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Aspartokinase of *Myxococcus xanthus*: "Feedback Stimulation" by Required Amino Acids

DAVID FILER, EUGENE ROSENBERG, AND S. H. KINDLER
Department of Microbiology, Tel Aviv University, Tel Aviv, Israel

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The aspartokinase activity found in extracts of the bacterium *Myxococcus xanthus* was subject to feedback inhibition and feedback repression by L-threonine and L-lysine. Both types of inhibition were essentially additive. The required amino acids, L-isoleucine and L-methionine, caused considerable increase in the activity of the enzyme. This phenomenon is referred to as "feedback stimulation." The polyamine, spermidine, exerted strong enhancement of the activity even at 0.1 mM. Meso-diaminopimelate, although not inhibitory by itself, abolished the activation exerted by either L-isoleucine or L-methionine. The possible physiological significance of interactions between the various effectors is discussed.

Myxococcus xanthus is commonly found in nature on decaying material, satisfying its nutritional requirements by preying on the other bacteria. In the laboratory, *M. xanthus* can be grown on peptone media (7) or defined media containing several amino acids (7, 8, 18). Deprivation of one of the required amino acids, methionine, or addition of 0.1 M threonine plus 0.1 M isoleucine was reported to lead to the formation of myxospores (18). Since all three amino acids are members of the aspartate family, it seemed worthwhile to investigate the control of the aspartokinase of *M. xanthus*.

The enzyme aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) catalyzes the first reaction in the pathway that leads to the biosynthesis of L-lysine, L-isoleucine, L-methionine, L-threonine, and α-ϵ-diaminopimelic acid (DAP).

Numerous studies on the regulation in microorganisms of the biosynthesis of amino acids of the aspartic family have revealed different control patterns. In *Escherichia coli* precise control is achieved by three isoenzymes (9). Many organisms have only a single aspartokinase regulated by interactions of several end products (1, 2, 4, 5, 10, 15). An intermediate in the biosynthetic pathway is an inhibitor of the enzyme in *Rhodopseudomonas spheroides* (6). A combination of all three kinds of control was found in the Marburg strain of *Bacillus subtilis* (13). In addition to feedback inhibition of the enzyme, aspartokinase has been reported to be repressed by one or more of the end products.

The present report describes the effect of a number of amino acids on the aspartokinase of *M. xanthus*. The most significant findings were that L-threonine and L-lysine repressed and feedback-inhibited the enzyme, whereas L-methionine and L-isoleucine greatly stimulated the activity. Since methionine and isoleucine are required amino acids for *M. xanthus* and consequently not really end products of the pathway, the term "feedback stimulation" is in quotes. The possible physiological significance of "feedback stimulation" by required amino acids is discussed in this communication, whereas evidence for the specific role of aspartokinase in the initiation of the developmental cycle of *M. xanthus* is reported in the accompanying paper (12).

(This report was taken from a dissertation submitted by David Filer to Tel Aviv University in partial fulfillment of the requirement for the M.Sc. degree. Part of this work was presented at the 41st Meeting of the Israeli Chemical Society, December 1971.)

MATERIALS AND METHODS

Organism and cultivation. *M. xanthus* strain FBmp (18) was used in this study. Casitone media, 1 CT and 0.1 CT (7), contained 1.0% and 0.1% casitone (Difco), respectively. Synthetic medium, M1, was as described previously (18) except that tryptophan was

omitted. In some cases additional amino acids were omitted, as indicated in the text.

The organism was grown in 2-liter Erlenmeyer flasks containing 400 ml of medium with vigorous gyratory shaking at 32 C. About 20 ml of an exponentially growing culture was used as inoculum. Growth was estimated by measuring the optical density (OD) of samples at 620 nm. The cells were harvested from cultures in the late exponential phase (OD = 0.5 to 1.0) by centrifugation at 4 C and washed twice with 0.02 M potassium phosphate buffer (pH 7.5) containing 0.03 M mercaptoethanol. Cells were occasionally stored frozen (−70 C) until used.

Enzyme preparation. Cells from 800 ml of culture were resuspended in 15 ml of the above buffer and disrupted by three 20-s exposures to sonic oscillations with a Branson B12 sonifier (setting 5). During this treatment the temperature of the cell suspension was not allowed to rise about 5 C. The crude extract was the supernatant fluid after centrifuging at 34,000 × *g* for 30 min. All steps of purification were conducted at 0 to 4 C. To the resulting supernatant solution, 1/10 vol of 10% (wt/vol) streptomycin sulfate solution was added, and after 30 min the resulting precipitate was removed by centrifugation at 10,000 × *g* for 10 min. The clear supernatant solution from the streptomycin precipitation was then fractionated by the addition of a saturated solution of ammonium sulfate. Protein precipitating at 30% saturation with ammonium sulfate contained no enzyme activity and was removed by centrifugation (16,000 × *g* for 10 min); to the supernatant fluid additional ammonium sulfate was added to yield a 50% saturated solution. The resulting precipitate was dissolved in 0.5 ml of the phosphate buffer containing mercaptoethanol and used as the source of the enzyme.

Enzyme assay. The aspartokinase activity was measured by hydroxamate procedure essentially as described by Stadtman et al. (14). The reaction mixture consisted of 10 mM adenosine 5'-triphosphate (ATP), 30 mM L-aspartate, 10 mM 2-mercaptoethanol, 100 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.5), 5 mM $MgSO_4$, and 800 mM NH_2OH (prepared by neutralization of $NH_2OH \cdot HCl$ with KOH, thus giving about 800 mM KCl in the assay). The reaction was initiated by the addition of enzyme to give 1.0 ml final volume and, after 40 min at 30 C, was terminated by the addition of a 1.0 ml solution containing 10% $FeCl_3$, 3.3% trichloroacetic acid, and 0.7 N HCl. After removing denatured protein by centrifugation, the absorbancy of the ferric hydroxamate complex was measured at 540 nm using a Gilford spectrophotometer. The molar absorbancy for β-hydroxamate was estimated using β-aspartylhydroxamate (Sigma) as a standard. Specific activity was expressed as nanomoles of β-hydroxamate produced per minute per milligram of protein. Protein was determined by the method of Warburg and Christian (17).

Incorporation of DL-aspartic-4-^{14}C into protein amino acids. *M. xanthus* FBmp was cultured for several days in M1 medium minus threonine, lysine, and asparagine; the doubling time under these conditions was 13 h. The culture was then exposed to 5 μg of aspartic acid per ml for 2 days to obtain 5.0×10^8 cells per ml. The culture was then diluted 1:3 into M1 medium minus threonine, lysine, and asparagine, and supplemented with 1 μCi of DL-aspartic acid-4-^{14}C per ml (4.12 Ci per mol, New England Nuclear Corp.). After the OD had increased 3 times, cells from 20 ml of the labeled culture were harvested and washed and subjected to extraction, hydrolysis, two-dimensional paper chromatography, radioautography, and counting procedures as described by Roberts et al. (11).

Chemicals. ATP was a product of Pabst Laboratories Biochemicals. Amino acids used for growth media were L-isomers puchased from Nutritional Biochemical Corp., Cleveland, Ohio. Amino acids used in the enzyme were products of Calbiochem. Corp., Los Angeles, Calif. Enzyme grade ammonium sulfate was a product of Mann Research Laboratories. Spermidine and putrescine were purchases from Serva Feinbiochemica, Heidelberg, Germany. All other chemicals were of the highest purity obtainable.

RESULTS

Effect of amino acids on aspartokinase activity. In crude extracts of *M. xanthus* FBmp, the aspartokinase obtained from cells growing on medium 1 CT had a very low activity. Using the hydroxamate assay, the enzyme could be detected only after 30-fold concentration. For this reason and also in order to free the preparation from endogeneous amino acids, the 30 to 50% ammonium sulfate fraction was used in this study. The enzyme assay was linear for at least 40 min and up to protein concentration of 1.0 mg/ml. It was found that 30 mM aspartate, 10 mM ATP, and 5 mM $MgSO_4$ were optimal. Assay values of controls lacking aspartate were substracted from values of experimental assays.

Amino acids of the aspartate family have different effects on the aspartokinase activity (Table 1). While L-threonine and L-lysine inhibited, L-isoleucine and L-methionine unexpectedly greatly enhanced the activity. None of these amino acids altered the control lacking aspartate. The strong inhibition by threonine (83%) was not overcome by methionine and decreased only slightly by isoleucine. The lesser inhibition produced by lysine (31%) was not affected either by methionine or isoleucine. Meso-diaminopimelic acid, which by itself did not influence the enzyme activity, abolished the methionine stimulation and decreased that produced by isoleucine. The following amino acids tested at the same concentration (1 mM) did not have any effect on aspartokinase activity: glycine, L-glutamic acid, L-arginine, L-serine, L-leucine, L-phenylalanine, and L-tryptophan. In the presence of isoleucine and methionine, the activation was about the mean of the values

TABLE 1. *Effect of certain amino acids on the aspartokinase of M. xanthus FBmp*

Additions (1 mM)	Sp act[a]
None	5.8
L-Methionine	7.9
L-Isoleucine	13.3
L-Threonine	1.0
L-Lysine	4.0
Meso-DAP	5.7
L-Methionine + L-threonine	1.0
L-Methionine + L-lysine	3.5
L-Methionine + meso-DAP	5.8
L-Methionine + L-isoleucine	11.5
L-Isoleucine + L-threonine	2.8
L-Isoleucine + L-lysine	3.7
L-Isoleucine + meso-DAP	7.9
L-Threonine + L-lysine	<0.1

[a] Cells were grown in 1.0 CT medium. Specific activities were determined on the 30 to 50% $(NH_4)_2SO_4$ fractions and are expressed as nanomoles of β-hydroxamate produced per minute per milligram of protein.

produced by each of them separately. On the other hand the simultaneous addition of threonine and lysine produced almost complete inhibition.

The stimulation effected by 1 mM isoleucine was more than twice that produced by 1 mM methionine. The dose response curves of both amino acids were sigmoidal, suggesting cooperative effects (Fig. 1). As indicated previously, threonine was a stronger inhibitor than lysine. This was particularly apparent at lower concentrations (Fig. 2).

Effect of polyamines upon aspartokinase. It had been reported previously (18) that microcyst formation could be induced by putrescine and inhibited by spermidine. Thus it seemed worthwhile to examine the effect of these polyamines on aspartokinase activity. From Table 2 it can be seen that spermidine at 0.1 mM was a potent stimulator of the aspartokinase of *M. xanthus*. Furthermore, it completely reversed the inhibition caused by 1 mM threonine. Spermidine did not further enhance methionine stimulation. Putrescine (1 mM) caused a small but significant (30%) inhibition of the activity.

Effect of growth medium composition upon aspartokinase biosynthesis. The amino acids lysine and threonine, although stimulatory for gorwth of *M. xanthus* FBmp are non-essential (18). When either lysine or threonine was absent from the growth medium, an eight- to ninefold rise in the specific activity of aspartokinase was observed (Table 3). Omission of both amino

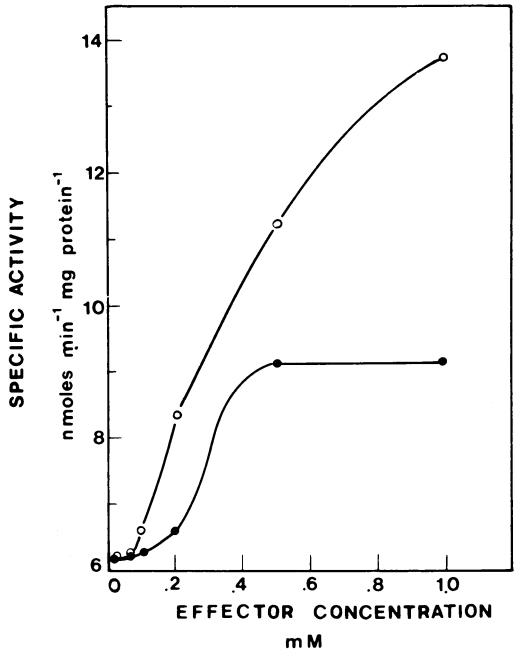

FIG. 1. *Aspartokinase activity of the 30 to 50% $(NH_4)_2SO_4$ fraction of M. xanthus strain FBmp grown in 1.0 CT. Activities were measured in the presence of varying amounts of* L-*isoleucine* (O) *and* L-*methionine* (●).

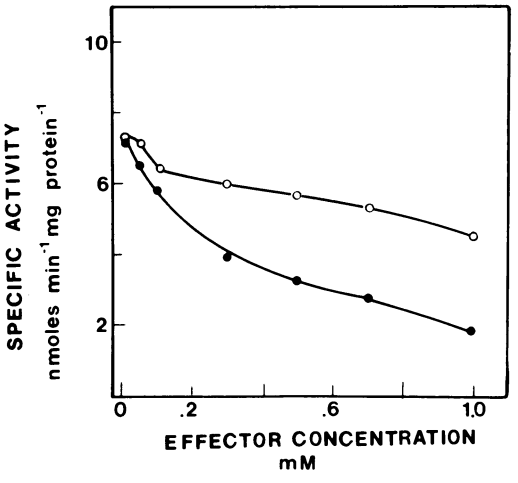

FIG. 2. *Aspartokinase activity of the 30 to 50% $(NH_4)_2SO_4$ fraction of M. xanthus strain FBmp grown in 1.0 CT. Activities were measured in the presence of varying amounts of* L-*lysine* (O) *and* L-*threonine* (●).

acids resulted in a 20-fold increase of specific activity. It would thus seem that both lysine and threonine not only inhibit the enzyme but also repress its synthesis. Again, the effect of the

two amino acids was additive, suggesting independent modes of action. It should be noted that the growth rate itself does not determine the level of aspartokinase, since depletion of lysine from the defined medium has no effect on doubling time. The previous report (18) concerning the longer generation time in the absence of lysine did not take into account the initial lag (presumably the time required for derepression).

The twofold-lower activity of the enzyme derived from cells grown in the synthetic medium M1, in comparison with the enzyme from cells grown in the complex medium CT, is probably due to higher levels of these amino acids in M1.

Biosynthesis of lysine and threonine by M. xanthus. The experiments described in this paper were predicated on the assumption that aspartic acid is the precursor for threonine and lysine in *M. xanthus*. Table 4 provides direct evidence that this assumption was correct. Of the two distinctive lysine pathways known to occur in nature, one of them has DAP as a key intermediate and the other, α-aminoadipic acid. By utilizing C4-labeled aspartic acid, the mode of lysine synthesis can be revealed (16). For example, in the case of the DAP-lysine pathway, as illustrated by the fern *Azolla caroliniana* (Table 4), the aspartate-*4*-^{14}C labels protein aspartic acid and lysine at approximately equal specific activities. On the other hand, with the α-aminoadipic acid-lysine path, as exemplified by the mushroom *Coprinus radians*, no radioactivity is found in lysine under these labeling conditions. *M. xanthus*, like other prokaryotic cells, clearly utilizes the DAP-lysine pathway.

TABLE 2. *Effect of spermidine on the aspartokinase activity of M. xanthus FBmp*

Addition[a]	Sp act[b]	Stimulation (%)	Inhibition (%)
None	25.0		
Spermidine	47.0	88	
Met	48.5	94	
Met + spermidine	47.2	89	
Thr	4.7		81
Thr + spermidine	48.5	94	

[a] The unsupplemented reaction mixture is described in Materials and Methods. Spermidine was 0.1 mM, while Met and Thr were 1.0 mM each.

[b] The 30 to 50% $(NH_4)_2SO_4$ fraction was prepared from a culture of *M. xanthus* FBmp grown on 0.1 CT medium. Specific activity expressed as nanomoles of β-hydroxamate produced per minute per milligram of protein.

DISCUSSION

The specific activity of the aspartokinase of *M. xanthus* is low when the cells are grown in media such as 1 CT or M1 containing relatively high concentrations of amino acids. This is consistent with the notion that under these circumstances the only role of this enzyme is in the biosynthesis of DAP for cell wall. The repression and feedback inhibition exerted by lysine and threonine would be expected to serve

TABLE 4. *Incorporation of* DL*-aspartic-4-*^{14}C *into protein amino acids*[a]

Organism	Amino acid		
	Asp	Thr	Lys
Azolla caroliniana[b]	100	80	91
Coprinus radians[b]	100	115	0
Myxococcus xanthus[c]	100	104	109

[a] The data are expressed as specific radioactivity (on a molar basis) relative to the respective protein aspartic acid values taken as 100.

[b] Data from Vogel (16).

[c] *M. xanthus* was grown in M1 medium minus lysine, threonine, and asparagine and containing limiting quantities of aspartic acid (see Materials and Methods).

TABLE 3. *Effect of growth medium composition on the specific activity and properties of aspartokinase from M. xanthus FBmp*

Growth medium[a]	Doubling time (h)	Sp act (counts/min)	Stimulation (%) by:		Inhibition (%) by:	
			Methionine[b]	Isoleucine[b]	Threonine[b]	Lysine[b]
1.0 CT	4.4	6.7	85	230	63	52
M1	6.5	3.2	84	100	31	36
M1-lysine	6.5	29.0	160	157	79	71
M1-threonine	9.5	25.1	32	50	43	29
M1-lysine-threonine	11.5	63.2	73	76	63	39

[a] The composition of the 1.0 CT and M1 media are described in Materials and Methods.

[b] Amino acids added to the enzyme assay mixture were 1.0 mM each.

as self-regulating devices, since the organism can synthesize these amino acids.

The activation of aspartokinase by methionine and isoleucine is especially interesting since these two amino acids, although members of the aspartate family, are required for the growth of *M. xanthus* (12, 18). The organism may have lost its ability to synthesize these amino acids through retrograde evolution, but retained a vestige of its former control mechanism. However, instead of exhibiting the characteristic inhibition by the end product on the first enzyme of the pathway, isoleucine and methionine activate the aspartokinase of *M. xanthus*. This phenomenon is referred to as "feedback stimulation." The activation by methionine and isoleucine is probably not fortuitous since, of eleven amino acids examined, only the four members of the aspartate family affected the aspartokinase. Mutation to end-product activation has previously been reported in the phenylalanine biosynthetic pathway in *B. subtilis* (3).

"Feedback stimulation" may help to insure adequate synthesis of DAP when the aspartokinase is partially inhibited and to increase the supply of threonine or lysine under conditions when these amino acids are depleted. The complete reversal of isoleucine and methionine activation by meso-DAP supports the hypothesis that the former amino acids have a significant role in the regulation of DAP biosynthesis.

It should be emphasized that, in this study, interactions between stimulators and inhibitors were measured only at one concentration (1 mM) of each amino acid. Exact interrelationships were not studied further at this stage because of the likelihood that more than one aspartokinase is present in our preparations. This supposition is strongly supported by the additive derepression observed in the absence of lysine and threonine from the growth medium. Furthermore, the activity obtained from cells grown in the absence of lysine exhibited far greater stimulations and inhibitions than the activity obtained when the medium was deprived of threonine. Further studies now in progress should help to resolve the question whether indeed there are two enzymes, one repressed by lysine and the other by threonine.

The activation of aspartokinase by spermidine has not been reported previously. Spermidine at 0.1 mM was the most potent activator examined; moreover, the spermidine stimulation could not be reversed by 1.0 mM threonine. The role of spermidine cannot be easily explained at present. It should be mentioned, however, that methionine is required for the conversion of putrescine to spermidine.

From the wide variety of microorganisms examined, the aspartokinase activity of *M. xanthus* resembles most closely that of *Rhodospirillum rubrum* (4). The aspartokinase of *R. rubrum* is stimulated by isoleucine and methionine and inhibited only by threonine. This was interpreted to indicate that lysine and methionine biosynthesis is controlled by the isoleucine/threonine ratio. It would thus seem that, although the general action of effectors on aspartokinase may be similar, the regulation of this enzyme in each organism is geared to its peculiar metabolic state and environmental conditions.

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