Aspartokinase of an Extreme Thermophile *Thermus flavus* Partial Purification and Some Properties

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Aspartokinase (ATP: L-aspartate 4-phosphotransferase) was extracted and partially purified 11-fold from an extreme thermophile, *Thermus flavus* AT-62. The enzyme has a temperature optimum near 75°C and a pH optimum of 7 to 8. The enzyme activity was feed-back inhibited 80% by L-threonine at the concentration of 0.1 mM at 60°C. No concerted effect of L-threonine with any other aspartate family amino acids was observed. The aspartokinase and homoserine dehydrogenase activities were eluted at different concentrations of KCl from DEAE-cellulose column. The aspartokinase was not inactivated after 30 min at 70°C, but 30% of the original activity was lost after 30 min at 80°C and rapid inactivation occurred above 85°C. The allosteric sensitivity of the enzyme was maintained even at 60~80°C but was reduced with the increase of temperature, accompanying desensitization above 80°C. The heat stability of the enzyme activity and of the allosteric sensitivity was discussed in comparison with other allosteric enzymes of thermophiles.

Aspartokinase (ATP: L-aspartate 4-phosphotransferase, EC 2.7.2.4) is an allosteric enzyme which catalyzes the first reaction in the branched pathway for the biosynthesis of the amino acids of the aspartate family, starting with L-aspartate and leading to L-lysine, L-threonine, and L-methionine. Various control patterns are known with the biosynthesis of aspartate family amino acids. For example, *Escherichia coli* (K-12) has three distinct aspartokinases, one of which is inhibited and repressed by L-lysine, a second is inhibited by L-threonine and repressed by L-threonine plus L-isoleucine, and a third one is repressed but not inhibited by L-methionine. In addition, in *Escherichia coli*, aspartokinase and homoserine dehydrogenase activities are associated in a complex protein.¹⁾ On the other hand, in several species of *Pseudomonas*,²⁾ *Bacillus*,^{3,4)} and *Brevibacterium*⁵⁾ a single aspartokinase is known to be under concerted feedback regulation by L-lysine and L-threonine.

One of the important properties of allosteric enzymes is that many of them lose their allosteric sensitivity by heat treatment above 50°C.

Thus it is an interesting problem whether allosteric enzymes of thermophilic organisms can maintain their allosteric sensitivity even at the optimum temperature range for their growth (above 50°C). Kuramitsu described that the aspartokinase from a moderate thermophile *Bacillus stearothermophilus* is feed-back inhibited by both L-lysine and L-threonine in a concerted manner above 50°C, but that the feedback sensitivity decreases as the assay temperature is increased and is almost completely lost above 60°C, while the optimum temperature for the growth of the organism is about 60~65°C.⁶⁾ We described in 1970 that the aspartokinase of an extreme thermophile *Thermus flavus* AT-62 (the optimum temperature for growth is about 70°C) exhibited allosteric sensitivity even at above 60°C.⁷⁾

This paper describes the partial purification of the aspartokinase of an extreme thermophile *Thermus flavus* AT-62 and its allosteric nature at elevated temperatures.

MATERIALS AND METHODS

Growth of bacteria. *Thermus flavus* AT-62 was aerobically grown as described previously.⁸⁾ Cells

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were harvested near the end of the logarithmic growth phase and were kept in a deepfreeze at -20°C .

Enzyme assays. Aspartokinase activity was measured at 60°C by aspartylhydroxamate formation as described by Stadtman *et al.*⁹⁾ The amount of reaction product was calculated by comparison with DL-aspartylhydroxamate as standard. Homoserine dehydrogenase activity was measured at 60°C as described previously.⁸⁾ One milliunit of aspartokinase and homoserine dehydrogenase is defined as the amount of enzyme forming 1.0 nmol of reaction product per min under the standard assay conditions.

Chemicals. L-Homoserine was a product of Kyowa Hakko Kogyo Co., Ltd., and all other amino acids used were purchased from Nippon Rikagakuyakuhin Co., Ltd. NADP and DL-aspartylhydroxamate were obtained from Oriental Yeast Co., Ltd., and from Sigma Chemical Co., respectively.

Enzyme preparation

Cell extract. The frozen cells (40 g) were thawed and suspended in 50 ml of 10 mM potassium phosphate buffer, pH 7.0, and were sonicated for 7 min at 10 kc using a Hitachi ultrasonic generator KMS-250 (Kubota Seisakusho, Ltd., Tokyo). The supernatant solution was retained and filled up to 100 ml with the same phosphate buffer (Fraction I, Table I).

DEAE-cellulose column chromatography. Fraction I was adsorbed onto a column, 3.8×24 cm of DEAE-cellulose, previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After the column was washed with the phosphate buffer, elution was carried out with a linear gradient of 0~1.0 M KCl in 2.0 liters of the buffer; 20 ml of fractions were collected. The elution pattern is shown in Fig. 1. The fractions containing aspartokinase activity were pooled and were dialysed against 3 liters of the phosphate buffer overnight (Fraction II).

2nd DEAE-cellulose column. Fraction II (72 ml) was adsorbed onto a DEAE-cellulose column (3.1×31 cm), previously equilibrated with the phosphate buffer. After the column was washed with the phosphate buffer, a linear 1.0 liter gradient from 0 to 0.8 M KCl in the phosphate buffer was applied. Fractions

FIG. 1. Elution Pattern of Aspartokinase and Homoserine Dehydrogenase on a DEAE-cellulose Column.

A 100-ml of crude extract (Fraction I) was applied to a DEAE-cellulose column (3.8×24 cm), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After the column was washed with the phosphate buffer, elution was carried out with a linear gradient of 0~1.0 KCl in 2.0 liters of the buffer; 20 ml of fractions were collected. -----, absorbance at 280 nm, ●-●, aspartokinase activity; ○-○, homoserine dehydrogenase activity; ---, concentration of KCl.

with high aspartokinase activity were pooled and concentrated by Diaflo ultrafiltration. The enzyme solution was dialyzed against 3 liters of 10 mM phosphate buffer, pH 7.0, overnight and then against 500 ml of 100 mM phosphate buffer, pH 7.0, overnight. The preparation (Fraction III) was stored at -20°C .

As a summary of the purification is given in Table I, aspartokinase activity was purified about 11-fold. It is demonstrated in Fig. 1 that aspartokinase and homoserine dehydrogenase activities could be separated each other in DEAE-cellulose column.

RESULTS

Effect of aspartate family amino acids

Among the amino acids of aspartate family only L-threonine exhibited inhibition of the aspartokinase and no concerted effect was observed (Table II). It is shown in Fig. 2 that the maximum inhibition by L-threonine is about 85% at 1.0 mM concentration.

TABLE I. PURIFICATION OF *Thermus flavus* ASPARTOKINASE

Step	ml	Total protein E_{280}	Total activity milliunits	Specific activity milliunits per E_{280}	Recovery %
I. Sonic extract	100	5300	28000	5.3	100
II. DEAE-cellulose	72	520	12700	24.4	45
III. 2nd DEAE-cellulose	40	164	9610	58.6	34

FIG. 2. Effect of L-Threonine Concentration on the Aspartokinase Activity.

Assays were performed at 60°C in the standard assay system added with L-threonine as variable.

TABLE II. EFFECT OF ASPARTATE FAMILY AMINO ACIDS ON THE ASPARTOKINASE OF *Thermus flavus* AT-62

Aspartokinase activity was measured at 60°C with additives indicated in Table. Each reaction mixture contained 0.2 ml of the partially purified enzyme preparation.

Amino acid	Concentration mM	Increase at 540 nm (60°C, 60 min)
None		0.180
Threonine	0.1	0.035
	1.0	0.035
	10	0.025
Lysine	0.1	0.180
	1.0	0.180
	10	0.175
Methionine	0.1	0.190
	1.0	0.190
	10	0.185
Isoleucine	0.1	0.200
	1.0	0.200
	10	0.190
Homoserine	0.1	0.210
	1.0	0.190
	10	0.135
Threonine + Lysine	0.1 (each)	0.035
	1.0	0.040
	10	0.020
Threonine + Methionine	0.1 (each)	0.040
	1.0	0.030
	10	0.025
Threonine + Isoleucine	0.1	0.065
	1.0	0.035
	10	0.040

Effect of temperature

The optimum temperature of the enzyme activity, when measured with reaction time of 30 min, was observed to be 75°C (Fig. 3). As shown in Fig. 4 the aspartokinase was very heat stable, not losing the activity after 30 min at or below 70°C, but was rapidly inactivated above 80°C which is the upper limit temperature for the growth of the organism.

The feedback sensitivity of the aspartokinase

FIG. 3. Effect of Temperature on the Aspartokinase Activity and on the Feedback Inhibition.

Aspartokinase activity was measured in the presence (○—○) or absence (●—●) of 0.1 mM L-threonine at various temperatures. ×---× indicates the ratio of both activities.

FIG. 4. Heat Stability of Aspartokinase.

After heat treatment at various temperatures in 50 mM potassium phosphate buffer, pH 7.0 (adjusted at 60°C), for 30 min, the residual activity of the aspartokinase was measured at 60°C.

to L-threonine, even at 60°C, is as high as that observed in *Escherichia coli* K-12 at 30°C,¹⁾ namely the concentration of L-threonine giving the half of maximum inhibition is about 0.02 mM (Fig. 2). However, the feedback sensitivity of the enzyme decreased with the rise of temperature; about 80% inhibition at 0.1 mM L-threonine at 60°C decreased to 60% at 70°C and to 50% at 75°C, and finally desensitization occurred above 80°C. Heat inactivation as well as desensitization is considered to occur above 80°C and L-threonine seems to have protecting effect against heat inactivation of the enzyme.

pH stability and optimal pH

The aspartokinase was stable at slight alkali to acidic pH range and was most stable at about pH 7. The enzyme was unstable at high alkali pH range, losing 90% of its original activity after 30 min at 70°C at pH 10.0 (Fig. 5). The optimum pH range for

FIG. 5. Effect of pH on the Stability of the Aspartokinase.

The enzyme was incubated at 70°C for 30 min in buffers of various pHs adjusted at 70°C, and then residual activities were measured with the standard assay procedure.

○—○, 0.1 M potassium phosphate buffer; ●—●, 0.1 M borate buffer.

allosteric sensitivity to L-threonine was pH 6.5 to 8 and significant desensitization occurred below pH 6 (Fig. 6).

FIG. 6. Effect of pH on Activity and Feedback Sensitivity of the Aspartokinase.

The aspartokinase activity was measured with the standard assay procedure except that the $\text{NH}_4\text{OH} \cdot \text{HCl}$ was adjusted to the pH of each buffered mixture at 60°C. ●—●, enzyme activity in 0.1 M phosphate buffer; ●---●, enzyme activity in 0.1 M phosphate buffer in the presence of 0.02 mM L-threonine; ○—○, enzyme activity in 0.1 M borate buffer; ○---○, enzyme activity in 0.1 M borate buffer in the presence of 0.02 mM L-threonine.

DISCUSSION

The aspartokinase was purified about 11-folds from *Thermus flavus* AT-62 cells. It was difficult to purify the enzyme to homogeneity because of both the low intracellular enzyme level and the presence of viscous lipid components. The attempt to purify the enzyme using affinity adsorbent such as N- ω -amino-hexyl-L-aspartic acid¹⁰⁾ (kindly provided by Dr. I. Chibata, Tanabe Seiyaku Co., Ltd., Osaka), ϵ -aminocaproyl-L-aspartic acid and ϵ -aminocaproyl-L-threonine, each of which was coupled to Sepharose 4-B, did not produce satisfactory results. From the results of purification it seems, unlike the case of *Escherichia coli*,¹⁾ that the organism has a single aspartokinase regulated by L-threonine alone, without forming complex with homoserine dehydrogenase.

In the previous paper⁷⁾ we reported that the aspartokinase is regulated by L-threonine plus L-lysine in concerted manner in the experiments using crude cell extract preparation. The presence of high concentration of viscous lipid substance in crude preparation might

interfere the development of color of the reaction product, leading to wrong conclusion.

It is clear that the aspartokinase of an extreme thermophile *Thermus flavus* AT-62 is far more heat stable compared with that of *Bacillus stearothermophilus*⁶⁾ in the respects of heat stability of the enzyme activity as well as of allosteric sensitivity. In spite of such an strong heat stability allosteric sensitivity of the enzyme decreased with the rise of temperature above 60°C and desensitization occurred above 80°C (the optimum temperature for growth of the organism is about 70°C). We described elsewhere about the same behavior to temperature of isocitrate dehydrogenase of *Thermus flavus* AT-62.^{11,12)} As already described in introduction the feedback sensitivity of the aspartokinase of moderate thermophile *Bacillus stearothermophilus* is almost completely lost above 60°C, while the optimum temperature for the growth of the organism is 60~65°C.

These facts may imply that certain enzymes of thermophiles are not ones adapted well to high temperature circumstances in respects to regulatory functions or to affinity for substrates or cofactors, in spite of the remarkable heat stability of enzymatic activity. Alternative possibility is that there might be some stabilizing or protecting factors *in vivo*. In addition far higher protein concentration *in vivo* than that in experimental conditions may contribute to the heat stability of most intracellular enzyme proteins.

It is interesting that the L-threonine deaminase of *Bacillus stearothermophilus* has an higher heat stability than that of the aspartokinase of the same organism in respect to allosteric sensitivity.¹³⁾ The fructose-bis-phosphatase of *Thermus thermophilus* (the optimum and maximum temperatures for growth are 70~75 and 85°C, respectively) has also higher heat stability in their enzymatic activities and in regulatory functions,¹⁴⁾ although their allosteric sensitivity also decreases above 75°C

with the increase of temperature.

It seems that there are many types of thermophilic or thermostable enzymes of thermophiles in respect to their enzymatic activity (V_{max}) or to affinity (or sensitivity) for ligands (K_m or K_i) including allosteric sensitivity. Relationship between temperature dependent kinetic properties of purified thermophilic enzymes and physiological concentrations of substrates and effectors *in vivo* at elevated temperatures remains to be resolved.

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