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Properties and Regulation of Aspartate Kinase from Barley Seedlings (*Hordeum vulgare* L.)

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ABSTRACT

Aspartate kinase (EC 2.7.2.4) has been purified 8-fold and characterized from germinating barley (*Hordeum vulgare*) seedlings. The enzyme is inhibited 50% by 0.7 mM L-lysine and almost completely at 5 mM. L-Methionine does not affect the enzyme on its own, but at low concentrations (0.1–1 mM) increases the inhibition in the presence of lysine, indicating that the two amino acids act as cooperative feedback regulators.

Aspartate kinase (ATP: L-aspartate 4-phosphotransferase, EC 2.7.2.4) catalyzes the ATP-dependent conversion of aspartate to β-aspartylphosphate, the first reaction in the biosynthesis of the aspartate family of amino acids—lysine, methionine, threonine, and isoleucine (16).

The enzyme is subject to feedback control by its amino acid end products, and a variety of regulatory patterns have been demonstrated for the enzyme from different plant tissues. The enzyme from maize is sensitive to L-lysine (5, 6, 14), whereas in *Pisum sativum*, the main effector is L-threonine and lysine is inactive (3). In contrast, Wong and Dennis (22, 23) suggested that the enzymes from wheat germ and *Lemna minor* were inhibited cooperatively by lysine and threonine. In both cases, however, their experiments were carried out with very low activity preparations, and high (1–3 mM) concentrations of threonine were required to cause appreciable inhibition. The wheat enzyme was inhibited 60% by 1 mM lysine on its own. Recently, Aarnes (2) studied the enzyme from four green plants and two algae and demonstrated that all were inhibited to varying extends by lysine and/or threonine.

In the present paper, we describe the partial purification and properties of an enzyme from barley which is sensitive to inhibition by lysine and to cooperative inhibition by lysine and methionine. Methionine has not previously been demonstrated to have a role in the regulation of aspartate kinase in higher plants.

MATERIALS AND METHODS

Chemicals. Amino acids and cofactors were purchased from Sigma. Amino acid analogues were purchased from Sigma, Calbiochem, Aldrich Chemical Co. Inc., and R. N. Emanuel Ltd. Those not commercially available were generous gifts from L. Fowden and P. J. Lea (Rothamsted). L-[U-^{14}C]aspartic acid (180 μCi/μmol) was obtained from the Radiochemical Centre, Amersham.

Enzyme Extraction and Purification. Untreated seeds of barley (*Hordeum vulgare* [L] cv. Julia) were germinated in the dark in moist vermiculite at 20 C for 7 days. Shoots were blended at 5 C with a 1:1 (w/v) ratio of 0.2 M tris-HCl (pH 8) containing 0.1 M KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 30% (v/v) glycerol. After filtration through gauze, the extract was centrifuged at 2,500*g* for 45 min. Solid $(NH_4)_2SO_4$ was added to the extract to 60% saturation and the precipitate was collected by centrifugation. This was dissolved in 0.05 M K-phosphate buffer (pH 7.5) with 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20% (v/v) glycerol, and dialyzed overnight against the same buffer. The dialyzed extract was shaken with DEAE-cellulose which was then eluted batchwise with phosphate buffer + 0.2 M NaCl (4 ml/g DEAE-cellulose). The active eluate was concentrated by $(NH_4)_2SO_4$ precipitation (60%), and the precipitate was redissolved in phosphate buffer and dialyzed overnight. The extract was finally centrifuged at 70,000*g* for 60 min. The preparation was stable at 5 C for several days and at −15 C for at least a week.

Enzyme Assays. The hydroxamate assay for aspartate kinase was based on that of Bryan *et al.* (5). The standard assay contained 50 mM L-aspartate, 20 mM $MgSO_4$, 20 mM ATP, 125 mM $NH_2OH \cdot HCl$ (adjusted to pH 7 with KOH immediately prior to the assay), 100 mM K-phosphate (pH 7.5), and enzyme in a total volume of 1 ml. The aspartate concentration was 25 mM in the amino acid feedback experiments. The reaction was linear for 60 min and over the range of enzyme concentration used (0.8–2.2 mg/assay). After incubation at 30 C for 60 min, the reaction was terminated by the addition of 0.5 ml of 15% trichloroacetic acid. The precipitated protein was removed by centrifugation, and 1 ml acid ferric chloride reagent was added to the supernatant. The absorbance at 505 nm was measured after 20 min, and the concentration of β-aspartyl hydroxamate was calculated from a standard curve prepared using the authentic compound. Coupled assays for aspartate kinase were based on those of Cheshire and Miflin (6) and Bryan *et al.* (5). Asparagine synthetase was determined by the specific radiochemical assay of Lea and Fowden (12) using NH_4^+ and glutamine as amino (amide) donors.

Protein was determined by the method of Lowry *et al.* (13).

RESULTS

Characteristics of the Enzyme. In typical experiments, 2 kg of fresh tissue yielded approximately 125 mg of protein with a specific activity of 2 to 4 nmol/min·mg protein under standard assay conditions. Modifications to the buffer systems, including alterations in pH, ionic strength, buffer type, and the inclusion of 1 mM lysine and 1 mM threonine had no effect on the amount of activity extracted. The inclusion of phenylmethylsulfonylfluoride, a protease inhibitor, in the extraction buffer was also without effect. We suggest, therefore, that the amount of activity extracted may have represented a fairly low endogenous level of active enzyme. The over-all purification was 8- to 9-fold, and the specific activity of the final preparation was similar to those of the preparations used by some other workers (3, 5, 6). The results are from 20 separate enzyme isolations, and the proper-

ties of the enzyme were very reproducible between experiments.

Enzyme activity was dependent upon aspartate, ATP, and Mg^{2+} or Mn^{2+}. Maximum activity was exhibited in K-phosphate buffer at pH 7 to 7.5. Glycerol was essential for stability during overnight dialysis at 5 C. Sulfhydryl compounds were not required for maximum activity or for stability of the purified preparation. The enzyme was sensitive to sulfydryl inhibitors, pCMB and $HgCl_2$, causing 90% inhibition at 0.1 and 0.05 mM, respectively.

At nonsaturating concentrations of Mg^{2+} and ATP, maximum activity was exhibited with a small (5 mM) excess of Mg^{2+}. In the presence of 10 mM ATP, 5 mM and 10 mM Mg^{2+} gave 35 and 75%, respectively, of the activity exhibited with 15 mM. Manganese at concentrations of 2.5, 5, and 10 mM gave 66, 84, and 84% respectively, of the activity with 15 mM Mg^{2+}.

The enzyme utilized GTP with 33% of the activity exhibited with ATP (10 mM concentrations). CTP, UTP, and ITP were not utilized. Lineweaver-Burk plots of enzyme activity against ATP/Mg concentration were nonlinear. A direct plot of activity against substrate concentration demonstrated that half-maximum velocity was exhibited with approximately 5 mM of ATP/Mg.

The enzyme showed two approximately linear phases of activity when the concentration of aspartate was varied (Fig. 1). These were between 1 and 10 mM and between 20 and 50 mM, at which concentration the enzyme was still not saturated. Lineweaver-Burk plots were nonlinear at several concentration ranges of aspartate, indicating non-Michaelis-Menten kinetics.

The identity of the product was confirmed using the coupled assay procedures of Bryan *et al.* (5) and Cheshire and Miflin (6). The reaction rates were low and difficult to reproduce accurately due to high background rates of NADPH oxidation. Asparagine synthetase will also catalyze the synthesis of β-aspartylhydroxamate utilizing aspartate, Mg^{2+}, ATP, and hydroxylamine (19). The absence of this enzyme was demonstrated using a specific radioassay (12) with either glutamine or NH_4^+ as amino (amide) donors and a range of ATP, Mg^{2+}, and aspartate concentrations. The activity of the enzyme preparation was not affected by 5 mM $Na_4P_2O_7$, a concentration sufficient to cause 45% inhibition of the asparagine synthetase of *Lupinus albus* (12).

Regulatory Properties. The enzyme was inhibited by L-lysine, the inhibition being 30% at 0.5 mM and almost total at 5 mM (Fig. 2a). Other amino acids derived from aspartate (methionine, threonine, homoserine, and isoleucine) had no effect at 10 mM concentrations (Table I). Valine and leucine (which are biosynthetically related to isoleucine) and alanine were also inactive. L-Methionine, however, was inhibitory in the presence of lysine (Table I; Fig. 2a). The concentration of methionine required to cause maximum inhibition was the same irrespective of lysine concentration (Fig. 2b). Threonine had no effect either in the presence of lysine, lysine and methionine, or methionine.

S-(β-Aminoethyl)-cysteine, an analogue of lysine (18), was inhibitory when supplied at high concentrations (Fig. 2a). Inhibition was 35% at 5 mM and 76% at 10 mM. The inhibition by lysine and AEC[1] was competitive with respect to aspartate. Other analogues were also tested for their ability to cause false feedback inhibition. L-Lysine tetrazole caused 23% inhibition at 10 mM while 16 other analogues (including D-lysine, α-aminocaprylic acid, *N*-α-acetyllysine, and several separate isomers of γ-hydroxylysine) had little or no effect at concentrations of 1 mM and 10 mM. False feedback inhibition was also caused by the methionine analogue (18), seleno-D,L-methionine. When supplied at 1 and 5 mM, seleno-D,L-methionine caused 18 and 26% inhibition in the presence of 0.5 mM L-lysine, and 21 and 28% inhibition in the presence of 4 mM AEC. The corresponding values for 1 and 5 mM L-methionine were 33 and 33% with lysine, and 26 and 32% with AEC. D,L-Ethionine at 10 mM caused approximately 10% inhibition in the presence of lysine and AEC. The following methionine analogues had no effect at 10 mM concentrations:- D,L-norleucine, D,L-methionine sulfoxide, D,L-methionine sulfone, L-methionine-D,L-sulfoximine, seleno-D,L-ethionine, *S*-ethyl-L-cysteine, and *S*-methyl-L-cysteine.

Allosteric modifiers are often capable of protecting an enzyme against thermal inactivation (7, 21). The ability of lysine and methionine to protect aspartate kinase was investigated by exposing the enzyme to a temperature of 45 C for 20 min. After heating, the enzyme was rapidly cooled and dialyzed overnight against buffer without amino acids. The results of the experiment are given in Table II. Lysine at 1 mM was effective in protecting the enzyme against heat inactivation whereas methionine had no effect. Lysine and methionine together were slightly more active than lysine alone. Neither amino acid, however, was able to protect the enzyme against desensitization to feedback inhibition. In a further experiment, the time course of heat inactivation was studied (Table III). In the absence of amino acids, the enzyme was completely inactivated after 60 min at 45 C, whereas in the presence of 1 mM lysine and 1 mM methionine, 15% of the activity was still remaining after 90 min. The degree of feedback inhibition was similar after 15 and 90 min

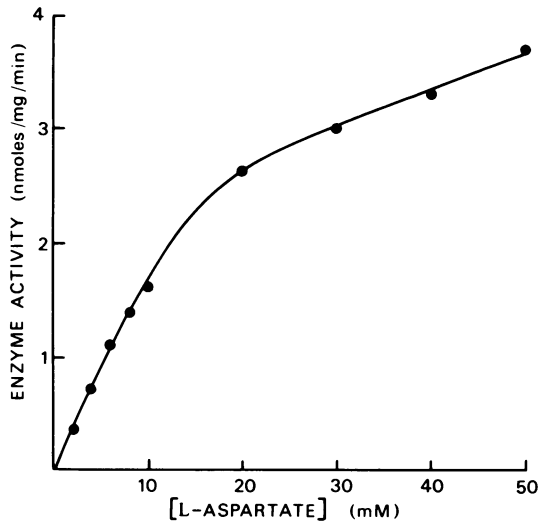

FIG. 1. Effect of L-aspartate concentration on the activity of aspartate kinase. The enzyme (1.8 mg protein) was assayed under the standard assay conditions. The activity at 50 mM aspartate represents an absorbance in the hydroxamate assay of 0.130.

TABLE I:

The effect of amino acids on the activity of aspartate kinase.

The enzyme (1.4 mg protein, specific activity equal to 4.2 nmol/mg min) was assayed under the standard assay conditions with 25 mM aspartate. Enzyme activity is expressed as a percentage of that in the absence of any added amino acids. 100% activity represents an absorbance in the hydroxamate assay of 0.114.

	Enzyme Activity	
	amino acid alone	amino acid + 0.5 mM lysine
	%	
0.5 mM L-lysine	67.2	-
10 mM L-threonine	107.6	68.5
10 mM L-methionine	100.0	26.0
10 mM L-valine	100.0	69.7
10 mM L-homoserine	101.7	70.6
10 mM L-alanine	108.8	73.9
10 mM L-isoleucine	104.6	71.4
10 mM L-leucine	97.5	71.4

[1] Abbreviation: AEC: S-(β-aminoethyl)-cysteine.

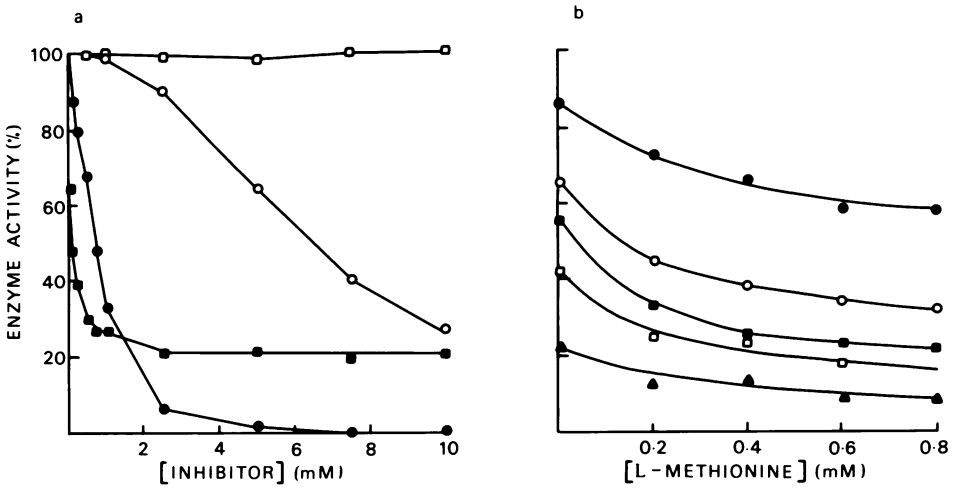

FIG. 2. a: Effect of varying concentrations of L-lysine (●), L-methionine (□), AEC (○), and L-methionine in the presence of 0.5 mM L-lysine (■) on the activity of aspartate kinase. The enzyme (1.2 mg protein, specific activity 2.9 nmol/mg·min) was assayed under standard conditions with 25 mM aspartate. Activity is expressed as a percentage of that in the absence of added amino acids. One hundred per cent activity represents an absorbance in the hydroxamate assay of 0.068. b: Effect of varying concentrations of L-lysine and L-methionine on the activity of aspartate kinase. The enzyme (1.4 mg protein, specific activity 2.9 nmol/mg·min) was assayed under standard conditions with 25 mM aspartate, 0 to 0.8 mM methionine, and 0.2 (●), 0.4 (○), 0.6 (■), 0.8 (□), and 1 (▲) mM lysine. Activity is expressed as a percentage of that in the absence of lysine and methionine. One hundred per cent activity represents an absorbance in the hydroxamate assay of 0.082.

TABLE II:

The effect of amino acids on the heat inactivation and desensitization of aspartate kinase.

Aliquots of the enzyme preparation were held at 45 C for 20 min in the absence and presence of 1 mM L-lysine and 1 mM L-methionine. They were then rapidly cooled and dialyzed overnight against buffer without amino acids. Assays were carried out under the standard conditions with 25 mM aspartate and in the absence and presence of 0.5 mM lysine and 0.5 mM methionine. Each assay contained 1.3 mg protein of specific activity 3.9 nmol/mg min. Enzyme activity is expressed as a percentage of that exhibited by an untreated aliquot of the preparation. 100% activity represents an absorbance in the hydroxamate assay of 0.104. Inhibition is expressed as a percentage of the activity in the absence of added amino acids.

Treatment	Enzyme Activity	Inhibition by 0.5 mM L-lysine	Inhibition by 0.5 mM L-lysine +0.5 mM L-methionine
	%	%	%
unheated	100	25	53
heated without amino acids	75	19	34
heated with 1 mM L-lysine	89	17	26
heated with 1 mM L-methionine	75	20	27
heated with 1 mM L-lysine and 1 mM L-methionine	93	18	31

and was not affected by the presence of lysine and methionine. Aspartate had no effect on the inactivation or the desensitization when supplied at substrate concentration (25 mM).

To confirm that a threonine-sensitive enzyme was not being inactivated or desensitized during the purification procedure, the enzyme was prepared using 1 mM threonine in all buffers. All $(NH_4)_2SO_4$ and DEAE-cellulose fractions were retained and assayed for threonine-sensitive aspartate kinase after rapid dialysis against threonine-free buffer. No threonine-sensitive enzyme was detected.

The enzyme was also inhibited by AMP and ADP. In the presence of 10 mM ATP and 15 mM Mg^{2+}, AMP at 1, 5, and 10 mM caused 9, 33, and 47% inhibition, respectively. The corresponding values for ADP were 9, 35, and 51%. The degree of inhibition was the same in the presence of 5 mM ATP and 10 mM Mg^{2+}, indicating that the inhibition was noncompetitive. The inhibition was also not affected by the addition of equimolar or excess Mg^{2+} which suggests that it was not a result of chelation by the nucleotides.

DISCUSSION

The enzyme is similar in its basic properties to those isolated from other tissues—maize (5, 6), pea (3), wheat (22), and *L. minor* (23). The effect of aspartate concentration on the activity of the enzyme is neither unique to barley nor an artifact of the hydroxamate assay procedure. Cheshire and Miflin (unpublished data) obtained similar results for the maize enzyme using a coupled spectrophotometric assay. They reported (6) that the Lineweaver-Burk plot deviated from a straight line at low concentrations and that the apparent *Km* for aspartate was very high (9 mM). Bryan *et al.* (5), using the hydroxamate assay reported, two *Km* values for the maize enzyme whereas other workers (3, 22) attributed their high *Km* values for the pea and wheat enzymes to the hydroxamate assay. The physiological signifi-

TABLE III:

The effect of amino acids on the time course of heat inactivation and desensitization of aspartate kinase.

Aliquots of the enzyme preparation were held at 45 C for varying time periods in the absence and presence of 1 mM L-lysine + 1 mM L-methionine or 25 mM L-aspartate. They were rapidly cooled, dialyzed overnight against amino acid-free buffer and assayed under standard assay conditions with 25 mM aspartate. Each assay contained 2.2 mg protein of specific activity 2.7 nmol/mg min. Enzyme activity is expressed as a percentage of that exhibited by an unheated aliquot of the preparation. 100% activity represents an absorbance in the hydroxamate assay of 0.088. Inhibition by L-lysine and L-methionine is expressed as a percentage of the activity in the absence of added amino acids.

Treatment	Duration of heating min	Enzyme activity %	Inhibition by 0.5 mM L-lysine + 0.5 mM L-methionine %
unheated	...	100	71
no amino acids	15	42	47
	30	15	41
	60	4	44
	90	0	...
1 mM L-lysine and 1 mM L-methionine	15	62	36
	30	40	28
	60	23	35
	90	15	37
25 mM L-aspartate	15	39	39
	30	19	24
	60	8	36
	90	0	...

cance of the relationship of enzyme activity to aspartate concentration is difficult to assess without data on the concentration of aspartate at the site of enzyme action. Recent work has demonstrated that some enzymes of amino acid biosynthesis and metabolism are present inside plastids (8, 17). These include two enzymes of the aspartate family – homoserine dehydrogenase in maize (Matthews and Bryan, unpublished data), and *Vicia faba* (Bryan and Miflin, unpublished data) and diaminopimelate decarboxylase in *V. faba* (15). It might be assumed, therefore, that aspartate kinase is also present inside the plastid. The instability of the two intermediates between aspartate kinase and homoserine dehydrogenase (β-aspartylphosphate and aspartic-β-semialdehyde) suggests that the two enzymes are close together in the same compartment, with the intermediates possibly being enzyme-bound. Aach and Heber (1) have shown that aspartate is present at concentrations from 20 to 70 nmol/mg dry weight in the plastids of several dicotyledonous plants. This may represent a concentration within the living plastid of 1 to 10 mM, which is within the lower range of activity of barley aspartate kinase. The significance of the stimulation of aspartate kinase by high concentrations of aspartate (10–50 mM) is not known.

The barley enzyme differs from those extracted from other plant tissues in that methionine is a cooperative feedback regulator. The results indicate that lysine and methionine act on the same enzyme, and we were unable to detect an isoenzyme which was sensitive to threonine. Other workers have also detected only one feedback-sensitive isoenzyme (3, 6) in contrast to some bacterial aspartate kinases (7, 21). Aach and Heber (1) showed that the lysine concentration in plastids was 0.7 to 3.1 nmol/mg dry weight, indicating that the physiological concentration is probably between 0.05 and 0.5 mM. This is within the range at which *in vitro* regulation of barley aspartate kinase occurs. Aach and Heber (1) give no data for the concentration of methionine in plastids.

The structural requirements for inhibitory activity were very strict. The only analogue which substituted for lysine was AEC, and this was only inhibitory at high concentrations. The ability of selenomethionine to substitute for methionine is to be expected in view of the inability of many enzyme systems to discriminate between Se and S (20).

Unlike the maize (5, 6) and pea (3) enzymes, the enzyme from barley was not activated by isoleucine, valine, or alanine. The inhibition of the enzyme by ADP and AMP is similar to that reported for the pea enzyme (3), where the inhibition again appears to be noncompetitive. It is possible that the enzyme is regulated *in vivo* by the changes in the ratio of ATP to ADP and AMP which occur when the plant is transferred from dark to light (9–11).

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