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CONTROL OF ASPARTOKINASE DURING DEVELOPMENT OF *BACILLUS LICHENIFORMIS*

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SUMMARY

Extracts prepared from late exponential phase *Bacillus licheniformis* cells contain an aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) activity that is 4-fold greater than that from early exponential phase cells. This increase occurs in cells grown on several different media. After growth has ceased, the specific activity decreases at a rate much greater than would be expected by turnover.

The enzyme activity is subject to feedback inhibition by L-lysine and L-aspartic-β-semialdehyde. L-Threonine has no effect on the activity when added alone but produces a concerted inhibition with L-lysine. The aspartic-β-semialdehyde effect may be an example of sequential control.

INTRODUCTION

During the process of bacterial sporulation in *Bacillus licheniformis*, spores are formed from vegetative cells at the expense of endogenous nutrients[1]. Since this microorganism does not contain detectable amounts of carbohydrate or lipid polymers, it is thought that intracellular degradation of protein followed by the oxidation of amino acids is necessary to provide the sporulating cell with monomers and energy for spore synthesis[1,2]. Under these conditions, the *in vivo* function of amino acid biosynthetic enzymes is ambiguous. Some are not required by the sporulating cell[2], and thus, the presence of these enzymes may be detrimental in that their operation requires energy, and unneeded metabolites are formed[3].

Considerable information already exists concerning the control of biosynthetic enzymes. The *in vivo* function of feedback inhibition of allosteric enzymes has been emphasized[4], but models previously proposed have not been concerned with the process of development. Because of the unique problem faced by developing systems

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in regard to control of biosynthetic enzymes and because *B. licheniformis* appears to reverse the role of amino acids upon initiating sporulation metabolism[2], we felt that it would be especially worthwhile to investigate one such system.

This report outlines our findings concerning the enzyme aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4). Aspartokinase is the first enzyme on the pathway of the aspartic acid family of amino acids (lysine, methionine, threonine and isoleucine)[5], and catalyzes the phosphorylation of aspartic acid with ATP, forming β-aspartyl phosphate and ADP. In *Escherichia coli*, there are at least two separately controlled aspartokinases, but this does not appear to be the case in Bacillus. The enzyme has been shown to have allosteric characteristics in *B. polymyxa*[6], and purification of the activity from *B. licheniformis*[7], shows only one enzyme with the properties reported here. A preliminary report of some of our work has been presented previously[8].

METHODS AND MATERIALS

Culturing of the microorganism

B. licheniformis A-5, was used throughout this study. Unless otherwise stated, the medium and conditions employed in culturing the organism were those specified previously[9], with the only modification being that the salts mixture included 0.1 g $CaCl_2 \cdot 2\ H_2O$ per l of medium.

Preparation of cell-free extracts

The cells were harvested at the desired time by centrifugation for 5 min at 10 000 × *g* at 0°, washed with buffer (5 mM Tris (pH 8.0); 1 mM mercaptoethanol; 1 mM $MgSO_4$; 0.1 M KCl), centrifuged again, and resuspended in the same buffer. The cells were broken either by passage through an Aminco-French pressure cell or by sonic oscillation (1.9-cm diameter probe of a 20 kcycles MSE sonic oscillator). After removal of whole cells and cell debris by centrifugation at 10 000 × *g* for 15 min, the supernatant solution was extensively dialyzed against the previously mentioned buffer. The extracts were either assayed immediately after dialysis or were stored at −20°.

Aspartokinase assay

The aspartokinase assay employed was that of STADTMAN *et al.*[5] except that mercaptoethanol was omitted. Incubation was at 37° for 30 min. The assay involves trapping of the product, aspartyl phosphate, as the hydroxamate and detection of this compound by reaction with the ferric chloride reagent of LIPMANN AND TUTTLE[10]. The amount of aspartyl hydroxamate formed was calculated from a standard curve established with synthetic DL-aspartic acid hydroxamate (Sigma Chemical). Specific activity is defined as mμmoles of aspartyl hydroxamate formed per min per mg of protein.

Synthesis of aspartic β-semialdehyde

Both L- and DL-aspartic-β-semialdehyde were synthesized by the methods outlined by BLACK AND WRIGHT[11]. Hog kidney acylase used in one of the steps of this procedure was purchased from Nutritional Biochemical. Aspartic-β-semialdehyde

was assayed enzymatically with homoserine dehydrogenase prepared from *E. coli* $-K_{12}$ that had been grown in a minimal medium[12]. Extracts of *E. coli* were prepared in the same manner as those of *B. licheniformis* except that a different buffer (5 mM Tris (pH 7.0), 1 mM mercaptoethanol, 0.1 M KCl) was employed. The aspartic-β-semialdehyde was stored as a solution in 4 M HCl at $-20°$ and was neutralized prior to utilization.

Resolution of radioactive amino acids

Samples of cells, pre-labeled with radioactive amino acids, were precipitated and washed with 5% trichloroacetic acid at 0°, followed by 2 washes with water. The cells were taken up in 6 M HCl, sealed under vacuum in a glass tube and heated at 105° for 24 h. The resultant hydrolysate was evaporated to dryness repeatedly in a desiccator and dissolved in water. Duplicate samples were spotted on 10 inch × 10 inch sheets of Whatman No. 3 MM paper, and resolved by high voltage electrophoresis in a Pherograph DBGM-1713858 instrument, using a pyridine, acetate buffer at pH 5.2. After drying the sheets, chromatography was performed in the other direction using either a methanol–pyridine–H_2O (40:10:1, by vol.) or a *n*-butanol–acetate–H_2O (12:3:5, by vol.) system on duplicate samples. After identifying the areas of the amino acids by spraying with 0.2% ninhydrin in butanol, strips were cut from the chromatograms and the radioactivity was determined with a Baird Atomic Strip Scanner Model PSC 363.

Materials

All chemicals were reagent grade and were purchased from commercial sources. The L-amino acids were chromatographically pure. Uniformly labeled L-[^{14}C]aspartic acid was purchased from Nuclear Chicago.

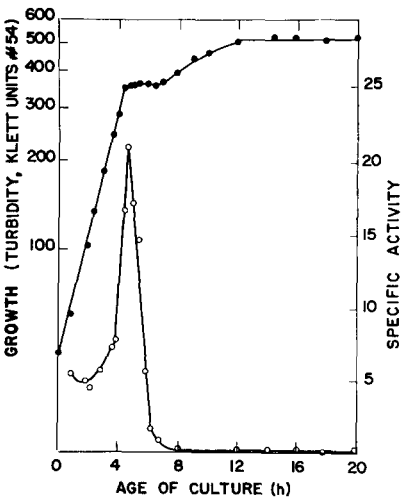


Fig. 1. The specific activity in mμmoles/min per mg protein, of aspartokinase in extracts of cells at various stages of development. At the indicated times, 250-ml samples of cells were harvested and extracts prepared as described in METHODS AND MATERIALS. The closed circles (●) are growth and the open circles (○), specific activity.

RESULTS

Aspartokinase activity during life cycle

The apparent specific activity of the aspartokinase in extracts of cells collected at various stages of growth and sporulation was determined. Fig. 1 shows the results of a typical experiment. In this case, growth was complete at about 4 h, spores first appeared at 14 h and 50% sporulation had occurred by 20 h. During late exponential phase growth, the aspartokinase activity increased approx. 4-fold and after reaching this peak, rapidly decreased to a non-detectable level. Essentially the same pattern of enzyme activity *vs.* culture age occurred whether the cells were grown on the normal medium containing glucose (20 mM) and ammonium lactate (50 mM) (Fig. 1) or on glutamate (40 mM), or pyruvate (20 mM) and NH_4Cl (50 mM). In each case the peak in activity occurred just as growth was complete and probably just as the main carbon source was exhausted. In the case of glucose-grown cells, it has been shown that cessation of growth coincides in time with exhaustion of the glucose from the medium[9]. When cells were grown in this normal glucose–ammonium lactate medium by continuous culture to a stage at which the aspartokinase activity was high, and then maintained at a steady state with fresh normal medium, the activity in crude extracts obtained from cells sedimented 2 or more h later was very low (<2.5 mμmoles/min per mg protein). Substitution of 12 mM glucose for the 20 mM glucose in the medium reservoir resulted in a maintainance of enzyme activity at a high level (15–20 mμmoles/min per mg protein). It thus appears probable that attainment of a low level of the carbon source is necessary for the observed increase in activity.

Although biosynthetic enzymes as a rule are under repression control, it seemed possible that this or a separate aspartokinase was induced when glucose was nearly exhausted from the medium. The substrate, aspartate, might be present in the intracellular pool at a higher concentration than normal when glucose is nearly gone, since the citric acid cycle becomes activated as glucose disappears from the cells[13], and aspartate could then be derived from oxaloacetate by the action of glutamate oxaloacetate transaminase. Alternatively, some unique physiological situation based on glucose catabolism may be responsible for an allosteric inactivation–reactivation of the enzyme activity[14]. These possibilities were ruled out by the finding that growth on glutamate or pyruvate gave the same pattern of aspartokinase activity *vs.* stage of development as did growth on glucose–ammonium lactate. Under these conditions of growth, the citric acid cycle must be operative in order to obtain sufficient energy for growth. More convincing than the above is the fact that supplementation of the normal medium with 0.01 M aspartate did not increase the enzyme activity during early exponential growth.

The increase in aspartokinase activity could presumably be due to derepression rather than to induction. To determine whether the enzyme was subject to end product repression, L-lysine, L-threonine, L-methionine, L-isoleucine, L-valine, and L-leucine, each at 0.01 M, were included in the normal growth medium. The specific activity in extracts taken at the end of the logarithmic phase was decreased to 50%, whereas the activity from early logarithmic phase cells was unaffected. However, a change in the amount of enzyme protein was not measured so it is not possible to definitively consider this a derepression (see DISCUSSION).

The other facet of the pattern of aspartokinase activity *vs.* stage of development that needs elucidation is the rapid decrease in activity after the peak has been obtained. Since growth is no longer occurring at this time, the decrease is obviously more rapid than can be accounted for by the dilution that would occur with protein turnover. One could hypothesize that the decrease is due to protease activity. Indeed, an active extracellular protease is induced by *B. licheniformis* at this time[15] that is catabolite repressed during growth on the normal medium[16]. If the decline in activity were due to protease activity, one would expect that addition of 12-h extract to an extract exhibiting high activity would render the latter extract inactive or at least cause a decrease in its activity. No such inactivation was observed.

Another possible explanation for the apparent lack of aspartokinase activity in extracts of pre-sporulating cells would involve an activity operating at a pH different from pH 8.1, the value at which the assay was conducted. However, no activity was detected in extracts of presporulating cells at pH values extending from pH 6.0 to 9.0.

The apparent lack of aspartokinase activity during pre-sporulation was particularly surprising since it is possible that the synthesis of dipicolinic acid, that substance in spores thought to be necessary for heat resistance, is dependent on the activity of aspartokinase[17,18]. Experiments were performed to demonstrate the presence of aspartokinase activity in pre-sporulating and sporulating cells by observing the *in vivo* utilization of [^{14}C]aspartate for the synthesis of its product amino acids. An excess of [^{14}C]aspartate was added to separate cultures during early exponential growth and during pre-sporulation. After sufficient time had elapsed for incorporation of the isotope, the protein was precipitated with 5% trichloroacetic acid, hydrolyzed with 6 M HCl, and the radioactivity of the amino acids was determined following resolution by high voltage electrophoresis and paper chromatography. From the data obtained, it appears that aspartokinase activity must be present during pre-sporulation, since aspartate is converted by post-logarithmic phase cells to several of the end product amino acids and to dipicolinic acid. The extent of this conversion during pre-sporulation was roughly 1/4 of that occurring during growth. Thus, the radioactive amino acid interconversion data are consistent with a significant decrease in aspartokinase activity during pre-sporulation but not with its disappearance. Recently, it has been possible to detect the activity in extracts of presporulating cells prepared by very short (15 sec) sonication times[7], suggesting that the enzyme may be more unstable during this period.

Feedback control of aspartokinase activity

Aspartokinase activity resident in extracts prepared from late exponential phase (OD 300-320) cells of *B. licheniformis* was assayed in the presence of various end products and intermediates of the aspartic acid family of amino acids, in order to determine the spectrum of control by feedback inhibition (Table I). Of the single amino acids, only aspartic-β-semialdehyde and lysine produced significant inhibition (67 and 50% respectively). A combination of lysine and aspartic-β-semialdehyde resulted in 82% inhibition, a greater extent of inhibition than was produced by either amino acid separately. This inhibition, not being complete, is apparently an example of cumulative feedback inhibition ($(A + B) < (A) + (B)$) seen originally with glutamine synthetase[19]. When the combined inhibitory effect of lysine and

TABLE I

FEEDBACK INHIBITION ON THE *B. licheniformis* ASPARTOKINASE ACTIVITY

*Compounds tested**	*Inhibition*** (%)
L-Lysine + DL-aspartic-β-semialdehyde	82
DL-Aspartic-β-semialdehyde	67
DL-Aspartic-β-semialdehyde + L-homoserine	65
L-Lysine	50
L-Lysine + L-threonine + L-methionine	64
L-Lysine + L-threonine	62
L-Lysine + L-threonine + L-methionine + L-isoleucine	56
L-Lysine + L-homoserine	53
L-Lysine + L-methionine	52
L-Lysine + L-isoleucine	51
L-Threonine + L-methionine	19
L-Threonine + L-homoserine	15
L-Isoleucine + L-homoserine	13
L-Threonine	12
L-Threonine + L-isoleucine	12
L-Methionine + L-homoserine	11
L-Methionine + L-isoleucine	<10
L-Methionine	<10
L-Homoserine	<10
L-Valine	<10
L-Leucine	<10
L-Isoleucine	<10

* All amino acids were added at a concn. of 10 mM except DL-aspartic-β-semialdehyde which was added at a concn. of 26 mM.

** Average of 3 experiments.

aspartic-β-semialdehyde was tested at varying concentrations of either of these amino acids, no concerted diminution of the enzyme activity was observed. At low concentrations the inhibition appeared to approach an additive value while at higher concentrations this became cumulative, as mentioned above. Inclusion of threonine with lysine only slightly enhanced the extent of inhibition observed with lysine alone, while the other combinations including lysine produced approximately the same inhibition as lysine alone. It appears that in cell extracts, the *B. licheniformis* aspartokinase is inhibited by lysine and aspartic-β-semialdehyde with very little effect being observed with threonine either alone or in combination with lysine. It has been reported[6] that the aspartokinases of *B. subtilis* and *B. polymyxa* were subject to a concerted threonine–lysine feedback inhibition. No inhibition occurred with either amino acid alone except at very high concentrations. Therefore, we were prompted to look more closely at the lysine effect and for concerted threonine–lysine inhibition in our enzyme preparations.

Table II shows the effect of storage on aspartokinase activity and on its sensitivity of lysine and aspartic-β-semialdehyde inhibition. Specific activity decreased markedly during storage at 0° or −20°. This loss in activity could not be prevented by storage at pH 6.75 rather than pH 8.0 as was reported to be the case with *E. coli* aspartokinase[5]. Storage of the enzyme also resulted in a partial desensitization to lysine inhibition, while no such effect occurred with aspartic-β-semialdehyde. After storage of the enzyme under the conditions indicated, lysine inhibition of the remaining activity is decreased to about 50% of that of fresh cell-free extracts.

TABLE II

THE EFFECT OF STORAGE OF CRUDE CELL-FREE EXTRACT ON THE ACTIVITY OF ASPARTOKINASE AND ITS SUSCEPTIBILITY TO FEEDBACK INHIBITION

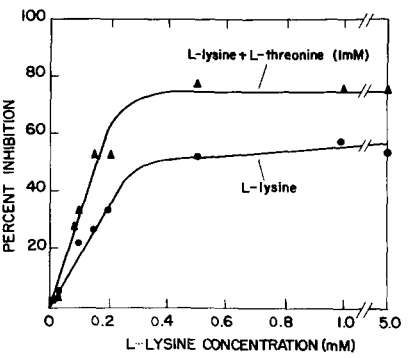
Expt. No.	*Time of storage of cell-free extract*	*Specific activity of control (mμmoles/min per mg protein)*	*Inhibition (%) by**	
			L-*Lysine*	DL-*ASA*
1	0 days	18.0	50	69
	3 days at −20°	11.5	37	61
2	0 days	21.3	50	68
	3 days at 0°	10.9	26	65
3	0 days	6.17	60	—
	2 days at 0°	4.63	24	—

Abbreviation: DL-ASA, DL-aspartic-β-semialdehyde.
* L-Lysine was added at 10 mM and DL-aspartic-β-semialdehyde at 26 mM.

The sensitivity to aspartic-β-semialdehyde, on the other hand, appears to be relatively unaffected by storage. These data suggest that there are separate sites on the enzyme for lysine and aspartic-β-semialdehyde.

When lysine, at a concn. of 10 mM, was added to the partially desensitized enzyme, an inhibition of about 25% occurred. No inhibition was observed with threonine alone. However, inclusion of lysine and threonine simultaneously resulted in about 50–55% inhibition, thus giving evidence for concerted threonine–lysine feedback inhibition in our enzyme.

Efforts were next made to examine fresh extracts more carefully for threonine–lysine concerted feedback inhibition. The data in Fig. 2 indicate that lysine produced a maximum inhibition of about 55%, half maximal inhibition occurring at a concn. of 0.16 mM. Threonine (1 mM) produced no inhibition when supplied alone. In combination with lysine at low concentration, however, a definite enhancement of



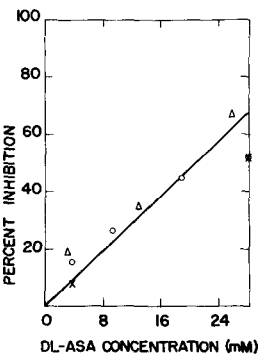


Fig. 2. Concerted feedback inhibition of the *B. licheniformis* aspartokinase by L-lysine and L-threonine. Details are presented in the text.

Fig. 3. DL-Aspartic-β-semialdehyde inhibition of aspartokinase activity. Except for the addition of DL-aspartic-β-semialdehyde as indicated, the assay conditions were as described in the text. The circles, triangles, and squares represent results of 3 separate experiments in which different enzyme preparations were utilized.

inhibition occurred. No greater inhibition occurred if threonine was added at a concn. of 10 mM. Thus, the aspartokinase of *B. licheniformis* is similar to those of *B. subtilis* and *B. polymyxa*[6] in that lysine–threonine concerted feedback inhibition is observed. The enzymes in the latter 2 organisms differ from ours, however, in that they are not inhibited by lysine alone except at very high concentrations.

The effect of differing concentrations of aspartic-β-semialdehyde on aspartokinase activity is presented in Fig. 3. As can be seen, the aspartic-β-semialdehyde must be present in high concentration in order to observe significant inhibition. Use of L-aspartic-β-semialdehyde instead of DL-aspartic-β-semialdehyde (at 1/2 the concn. of the DL-aspartic-β-semialdehyde) did not result in a greater extent of inhibition. Thus, it can be inferred that about 10 mM L-aspartic-β-semialdehyde is required to produce a 50% inhibition.

It has been reported[20] that the *Salmonella typhimurium* α-isopropylmalate synthetase, the first enzyme in the pathway to leucine, possesses a pH optimum for leucine inhibition which is different from that for enzymatic activity. At pH 8.5 (optimal for activity) it required over 30 times as much leucine for 50% inhibition as was required at pH 6.5 (optimal for leucine inhibition). To determine whether an analogous situation existed with aspartic-β-semialdehyde inhibition of *B. licheniformis* aspartokinase, enzymatic activity and L-aspartic-β-semialdehyde inhibition (20 mM) were determined at various pH values between 6.0 and 9.0. The enzyme appeared to be of approximately the same sensitivity to L-aspartic-β-semialdehyde at all pH values.

DISCUSSION

WRIGHT has elegantly discussed the relationships between apparent enzymatic activities and the life cycle in developing systems[21]. These relationships can be summarized as follows: (1) An increase or decrease in activity can be correlated with an *in vivo* change in the rate of product formation; (2) At substrate concentrations below saturating levels, product formation is a function of substrate concentration, at constant enzyme concentration; and (3) Inhibitors may accumulate that cause an *in vivo* decrease in product formation even under conditions in which enzyme and substrate increase.

It is premature to attempt to relate the control of aspartokinase with its *in vivo* function. This is especially true as this enzyme has been shown to be allosterically affected in several members of the genus Bacillus. Thus, the aspartokinase of *B. licheniformis* is inhibited by lysine alone, by concerted threonine–lysine inhibition, and by aspartic-β-semialdehyde. Recently[22], an aspartic-β-semialdehyde inhibition of the single aspartokinase from *Rhodopseudomonas spheroides* has also been observed. This type of inhibition, *i.e.* inhibition by a branch-point metabolite, has been reported in the aromatic amino acid pathway of *B. subtilis*[23]. In this case, prephenic and chorismic acids were shown to be potent inhibitors of the first enzyme in the biosynthetic pathway, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthetase. The aromatic pathway of *B. subtilis* was shown to be comprised of units of control which interact in a sequential fashion so that the action of a terminal branch feedback inhibitor (amino acid end products) sequentially initiates the action of another feedback inhibitor (intermediate) which inhibits the first enzyme in the pathway[23,24].

Whether a similar situation involving sequential inhibition exists in the aspartate pathway in *B. licheniformis* remains to be determined. The observed aspartic-β-semialdehyde inhibition suggests that this pattern of control may be present. It should be indicated that in another sporulating organism, yeast, aspartokinase is inhibited by homoserine, a branch point metabolite between methionine and threonine[25].

The level of aspartokinase activity at different stages of development was shown to vary drastically. As the carbon source was depleted from the medium, the activity increased at least 4-fold. This peak in activity was followed by a rapid decline in activity. The rate of activity decrease was greater than would be expected by dilution due to normal protein turnover. Evidence has been presented[3] that in *Saccharomyces cerevisiae* additions of arginine to the medium resulted in production of a specific regulatory binding protein for ornithine transcarbamylase, resulting in a rapid disappearance of enzyme activity in permeabilized cells. In addition, a number of other systems seem to fit this same pattern that has been termed 'inactivation-repression'[14]. In Bacillus the facilitated disappearance of lysine decarboxylase has been observed[26], as has isocitric dehydrogenase[27]. These data led to the idea that if certain end products were caused to increase in concentration in the intracellular pools of *B. licheniformis* cells at the time of growth cessation, a similar 'binding protein' might be produced which would cause the observed rapid decline in activity. However, if this were the case, one would expect that addition of pre-sporulating cell 'inactive enzyme' to an enzyme preparation of high activity would render the latter inactive or at least reduce its activity. As indicated previously, no such effect was observed. Although it is unlikely that we are observing the effect of a regulatory binding protein, the possibility cannot be dismissed. If the regulatory proteins were stable in cell-free extracts only when bound to their specific enzyme, then no effect would be expected in the mixed extract experiment. An alternative proposal to the above would assume that a metabolite present in actively growing cells is necessary to prevent an inactivation of the aspartokinase. In this regard, the threonine dehydratase of *B. licheniformis*[28] is inactivated with kinetics similar to those presented here for the aspartokinase. Experiments with antiserum prepared against purified threonine dehydratase have demonstrated that the activity changes during the life cycle but the amount of enzyme protein remains constant. Thus, it is possible that the aspartokinase activity is controlled during conmittment to sporulation by a mechanism that does not involve protein synthesis or degradation.

In any case, developing cells seem to have a mechanism for eliminating enzymatic activites that appear to function during growth but are superfluous or even detrimental during differentiation. Further study of allosteric enzymes in this context may allow an evaluation of the utility of such plastic systems on a much larger scale.

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