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THREONINE-SENSITIVE ASPARTATE KINASE AND HOMOSERINE DEHYDROGENASE FROM *PISUM SATIVUM*

HALVOR AARNES and SVEN ERIK ROGNES

Botanical Laboratory, University of Oslo, P O Box 1045, Blindern, Oslo 3, Norway

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Abstract—Aspartate kinase and two homoserine dehydrogenases were partially purified from 4-day-old pea seedlings A sensitive method for measuring aspartate kinase activity is described. Aspartate kinase activity was dependent upon ATP, Mg^{2+} or Mn^{2+}, and aspartate. The aspartate kinase was inhibited in a sigmoidal manner by threonine and K_i for threonine was 0 57 mM The enzyme could be desensitized to the inhibitor and threonine protected the enzyme against thermal inactivation Aspartate kinase activity was enhanced by isoleucine, valine and alanine Homoserine, methionine and lysine were without effect The homoserine dehydrogenase activity which was associated with aspartate kinase during purification could be resolved into two peaks by gel filtration The activity of both peaks was inhibited by aspartate and cysteine and one was inhibited by threonine

INTRODUCTION

ASPARTATE kinase (ATP:L-aspartate 4-phosphotransferase, E.C. 2.7.2.4) converts aspartate to β-aspartylphosphate in the presence of ATP and Mg^{2+} Homoserine dehydrogenase (L-homoserine:NAD^+($NADP^+$) oxidoreductase, E.C. 1.1.1.3) catalyzes the conversion of aspartate-β-semialdehyde to homoserine. These are the first and the third, respectively, of the enzymes in the pathway leading to methionine, threonine and isoleucine in the aspartate family of amino acids. The regulatory properties of several of the highly purified bacterial enzymes have been thoroughly studied.[1–3]

Information about these enzymes and their regulation in higher plants is rather limited.[4–6] A lysine-sensitive aspartate kinase[7] and a homoserine dehydrogenase[8] were partially purified and characterized from *Zea mays* Aspartate kinases from wheat germ and *Lemna minor* have recently been described [9,10]

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PHYTO 13/12-G

It is well known that peas synthesize and accumulate homoserine in large amounts during germination [11-14] Aspartate-β-semialdehyde dehydrogenase (E C 1 2 1 11) and homoserine dehydrogenase, the second and third enzyme in the pathway, were detected in pea seedlings by Sasaoka,[15 16] but no studies of regulatory properties were made Here we report the purification and properties of a threonine-sensitive aspartate kinase from pea seedlings, which is inhibited and activated by nucleotides and several amino acids Evidence for the existence of two homoserine dehydrogenases is also given

RESULTS

A summary of the purification procedure designed for aspartate kinase is given in Table 1 The overall purification was about 85-fold for aspartate kinase and 15-fold for homoserine dehydrogenase

TABLE 1 PURIFICATION OF PEA ASPARTATE KINASE AND HOMOSERINE DEHYDROGENASE

Stage of purification*	Volume (ml)	Total protein (mg)	Aspartate kinase Total activity (units*)	Aspartate kinase Specific activity (units/mg protein)	Homoserine dehydrogenase Total activity (units*)	Homoserine dehydrogenase Specific activity (units/mg protein)
(1) Crude extract	200	10600	294	0 027	55700	5 3
(2) First $(NH_4)_2SO_4$ ppt and dialysis	79	2050	506	0 250	27900	13 6
(3) DFAE-Sephadex A-50 and second $(NH_4)_2SO_4$ ppt	7 5	300	242	0 807	5600	18 8
(4) Sephadex G-200	40	92	213	2 33	7300	79 2

* For details of purification and units of enzyme activities see Experimental

Requirements and product of the aspartate kinase reaction

When the purified pea enzyme was incubated under standard assay conditions the formation of β-aspartylhydroxamate-[^{14}C] was completely dependent upon the presence of ATP, Mg^{2+} and NH_2OH The synthesis of β-aspartylhydroxamate was linear with time for at least 90 min in the presence of 0 2 mM threonine In the absence of NH_2OH β-aspartylphosphate was identified as a product by a coupled assay[7] (Table 2).

Effect of substrate concentration on aspartate kinase activity

Mn^{2+} in concentrations of 1 6, 4 and 8 mM gave 80, 51 and 36% respectively, of the activity with 12 mM Mg^{2+} The complex $MgATP^{2-}$ was indicated as a substrate Slightly higher concentrations of Mg^{2+} than those of ATP gave optimal results Up to 10 mM excess of Mg^{2+} did not inhibit the reaction By varying the ATP concentration with a 4 mM excess of Mg^{2+}, a K_m for ATP of 0 7 mM was calculated (enzyme isolated in Tris buffer). When the enzyme was isolated in phosphate buffer a higher K_m for ATP was obtained (2 8 mM) The K_m for aspartate was found to be *ca* 6 mM Such a high value

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is probably partly due to the assay method, because of the high concentration of NH_2OH present.[17] The reciprocal plots were essentially linear for all the substrates, indicating the absence of cooperative effects and the studies indicated a sequential mechanism[18] of binding for the substrates ATP and aspartate.

TABLE 2 IDENTIFICATION OF β-ASPARTYLPHOSPHATE AS A PRODUCT OF THE ASPARTATE KINASE REACTION

Reaction mixture	NADPH oxidation	
	$\Delta A_{340\,nm}$	nmol
Complete system	0·948	153
Minus aspartate	0·127	20
Minus ATP	0·195	31
Minus aspartate, minus ATP	0	0

A reaction mixture containing 25 mM Na-aspartate (pH 8), 5 mM ATP, 6 mM $MgCl_2$, 7·6 mg enzyme protein (fraction 3) and H_2O in a final vol of 2 ml (complete system) was incubated for 60 min at 20° together with control tubes from which aspartate and ATP were omitted as indicated. The mixtures were then centrifuged for 10 min at 44000 *g* at 4°. The β-aspartylphosphate produced by this reaction was used as a substrate for yeast aspartate-β-semialdehyde dehydrogenase. 0·8 ml supernatant was added to a cuvette containing 1·7 mg yeast enzyme and 0·6 mg NADPH in a final vol of 1 ml. The oxidation of NADPH was followed at 340 nm for 2 hr at 20°.

Stability of aspartate kinase

For preservation of enzyme activity the addition of mercaptoethanol to the buffer was essential. In phosphate buffer with glycerol and 1 mM threonine included (buffer PB) the enzyme was fairly stable for at least 3 weeks at −25°. The enzyme was much less stable in Tris buffer. In Tris buffer lacking threonine a variable proportion of the catalytic activity was lost, and after Sephadex G-25 and DEAE-Sephadex A-50 treatments the enzyme became desensitized to the inhibitor threonine.

Threonine protected the enzyme against heat inactivation. Purified enzyme was freed of threonine by passage through a Sephadex G-25 column equilibrated with buffer PB minus threonine. In the absence of threonine the half-life of the enzyme at 40° was 3 min with less than 10% of the original activity remaining after 7 min. In the presence of 1 mM threonine the corresponding values were 6 min and 40%.

Inhibition and activation of aspartate kinase

As little as 0·8 mM threonine produced 86% inhibition of activity. The enzyme was also very sensitive to inhibition by threonine at all stages of purification. A sigmoidal relationship between inhibitor concentration and reaction velocity was established in several experiments. By use of a computer program[19] V_m values could be estimated, and a Hill plot ($\log[(V_0 - v)/(v - V_m)]$ vs log[inhibitor]) gave an interaction coefficient $n_H = 1{\cdot}8$ and an inhibitor constant $K_i = 0{\cdot}57$ mM for threonine. In this particular experiment V_0 had to be approximated. Other experiments, in which V_0 was determined experimentally, gave the same n_H value but a slightly lower K_i (0·3 mM).

Leucine, in the presence or absence of threonine, also inhibited the enzyme (Table 3). The response curve with increasing leucine concentration showed sigmoidicity. The interaction coefficient n_H was 1·5 and K_i was estimated to 8·4 mM (0·2 mM threonine present during the assays).

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The enzyme was markedly activated by isoleucine, valine and alanine (Table 3). Alanine appeared to have an activating effect different from that of isoleucine and valine. In the presence of isoleucine or valine, threonine inhibited the activity When alanine was present, threonine was unable to cause inhibition. Homoserine, methionine and lysine (up to 8 mM) were without effect. Threonine plus lysine gave no greater inhibition than threonine alone To ascertain that small amounts of a possible lysine-sensitive enzyme were not undetected during purification, the first two purification steps were also carried out using a buffer which, in addition to threonine, also contained 1 mM each of lysine and homoserine These conditions did not reveal any lysine-sensitive fraction of the activity.

TABLE 3 ACTIVATION AND INHIBITION OF ASPARTATE KINASE BY AMINO ACIDS

Amino acid added	Aspartate kinase activity (units × 10^3) Threonine concentration (mM) 0	0·2	0·4
Experiment 1			
None	133	93	17
8 mM isoleucine	240	157	37
8 mM valine	263	157	40
8 mM leucine	80	*	*
Experiment 2			
None	87	*	*
8 mM alanine	160	207	182

* Not determined

Standard assay conditions were employed with amino acid additions as indicated Prior to assay threonine was removed from the enzyme preparation by Sephadex G-25 gel filtration with buffer PB minus threonine s.e. = ±0·01 units

The nucleotides ADP and AMP inhibited the reaction. The kinetic response curve had a sigmoidal shape for both ADP and AMP, indicating cooperative interactions in binding of these inhibitors. V_m values were calculated according to a computer program [19] In the Hill plots the slope of the linear ADP curve was 1·9 and K_i for ADP was 0·95 mM The corresponding values for AMP were 2·4 and 0·38 mM. However, when 0·6 mM ADP was present, the saturation curve for AMP changed to a hyperbolic one The reciprocal plot was then linear, indicating Michaelis kinetics In this case K_i for AMP was estimated to 0·24 mM and the interaction coefficient $n_H = 1·0$ With AMP (0·2–2·0 mM) included the saturation curve for ADP also changed to a less sigmoidal one Thus, the presence of ADP seemed to diminish cooperation in the binding of AMP and vice versa

Results of an experiment in which the ATP concentration was varied at several fixed levels of ADP and AMP suggested that inhibition by ADP and AMP is non-competitive with respect to ATP

Evidence for two homoserine dehydrogenases

The elution diagram after fractionation by Sephadex G-200 gel filtration consistently showed two peaks of homoserine dehydrogenase activity (Fig. 1) The first peak, termed HSDH I, coincided with the single peak of aspartate kinase activity, whereas a second, slightly smaller peak, HSDH II, was trailing it by four fractions The addition of 5 mM threonine to the standard assay eliminated a large portion of the activity of the first peak, the second part of the curve remaining more or less unchanged. This suggested the presence of two forms of homoserine dehydrogenase with different MWs and different sensi-

tivity toward threonine. The properties of representative fractions from both peaks were further compared

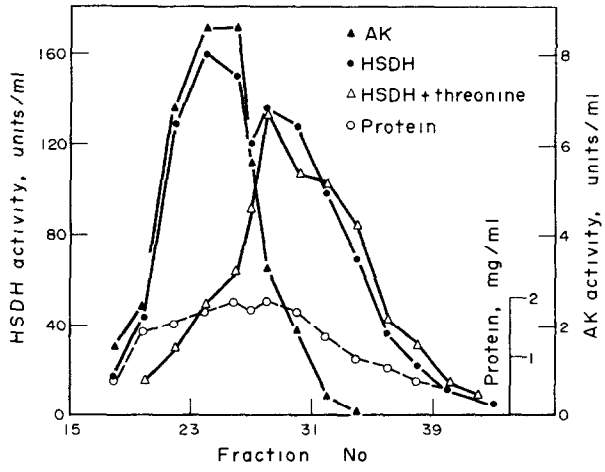

FIG 1 ELUTION PATTERN FOR ASPARTATE KINASE (AK) AND HOMOSERINE DEHYDROGENASE (HSDH) DURING SEPHADEX G-200 GEL FILTRATION

Aspartate kinase activity was measured under standard conditions ● Homoserine dehydrogenase activity measured under standard conditions, △· homoserine dehydrogenase activity measured with 5 mM threonine added to the standard assay

HSDH I was inhibited by threonine, HSDH II was not. Both were inhibited by aspartate (Table 4). Up to 10 mM methionine was without effect, but 5 mM cysteine caused a complete inhibition of both HSDH I and HSDH II. The affinities for the substrate aspartate-β-semialdehyde were significantly different. K_m for HSDH I was estimated to be 0·5 mM, the value for HSDH II was 0·08 mM. After disc gel electrophoresis and staining for homoserine dehydrogenase activity HSDH I (fraction 22 in Fig. 1) gave one single band, HSDH II (fraction 34) another one, faster migrating band, and mixtures of both two distinctly separated bands of activity.

TABLE 4 EFFECT OF AMINO ACIDS ON THE TWO FORMS OF HOMOSERINE DEHYDROGENASE

Amino acid added	Homoserine dehydrogenase activity (% of control) HSDH I	HSDH II
None	100	100
5 mM threonine	44	100
10 mM threonine	37	100
5 mM aspartate	61	56
10 mM aspartate	54	53

Standard assay conditions were employed with amino acids added as indicated HSDH I was represented by fraction 23, HSDH II by fraction 33 in Fig 1

DISCUSSION

A threonine-sensitive aspartate kinase has been partially purified from etiolated pea seedlings. The nearly complete inhibition caused by threonine and the elution of activity in a single peak during Sephadex G-200 gel filtration indicated that the activity of only one

aspartate kinase was measured. Since lysine had no inhibitory effect at any stage of purification, this aspartate kinase is different from the enzymes previously described from other higher plants, all Monocotyledons [7,9,10] They were all very sensitive to lysine and only slightly inhibited by threonine It would be of interest to isolate aspartate kinase from other dicotyledonous plants to see whether this difference in feedback sensitivity is a general one

The pea enzyme behaved like a typical allosteric enzyme,[20] displaying a complex regulatory pattern The enzyme could be desensitized to the inhibitor threonine and there was a sigmoidal relationship between inhibitor concentration and reaction velocity The fairly strong cooperative interaction between threonine molecules would suggest more than one threonine-binding site Like the maize enzyme[7] the pea aspartate kinase was activated by isoleucine, valine and alanine. Leucine had no effect on the maize enzyme studied by Bryan *et al.*,[7] however, Cheshire and Miflin[21] have later reported a slight activation by this amino acid The pea enzyme differs in this respect, being inhibited by leucine

ADP and AMP inhibited the enzyme in a complex manner, suggesting that the energy balance of the cell is important in the regulation of aspartate kinase activity [17] These negative effectors also showed cooperative kinetics In the case of ADP the Mg-complex probably acted as an inhibitor Regarding AMP, it is not clear whether inhibition is due to free AMP or the Mg-complex, the situation may be compared to that for crayfish arginine kinase.[22] It is not likely that the inhibition by AMP is an artifact resulting from the presence of adenylate kinase in the enzyme preparation.

Some of the total homoserine dehydrogenase activity was associated with aspartate kinase during purification of the latter. The existence of two homoserine dehydrogenases with different properties was clearly indicated. It may be possible that HSDH II represents a desensitized dehydrogenase, since Bryan[8] has shown that the *Zea mays* enzyme could be desensitized to the inhibitor threonine However, it is not very likely in this case since threonine was included in the buffers throughout Studies of the native and desensitized forms of the threonine-sensitive homoserine dehydrogenase from *E coli*[23] showed that the two forms of the enzyme had almost equal K_m values for aspartate-β-semialdehyde HSDH I and HSDH II had quite different K_m values for this substrate. The value for HSDH I (0 5 mM) is equal to that found by Sasaoka[16] (0·46 mM)

The threonine-sensitive aspartate kinase and the threonine-sensitive homoserine dehydrogenase were eluted in one peak during Sephadex G-200 gel filtration. Since the pea homoserine dehydrogenase was also inhibited by aspartate, the relationship between the two enzymes bears a resemblance to the threonine-sensitive aspartate kinase- homoserine dehydrogenase complex in *E coli* [24] Bryan[8] unsuccessfully tried to detect aspartate kinase activity in his homoserine dehydrogenase preparation from *Zea mays*, which was also inhibited by threonine, aspartate and cysteine Further purification and more detailed studies will be necessary before the existence of such an enzyme complex in pea seedlings is established

EXPERIMENTAL

Materials Pea seeds (*Pisum sativum* cv Pilert Fenomen) were obtained from A/S Norsk Frø L-Amino acids and nucleotides were purchased from Sigma Uniformly labelled L-aspartic acid-[^{14}C] was a product of The

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Radiochemical Centre, Amersham DL-Aspartate-β-semialdehyde was prepared by ozonolysis of DL-allylglycine (Sigma) according to the method of Black and Wright [25] Its concentration was estimated enzymatically β-Aspartylhydroxamate was synthesized by the method of Roper and McIlwain [26] Aspartate-β-semialdehyde dehydrogenase (free of aspartate kinase activity) was purified from baker's yeast as described by Black and Wright.[25]

Germination The seeds were surface sterilized with a hypochlorite soln, rinsed in H_2O and allowed to imbibe H_2O for 24 hr The seeds were washed again before germination in moist vermiculite for 3 days in darkness at 22°

Extraction, first $(NH_4)_2SO_4$ *fractionation and dialysis* The following steps were carried out at 0–5° Seedcoats were removed and the seedlings homogenized with 2 vol of buffer PB [containing 50 mM K phosphate (pH 7 2), 15% by vol glycerol, 1 mM EDTA, 1 mM threonine and 14 mM 2-mercaptoethanol] in a Waring Blendor for 2 × 30 sec The homogenate was filtered through gauze before centrifugation for 30 min at 39000 *g* Solid $(NH_4)_2SO_4$ (35 1 g/100 ml) was added to the supernatant to 55% saturation After 60 min the precipitated protein was collected by centrifugation (20000 *g*, 20 min) The ppt was dissolved in buffer PB and dialyzed overnight against a large vol of buffer PB

DEAE-Sephadex chromatography and second $(NH_4)_2SO_4$ *fractionation* The dialyzed extract was adsorbed onto a DEAE-Sephadex A-50 column (6 5 × 16 cm), equilibrated with buffer PB The column was washed with buffer PB to remove unadsorbed protein and eluted with a 500 ml continuous, linear gradient 0–1 M NaCl in buffer PB The most active fractions were concentrated by precipitation with $(NH_4)_2SO_4$ to 60% saturation (39 g/100 ml) The ppt was centrifuged down and dissolved in minimal vol of buffer PB

Sephadex G-200 gel filtration The soln from the preceding step was applied to a Sephadex G-200 column (3 1 × 28 5 cm), equilibrated with buffer PB, and eluted with the same buffer The most active fractions were concentrated by $(NH_4)_2SO_4$ precipitation (0–60%) and after 60 min the ppt was collected and dissolved in a small amount of buffer PB The enzyme was stored frozen at −25° Unless otherwise stated, this fraction was used in the studies reported

Assay of aspartate kinase The colorimetric method of Black and Wright[27] was inaccurate and not sensitive enough for measuring significant aspartate kinase activity in the early fractions of the purification procedure An assay was therefore designed, using ^{14}C-labelled aspartate, which in the presence of NH_2OH gave β-aspartylhydroxyamate-[^{14}C] Neither assay method is specific for aspartate kinase, since asparagine synthetase[9 28] would also catalyze aspartylhydroxamate formation under these conditions However, we found no evidence for interference by any asparagine synthetase during the present study

The standard assay contained 12 mM L-aspartate-[U-^{14}C] (sp act 0 33 μCi/μmol), adjusted to pH 8 with NaOH, 12 mM $MgCl_2$, 8 mM ATP, 11 mM 2-mercaptoethanol, 400 mM NH_2OH HCl (adjusted to pH 6 2 with KOH), 25 μl of enzyme extract and H_2O to a final vol of 125 μl If threonine was not removed from the enzyme preparation, its final concentration during the assay was 0 2 mM Incubation was at 20° for 60 min The reaction was stopped by the addition of 25 μl of 10% (w/v) TCA, and 10 μl of cold 30 mM β-aspartylhydroxamate was added as a carrier 25 μl Portions of the reaction mixtures were applied to Whatman no 3 paper and β-aspartylhydroxamate-[^{14}C] was separated from aspartate-[^{14}C] by electrophoresis [28] The β-aspartylhydroxamate spot was located close to the origin and its position was detected by spraying control strips with 0 2% (w/v) ninhydrin in EtOH Strips (2 8 × 4 8 cm) bearing the β-aspartylhydroxamate spot were cut out and the radioactivity measured in a liquid scintillation analyzer The counting efficiency was about 70% The activity was corrected for the value of control tubes (enzyme omitted) 500 cpm correspond to *ca* 1 nmol of β-aspartylhydroxamate A unit of aspartate kinase activity catalyzes the formation of 1 nmol min^{-1} of β-aspartylhydroxamate under standard conditions

Assay of homoserine dehydrogenase The standard assay (forward direction) contained 0 5 mM DL-aspartate-β-semialdehyde (neutralized with $KHCO_3$ immediately before use), 0 16 mM NADPH, 50 μl of enzyme extract and H_2O in a final vol of 1 ml The decrease in A at 340 nm was measured at 20° with H_2O as a blank and appropriate controls A unit of homoserine dehydrogenase activity catalyzes the aspartate-β-semialdehyde-dependent oxidation of 1 nmol min^{-1} of NADPH under standard conditions ($\epsilon^{NADPH}_{340\,nm} = 6\,22 \times 10^3\ M^{-1}\ cm^{-1}$)

Protein determination Protein was determined by the method of Lowry *et al*.,[29] with crystalline bovine serum albumin as a standard

Disc gel electrophoresis Polyacrylamide gels and solns were prepared according to Davis [30] Electrophoresis was carried out at 5° with 1 mM threonine included in the buffers The gel rods were then assayed for homoserine dehydrogenase activity[31] by immersion in 15 ml of a staining soln containing 5 mg nitroblue tetrazolium, 0 3

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mg phenazine methosulfate, 25 mM DL-homoserine, 2 5 mM $NADP^+$, 200 mM Tris-HCl (pH 9 0) and H_2O Incubation was at 30° in the dark Activity was shown by the homoserine-dependent appearance of dark bands

Treatment of kinetic data The calculation of kinetic constants was performed on a CD 3 300 computer and was based upon a computer program[19] kindly provided by Dr H -J Wieker In the inhibition studies V_m is the reaction velocity with an infinitely high concentration of inhibitor V_0 is the reaction velocity in the absence of inhibitor v Is the reaction velocity with a non-saturating concentration of inhibitor