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Aspartokinase from Wheat Germ

ISOLATION, CHARACTERIZATION, AND REGULATION[1]

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ABSTRACT

Aspartokinase has been isolated from wheat germ and a preliminary survey made of its properties in a partially purified extract. The enzyme has an absolute requirement for ATP and a divalent metal ion. The phosphate donor can be either ATP or GTP, but other nucleotides are ineffective. Both magnesium and manganese will activate the enzyme, whereas calcium shows a trace amount of activity. The enzyme has a Km of 16.7 mM for aspartate, 1.2 mM for ATP, and 3.3 mM for $MgCl_2$. Lysine inhibits the reaction at fairly low concentrations, and threonine inhibits at high concentrations. Other amino acids which are derived from aspartate (methionine, homoserine, threonine, and isoleucine) have little effect. When lysine and threonine are added together, they show a concerted inhibition of the reaction. The enzyme is also stabilized against heat inactivation by lysine and threonine together but not by either when added separately. It is suggested that aspartokinase from plants is a regulatory enzyme and exhibits a concerted feedback mechanism.

In higher plants and bacteria the amino acids lysine, homoserine, threonine, isoleucine, and methionine are derived from aspartic acid (6–8, 13, 19). The first reaction of the branched pathway is:

$$\text{L-Aspartate} + \text{ATP} \xrightleftharpoons{Mg^{2+}} \text{L-Aspartyl-phosphate} + \text{ADP}$$

and is catalyzed by aspartokinase (ATP: L-aspartate 4-phosphotransferase, EC 2.7.2.4). This enzyme has been extensively studied in bacteria but has not been definitely detected in plants. The enzymes from bacteria show considerable diversity, especially in their regulatory properties. In *E. coli*, there are three aspartokinases; one of which is inhibited by lysine, another by threonine, and the third shows no feedback regulation but is repressed by methionine (14, 18). Other bacteria possess only one aspartokinase, but this is regulated in a variety of ways. The enzyme from *Rhodopseudomonas spheroides* is inhibited by aspartic semialdehyde (5), whereas the enzyme from *Rhodospirillum rubrum* is inhibited by threonine (3). In other bacteria such as *Rhodopseudomonas capsulatus* (3, 4), *Brevibacterium flavum* (17), and some *Bacillus* species (10, 15), the enzyme is inhibited by the concerted action of lysine and threonine.

There have been a number of attempts to detect this enzyme in plants, but it has never been positively identified. Webster and Varner (21) have reported an aspartylhydroxamate forming system in extracts of lupine, pea, and wheat germ, and there were recent attempts to find this enzyme in extracts of maize roots (2) and *Marchantia* (9). The present investigation shows the presence of aspartokinase in wheat germ and demonstrates that the higher plant enzyme is subjected to feedback regulation by lysine and threonine in a concerted manner.

MATERIALS AND METHODS

Preparation of Aspartokinase. Wheat germ (Sigma Chemical Company) was homogenized in a Waring Blendor for 0.5 min. at 4 C with acetone precooled to −15 C. The husk was allowed to settle to the bottom of the homogenate, and the fine powder was decanted into a Büchner funnel. The powder was washed thoroughly with the cold acetone until the filtrate was colorless and was then dried and stored at −15 C.

The acetone powder was suspended in 0.05 M TES-KOH buffer (pH 8.0) in a ratio of 1:5 (w/v) and stirred for 30 min. The suspension was passed through eight layers of cheesecloth and centrifuged at 20,000*g* for 20 min. The clear supernatant was fractionated with a saturated solution of ammonium sulphate. The protein precipitating between 0.78 M and 1.56 M ammonium sulfate saturation was collected by centrifugation and dissolved in 0.05 M TES-30% glycerol (pH 8.0). This extract was either passed through Sephadex G-25 column (2.5 cm × 30 cm) equilibrated with the same buffer or dialyzed for 15 hr in the same buffer. All procedures were carried out at 4 C. This enzyme preparation was used in all the subsequent experiments without further purification. Protein was estimated by the biuret method with bovine serum albumin as standard (12).

Assay for Aspartokinase Activity. A modification of the method of Stadtman *et al.* (18) was used to assay the aspartokinase. Typically, the reaction mixture contained 48 mM TES, 8 mM $MgCl_2$, 4 mM β-mercaptoethanol, 320 mM KCl, 480 mM neutralized hydroxylamine, 160 mM L-aspartate, 8 mM ATP, and 5 to 6 mg of protein in a total volume of 2.5 ml. The final pH of the reaction mixture was 7.8. After 30 min at 27 C, the reaction was stopped by the addition of 0.5 ml of a solution of 15% TCA-3 N HCl, followed by 0.5 ml of a solution of 5% $FeCl_3$-6 H_2O in 1 N HCl. The mixture was centrifuged for 5 min at 20,000*g*, and the absorbance of the supernatant was measured at 540 nm. Blank reaction mixtures containing all the components except aspartate were always included as controls. In some experiments the complete reaction mixture at zero time was used as a control. It was found in studies with authentic aspartylhydroxamate that the color development in the assay was very dependent upon the final pH. In all experiments this pH was maintained at 1.1. It was found under

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these conditions that authentic aspartylhydroxamate had a molar extinction coefficient of 528.

In some experiments a coupled assay using homoserine dehydrogenase was used. This enzyme was prepared from yeast as described by Black and Wright (1). The activity of this enzyme was checked by using aspartate semialdehyde as substrate. When this enzyme was used to assay aspartokinase, the reaction mixture contained 1 mM NADPH, 1.8 mg of the yeast extract protein, and all the components of the reaction mixture described in "Materials and Methods" except hydroxylamine.

Reagents. Sephadex G-25 gel was purchased from Pharmacia, Montreal. Hydroxylamine was obtained from Fisher Scientific Company, and all biochemicals were purchased from the Sigma Chemical Company. Aspartic semialdehyde was prepared according to the method of Black and Wright (1). All amino acids used were the L-isomers unless stated otherwise. All reagents were of the highest grade available and were used without further purification.

RESULTS

Under the conditions of the assay the reaction was linear for periods up to 40 min (Fig. 1). The amount of aspartylhydroxamate formed was also linear with protein concentrations up to 5 mg (Fig. 2). The requirement for such a high protein concentration is partly due to the low sensitivity of the assay system. However, the enzyme from a number of sources is found to have a low turnover number so that a high concentration of protein over long periods of time is usually used. More sensitive assays, such as the pyruvate kinase-lactic dehydrogenase coupled system, cannot be employed due to the presence of ATPase, phosphoenolpyruvate carboxylase, and malate dehydrogenase in the enzyme preparations which interfere with this assay.

The hydroxylamine used in the assay always contains some ammonium ions which could be used by asparagine synthetase to form asparagine. This will give the same color as aspartylhydroxamate with ferric chloride. It was, therefore, essential to show that the activity which was measured was due to aspartokinase and not to asparagine synthetase which may also be present. This was demonstrated by the following method. One reaction mixture was set up exactly as described in "Materials and Methods." Another reaction mixture contained all the components except hydroxylamine. The reactions were allowed to proceed for 30 min at 27 C, when 8 mM EDTA was added to both mixtures to chelate the magnesium and stop the reactions. Neutralized hydroxylamine (480 mM) was added to the mixture without this compound, and the mixtures were incubated another 10 min at 27 C to allow the hydroxamate to form. The trichloroacetic acid-HCl and $FeCl_3$-6 H_2O mixtures were then added in the usual manner. It was found that both reactions gave identical absorbance readings showing that ammonium ions from hydroxylamine did not interfere with the aspartokinase assay and asparagine synthetase was not contributing to the reaction.

A color reaction was also found when the aspartate was replaced by glutamate in the reaction mixture. This was a result of glutamine synthetase in the partially purified enzyme and was not the same activity as aspartokinase. It was shown that the glutamine synthetase activity had an absolute requirement for ammonium ions which were being supplied by the hydroxylamine. Reaction mixtures were set up with and without hydroxylamine; the reaction was stopped by adding EDTA and hydroxylamine was added to the mixture which was without this compound. The hydroxamate was estimated by the procedure described above and it was found that in the absence of hydroxylamine during the incubation period no hydroxamate was formed, indicating that glutamine synthetase has an absolute requirement for ammonium ions derived from hydroxylamine. The formation of a hydroxamate from glutamate was not inhibited by lysine or threonine either alone or in combination, again indicating that this reaction is quite distinct from the aspartokinase reaction which is inhibited by these amino acids as will be described later.

The aspartokinase reaction was further confirmed by coupling the reaction with NADPH and an excess of aspartic semialdehyde dehydrogenase prepared from yeast (1). It was found that the oxidation of NADPH was completely dependent on ATP, aspartate, and the wheat germ extract. In a typical experiment 58 nmoles of NADPH was oxidized as compared to 55 nmoles of aspartylhydroxamate produced as measured by the standard assay. The coupled assay was not used rou-

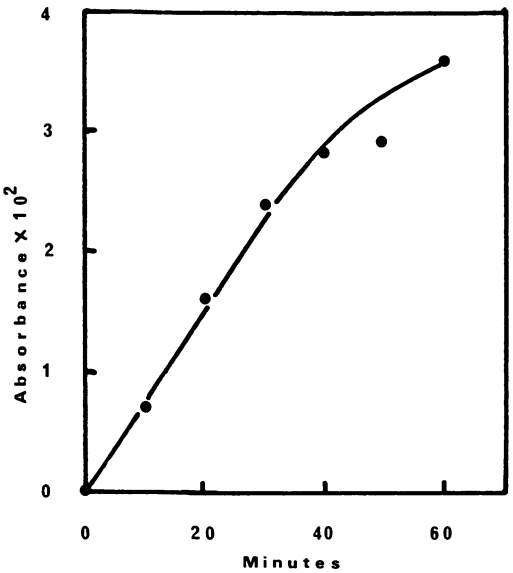

FIG. 1. Time course of aspartylhydroxamate production. Enzyme activity was assayed as described in "Materials and Methods." Each assay contained 5.5 mg of extract protein.

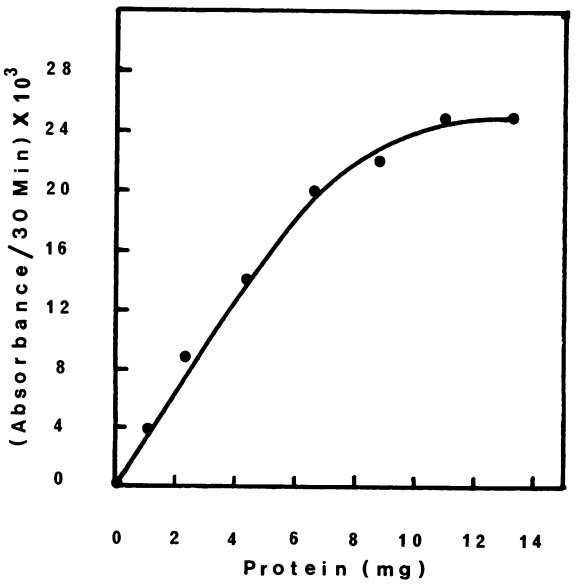

FIG. 2. Effect of enzyme protein concentration on aspartokinase activity. Enzyme activity was assayed as described in "Materials and Methods."

tinely because of the possibility of homoserine dehydrogenase being present in the extracts in variable amounts. Assays for homoserine dehydrogenase showed that trace amounts were present.

Stability of the Enzyme. When aspartokinase of wheat germ was dissolved in 0.05 M TES buffer, pH 8.0, it lost approximately 70% of its original activity after 15 hr at 4 C. However, when the enzyme was dissolved in 0.05 M TES buffer, pH 8.0, containing 30% glycerol the activity remained unchanged after 6 days at 4 C, and when such a preparation was stored at −15 C, the activity remained unchanged after 2.5 months.

Effect of Substrates. In the assay method used, all the substrates showed normal Michaelis-Menten kinetics and double reciprocal plots were linear. The *Km* for L-asparatate is 16.7 mM. The requirement for such a high concentration of the amino acid for activity may be partly due to the assay method, since it has been found in bacterial systems that the pyruvate kinase-lactic dehydrogenase assay gives lower *Km* values for aspartate (20). The *Km* values for ATP and $MgCl_2$ are 1.3 mM and 3.3 mM respectively.

In addition to ATP, GTP will act as a phosphate donor in the reaction giving a rate 70% that observed with ATP. No reaction is observed with ITP, CTP, or UTP. The reaction has an absolute requirement for divalent metal ions. At the concentrations tested, Mg^{2+} is the most effective activator with Mn^{2+} giving 75% of the activity. Ca^{2+} showed a trace of activity and Zn^{2+} was inactive (Table I).

Feedback Inhibition by End Products. The enzyme was also examined for inhibition by the amino acids derived from aspartate. Homoserine, methionine, threonine, and isoleucine have little effect at a concentration of 8 mM (Table II). An intermediate in the synthesis of lysine, diaminopimelic acid (added as the D,L-meso isomers) and a lysine analogue, N-ε-formyl-L-lysine inhibit the activity 21%. However, lysine is found to be a potent inhibitor at low cencentrations, and 60% inhibition is obtained at 1 mM, although increasing the concentration gives no greater inhibition (Fig. 3). When these data are plotted in the form of a Hill plot, a straight line is obtained with a slope of 2 which indicates that there may be cooperative binding (Fig. 4).

The inhibition by 0.3 mM lysine is greatly increased when 2 mM threonine is added to the reaction mixture (Table III). This concentration of threonine when added alone is not inhibitory. This effect is specific for threonine, since 2 mM homoserine or methionine had little effect of isoleucine appeared to slightly reduce the inhibition by lysine. The effect of increasing concentrations of lysine and threonine are shown in Figure 5. Threonine alone appeared to be slightly inhibitory at higher concentrations, but the maximum inhibition ob-

Table I. *Effect of Metal Ions on the Activity of Aspartokinase*

Aspartokinase activity was assayed as described in "Materials and Methods," except that Mg^{2+} was replaced with other metal ions.

Metal Ions	Enzymic Activity
8 mM	*A/30 min × 10³*
None	0
Mg^{2+}	20
Mn^{2+}	15
Ca^{2+}	3
Zn^{2+}	0

Table II. *Effect of Amino Acid on Aspartokinase Activity*

Enzyme activity was assayed according to the method described in "Materials and Methods."

Amino Acid Added	Inhibition
8 mM	%
D-Lysine	7
D-Threonine	0
L-Lysine	57
L-Threonine	10
L-Homoserine	0
L-Methionine	0
L-Isoleucine	0
DL,α-ε-Diaminopimelate	21
N-ε-Formyl L-lysine	21

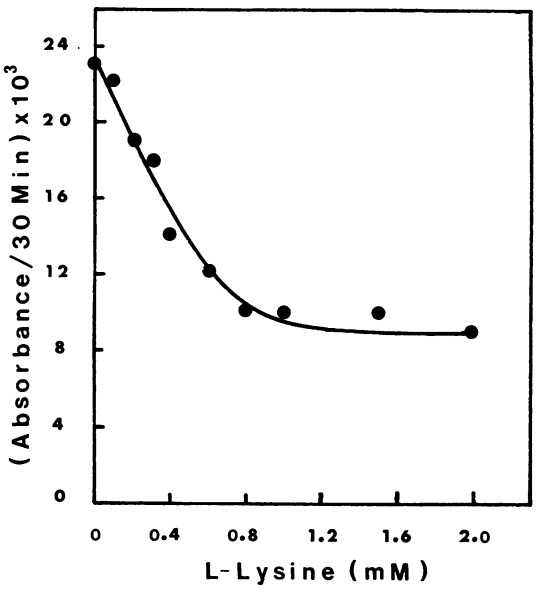

FIG. 3. Effect of lysine concentration on the inhibition of aspartokinase activity. Enzyme activity was assayed as in "Materials and Methods."

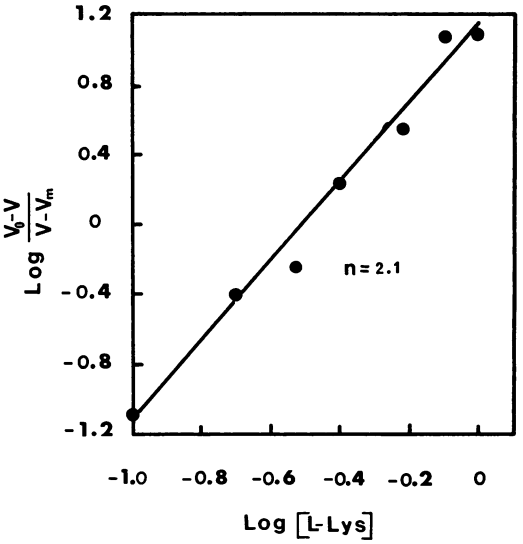

FIG. 4. Hill plot of lysine inhibition from the results of Figure 6 up to 1 mM lysine concentration. Vo is the rate in the absence of the inhibitor, V is the rate in the presence of nonsaturating concentrations of the inhibitor, and Vm is the rate at saturating concentration of the inhibitor.

Table III. *Effect of L-Lysine and Other End Product Amino Acids on Aspartokinase Activity*

Aspartokinase activity was assayed as described in "Materials and Methods." The concentration of L-lysine was at 0.3 mM, whereas other amino acids were each at 2 mM.

Amino Acids Added	Inhibition
	%
L-Lysine	22
L-Lysine + L-threonine	52
L-Lysine + L-isoleucine	13
L-Lysine + L-methionine	26
L-Lysine + L-homoserine	30

tained was 10%. Lysine alone showed strong inhibition, but this was significantly increased when threonine and lysine were varied together. In this experiment the threonine concentration was 10-fold greater than the lysine. The maximum inhibition which occurred under these conditions was 60%. The inhibition when threonine and lysine are varied together appeared not to be sigmoidal.

Heat Inactivation of the Enzyme. In the absence of any amino acids, the enzyme lost 70% of its original activity after heating at 45 C for 15 min (Table IV). This loss of activity is partially prevented by the presence of both lysine and threonine. Lysine and threonine individually however, are ineffective.

DISCUSSION

The presence of aspartokinase in plants has been postulated, but there was no direct evidence for this enzyme in plants until this study which clearly indicates that aspartokinase is present in wheat germ. Aspartylhydroxamate is produced when ATP, aspartate, a magnesium ion, and hydroxylamine are incubated in the presence of wheat germ extract. The possibility that the reaction could be due to asparagine synthetase was excluded, since equal amounts of aspartohydroxamate were obtained in the presence and absence of hydroxylamine, a possible source of ammonium ions, during the incubation period. Additional evidence for the enzyme was obtained by coupling the reaction to a partially purified preparation of aspartic semialdehyde from yeast. The oxidation of NADPH stoichiometric with the production of aspartylhydroxamate indicates that β-aspartylphosphate must be produced to support the aspartic semialdehyde dehydrogenase reaction.

In bacteria and plants, lysine, homoserine, threonine, isoleucine, and methionine are all synthesized from aspartic acid. The pathway is complex and is regulated in different ways by various organisms. In *E. coli* there are three isoenzymes each of which is regulated by different end products. So far there is no evidence for more than one aspartokinase in plants. Attempts are being made to obtain a more highly purified preparation of aspartokinase, and this may reveal the presence of isoenzymes.

In other organisms such as *R. capsulatus* (4) and *B. polymyxa* (15), only one aspartokinase is present regulated by a concerted feedback mechanism involving lysine and threonine. The enzyme from wheat germ appears to be regulated in a similar manner. Only lysine of all the amino acids tested inhibited the activity, but even at high lysine concentrations this was not complete. The maximum inhibition which could be observed was 60%. It is possible that the remaining 40% of the activity was due to a different enzyme, but there is no direct evidence for this. Threonine alone did not inhibit the enzyme and in the presence of high concentrations of lysine did not increase the amount of inhibition above the 60% due to lysine alone. However, at lower concentrations of lysine, threonine significantly increased the inhibition. It is suggested, therefore, that the wheat germ aspartokinase shows a concerted feedback mechanism, and this is effective at fairly low concentrations of lysine and threonine. Diaminopimelate also inhibited the enzyme but not to the same extent as lysine. Diaminopimelate has been implicated in the regulation of the enzyme from *Bacillus subtilis* (11, 16). In these bacteria, however, diaminopimelate is important in cell wall formation.

The inhibition data suggest that there are two sites on the enzyme, one for lysine and one for threonine. This is supported by the heat inactivation studies where both amino acids were more effective in stabilizing the enzyme to heat than either one alone. It is suggested, therefore, that plants contain a regulatory aspartokinase. The following paper (22) describes an *in vivo* and *in vitro* study of aspartokinase from *Lemna minor*, which indicates that the enzyme responds in the intact plant in a similar manner to the isolated enzyme.

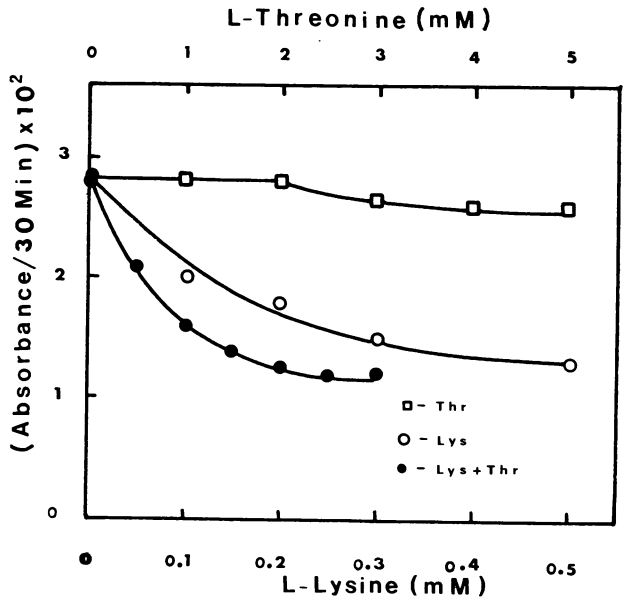

FIG. 5. Effect of lysine and threonine concentration on the inhibition of aspartokinase activity. Enzyme activity was assayed as in "Materials and Methods." □: Threonine concentration varied; ○: lysine concentration varied; and ●: lysine and threonine concentrations varied.

Table IV. *Effect of Amino Acids against Heat Inactivation*

Enzyme preparation (100 mg of protein in 0.05 M TES-30% glycerol, pH 8.0) containing the amino acids in a final volume of 6 ml were incubated at 45 C for 15 min. The heated mixtures were rapidly cooled in ice and then dialyzed thoroughly for 15 hr in 0.05 M TES-30% glycerol, pH 8.0. Enzyme activity of each mixture was assayed according to the method described in "Materials and Methods."

Amino Acids	Activity Remaining
	%
None	30
L-Lysine (8.4 mM)	34
L-Threonine (8.4 mM)	24
L-Lysine (4.2 mM) + L-threonine (4.2 mM)	71

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