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ASPARTOKINASE ISOENZYMES OF THE FRUITING MYXOBACTERIUM *MYXOCOCCUS XANTHUS*

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Summary

Two isoenzymes of aspartokinase can be found in extracts of the differentiating bacterium *Myxococcus xanthus*. Aspartokinase I is repressed by L-lysine and feedback is inhibited by meso-diaminopimelate and by low concentrations of L-lysine. However, the inhibition by L-lysine is no longer observed at high concentration of this amino acid. Aspartokinase II is repressed and feedback inhibited specifically by L-threonine. Both enzymes are stimulated significantly by L-methionine and L-isoleucine; the effect is greater with aspartokinase I.

The role of these enzymes in relation to growth conditions of the organism is discussed and a correlation with life cycle activity is indicated.

Introduction

The enzyme aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) is regulated in a unique fashion in many bacterial species. In some microorganisms such as *Escherichia coli* and *Bacillus subtilis*, the control is made more precise by the existence of 2 or 3 isofunctional enzymes [1,2]. In other cases the control is exerted through the concerted action of two metabolites on the activity of a single enzyme [3—6]. Recent studies from this laboratory have shown that the aspartokinase of the differentiating bacterium *Myxococcus xanthus* is subject to feedback inhibition as well as repression by the non-essential amino acids L-lysine and L-threonine [7]. On the other hand, the required amino acids, L-isoleucine and L-methionine, stimulated considerably the activity of this enzyme. When either lysine or threonine was absent from the growth medium about a 9-fold increase in the specific activity of aspartokinase was observed. Omission of both amino acids resulted in 20-fold rise in specific activity. Since the derepression was additive, the existence of isoenzymes was suspected.

Myxobacteria of the genus *Myxococci* are commonly found in nature on decaying material, satisfying their nutritional requirements by preying on other microorganisms. Under nutritionally limiting conditions, the cells agregate to form a fruiting body within which the individual cells undergo morphogenesis, becoming refractile resting cells called either microcysts or myxospores [8]. Myxospores differ in many ways from endospores, including the fact that they lack calcium dipicolinic acid [8]. Previously we demonstrated [9] that two required amino acids of *M. xanthus*, L-methionine and L-isoleucine, inhibited fruiting body formation, whereas L-threonine, a potent feedback inhibitor of the aspartokinase stimulated fruiting body formation. These data indicated a correlation between aspartokinase activity and the induction of the developmental cycle of *M. xanthus*.

In this paper we describe the separation and properties of two aspartokinases from *M. xanthus*.

Materials and Methods

Chemicals. All amino acids were obtained from Calbiochem. ATP was a product of Pabst Laboratories. Streptomycin sulfate was purchased from Rafa Laboratories. Enzyme grade ammonium sulfate was a product of Mann Research Laboratories. *meso*-Diaminopimelic acid was acquired from Calbiochem. All other chemicals were of the highest grade available commercially.

Organism and cultivation. M. xanthus strain FBmp [10] was used in this study. Cells were maintained in casitone medium [11]. The synthetic medium 1 was described previously [10], except that tryptophan was omitted. In order to obtain derepressed cultures, the following media were prepared: L-lysine- and L-threonine-free medium 1; and lysine-free and threonine-free medium 1. Inocula were prepared by growing 20 ml cells in 100 ml Erlenmeyer Flasks to an absorbance of 0.8—1.2 at 560 nm (corresponding to about $5 \cdot 10^8$ cells/ml). For the preparation of the enzyme, the organism was grown in 2-liter Erlenmeyer Flasks, containing 400 ml of one of the synthetic media with vigorous gyratory shakings at 32° C. The flasks were inoculated with 20 ml of a culture grown under identical conditions.

In general, cells were harvested from cultures in the late exponential phase after 3—4 days of growth, when an absorbance of 0.8—1.2 was reached. The cells were centrifuged at 4° C and washed twice with 0.02 M potassium phosphate buffer (pH 7.5) containing 0.03 M mercaptoethanol.

Enzyme preparation. Accumulated cells from 4 liters of culture were resuspended in 10 ml of the above buffer and disrupted by four 30-s exposures to sonic oscillations with a Branson B 12 Sonifier (setting 5). This and all further steps were conducted at 4° C. The sonicate was centrifuged at 34 000 × *g* for 30 min. To the resulting supernatant solution 1/10 vol of 10% (w/v) streptomycin sulfate solution was added, and after 30 min the precipitate was removed by centrifugation at 10 000 × *g* for 10 min. The clear supernatant solution was then fractionated by the addition of a saturated solution of ammonium sulfate. The material which precipitated between 30 and 50% saturation was redissolved in 0.02 m potassium phosphate buffer, pH 7.5, to the desired protein concentration.

Enzyme assay. The aspartokinase activity was measured by hydroxamate procedure essentially as described by Stadtman et al. [12]. 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 M HCl. After removing denatured protein by centrifugation, the absorbance of the ferric hydroxamate complex was measured at 540 nm using a Gilford spectrophotometer. The molar absorbance for β-hydroxamate was estimated using β-aspartohydroxamate (Sigma) as a standard. Specific activities are expressed as nmol of β-hydroxamate produced per min per mg of protein. Protein was determined by the method of Warburg and Christian [13].

Results

Isoenzymes

In order to ascertain that the aspartokinase activity of *M. xanthus* is due to more than one enzyme, further purification was undertaken. For this purpose advantage was taken of the large increase of activity in derepressed cultures. The 30—50% ammonium sulfate fraction from a culture grown in lysine + threonine-free medium 1 was subjected to gel filtration on Sephadex G 200. As can be seen from Fig. 1, two well separated peaks of aspartokinase activity were obtained. The most active fractions from each peak were then combined and their properties with regard to the various effectors were determined (Table I). The activity from the earlier peak (aspartokinase-I) was inhibited 31% by 1 mM L-lysine and 14% by 1 mM L-threonine; whereas the activity from the

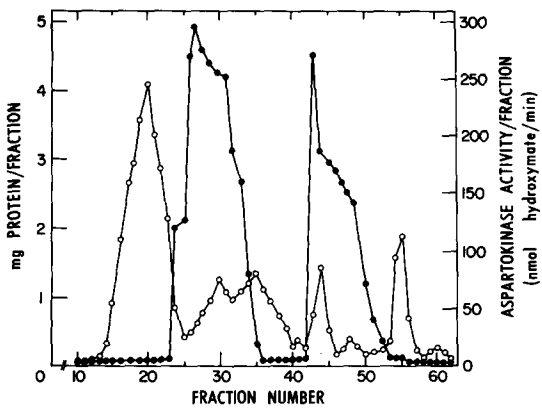


Fig. 1. Separation of aspartokinase I and II. *M. xanthus* cells grown in lysine + threonine-free medium 1 were disrupted by sonication, and the 30—50% ammonium sulfate fraction, prepared as described in Methods, was dissolved in 0.02 M potassium phosphate buffer, pH 7.5, to give a protein concentration of 11 mg per ml. 5 ml of this fraction was applied to a column (2.5 × 110 cm) of Sephadex G-200 (void volume 62 ml) equilibrated with the same buffer at 4° C. The column was eluted with the above buffer. Fractions (6 ml) were collected and assayed for aspartokinase activity (●———●) and for protein (○———○). Flow rate 25 ml/h.

TABLE I

ACTION OF EFFECTORS ON ASPARTOKINASE ACTIVITY BEFORE AND AFTER GEL FILTRATION

M. xanthus FBmp was grown on the synthetic lysine + threonine-free medium. The 30—50% ammonium sulfate fraction was prepared as described in Methods and then fractionated on Sephadex G-200 column (2.5 × 110 cm).

Effector present (1 mM)	Specific activity of enzyme before gel filtration		Specific activity of peak 1		Specific activity of peak 2	
None	65.4	—	79.3	—	82.1	
L-Methionine	112.0	(75)*	117.8	(48)*	85.7	(4)*
L-Isoleucine	128.3	(97)*	120.3	(52)*	91.4	(11)*
L-Threonine	20.5	(63)**	68.9	(14)**	1.4	(98)**
L-Lysine	38.0	(36)**	54.6	(31)**	77.0	(5)**

* % stimulation.
** % inhibition.

second peak (aspartokinase-II) was almost completely inhibited by 1 mM L-threonine (98%) but only slightly by 1 mM L-lysine (5%). Furthermore, the activity of aspartokinase-I was stimulated much more by 1 mM L-methionine or 1 mM L-isoleucine than the activity of aspartokinase-II under conditions employed in this experiment. In this case the specific activity of the concentrated fractions from the two peaks was not increased due to loss of enzymatic activity. This was avoided subsequently by using shorter elution time and 0.05 M phosphate buffer, pH 8.0.

In order to confirm that the two peaks of activity did indeed correspond to different enzymes repressed by either lysine or threonine, cultures of *M. xanthus* were grown in synthetic media lacking either lysine or threonine. When partially purified extracts from either of these cultures were placed on Sephadex G-200 columns, only one peak of activity was obtained in each case (Figs 2

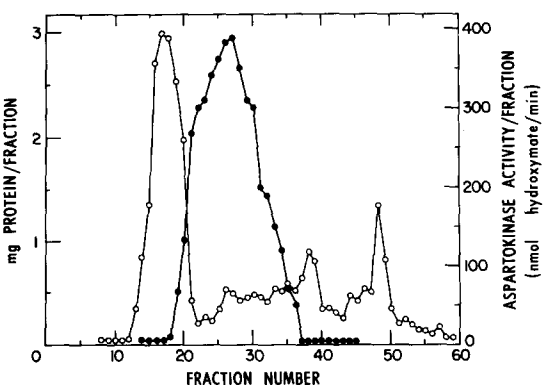


Fig. 2. Gel filtration of aspartokinase I on Sephadex G-200. *M. xanthus* FBmp cells grown in lysine-free medium 1 were disrupted by sonication and the 30—50% ammonium sulfate fraction was prepared as described in Methods. A sample containing 30 mg protein was applied to the column (2.5 × 85 cm) (void volume 64 ml) equilibrated with 0.05 M potassium phosphate buffer, pH 8.0 at 4°C. The column was eluted with the same buffer, fractions (4 ml) were collected and assayed for aspartokinase activity (●———●) and for protein (○———○). Flow rate 30 ml/h.

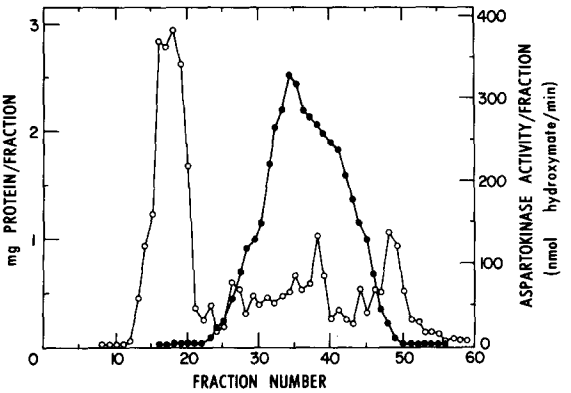


Fig. 3. Gel filtration of aspartokinase II on sephadex G-200. *M. xanthus* FBmp cells grown on threonine-free medium 1 were disrupted by sonication and the 30—50% ammonium sulfate fraction was prepared as described in Methods. A sample containing 30 mg protein was applied to the column (2.5 × 85 cm) (void volume 65 ml) equilibrated with 0.05 M potassium phosphate buffer, pH 8.0, at 4°C. The column was eluted with the same buffer, fractions (4 ml) were collected and assayed for aspartokinase activity (●———●) and for protein (○———○).

and 3). As can be seen from Table II, aspartokinase-I and aspartokinase-II were purified 30 and 40 fold, respectively, by this procedure.

Lysine repressed enzyme

Aspartokinase-I was very sensitive to inhibition by either L-Lysine (1 mM) or *meso*-diaminopimelic acid (0.1 mM) 78 and 61%, respectively (Table III). Both effectors decreased V without altering K_m for L-aspartate (Fig. 4). The enzyme was stimulated greatly by L-methionine and by L-isoleucine. While the former affected only V, the latter also decreased by 10-fold K_m for aspartate (Fig. 4). The inhibitory effect of L-lysine was peculiarly dependent on concentration. Although 50% inhibition can be obtained already at 0.1 mM L-lysine,

TABLE II

PARTIAL PURIFICATION OF ASPARTOKINASE I AND II

The enzymes were purified from cultures of *M. xanthus* FBmp grown on media allowing selective derepression of isozymes; lysine-free medium 1 for aspartokinase I and threonine-free medium 1 for aspartokinase II.

Enzyme	Fraction	Total protein (mg)	Total activity (unit*)	Specific activity (unit/mg)	Yield (%)
Aspartokinase I	Crude extract	996.8	6370	6.3	100
	$(NH_4)_2SO_4$ fraction	150.7	4825	32.0	76
	Sephadex G-200** fraction	4.3	820	190.7	13
Aspartokinase II	Crude extract	1125.8	6530	5.8	100
	$(NH_4)_2SO_4$ fraction	180.3	5320	29.5	81
	Sephadex G-200** fraction	5.2	1280	246.1	20

* nmol hydroxamate/min.

** Concentrated eluted fractions from Sephadex G-200 column (2.5 cm × 85 cm); see Figs 2 and 3.

TABLE III

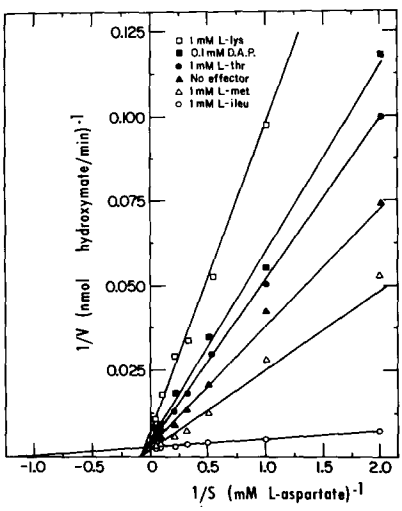
RESPONSE TO EFFECTORS OF ASPARTOKINASE I AND II

The partially purified enzymes were prepared from selectively derepressed cultures of *M. xanthus* FBmp as described in Methods and Table II. Final concentration of the effectors was 1 mM with the exception of *meso*-diaminopimelic acid which was 0.1 mM.

Effector present	Specific activity			
	Aspartokinase I		Aspartokinase II	
None	205.0	—	256.0	—
L-Methionine	370.0	(81)*	308.0	(21)*
L-Isoleucine	470.0	(130)*	336.0	(32)*
L-Threonine	160.0	(22)**	36.0	(86)**
L-Lysine	45.0	(78)**	244.0	(6)**
meso-Diaminopimelic acid	79.0	(61)**	252.0	(1.4)**

* % stimulation.
** % inhibition.

almost no inhibition can be seen at 2 mM (Fig. 5). A similar decrease in sensitivity to effector at high concentration was seen with L-isoleucine, which produced 130% activation at 1 mM, but only 6% at 10 mM. On the other hand, the inhibitory effect of L-threonine increased from 22% at 1 mM to 60% at 10 mM (Table IV).



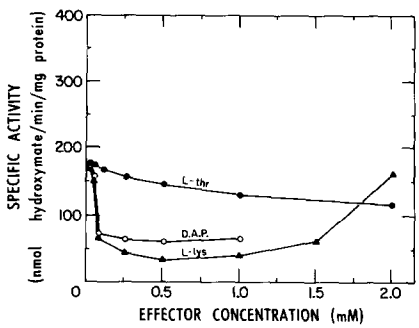


Fig. 4. Effect of inhibitors and activation on aspartokinase I at various aspartate concentrations. The enzyme after Sephadex G-200 column purification (0.5 mg protein) was assayed in the absence of effectors (▲———▲) and in the presence of 1 mM L-lysine (□———□), 0.1 mM *meso*-diaminopimelic acid (■———■), 1 mM L-threonine (●———●), 1 mM L-methionine (△———△) and 1 mM L-isoleucine (○———○).

Fig. 5. Effect of inhibitors at various concentrations on aspartokinase I. The enzyme after Sephadex G-200 purification was assayed in presence of rising amount of L-threonine (●———●); *meso*-diaminopimelic acid (○———○); and L-lysine (▲———▲).

TABLE IV

EFFECT OF HIGH CONCENTRATION OF L-ISOLEUCINE AND L-THREONINE ON ASPARTOKINASE I AND II

Preparation of enzymes as described in Table III.

Effector present	Concn (mM)	Specific activity			
		Aspartokinase I		Aspartokinase II	
None		225.0		240.0	
L-Isoleucine	5.0	310.0	(37)*	320.0	(33)*
	7.5	275.0	(22)*	290.0	(20)*
	10.0	240.0	(6)*	280.0	(16)*
L-Threonine	5.0	140.0	(37)**	30.0**	(87)**
	7.5	125.0	(44)**	25.0**	(89)**
	10.0	91.0	(60)**	12.0**	(95)**

* % stimulation.
** % inhibition.

When higher enzyme concentrations were used, the stimulatory effect of L-isoleucine decreased markedly while the inhibition by L-threonine and L-lysine increased (Fig. 6).

Threonine repressed enzyme

Aspartokinase-II was specifically inhibited by L-threonine, 86% at 1 mM (Table III). L-lysine (1 mM) and *meso*-diaminopimelic acid (0.1 mM) lowered

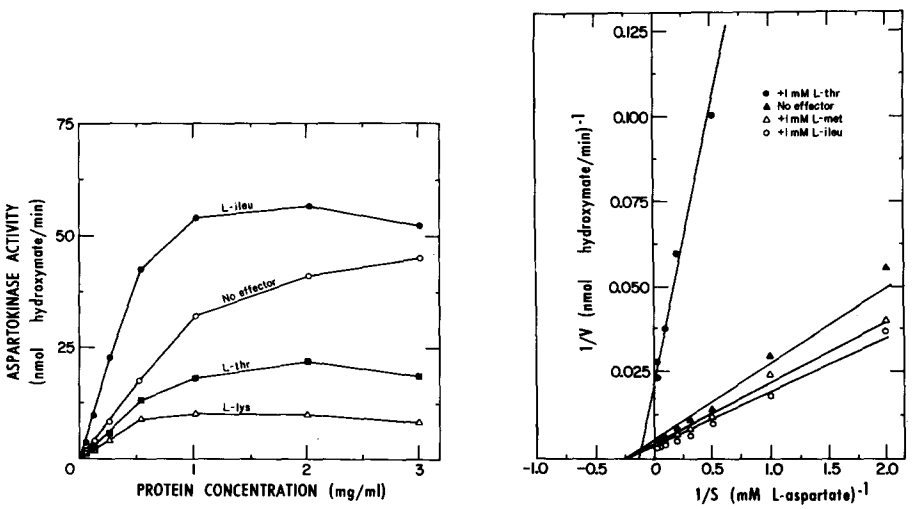


Fig. 6. Effect of inhibitors and activator on aspartokinase I at various protein concentrations. The enzyme after Sephadex G-200 column purification was assayed in absence of effectors (○———○), and in the presence of 1 mM L-isoleucine (●———●), 1 mM L-threonine (■———■), and 1 mM L-lysine (△———△).

Fig. 7. Effect of inhibitors and activators on aspartokinase II at various aspartate concentrations. The enzyme after Sephadex G-200 column purification (0.5 mg protein) was assayed in the absence of effectors (▲———▲) and in the presence of 1 mM L-threonine (●———●), 1 mM L-methionine (△———△), and 1 mM L-isoleucine (○———○).

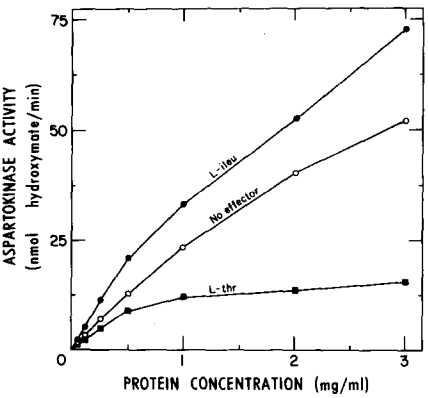


Fig. 8. Effect of L-isoleucine and L-threonine on aspartokinase II at different enzyme concentrations. The enzyme after Sephadex G-200 column purification was assayed in the absence of effectors (○———○), and in the presence of L-isoleucine (●———●) and L-threonine (■———■).

the activity only slightly. This enzyme was also activated by L-methionine (21%) and L-isoleucine (32%). L-Threonine decreased V considerably and also decreased 2-fold the affinity for aspartate. On the other hand, the stimulatory amino acids, L-methionine and L-isoleucine only increased V (Fig. 7).

The inhibitory effect of L-threonine increased with rising enzymes concentration, but such effect was not seen with the stimulation produced by L-isoleucine (Fig. 8).

Discussion

The two aspartokinases of *M. xanthus* show marked differences in several respects, especially in their response to the effectors. Aspartokinase I, which is repressed by L-lysine, is inhibited by low concentrations of L-lysine and *meso*-diaminopimelic acid, whereas much higher concentrations of L-threonine are required to decrease the activity of this enzyme. The very dramatic decrease of inhibition by higher concentration of L-lysine is a unique property of this aspartokinase. On the other hand, aspartokinase II, which is specifically repressed by L-threonine, is feedback-inhibited significantly only by this amino acid. Although both isoenzymes are stimulated by the essential amino acids, L-isoleucine and L-methionine, the effect is more pronounced with aspartokinase I.

In minimal media, the function of the two aspartokinases of *M. xanthus* is to ensure the synthesis of L-threonine, L-lysine as well as its cell wall component *meso*-diaminopimelic acid. In rich media, in which the organism usually finds itself in nature [8], there is only a low aspartokinase activity since both enzymes are repressed and aspartokinase II greatly inhibited, by L-threonine. Under this condition aspartokinase I would ensure the synthesis of *meso*-diaminopimelic acid, which is required in relatively small amounts. The dual control of aspartokinase I by L-lysine and *meso*-diaminopimelic acid is a little puzzling at first since both amino acids inhibit equally at low concentrations. However, when L-lysine is present at high concentrations it no longer inhibits

this enzyme, in which case *meso*-diaminopimelic acid would be the critical effector. This is supported by the observation that *meso*-diaminopimelic acid inhibits the enzyme also in the presence of high concentrations of L-lysine. In this respect aspartokinase I of *M. xanthus* resembles the aspartokinase I of *B. subtilis*, which is specifically inhibited by *meso*-diaminopimelic acid [2]. On the other hand both aspartokinase of *M. xanthus* share an unusual property with the aspartokinase of *Rhodospirillum rubrum*, being significantly stimulated by methionine and isoleucine [14].

The activation of both *M. xanthus* aspartokinases by methionine and isoleucine is particularly interesting since these two amino acids, although members of the aspartate family, are essential for the growth of *M. xanthus* [9,11]. Apparently, the organism 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 aspartokinases of *M. xanthus*. 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 aspartokinase activity. We refer to this phenomenon as "feedback stimulation". More generally, it is reasonable to assume that following mutation involving loss of synthetic ability, there would occur a strong selective pressure for a second wave of regulator mutations. In this particular case, "feedback stimulation" would have the selective advantage of insuring adequate synthesis of *meso*-diaminopimelic acid in a rich medium and reducing its production during starvation of the required amino acids. Mutation and end-product activation has previously been reported in the phenylalanine pathway in *B. subtilis* [15].

In a previous communication [9] we have discussed the strong correlation between aspartokinase activity and the induction of the developmental cycle in *M. xanthus*. Amino acids which modified the activity in vitro affected fruiting body formation in a reciprocal manner in vivo. For example, the two essential amino acids, methionine and isoleucine, stimulated enzyme activity in vitro but prevented aggregation and subsequent fruiting body formation on solid media (which normally support the complete developmental cycle). The correlation between enzyme activity and induction of the developmental cycle is further strengthened by the fact that (A) *meso*-diaminopimelic acid which stimulates fruiting body formation [16], is a strong inhibitor of aspartokinase I (Fig. 4), and (B) elevated concentrations of lysine neither effect fruiting body formation nor aspartokinase activity (Fig. 5). Certainly much more has to be known about general amino acid metabolism, metabolic interlocks [17] and chemotaxis in *M. xanthus* before any cause and effect relationship can be ascribed to this striking correlation.

Acknowledgement

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