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Regulation of Cephamycin C Synthesis, Aspartokinase, Dihydrodipicolinic Acid Synthetase, and Homoserine Dehydrogenase by Aspartic Acid Family Amino Acids in *Streptomyces clavuligerus*

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The effect of the cephalosporin precursors and amino acids of the aspartic acid family on antibiotic production by *Streptomyces clavuligerus* was investigated. DL-*meso*-Diaminopimelate and L-lysine each stimulated specific antibiotic production by 75%. A fourfold increase in specific production was obtained by simultaneous addition of the two compounds. The stimulation could be further increased by adding valine to the two effectors. In the streptomycetes the α-aminoadipyl side chain of the cephalosporin antibiotics is derived from lysine. Streptomycetes, like other bacteria, are expected to produce lysine from aspartic acid; therefore, the feedback control mechanisms operating in the aspartic acid family pathway of *S. clavuligerus*, which may affect the flow of carbon to α-aminoadipic acid, were investigated. Threonine inhibited antibiotic production by 41% when added to minimal medium at a concentration of 10 mM. Simultaneous addition of 10 mM lysine completely reversed this inhibition. The aspartokinase of *S. clavuligerus* was found to be subject to concerted feedback inhibition by threonine and lysine. Threonine may act to limit the supply of lysine available for cephamycin C biosynthesis via this concerted mechanism. Single or simultaneous addition of any other amino acid of the aspartate family in the in vitro assay did not inhibit aspartokinase activity. Activity was stimulated by lysine. Aspartokinase biosynthesis was partially repressed by methionine or isoleucine at concentrations higher than 10 mM. Methionine, but not isoleucine, inhibited cephamycin C synthesis by 27% when added to minimal medium at a concentration of 10 mM. Dihydrodipicolinate synthetase, the first specific enzyme of the lysine branch, was not inhibited by lysine but was partially inhibited by high concentrations of 2,6-diaminopimelate and α-aminoadipate; it was slightly repressed by diaminopimelic acid. Homoserine dehydrogenase activity was inhibited by threonine and partially repressed by isoleucine. It appears that *S. clavuligerus* aspartokinase is a key step in the control of carbon flow toward α-aminoadipic acid.

α-Aminoadipic acid (AAA) is a direct precursor for the cephalosporin-type molecules produced by both fungi (31, 33) and actinomycetes (35), and it is also an intermediate in the biosynthesis of penicillin (7, 14). The regulatory mechanisms operating at the level of primary metabolism which affect the biosynthesis of AAA in fungi have been shown to determine the availability of this compound for antibiotic synthesis, thus determining the rate and extent of antibiotic production (9). In fungi such as *Penicillium*, *Cephalosporium*, and *Paecilomyces*, AAA is an intermediate in the lysine pathway (32). Thus, lysine and the β-lactam antibiotics are end products of a branched pathway which shares AAA as a common intermediate. It has been shown that, by feedback inhibition and repression of homocitrate synthetase, the first step in the common part of the pathway, lysine reduces the availability of AAA for penicillin production (7, 8, 13, 15, 22, 23). Lysine was also shown to inhibit cephalosporin C production by *Paecilomyces persicinus* P-10 (4) and *Cephalosporium acremonium* (25) when added at high concentrations. However, when lysine was added to a culture of *C. acremonium* at a low concentration (1 mg/ml), it enhanced cephalosporin C production (25).

The AAA moiety of the cephalosporins produced by actinomycetes (which include the cephamycins), however, has been shown to be derived from lysine. L-[^{14}C]lysine is incorporated into the α-aminoadipyl side chain of these antibiotics (35). Kern et al. (19) characterized an

L-lysine ε-aminotransferase activity in extracts of *Streptomyces lactamdurans*, which catalyzes the conversion of L-lysine to L-α-aminoadipic acid.

In bacteria lysine is synthesized from aspartic acid via a pathway which involves diaminopimelic acid (DAP) as an intermediate. Kirkpatrick et al. (20) demonstrated that DAP was an intermediate for lysine biosynthesis in *Streptomyces lipmanii*. In the *S. lipmanii* study supplementation of the medium with high levels of lysine was required, by both wild type and a lysine auxotroph, to produce cephalosporins. In the present study we demonstrated that both L-lysine and DL-*meso*-DAP stimulated the production of a cephalosporin antibiotic, cephamycin C, in *Streptomyces clavuligerus*. Since several branch points exist in the aspartate family pathway, the flow of carbon from aspartate through lysine to the α-aminoadipyl side chain of the cephalosporin molecules might be controlled by regulatory mechanisms operating at the initial and branching steps of the pathway. We have made a study of the regulation of activity and biosynthesis of three key enzymes in *S. clavuligerus*: (i) the first common step in the pathway, aspartokinase (AK), which catalyzes the formation of β-aspartyl phosphate from aspartate; (ii) dihydrodipicolinic acid synthetase (DDPS), which catalyzes the condensation of aspartic semialdehyde (ASA) with pyruvate and is the first specific step in the lysine branch; and (iii) homoserine dehydrogenase (HSD), which catalyzes the formation of homoserine from ASA in the first step of the other branch of the pathway leading to threonine, isoleucine, and methionine. Preliminary results have been presented (S. Mendelovitz and Y. Aharonowitz, Abstr. 6th Int. Ferment. Symp. Canada, 1980, F-4.10, p. 19). In this communication we describe our observations that the addition of amino acids, members of the lysine branch of the aspartic acid family, stimulated antibiotic production in *S. clavuligerus* fermentations. Some of the feedback effects of certain amino acids on AK, DDPS, and HSD are also described.

MATERIALS AND METHODS

Bacteria cultivation. *S. clavuligerus* NRRL3585 (17) was used throughout this study. The chemically defined medium and growth conditions employed were reported previously (1). Supplements added to fermentation medium at 0, 24, and 48 h were added as concentrated sterile solutions so that the volume of the culture was not changed significantly. Spores were prepared on solid medium which contained (in 1 liter): 10 g of malt extract (Difco Laboratories), 4 g of yeast extract, 4 g of glycerol, 100 μg of $CoCl_2$, and 20 g of agar, adjusted to pH 7.0.

Cell extracts and enzyme preparations. Cells were harvested by centrifugation at 4°C. For the AK assay, they were washed three times in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.03 M mercaptoethanol. For the DDPS and HSD assays, they were washed in 0.1 M potassium phosphate buffer (pH 7.0). Washed cells were stored at −20°C. Extracts were prepared by ultrasonic disruption in a Branson B-12 Sonifier for a total of 90 s in 15-s pulses at 0°C. Cell debris was removed by centrifugation for 10 min at 4°C at 10,000 × *g* in a Sorvall centrifuge; the supernatant fluid constituted the crude extract. AK was precipitated in the 30 to 65% saturated ammonium sulfate fraction, and DDPS and HSD were precipitated in the 30 to 55% saturated ammonium sulfate fraction.

When a higher degree of purification was required for the AK, streptomycin sulfate was added to the crude extract to a final concentration of 1%. The precipitate formed after 30 min at 0°C was collected by centrifugation and discarded. Ammonium sulfate was added to the supernatant fluid, and the 30 to 65% ammonium sulfate fraction was dissolved in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.03 M mercaptoethanol and 0.01 M L-threonine (PMT) and applied to a column (2.6 by 90 cm) of Sephacryl S-300 (superfine) equilibrated in the same buffer. The enzyme was eluted with PMT buffer, and active fractions were applied to a DEAE-cellulose column (1.9 × 20 cm) equilibrated in PMT buffer. The column was washed with PMT buffer containing 0.1 M KCl following by elution with a linear gradient of 0.1 to 0.5 M KCl in PMT buffer with 80 ml of elution buffer in each compartment. The fractions containing AK activity were combined, and the enzyme was concentrated by ammonium sulfate precipitation to 75% saturation. The precipitate was dissolved in PMT buffer for further determinations.

DL-ASA preparation. DL-ASA was prepared by ozonolysis of DL-allylglycine by the method of Black and Wright (2). Stock solutions of DL-ASA were maintained in 1 N HCl. DL-ASA was neutralized immediately before use in the enzyme assay.

Enzyme assays. AK activity was measured by the formation of aspartylhydroxamate (29). The reaction mixture contained (in 1 ml): 0.1 M Tris-hydrochloride buffer (pH 7.6), 0.02 M ATP, 0.02 or 0.05 M L-aspartate, 0.01 M $MgSO_4$, 1.4 mM β-mercaptoethanol, 0.8 M NH_2OH-HCl neutralized with KOH, and enzyme as indicated. Incubation was at 30°C. Specific activity is expressed as nanomoles of aspartylhydroxamate formed per minute per milligram of protein.

DDPS activity was measured as described by Yugari and Gilvarg (38). The reaction mixture contained (in 1 ml): 0.1 M imidazole buffer (pH 7.8), 5 mM sodium pyruvate, and 1 mM ASA freshly prepared by neutralizing a sample from a stock solution kept in 1 N HCl. When the effect of amino acids on DDPS activity was studied, reduced concentrations of both substrates were used as indicated in the text. Enzyme was added as indicated, and incubation was carried out at 30°C. After a brief lag, the increase in absorption at 270 nm was followed with time. One unit of enzyme activity is that amount of protein which catalyzes an increase of one unit of optical density at 270 nm per min. For comparison of the specific activity of DDPS to those of other enzymes, the rate of conversion of pyruvate to dihydrodipicolinic acid was measured in micromoles per minute per milligram of protein. Lactic dehydrog-

enase was used to measure the amount of pyruvate remaining in the DDPS reaction mixture at any given time.

HSD activity was measured by determining the initial rate of decrease of absorbance at 340 nm. The standard reaction mixtures contained (in 1 ml); 0.1 M potassium phosphate buffer (pH 6.7), 0.25 mM NADH, 1 mM ASA, 0.001% cetyltrimethyl-ammonium bromide when crude enzyme preparations were used, and enzyme as indicated. Incubation was at 30°C. Units of enzyme activity were defined as micromoles of NADH oxidized per minute. Protein was estimated by the method of Lowry et al. (21) with bovine serum albumine as the standard.

Antibiotic assay. Samples were assayed for the production of cephalosporins by the standard disk agar plate technique, using as the assay organism an *Escherichia coli* strain that is supersensitive to β-lactam antibiotics. In this assay we detected only the cephalosporins. Clavulanic acid had only 5 to 10% the activity of cephalosporin C in the assay (1).

RESULTS

Effect of amino acids on antibiotic production. We have begun to study the effect of amino acids of the aspartic acid family and related intermediates on the production of antibiotics by *S. clavuligerus*, a species known to produce β-lactam antibiotics (17). In previous studies, a chemically defined fermentation medium was devised in which antibiotics were produced in the absence of exogenous lysine (1). This medium contained glycerol, L-asparagine, morpholinopropane sulfonic acid buffer, and mineral salts. Cephamycin C was found to be the dominant antibiotic product in the fermentation of *S. clavuligerus* in this medium (1). In the present study three direct precursors of cephamycin C (AAA, cysteine, and valine) each had no marked stimulatory effect on specific production when added to the chemically defined medium (Table 1). Cysteine reduced growth and completely inhibited production. However, L-lysine and DL-*meso*-DAP, both intermediates in the specific branch leading to AAA markedly stimulated antibiotic formation. The stimulatory effect of L-lysine could be further increased by combining it with threonine or valine. A similar effect was shown by combining AAA with valine and threonine. Further-

TABLE 1. Effect of amino acids members of the aspartate family and cephamycin precursors on antibiotic production[a]

Amino acid added (10 mM)	DCW[b] (mg/ml)	Maximum antibiotics (μg/ml)	Specific production of antibiotics[c] (μg/mg of DCW)	Relative specific production
No addition	1.78	52	29.2	1.00
L-Lysine	1.74	86	49.4	1.69
DL-*meso*-DAP	2.11	115	54.5	1.86
AAA	2.16	70	32.4	1.11
L-Threonine	2.06	36	17.4	0.59
L-Isoleucine	1.44	53	36.8	1.26
L-Methionine	1.49	32	21.4	0.73
D-Lysine	2.21	60	27.1	0.92
L-Valine	1.54	52	33.6	1.15
L-Cysteine	1.30	0	0	
L-Lysine + L-threonine	1.94	122	62.8	2.15
L-Lysine + L-methionine	2.21	88	39.8	1.36
L-Lysine + L-isoleucine	2.31	115	49.7	1.70
L-Lysine + L-valine	1.03	68	66.0	2.26
DAP + L-lysine	2.30	335	145.6	4.98
DAP + L-threonine	2.32	151	65.3	2.23
DAP + AAA	2.49	220	88.3	3.02
DAP + L-valine	2.19	312	142.4	4.87
DAP + L-isoleucine	2.26	175	77.4	2.65
AAA + L-valine	1.10	84	76.4	2.61
AAA + L-threonine	2.31	220	95.2	3.26
L-Isoleucine + L-methionine	2.50	58	23.6	0.80
L-Isoleucine + L-threonine	1.96	53	27.0	0.92
L-Threonine + L-methionine	2.87	35	12.1	0.41
DAP + L-lysine + L-valine	2.36	400	169.4	5.80
DAP + L-valine + L-cysteine	1.84	32	17.4	0.59
AAA + L-valine + L-cysteine	1.84	35	19.0	0.65

[a] The culture was grown in the chemically defined medium with and without an added amino acid. At various times, samples were taken for pH, biomass, and antibiotic analysis.

[b] DCW, Dry cell weight; the maximum was achieved during fermentation for 120 h.

[c] Maximum cephalosporins titer divided by the maximum dry cell weight achieved during fermentation.

TABLE 2. Effect of time of addition and concentrations of three amino acids on antibiotic production[a]

Amino acid added	Final concn (mM)	Specific production (μg/mg of dry cell weight) at time of addition (h):		
		0	24	48
No addition		27.0 (100)	27.0 (100)	27.0 (100)
L-Lysine	5	25.6 (94)	31.3 (115)	42.0 (155)
	10	45.2 (167)	47.2 (174)	53.8 (199)
	20	56.8 (210)	32.4 (120)	50.6 (187)
DL-*meso*-DAP	5	26.8 (99)	46.5 (172)	22.3 (82)
	10	44.9 (166)	63.2 (234)	23.1 (85)
	20	53.5 (198)	46.6 (172)	26.6 (98)
AAA	5	ND	43.8 (162)	32.3 (119)
	10	28.3 (104)	36.0 (133)	35.6 (131)
	20	25.7 (95)	40.9 (151)	36.4 (134)

[a] Cells were grown in the chemically defined medium for 120 h. At the times indicated, amino acids were added at different final concentrations.

[b] Specific production is the maximum antibiotic titer (in micrograms per milliliter) divided by the maximum biomass (in milligrams per milliliter) in each fermentation. The numbers within parentheses represent percentages of control. ND, Not detected.

more, the simultaneous addition of DL-*meso*-DAP with lysine or valine caused a four- to fivefold increase in the specific production. The combination of DL-*meso*-DAP with lysine and valine stimulated specific productivity to an even greater extent. In all experiments in which cysteine was added to the culture medium, antibiotic production was much lower than in the control (Table 1).

We next determined the effect of time of addition and the optimum levels of L-lysine, DL-*meso*-DAP, and AAA on antibiotic formation (Table 2). The addition of L-lysine to concentrations of 10 or 20 mM was equally effective in stimulating cephamycin C synthesis regardless of the time at which the addition was made. The addition of lysine to a concentration of 5 mM was effective in stimulating antibiotic synthesis only when added at 48 h. Earlier addition probably allowed consumption of lysine for growth thereby preventing its effect on antibiotic production. DL-*meso*-DAP stimulated production to the highest extent when added 24 h after the inoculation of the culture at 10 mM concentration. The addition of DL-*meso*-DAP at a later time had no stimulatory effect on antibiotic production. The addition of AAA stimulated production of antibiotics only when added at 24 or 48 h of growth; stimulation occurred at all concentrations tested.

Effect of amino acids on AK activity. The effect of four end products (lysine, methionine, threonine, and isoleucine), two key intermediates (homoserine and DAP), and a lysine catabolic product (AAA) on the activity of AK was examined. When added singly at 5 mM, only lysine displayed a significant activation effect on AK activity, but the other amino acids had no effect (Table 3). L-Threonine had no significant effect on AK activity over a wide range of concentrations (Fig. 1). However, the effect of L-lysine was highly significant; the addition of L-lysine at

TABLE 3. Effect of amino acids on AK activity[a]

Additions	Relative enzyme activity %
None	100
L-Threonine	106
L-Lysine	140
L-Methionine	101
L-Isoleucine	106
L-Homoserine	102
DL-*meso*-DAP	95
AAA	93
L-Lysine + L-threonine (1 mM each)	13
L-Lysine + L-threonine (5 mM each)	6
L-Lysine + L-isoleucine	135
L-Lysine + L-methionine	120
L-Lysine + L-homoserine	131
L-Threonine + L-methionine	108
L-Threonine + L-isoleucine	104
L-Threonine + DL-*meso*-DAP	97
L-Threonine + AAA	111
L-Methionine + AAA	92
L-Lysine + L-threonine + L-methionine	19
L-Lysine + L-threonine + L-isoleucine	15

[a] The standard hydroxamate assay was used. The enzyme preparation was the 30 to 65% ammonium sulfate fraction prepared from a 48-h culture. Each amino acid was present at 5 mM unless otherwise indicated. The protein content in each assay was 0.94 mg. In the reaction with no added amino acid, 21.6 nmol of aspartylhydroxamate per min was formed, representing 100% relative activity.

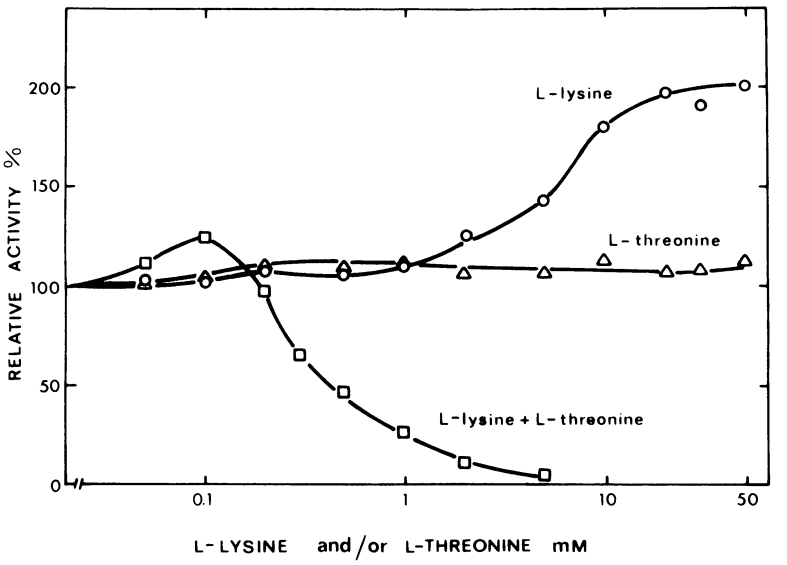

FIG. 1. Effect of lysine and threonine concentrations on AK activity. Each reaction mixture (see text) contained 0.8 mg of protein of the 30 to 65% ammonium sulfate fraction and various concentrations of L-lysine (○), L-threonine (△), or L-lysine plus L-threonine (□).

concentrations higher than 1 mM stimulated AK activity up to 200%.

A strong concerted feedback inhibition was exerted on AK activity when L-lysine plus L-threonine was added. The concerted feedback inhibition of lysine plus threonine with respect to concentration of both effectors was represented by a sigmoidal curve (Fig. 1) with 50% inhibition reached at 0.4 mM of each amino acid. However, at a very low concentration of the amino acid pair, a slight stimulation of activity was noted. Combinations of L-lysine with amino acids other than L-threonine did not diminish significantly the stimulatory effect that L-lysine had on AK activity. L-Methionine and L-isoleucine did not reverse the concerted feedback inhibition exerted by L-lysine plus L-threonine.

Effect of culture age on AK activity. The specific activity of AK found in crude cell extracts was dependent on the culture growth phase (Fig. 2). Maximum specific activity was obtained when extracts were prepared at the end of the exponential growth phase. As the cells approached the stationary growth phase, specific activity declined. The stimulatory effect of L-lysine on AK activity and the inhibition effect of lysine plus threonine on AK activity did not change significantly with culture age. The degree to which L-lysine stimulated AK activity varied between different experiments, similar to the reported effect of amino acid modifiers in other bacteria (36).

Effect of amino acids on AK biosynthesis. When different amino acids (10 mM) were added to the chemically defined medium, some repressive effects on AK were observed (Table 4). L-Isoleucine and L-methionine repressed AK synthesis by about 70 and 50%, respectively. Other amino acids had little effect, except for L-lysine which stimulated the synthesis of AK to 20% above control levels. The addition of L-lysine to the growth medium partially reversed the repressive effect of L-isoleucine. In addition to its repressive effect, L-isoleucine inhibited both the rate and the extent of growth by about 20 to 30%. The concerted feedback inhibition by lysine plus threonine on AK activity was similar in all the cultures. Cells grown in the presence of isoleucine exhibited AK activity that could be stimulated twofold by 8 mM lysine. Cells grown in the presence of other amino acids exhibited AK activity that was stimulated only 1.6-fold (approximately) by 8 mM lysine. AK biosynthesis in cultures grown in the presence of valine, leucine, or cysteine was repressed, but these three amino acids had no effect on AK activity when tested in vitro.

Properties of AK from *S. clavuligerus*. The optimum pH for the formation of aspartylhydroxamate was near pH 7.6 when assayed under standard conditions with no effectors. The enzyme was most sensitive to the concerted effect of lysine plus threonine between pH 7.5 and 8.0; lower feedback sensitivity could be observed by increasing the pH of the reaction to 8.5 to 9.0. The activity profile of AK in the presence or absence of effectors was not altered during the course of several purification steps. However, during purification and mainly upon dialysis, significant losses in activity were detected.

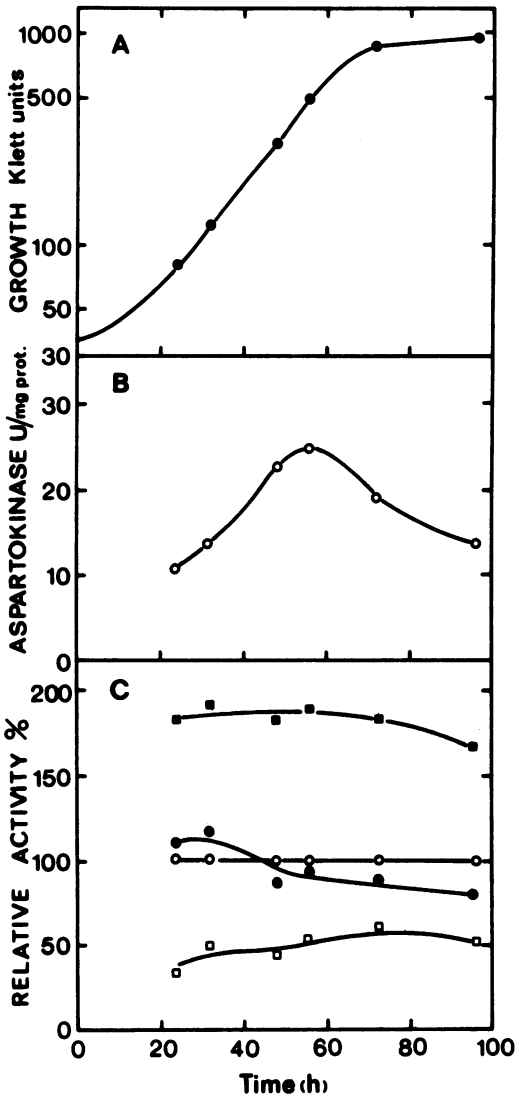

FIG. 2. Effect of culture age on AK specific activity and on the response of AK activity to amino acids. At time intervals samples were removed from the culture. Cells were washed and frozen. Thawed cells were broken, and crude extracts were fractionated with ammonium sulfate. Each reaction mixture for determining AK activity contained 0.5 to 1.0 mg of protein of the 30 to 65% ammonium sulfate fraction. Panels: A, cell growth; B, enzyme specific activity; C, relative enzyme activities when supplements were added to the following reaction mixtures: (○) none, (●) 8 mM threonine, (■) 8 mM lysine, (□) 0.4 mM each lysine and threonine.

These losses in activity could be partially prevented by the addition of 10 mM L-threonine to the buffer. At pH 7.6 the apparent K_m values for aspartate and ATP were 17 and 2.8 mM, respectively.

DDPS of *S. clavuligerus*. The pyruvate-ASA condensing enzyme is the first step in the lysine specific branch of the aspartate family pathway. Its activity was assayed in 30 to 55% ammonium sulfate fractions. The assay method used showed specificity for the two substrates pyruvate and ASA. The *S. clavuligerus* enzyme exhibited maximum activity at pH 7.8, with 50% reduction in activities at a pH of 7.2 and 8.4. The enzyme was very stable when stored in 0.1 M potassium phosphate buffer (pH 7.0) at room temperature for at least 1 week and for over 2 months when kept at 4°C. In the presence of 20% glycerol, the enzyme could be stored at −20°C, with repeated freezing and thawing. The apparent K_m values of DDPS with respect to ASA and pyruvate were 0.3 and 1 mM, respectively.

Effect of amino acids on DDPS activity. A variety of amino acids including amino acids of the aspartate family were tested for their effect on the DDPS activity. In these determinations substrate concentrations employed (0.03 mM ASA and 0.75 mM pyruvate) were insufficient to saturate the enzyme. Under these conditions DL-*meso*-DAP (a lysine intermediate) and AAA (a lysine catabolic product) exerted significant inhibitory effects (Fig. 3). L-Lysine and others

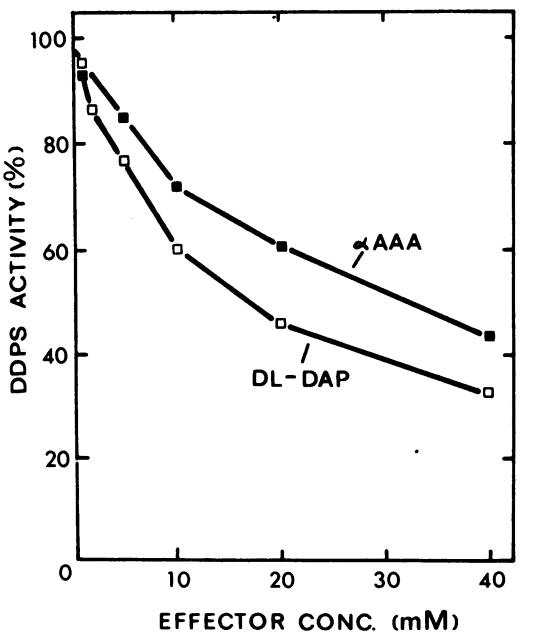

FIG. 3. Effect of AAA and DL-*meso*-DAP on DDPS activity. Protein (280 μg) from the 30 to 55% ammonium sulfate fraction of a 72-h culture was used in the standard assay for absorbance at 270 nm. Sodium pyruvate and ASA concentrations were 0.75 and 0.03 mM, respectively; 100% activity was equal to 0.01 U of DDPS activity.

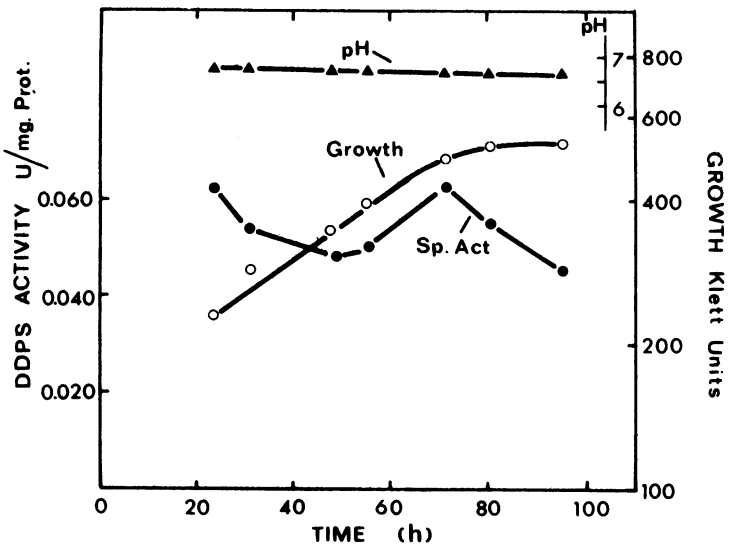

FIG. 4. DDPS specific activity during the growth cycle. Cells were grown in the chemically defined medium for 96 h (○). At various times after inoculation, cells were harvested and the 30 to 55% ammonium sulfate fraction was prepared. DDPS specific activity (●) was determined in the standard assay of absorbance at 270 nm.

had no effect when added at concentrations of 10 mM. Similar results were obtained with the DDPS of *Bacillus subtilis* (3), *Bacillus megaterium* (34), and *Brevibacterium lactofermentum* (30). In contrast, lysine inhibited DDPS activity both in *Escherichia coli* (37) and *Pseudomonas* species (16). The simultaneous addition of other end products of the aspartate family did not reverse the inhibitory effect of DL-*meso*-DAP or AAA.

Effect of the growth phase and medium composition on DDPS biosynthesis. Only minor changes in DDPS specific activity of about 25% were detected in extracts of cells harvested at different phases of growth (Fig. 4). To test specific regulatory effects of amino acids on DDPS biosynthesis, cells were grown in the chemically defined medium for 24 h, after which amino acids were added at concentrations of 10 mM each. DDPS specific activity was assayed 24 h

TABLE 4. Effect of amino acids on AK biosynthesis[a]

Amino acid added (10 mM)	AK activity		Relative activity[b] (%) in the presence of:		
	Specific (U/mg of protein)	Relative (%)	Lys	Thr	Lys + Thr
No addition	19.4	100	170	116	38
L-Lysine	23.6	122	165	128	36
L-Threonine	19.4	100	155	114	39
L-Methionine	9.0	46	166	90	35
L-Isoleucine	5.6	29	235	100	26
DL-*meso*-DAP	18.3	94	160	105	34
L-Homoserine	17.0	88	167	98	26
AAA	16.4	84	164	112	40
L-Aspartate	22.9	118	150	92	28
L-Lysine + L-threonine	24.4	126	155	118	39
L-Lysine + L-isoleucine	12.2	63	187	130	34
L-Methionine + L-threonine	14.7	76	170	119	33

[a] Cells were grown in the chemically defined medium. Amino acids were added at 10 mM final concentration. Specific activities were determined on the 30 to 65% ammonium sulfate fraction of each culture after 48 h of incubation. Each extract was assayed in the presence or absence of lysine, threonine, or both. The concentration of aspartate in the reaction mixture was 20 mM.

[b] Relative activities were obtained by dividing specific activity values obtained in presence of 8 mM lysine (Lys), 8 mM threonine (Thr), or 0.4 mM each lysine and threonine by the AK specific activity.

TABLE 5. Effect of amino acids on DDPS biosynthesis[a]

Amino acid added (10mM)	Relative sp act (%)	Relative activity (%) in the presence of:		
		10 mM Lys	10 mM AAA	10 mM DL-DAP
None	100	100	87	68
L-Lysine	100	97	87	74
DL-*meso*-DAP	77	99	76	63
AAA (5 mM)	108	95	91	68
L-Threonine	109	99	78	73
L-Methionine	103	96	82	65
L-Homoserine	100	96	83	75
L-Isoleucine	84	99	83	65
L-Lysine + L-threonine	126	108	81	77
L-Lysine + L-methionine	100	102	86	65
L-Lysine + L-isoleucine	94	103	85	63

[a] Cells were grown in the chemically defined medium for 24 h. Amino acids were added to each culture at the indicated final concentrations. The DDPS activity was determined 24 h after the addition of the amino acid in the 30 to 55% ammonium sulfate fraction of each cell extract; 100% relative activity represents a specific activity of 0.063 U/mg of protein.

after effector addition (Table 5). Neither L-lysine nor other end products of the aspartate family affected DDPS biosynthesis. However, DL-*meso*-DAP, L-leucine, and L-alanine (not shown) caused a 20% reduction in the specific activity when added singly, whereas the combination of L-threonine plus L-lysine caused an increase of about 25%.

HSD of *S. clavuligerus*. HSD, the first enzyme of the threonine, methionine, and isoleucine branch, utilizes the same substrate (ASA) as DDPS for its activity. Thus, the regulation of its activity and biosynthesis might determine the availability of ASA for the biosynthesis of DAP, lysine, and AAA and the levels of threonine which, together with lysine, might affect the AK activity as shown above. The reaction was specific for ASA as substrate and NADH rather than NADPH as cofactor. The pH optimum for enzymatic activity was 6.7, and the apparent K_m for ASA was 0.28 mM. The enzyme was found to be unstable, and all assays were carried out shortly after preparation of the extracts.

Effect of amino acids on HSD activity and biosynthesis. Among a variety of amino acids tested for their effect on HSD activity, 1 mM L-threonine inhibited 75% of the activity. Homoserine and L-methionine at the same concentration inhibited the activity of 50 and 20%, respectively. The presence of L-threonine (10 mM) in the chemically defined growth medium caused only a 15% decrease in the HSD levels. L-Lysine, which had no effect on HSD activity, stimulated its synthesis by 20%, and L-isoleucine repressed HSD activity by about 45%.

DISCUSSION

The elucidation of several steps in the formation of AAA in streptomycetes (19, 20) has established the importance of the aspartate pathway in providing AAA required for the biosynthesis of the cephalosporin-type antibiotics. Our own results suggest that the carbon flow through the lysine branch of the aspartic acid family in *S. clavuligerus* is a rate-limiting step in the formation of cephalosporins. Since DAP was reported to be a cell wall component of *S. clavuligerus* (17) and identified as the LL-DAP isomer (F. Kirnberg and Y. Aharonowitz, unpublished results), the availability of lysine and AAA for antibiotic production is probably strictly controlled. We observed that the addition of DAP 48 h after inoculation could not stimulate antibiotic production (Table 2), whereas the addition of lysine or AAA could. These observations suggest that at 48 h the cell content of DAP decarboxylase was insufficient to support maximal cephalosporin production. Alternatively, at 48 h DAP decarboxylase activity might become subject to feedback control mechanisms which would determine the ability of cells to convert DAP to lysine. The conversion of lysine to AAA is not dependent on DAP decarboxylase activity; therefore, lysine addition or direct addition of AAA could stimulate antibiotic production at later stages of growth. A third possibility is that transport of DAP at 48 h has diminished.

In vitro assay results presented in this communication (Fig. 5) suggest the possible involvement of feedback control mechanisms in regulation of the carbon flow from aspartate via the lysine branch to AAA in *S. clavuligerus*. The enzymes AK, HSD, and DDPS occupy key positions in the pathway which forms and metabolizes ASA (Fig. 5). Regulation of AK would effectively control the availability of ASA, whereas regulation of HSD and DDPS would effectively control the route by which ASA is metabolized. DDPS activity of *S. clavuligerus* was not inhibited by lysine, and DL-*meso*-DAP and AAA could inhibit its activity by only 30%.

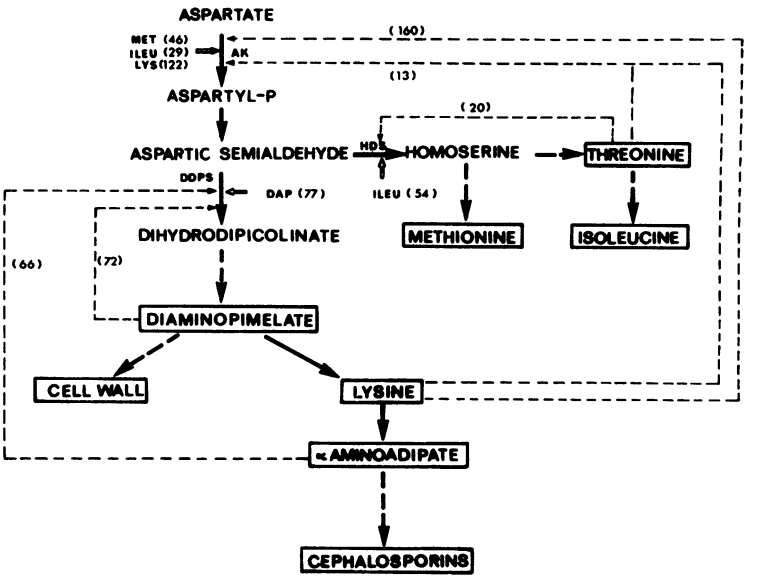

FIG. 5. Feedback control mechanisms operating in the aspartic acid family pathway of *S. clavuligerus*. Inhibitory or stimulatory effects are indicated by the dashed lines. Effects on enzyme biosynthesis are indicated by open arrows. The numbers in brackets indicate percentages of residual activities. Abbreviations not used in text: MET, methionine; ILEU, isoleucine; LYS, lysine.

Since LL-DAP is a cell wall component of *S. clavuligerus* (F. Kirnberg and Y. Aharonowitz, unpublished data), the lack of inhibition of DDPS by distal metabolites of the pathway may be of physiological significance for the cell, thus enabling the flow of carbon to DAP with no interference. A similar lack of metabolic inhibition of DDPS was reported for other organisms mainly of the genus *Bacillus* (3, 34).

In addition to its conversion to DDP, ASA is converted to homoserine by homoserine dehydrogenase. Our data show that the affinities of DDPS and HSD to ASA were quite similar, with K_m values for ASA of 0.3 and 0.28 mM, respectively. The HSD of *S. clavuligerus* was feedback inhibited by low concentrations of threonine and partially repressed by isoleucine. Thus, it is expected that more ASA would be converted to DAP upon elevation of threonine concentration. Furthermore, in all our experiments, we have found the specific activity of DDPS to be higher than that of HSD (data not shown). The greater specific activity of DDPS may suggest conversion of ASA to DDP rather than to homoserine in greater efficiency in *S. clavuligerus*. In *Brevibacterium flavum* the relative levels of these enzymes (28) are the reverse of the order observed in *S. clavuligerus*. A strict concerted feedback inhibition of aspartokinase (first enzyme in the pathway from aspartate to ASA) occurs in *S. clavuligerus* in the presence of a mixture of L-lysine and L-threonine. L-Threonine alone had no effect. L-Lysine alone stimulated AK activity. A similar stimulatory effect of lysine was detected in *Rhodopseudomonas capsulata* (5) and *Pseudomonas fluorescens* (10). Concerted feedback inhibition of AK by lysine plus threonine has been reported for several other organisms (5, 18, 24, 26, 28). In many of these organisms, AK was inhibited not only by the combined addition of lysine and threonine, but also to some extent by the single addition of lysine or threonine or other members of the aspartate family (27, 28). The activation of AK by the addition of a single amino acid has been reported in other organisms (6, 11, 12, 28). However, we are unaware of any previous example in which an amino acid shown to activate AK is inhibitory when combined with another end product of the pathway. Similar patterns of feedback inhibition or stimulation were observed in extracts prepared from cells grown in the presence of different members of the aspartate family pathway. This observation suggests that there is a single AK in *S. clavuligerus*. An exception occurred for cells grown in the presence of isoleucine. AK activity in extracts of these cells was stimulated by lysine to a higher extent than for cells grown in the presence of other amino acids. This observation may be related to the low specific activity of AK in these extracts. AK activity obtained in different purification steps responded similarly to effector addition. This observation also supports the suggestion that there is only a single AK enzyme in *S. clavuligerus*. However, the enzyme was quite

unstable and lost activity during purification. If more than one enzyme existed in the crude preparations, one might have been lost during purification.

AAA is a direct precursor of cephalosporins. Since, in *S. clavuligerus*, AAA is derived from lysine and lysine is derived from aspartate, aspartokinase is, in effect, the first step in the carbon flow from aspartate to AAA. Since AK in *S. clavuligerus* is inhibited in the presence of lysine plus threonine, under these conditions AK is likely to be the first rate-limiting step in the conversion of aspartate to AAA (and cephalosporins). Mutants of *S. clavuligerus* deregulated in their AK activity overproduced cephalosporins (manuscript in preparation). The importance of AK as a rate-limiting step and site of regulation in the carbon flow from primary metabolites to cephalosporin type antibiotics in *S. clavuligerus* is substantiated by the recent isolation of such AK deregulated mutants in this laboratory. Steps between DAP and AAA could also be targets for feedback regulation in *S. clavuligerus*. In *Streptomyces lipmanii* lysine appears to control the DAP decarboxylase step, thereby regulating its own biosynthesis without interfering with cell wall formation (20). L-Lysine ε-aminotransferase (19) may also be feedback regulated by AAA and other intermediates involved in the biosynthesis of cephalosporins. Regulation of DAP-decarboxylase and L-lysine ε-amino-transferase is therefore a topic of interest for future studies of factors regulating cephamycin C synthesis by *S. clavuligerus*.

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