BBA 65876

THE REGULATION OF ASPARTOKINASE IN *BACILLUS LICHENIFORMIS*

BEULAH H. GRAY AND ROBERT W. BERNLOHR
Department of Microbiology, University of Minnesota, Minneapolis, Minn. 55455 (U.S.A.)

(Received November 6th, 1968)

SUMMARY

The specific activity of aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) decreases rapidly to a 10- to 20-fold lower level at the end of the growth phase in *Bacillus licheniformis* grown on a minimal glucose–salts medium. Concurrent with this decrease in specific activity is a loss of sensitivity to feedback inhibition by L-lysine and a slower decrease in the concerted inhibition caused by L-lysine and L-threonine.

Growth of the cells in the minimal medium *plus* L-lysine and L-threonine caused the production of aspartokinase with characteristics similar to those of enzyme from cells harvested 4 h after the end of growth. Growth in the presence of either L-lysine or L-threonine did not affect the specific activity of the enzyme but did alter its inhibition sensitivity. The addition of L-lysine to the medium during growth on the minimal medium caused a rapid loss of sensitivity to lysine inhibition, 50% desensitization occurring in 15 min. These latter conditions did not produce an alteration in the specific activity of the enzyme or in the concerted inhibition by lysine and threonine.

Despite the marked alteration in feedback-inhibition properties, changes in physical or kinetic properties of purified aspartokinase were not observed. Throughout the purification steps there was no evidence that more than one aspartokinase was present in *B. licheniformis* cells. It is concluded that this enzyme is unusually plastic and assumes alternate forms depending on the physiology of the cell.

INTRODUCTION

Aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) catalyzes the first step in the synthetic pathway of lysine, threonine and methionine[1]. The pattern of feedback control of aspartokinase of different organisms has proved to be quite varied. In *Escherichia coli*[1] there are multiple enzymes: one specifically inhibited by L-lysine and its formation inhibited by growth in the presence of L-lysine; a second aspartokinase is inhibited by L-threonine, but not affected by growth in the presence of L-threonine; and a third enzyme is specifically inhibited by homoserine[2]. The fact that the inhibition of the aspartokinase of *E. coli* by lysine and threonine was independent and additive suggested to STADTMAN *et al.*[1] that there were at least two differ-

ent enzymes. The threonine-sensitive and lysine-sensitive enzymes proved to have different heat stabilities and to be easily separable by $(NH_4)_2SO_4$ precipitation.

The aspartokinases of *Bacillus polymyxa*[3], *Rhodopseudomonas capsalatum*[4] and *Brevibacterium flavum*[5] are subject to concerted inhibition. L-Lysine and L-threonine together produced nearly complete inhibition of aspartokinase while either amino acid alone had little effect. These results suggested that there was only one aspartokinase in these organisms.

The aspartokinase of *Saccharomyces cerevisiae*[1] is almost completely inhibited by threonine while the aspartokinase of *Rhodopseudomonas spheroides*[6] is not inhibited by any of the amino acid end products but is inhibited by aspartic-β-semialdehyde, the first branch point intermediate in the pathway fed by the aspartokinase reaction.

The aspartokinase of *B. licheniformis*[7] was shown to be inhibited by L-lysine and aspartic-β-semialdehyde and was subject to concerted feedback inhibition by L-lysine and L-threonine. The aspartokinase of *B. licheniformis*, therefore, appeared to be similar to that of several other organisms in that there is a single enzyme subject to concerted feedback inhibition by lysine and threonine.

In an earlier report from this laboratory[7] a rapid decrease in the specific activity of aspartokinase of *B. licheniformis* at the end of log phase was described. The inactivation of selected enzymes after nutritional change has been observed by other investigators using Bacillus[8,9], *E. coli*[10–13] and yeast[14–15]. LEITZMANN AND BERNLOHR[16] in their study of threonine dehydratase observed the loss of enzyme activity at the end of the growth phase and demonstrated immunochemically that the loss of activity was not accompanied by a similar loss in enzyme protein. These results indicate that in the case of threonine dehydratase, an inactivation, without loss of enzyme protein, takes place at the end of growth. Similar experiments have not been reported for other enzymes.

We have continued the study of the regulation of aspartokinase in *B. licheniformis* with the hope of elucidating the mechanism of rapid loss of activity at the end of the growth phase. During the course of the continued investigation, it was apparent that the regulation of aspartokinase in this organism is more complex than originally proposed. This report will deal with the feedback inhibition, purification and properties of aspartokinase of *B. licheniformis* cells grown under varied nutritional conditions. We will present evidence of altered patterns of feedback inhibition of aspartokinase dependent on growth conditions.

EXPERIMENTAL PROCEDURE

Materials

L-Lysine, L-threonine and L-aspartic acid were purchased from Calbiochem. Disodium ATP was from Sigma or P–L Biochemicals. Calcium phosphate gel was purchased from Sigma, Sephadex from Pharmacia, and DEAE-cellulose from Calbiochem. All other chemicals were of the highest purity available.

Methods

Enzyme assay

Aspartokinase activity was measured by the production of aspartylhydroxamate in a reaction mixture containing hydroxylamine, aspartic acid, ATP, $MgSO_4$, KCl and

mercaptoethanol as described by STADTMAN *et al.*[1]. The reaction mixture was incubated at 25° for 30 min, and aspartic acid was present at 10 mM unless otherwise designated. Specific activity is expressed as mμmoles of aspartylhydroxamate formed per min, per mg protein. Protein was estimated by the method of LOWRY *et al.*[17]. The rate of the aspartokinase reaction (Fig. 1) was constant over a time interval of 30 min. This was true at all enzyme concentrations used in this investigation.

Growth of cells

Bacillus licheniformis (A5) was grown on minimal salts *plus* glucose and ammonium lactate as previously described[18]. The amino acids, L-lysine and L-threonine when added were present in the growth medium at 0.01 M. Lots of 15 l of cells were grown under forced aeration. The cells reached the end of growth after 5–6 h. Maximum specific activity (40–50) of aspartokinase in crude extracts was reached 30–60 min before the end of growth and the specific activity decreased rapidly over a 60-min

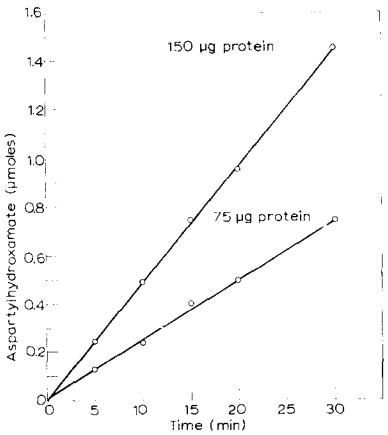

Fig. 1. The time course of the aspartokinase reaction at two concentrations of partially purified enzyme. The assay was as described in the text. The enzyme specific activity was 330.

period. The specific activity 4 h after the end of growth was < 10. Log-phase cultures were harvested 30 min before the end of growth phase and post-log cells were harvested 4 h after the end of growth. Absorbance was read in a Zeiss PMQ II spectrophotometer at 540 mμ.

Enzyme purification

Preparation of extract. Extraction of the enzyme from bacteria and all subsequent steps were carried out at 0–5°. Packed cells from a 15-l culture were suspended in 80 ml of 0.25 M potassium phosphate (pH 6.8) containing 0.03 M mercaptoethanol and broken by two passages through an Aminco–French pressure cell held at 10 000 lb · inch^{-2}. The suspension was sonicated for 1 min in a 20.0-kcycle MSE sonic oscillator in 10-ml volumes to decrease viscosity. The broken cell suspension was centrifuged at 105 000 × *g* for 60 min in a Spinco model-L centrifuge. The supernatant solution was brought to a volume of 500 ml (8 mg protein/ml) with distilled water.

$(NH_4)_2SO_4$ fractionation. Sufficient solid $(NH_4)_2SO_4$ was added to the supernatant solution to give a saturation of 40%. The mixture was stirred for 30 min and

precipitated material was removed by centrifugation at 13 000 × *g* during 20 min in a Servall centrifuge. Solid $(NH_4)_2SO_4$ was then added to 65% satn. and the precipitated material was removed. The 40–65% $(NH_4)_2SO_4$ fraction was suspended in 300 ml 0.002 M potassium phosphate buffer (pH 6.8) containing 0.003 M mercaptoethanol (7 mg protein/ml) and the same procedure was repeated to obtain material precipitating between 0–35, 35–40, 40–45, 45–50, 50–55 and 55–60% satn. Aspartokinase was concentrated in the 50–55% fraction. This was suspended in 10 ml 0.002 M phosphate buffer containing 0.003 M mercaptoethanol (29 mg protein/ml).

Gel filtration. The 50–55% $(NH_4)_2SO_4$ fraction was passed through a column of Sephadex G-100 (3 cm × 10 cm). The column had been equilibrated and was eluted with 0.002 M phosphate buffer. The protein-containing fraction in the void volume was absorbed to calcium phosphate gel.

Calcium phosphate. Enzyme was absorbed to the gel (25 mg protein/g wet wt. of gel) and eluted noncontinuously. The gel had been washed with 0.002 M phosphate buffer and washed again after protein was absorbed. The first elution solution used was 0.01 M phosphate buffer, and the gel was centrifuged at 17 000 × *g* during 5 min in a Servall centrifuge at each step. All buffers contained 0.003 M mercaptoethanol. The majority of the enzyme activity was eluted at 0.03 M phosphate buffer with a small amount in the 0.05 M fraction. The supernatant solutions from the 0.03 and 0.05 M elution were pooled (0.9 mg protein/ml) and absorbed to DEAE-cellulose.

DEAE-cellulose. A column (1.5 cm × 10 cm) was prepared from washed DEAE-cellulose and equilibrated with 0.002 M phosphate buffer containing 0.003 M mercaptoethanol. Enzyme from the gel elution was absorbed, the column was washed with 5 ml of same buffer and a linear gradient from 0 to 0.5 M KCl in 0.002 M phosphate buffer at pH 6.8 containing 0.003 M mercaptoethanol was applied. The total gradient volume was 400 ml, and 5-ml fractions were collected. Six fractions with high specific activities (Tubes 38–43) were pooled and precipitated at 60% $(NH_4)_2SO_4$ saturation. The precipitated material was suspended in 0.1 vol. 0.025 M phosphate buffer containing 0.003 M mercaptoethanol and brought to 60% satn. with solid $(NH_4)_2SO_4$ (1.9 mg protein/ml). This preparation was stored at −20° over a period of months with no significant loss in activity.

The specific activity of the crude supernatant solutions after centrifugation at 105 000 × *g* ranged from 10–20. The specific activity of the final enzyme preparation was from 300–700. When enzyme was purified from cells grown in the presence of amino acids or from cells harvested after the end of growth, the extent of purification and recovery at each step was very similar. However, the loss of activity during storage between purification steps results in different final recovery of enzyme activity. A summary of a representative purification of the logarithmic-phase activity is shown in Table I. Also included for comparison are purifications of the activity from cells grown in the presence of amino acids and from cells harvested after the end of growth.

$(NH_4)_2SO_4$ preparations for inhibitor studies

Because of loss of lysine sensitivity during purification, some inhibitor studies were performed using crude extracts precipitated with $(NH_4)_2SO_4$. Packed cells from a 1-l lot of cells suspended in 20 ml 0.025 M phosphate buffer (pH 6.8) containing 0.003 M mercaptoethanol were sonicated for 2 min in 10-ml volumes. The broken cell suspension was centrifuged at 105 000 × *g* for 60 min, and the supernatant solution was brought to a volume of 30 ml with 0.025 M phosphate buffer. The precipitate

TABLE I

PURIFICATION OF ASPARTOKINASE

Protein concentrations and volumes are given in the text.

Preparation	*Glucose–salts (log phase)*		*Glucose–salts plus* L-*lysine*		*Glucose–salts plus* L-*lysine and* L-*threonine*		*Glucose–salts (post-log phase)*	
	Specific activity	*Yield (%)*	*Specific activity*	*Yield (%)*	*Specific activity*	*Yield (%)*	*Specific activity*	*Yield (%)*
Crude extract	9	100	—	—	—	—	—	—
Supernatant solution 105 000 × *g*	23	—	—	—	—	—	—	—
40–65% $(NH_4)_2SO_4$	41	100	36	100	15	100	3	100
50–55% $(NH_4)_2SO_4$	65	41	89	34	25	49	7	37
$Ca_3(PO_4)_2$	161	16	212	17	48	14	17	24
DEAE-cellulose	412	7	470	9	95	8	40	11

formed between 35–60% satn. with $(NH_4)_2SO_4$ was collected and resuspended in one third the original volume. The specific activity and inhibition by lysine and threonine were determined immediately.

Sampling after L-*lysine addition during growth*

A zero-time sample (80 ml) was removed at the middle of the growth phase (*A* was approx. 1.35). L-Lysine to 0.01 M was added to the culture immediately, and 80-ml samples were removed at 5, 15 and 30 min after the addition of lysine. Packed cells were suspended in 4 ml 0.025 M phosphate buffer (pH 6.8) and broken by sonication for 30 sec. The broken cell suspension was centrifuged at 105 000 × *g* for 60 min and the supernatant solution brought to a volume of 10 ml. The material precipitated between 0 and 60% saturation with $(NH_4)_2SO_4$ was suspended in 1 ml 0.025 M phosphate buffer (pH 6.8).

Sucrose-gradient centrifugation

Sucrose-density gradients were prepared and sampled by the method of BRITTEN AND ROBERTS[19] and as previously described[16].

Sephadex G-200 gel filtration

The enzyme from different cultures was filtered through a Sephadex G-200 (1.5 cm × 10 cm) column which was equilibrated and eluted with 0.025 M phosphate buffer containing 0.003 M mercaptoethanol. 1-ml fractions were collected.

Heat treatment

1-ml samples from $(NH_4)_2SO_4$ preparations were heated at 50° for 10 min. L-Lysine and/or L-threonine were present at 0.01 M. Samples were brought to 60% satn. with a saturated $(NH_4)_2SO_4$ solution, and the sedimented fraction was suspended in 1 ml 0.025 M phosphate buffer.

RESULTS

Feedback inhibition and repression of aspartokinase

In an effort to determine if the synthesis of aspartokinase of *B. licheniformis* were repressible, cells were grown in minimal salts medium containing 0.01 M L-lysine,

TABLE II

FEEDBACK INHIBITION AND REPRESSION OF ASPARTOKINASE

Enzyme from cells grown on:	*Phase*	*Specific activity**	*% Inhibition by***		
			1 mM L-*lysine*	*1 mM* L-*threonine*	*1 mM lysine and threonine*
Glucose–salts	log	86	60	0	81
Glucose–salts and 0.01 M L-lysine	log	79	0***	0	76
Glucose–salts and 0.01 M L-threonine	log	69	74	0	80
Glucose–salts and 0.01 M L-lysine and L-threonine	log	15	0***	0	32
Glucose–salts	post-log	5	0***	0	35

* 10 mM aspartate was present in the reaction mixture.
** 30 mM aspartate was present in the reaction mixture.
*** The inhibition remained 0% at 10 mM L-lysine.

L-threonine or both. The specific activities and the sensitivity to feedback inhibitors of the enzymes in the 35–60% $(NH_4)_2SO_4$ fraction are shown in Table II. Growth in the presence of L-lysine resulted in a complete loss of inhibition of aspartokinase by lysine. There was no significant decrease in specific activity. Growth in medium containing L-threonine did not appear to influence the specific activity of aspartokinase, but sensitivity to feedback inhibition by lysine was increased. Growth in the presence of either L-lysine or L-threonine did not influence the concerted inhibition of aspartokinase by lysine *plus* threonine. Growth in the presence of both L-threonine and L-

TABLE III

EFFECT OF GROWTH CONDITIONS ON INHIBITION OF ASPARTOKINASE BY L-THREONINE

30 mM aspartate was present in the reaction mixture and in all cases except the last, the 36–60% ammonium sulfate fraction was used (specific activities shown in Table I).

Enzyme from cells grown on:	*Phase*	*Inhibition by 10 mM* L-*threonine*	*Inhibition by 30 mM* L-*threonine*
Glucose–salts	log	2	15
Glucose–salts and L-lysine	log	12	54
Glucose–salts and L-threonine	log	4	9
Glucose–salts and L-lysine and L-threonine	log	9	29
Glucose–salts - purified enzyme	log	8	48

lysine resulted in a 6-fold decrease in specific activity. The aspartokinase in this case was slightly inhibited by lysine and inhibited only 32% by lysine and threonine in combination. The $(NH_4)_2SO_4$ preparations from post-log phase cells were low in aspartokinase activity. The aspartokinase was not inhibited by lysine and the sensitivity to lysine and threonine inhibition was slightly decreased. The percent inhibition at the concentrations of amino acids used are maximal with the exception of threonine. Inhibition by L-threonine alone at higher concentrations is shown in Table III. Aspartokinase from cells grown in L-lysine was more sensitive to threonine inhibition at high concentrations. Aspartokinase from cells grown in L-lysine and L-threonine and purified enzyme from cells grown in minimal salts were also more sensitive to threonine inhibition.

Cold sensitivity of aspartokinase

The loss of aspartokinase activity and lysine sensitivity during purification and storage of lysine-sensitive enzyme led us to investigate the cold sensitivity of our preparations during a 4-h incubation at 0°. The results are shown in Table IV. Lysine sensitivity of enzyme from cells grown in the glucose–salts medium or in the presence

TABLE IV

SENSITIVITY OF ASPARTOKINASE TO INCUBATION AT 0°

An average of 3 experiments. Reaction mixtures contained aspartic acid at 30 mM.

Enzyme from cells grown on:	*Phase*	*Specific activity*		*Inhibition (%) by*			
				1 mM L-*lysine*		*1 mM* L-*lysine and* L-*threonine*	
		0 h	*4 h*	*0 h*	*4 h*	*0 h*	*4 h*
Glucose–salts	log	96	64	52	28	86	75
Glucose–salts and L-lysine	log	107	105	0	0	83	84
Glucose–salts and L-threonine	log	90	49	75	45	80	75

of L-threonine was decreased in the $(NH_4)_2SO_4$ preparations. Aspartokinase activity was also decreased in these preparations. Enzyme from lysine-grown cells did not change when incubated at 0°. The loss of lysine sensitivity of extracts from cells grown in glucose salts or in the presence of threonine did not take place at room temperature. The loss of total activity and lysine sensitivity in the cold was not accompanied by a change in sensitivity to concerted inhibition by lysine and threonine.

Effect of L-*lysine addition during growth*

The effect of the addition of lysine to cells growing in glucose–salts is shown in Table V. While the culture continued to grow after the addition of lysine and the specific activity of aspartokinase continued to increase, the sensitivity to lysine inhibition decreased rapidly. The loss of lysine sensitivity in two experiments is shown in Fig. 2. There was no coincident decrease in the sensitivity of aspartokinase to

TABLE V

EFFECT OF L-LYSINE ADDITION DURING GROWTH

Time (min) after lysine addition	*Absorbance*	*Specific activity**	*Inhibition (%)*	
			1 mM L-*lysine*	*1 mM* L-*lysine and* L-*threonine*
Expt. 1				
0	1.40	118	50	83
5	1.60	141	41	81
15	1.70	158	27	83
30	1.95	154	13	82

* Assayed in the presence of 30 mM L-aspartic acid.

inhibition by lysine *plus* threonine. The addition of lysine and threonine did not alter lysine sensitivity of aspartokinase but did lead to a slow decrease in specific activity. The addition of lysine and threonine to cultures during harvesting did not lead to any alteration in lysine sensitivity.

Enzyme properties

None of the enzyme preparations when tested at a 2- or 70-fold purification level was retarded by Sephadex G-200. All preparations shown in Table II were protected against heat inactivation at 50° for 10 min when lysine and threonine were present at 0.01 M. Lysine or threonine alone did not protect the activity. Sucrose-density-gradient centrifugation of the purified aspartokinase from cells growing on the minimal medium is shown in Fig. 3. The enzyme activity was located in Fractions 5–6 in all experiments. As will be shown subsequently, this purified enzyme (70-fold) is insensitive to L-lysine inhibition. $(NH_4)_2SO_4$ preparations (2-fold purification and lysine sensitive) from the same cells were also examined by gradient centrifugation and the enzyme activity was

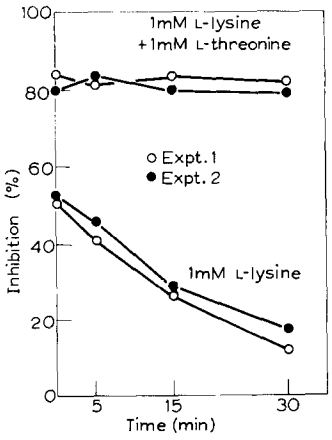

Fig. 2. The inhibition of aspartokinase by L-lysine or L-lysine *plus* L-threonine. Samples were taken before and 5, 15 and 30 min after the addition of L-lysine (0.01 M) to the culture.

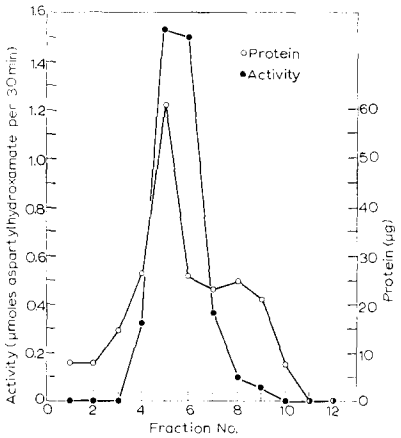

Fig. 3. Sucrose-density gradient of purified enzyme with a specific activity of 660. 30-drop samples were collected and assayed for enzyme activity and protein content as described under *Methods*.

again found in Fractions 5–6. Furthermore, enzyme from cells grown under the five conditions shown in Table II migrated during gradient centrifugation to the same position as indicated for the preparations above; Fractions 5–6.

These data, and the fact that all of the enzyme activities shown in Table II purify in a similar fashion (Table I), indicate that *B. licheniformis* produces only one aspartokinase, but that this activity is altered *in vivo* depending on the growth conditions.

The effect of lysine and/or threonine on activity of purified aspartokinase

The effects of increasing concentrations of lysine and/or threonine on aspartokinase activity of purified enzyme from log-phase cells grown in glucose–salts are shown in Fig. 4. Lysine inhibition in the presence of 1 mM threonine was maximal at 0.8 mM lysine. Threonine inhibition in the presence of 1 mM lysine was maximal at approximately the same concentration. Lysine or threonine alone were inhibitory only at much higher concentrations. Thus, lysine sensitivity is lost during the purification of the enzyme.

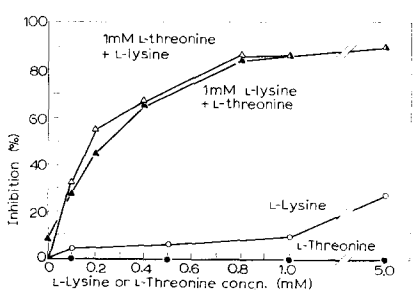

Fig. 4. The effect of L-lysine and L-threonine or both on inhibition of aspartokinase. Each reaction mixture contained 75 μg of enzyme of a specific activity of 330 and 30 mM L-aspartic acid.

The effect of substrate concentration on the purified aspartokinase activity

The effects of concentrations of aspartate and ATP on activity of partially purified enzyme are shown in Figs. 5 and 6. The reciprocal plots show normal Michaelis–Menten kinetics with an apparent K_m for aspartate of about 20 mM. The apparent K_m of ATP is about 1.6 mM. This value was not altered at concentrations of aspartate ranging from 10 to 40 mM.

The kinetics of inhibition of the purified aspartokinase by L-*lysine and* L-*threonine*

The effect of L-lysine and L-threonine on the apparent K_m is shown in Fig. 5. The data indicate that the inhibition of aspartokinase by lysine and threonine is

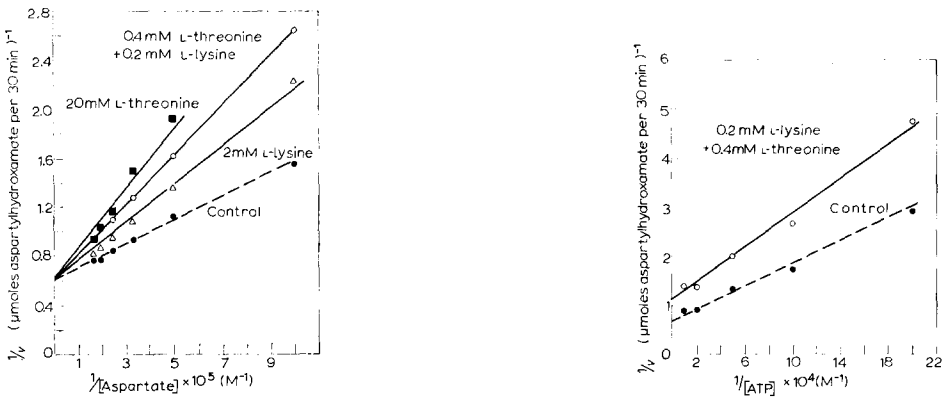

Fig. 5. Double reciprocal plots of reaction rates and aspartate concentrations in the presence of 10 mM ATP and feedback inhibitors as indicated. 75 μg of enzyme of specific activity of 330 was present in the reaction mixture.

Fig. 6. Double reciprocal plots of reaction rates and ATP concentrations in the presence of 30 mM L-aspartic acid and feedback inhibitors as indicated. 75 μg of enzyme of specific activity of 330 was present in the reaction mixture.

apparently competitive. Lysine and threonine in combination are also apparent competitive inhibitors of aspartokinase. The effect of lysine and threonine on the apparent K_m of ATP is shown in Fig. 6. Both lysine and threonine were apparent noncompetitive inhibitors.

The effect of growth conditions on kinetic properties of the purified aspartokinase

Purified enzyme from post-log phase cells and from cells grown in the presence of L-lysine, or L-lysine and L-threonine were examined at increasing concentrations of aspartate or ATP. There was no significant difference of the apparent K_m of ATP or aspartate (Table VI) when purified aspartokinase preparations from different growth conditions were compared.

The inhibition of aspartokinase by ADP and AMP

The effects of increasing concentrations of ADP and AMP are shown in Table VII. ADP was completely inhibitory at 30 mM. The inhibitor effect of ADP was dependent on the absolute concentration of ADP rather than the ratio of ADP to ATP. Double

TABLE VI

EFFECT OF GROWTH CONDITIONS ON KINETICS OF ASPARTOKINASE

Enzyme from cells grown on:	*Phase*	*Apparent $K_{m\ aspartate}$ (mM)*	*Apparent $K_{m\ ATP}$ (mM)*
Glucose–salts	log	20	1.0
Glucose–salts and L-lysine	log	25	1.2
Glucose–salts and L-lysine and L-threonine	log	24	1.6
Glucose–salts	post-log	16	

* Determined in the presence of 10 mM ATP.
** Determined in the presence of 30 mM aspartic acid.

reciprocal plots (not shown) of reaction rates and aspartate concentrations in the presence and absence of ADP revealed that inhibition of aspartokinase by ADP was noncompetitive.

DISCUSSION

We have demonstrated that growth of *B. licheniformis* in the presence of lysine yielded an enzyme which was no longer sensitive to inhibition by lysine but was fully sensitive to lysine and threonine in combination. There was no decrease in specific activity of aspartokinase. The aspartokinase of these preparations was more sensitive to threonine inhibition at high concentrations (10–30 mM). Growth in the presence of threonine also did not result in a decreased specific activity of enzyme but yielded an enzyme with increased sensitivity to lysine inhibition and normal inhibition by lysine *plus* threonine. Growth in the presence of lysine *plus* threonine decreased the specific activity of aspartokinase, and the enzyme was not sensitive to lysine inhibition and was inhibited only 32% by lysine and threonine in combination. Alteration in feedback-inhibition properties was also found to take place as the culture went out of log phase. Enzyme of low activity from post-log cells was not sensitive to lysine inhibition, and

TABLE VII

INHIBITION OF ASPARTOKINASE BY ADP AND AMP

Expt. 1		*Expt. 2*		*Expt. 3*		
*mM ADP**	*Inhibition (%)*	*mM AMP**	*Inhibition (%)*	*mM ADP*	*mM ATP*	*Inhibition (%)*
0	0	0	0	2	2	13
3	35	3	10	4	4	38
5	52	5	20	6	6	48
10	68			8	8	52
20	81	20	41			
30	99	30	53			

* ATP concentration at 10 mM.

sensitivity to inhibition by lysine and threonine was decreased. Aspartokinase lost lysine sensitivity during purification and was found to have increased sensitivity to inhibition by threonine. However, purified aspartokinase was fully sensitive to inhibition by lysine and threonine.

That the feedback inhibition of aspartokinase of *B. licheniformis* was not a typical case of concerted feedback inhibition was suggested by the finding of STAHLY AND BERNLOHR[7] that the aspartokinase activity of crude extracts was inhibited 50–60% by lysine alone. Our demonstration that enzyme preparations from cells grown in the presence of the end products, lysine or threonine have different patterns of feedback inhibition might suggest either a multiple-enzyme system similar to that in *E. coli* or an alteration *in vivo* of a single enzyme under different physiological conditions.

Our best evidence for enzyme alteration during growth in the presence of lysine, rather than a derepression of a lysine-insensitive aspartokinase, is the finding that lysine sensitivity is lost after addition of lysine to a growing culture. The loss of lysine sensitivity observed was far too rapid to be accounted for by synthesis of a new lysine-insensitive enzyme, the half-time of desensitization being about 15 min, while the generation time under these conditions is 60 min. In addition, the inoculation of cells containing lysine-insensitive enzyme into minimal medium produces lysine-sensitive enzyme within one generation (unpublished data).

In *B. licheniformis* there is no aspartokinase specifically inhibited by low levels of threonine as there is in *E. coli*. Some of our data could be interpreted as evidence for the presence of two enzymes: one inhibited by lysine and a second inhibited by lysine *plus* threonine. When cells were grown in the presence of lysine, lysine sensitivity of aspartokinase was lost, but the concerted effect of lysine and threonine was unchanged. In addition, enzyme from log-phase cells grown in glucose–salts medium lost lysine sensitivity during purification while inhibition by lysine and threonine was not altered. We would expect that if two enzymes were present, the inhibitory effect of lysine alone and lysine and threonine in combination would be additive. However, the loss of lysine sensitivity in every instance has not resulted in any alteration to concerted feedback inhibition. We might also expect that a lysine-sensitive enzyme would be protected by lysine against heat inactivation. However, under conditions when lysine-sensitive aspartokinase was effectively protected by lysine and threonine, lysine did not protect.

We have been unable to show a difference in properties, other than feedback control, of any enzyme preparation during purification or by other physical means. One exception was the difference in cold sensitivities. STAHLY AND BERNLOHR[7] have reported that the aspartokinase of crude extracts stored at −20° were less sensitive to inhibition by lysine. We observed that aspartokinase from cells grown in the presence of lysine was completely stable over a 4-h period at 0°. Aspartokinase from cells grown in the glucose–salts medium or in the presence of threonine lost activity during standing at 0° and at the same time lost lysine sensitivity. Cold sensitivity, therefore, appears to correlate in some way with lysine sensitivity. Perhaps the conformation of the lysine-insensitive aspartokinase is a more cold-stable structure.

In all cases where purified enzymes were studied, it was shown that the apparent K_m for aspartate and ATP were similar regardless of feedback-inhibition properties. This was also true of the K_m for aspartate of lysine-sensitive and lysine-insensitive aspartokinase in $(NH_4)_2SO_4$ preparations.

Our evidence suggests that there is a single enzyme, the feedback-inhibition properties of which can be markedly altered by growth in the presence of end products or by the termination of growth of the cell. To our knowledge this is the first report of such a specific conversion *in vivo*, although alterations in glutamine synthetase show some similarity[12,13].

In *B. licheniformis* a rapid decrease in aspartokinase activity occurs at the end of growth[7]. The decrease observed is too rapid to be accounted for by protein turnover, but aspartokinase may be specifically degraded or inactivated. We favor a hypothesis invoking inactivation based on the finding that threonine dehydratase of decreased activity does not show a coincident decrease in enzyme protein[16]. We have made repeated efforts to obtain antisera with precipitating activity for aspartokinase without success. Therefore, we have been unable to extend the findings in the case of threonine dehydratase to that of aspartokinase. It is possible that the enzyme exists in two or more active forms *in vivo*. An alteration of this type *in vitro* has been reported for the malate dehydrogenase of *B. subtilis*[20]. Growth of *E. coli* in medium containing glutamate or ammonia has been shown to result in glutamine synthetase of altered properties[10–13]. Growth of yeast in the presence or absence of O_2 leads to the activation or inactivation of cytochrome *c* peroxidase[21]. It has been proposed that the aspartokinase of *B. polymyxa*[22] assumes four different allosteric states *in vitro*. In the case of the *B. polymyxa*[3,23] and *Rh. capsalatum*[6] aspartokinases, inhibition by lysine alone was not observed, although purified preparations were used in both cases. As reported here, lysine sensitivity is lost during purification of the *B. licheniformis* enzyme.

Concerted feedback inhibition does not allow the regulation of the first enzymatic step in a branched pathway by individual end products[2]. Since the aspartokinase of *B. licheniformis* is very sensitive to inhibition by lysine alone, the synthesis of both methionine and threonine could be diminished by excessive production of lysine. However, the desensitization of aspartokinase to lysine inhibition when lysine is in excess could allow continued production of intermediates of the pathway. This type of desensitization control would regulate lysine production by the concerted feedback effect and would insure that the accumulation of lysine would not decrease the production of other end products of the pathway. When lysine and threonine are in excess during growth, the reduction of aspartokinase activity and synthesis may be very effective.

The rapid loss of enzyme activity at the end of growth was initially observed in studies on the relationship between activity and sporulation in *B. licheniformis*. From the results reported here, it is clear that changes in the total activity and the properties of the enzyme are closely related to the physiology of the cell. As the phenomenon of sporulation is coincident with changes in nutrition and physiology, it is not possible to assume that the decrease of specific activity and desensitization to lysine inhibition are integral parts of the sporulation process. However, the unusual plasticity of the aspartokinase may represent a more optimal type of control in a developing procaryot and may allow growth and sporulation to proceed in the absence of multiple genomes for a single activity.

ACKNOWLEDGMENTS

We thank Miss VIRGINIA CLARK for excellent technical assistance. This investi-

gation was supported by grant GB-7347 from the National Science Foundation and grant AI-05096 from the U.S. Public Health Service. R. W. B. is the recipient of Research Career Development Award GM-K3-7709.

REFERENCES

1 E. R. STADTMAN, G. N. COHEN, G. LEBRAS AND H. DE ROBICHON-SZULMAJSTER, *J. Biol. Chem.*, 236 (1961) 2033.
2 E. R. STADTMAN, *Advan. Enzymol.*, 28 (1966) 85.
3 H. PAULUS AND E. GRAY, *J. Biol. Chem.*, 239 (1964) 4008.
4 P. DATTA AND H. GEST, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 1004.
5 R. MIYAJIMA, S. OTSUKA AND I. SHIIO, *J. Biochem. Tokyo*, 63 (1968) 139.
6 P. DATTA AND L. PRAKASH, *J. Biol. Chem.*, 241 (1966) 5827.
7 D. P. STAHLY AND R. W. BERNLOHR, *Biochim. Biophys. Acta*, 146 (1967) 467.
8 J. MANDELSTAM, *J. Gen. Microbiol.*, 11 (1954) 426.
9 J. SZULMAJSTER, *Bull. Soc. Chim. Biol.*, 46 (1964) 443.
10 D. MECKE AND H. HOLZER, *Biochim. Biophys. Acta*, 122 (1966) 341.
11 H. S. KINGDON AND E. R. STADTMAN, *Biochem. Biophys. Res. Commun.*, 27 (1967) 470.
12 H. S. KINGDON AND E. R. STADTMAN, *J. Bacteriol.*, 94 (1967) 949.
13 H. HOLZER, H. SCHUTT, Z. MASEK AND D. MECKE, *Proc. Natl. Acad. Sci. U.S.*, 60 (1968) 721.
14 J. BECKET AND J. M. WIAME, *Biochem. Biophys. Res. Commun.*, 21 (1965) 226.
15 J. J. FERGUSON, JR., M. BOLL AND H. HOLZER, *European J. Biochem.*, 1 (1967) 21.
16 C. LEITZMANN AND R. W. BERNLOHR, *Biochim. Biophys. Acta*, 151 (1968) 461.
17 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
18 R. W. BERNLOHR, *J. Bacteriol.*, 93 (1967) 1031.
19 R. J. BRITTEN AND R. B. ROBERTS, *Science*, 131 (1960) 32.
20 W. H. MURPHY AND N. O. KAPLAN, *J. Biol. Chem.*, 242 (1967) 1560.
21 A. A. SELS AND C. CORIAMONT, *Biochem. Biophys. Res. Commun.*, 32 (1968) 192.
22 H. PAULUS AND E. GRAY, *J. Biol. Chem.*, 242 (1967) 4980.
23 H. PAULUS AND E. GRAY, *J. Biol. Chem.*, 243 (1968) 1349.