylase activity may be sufficient for lysine biosynthesis in *B. subtilis*. Lysine inhibition of diaminopimelate decarboxylase may therefore be the rule rather than the exception in the growing cell. Should severe lysine depletion ever occur, however, it is apparent that the large reserves of diaminopimelate decarboxylase activity which were found in cell-free extracts might be sufficient to replenish lysine pools in the bacterium in a relatively short period of time.

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