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Aspartokinase of *Lemna paucicostata* Hegelm. 6746

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ABSTRACT

A sensitive and specific method was developed for assay of aspartokinase (EC 2.7.2.4) in crude extracts of *Lemna paucicostata*. Lysine inhibited approximately 93%, and threonine approximately 6%; together, these amino acids inhibited 99%. Inhibition by lysine was synergistically increased by *S*-adenosylmethionine, which by itself had no effect on activity. Essentially complete inhibition of threonine-resistant activity was obtained with lysine, and of lysine-resistant activity with threonine. Inhibition by lysine and threonine was additive, with no indication of concerted inhibition. Aspartate concentration had no effect on the relative proportions of lysine- and threonine-sensitive activities. Aspartokinase activity was in large excess of that reported by other workers, the maximum capacity (V_{max}) far exceeding the *in vivo* requirements. Estimations of rates of aspartokinase *in vivo* suggest that the step catalyzed by this enzyme may not be the overall 'rate-limiting' one for entry of 4-carbon units into the aspartate family of amino acids, and that feedback inhibition of this enzyme by lysine and threonine may not be a major factor in regulating flux through this step.

Aspartokinase (EC 2.7.2.4) catalyzes the first committing step in the synthesis of the aspartate family of amino acids, lysine, methionine, threonine, and isoleucine (4, 19). In a recent review, Miflin and Lea (19) concluded that most plants contain two aspartokinase activities, one inhibited by threonine, the other by lysine. The two forms from a variety of plants have been physically separated (19, 23). The predominant portion of the aspartokinase from most plants studied is sensitive to lysine (4). The inhibition by lysine is synergistic with AdoMet[2] (24). Predominantly threonine-sensitive aspartokinase has been reported in peas, soybean, and *Sinapis alba* L. (4). The relative proportions can change also with the physiologic state of the tissue; rapidly growing cells have a higher proportion of lysine-sensitive activity than do older, nondividing cells (19). Aspartokinase of *Lemna* may be atypical in that inhibition by lysine and threonine has been claimed to be concerted for the enzyme from *L. minor* (32). Initial reports of concerted inhibition by lysine and threonine of aspartokinase from wheat (31) and *Cucumis* (1) have not been substantiated by subsequent studies (3, 24, 33).

It has been proposed that aspartokinase plays a regulatory role in the biosynthesis of the aspartate family of amino acids (19, 25). However, the nature and extent of the regulation *in vivo* remains to be clarified. In the present work we examined aspartokinase of *L. paucicostata*, a higher plant possessing many experimental advantages (9) and used in this laboratory for extensive studies on the regulatory patterns of biosynthesis of the aspartate family of amino acids (12, 13, 28). We developed a sensitive and specific assay suitable for crude extracts to elucidate the salient catalytic and regulatory features of aspartokinase in this plant. This paper presents initial evidence that questions the validity of two tenets of current schemes proposed for regulation of synthesis of the aspartate family of amino acids: (1) that aspartokinase is the overall 'rate-limiting'[3] step for entry of 4-carbon units into the aspartate family of amino acids (23, 25), and (b) that feedback inhibition of aspartokinase by lysine and threonine is a major factor in regulating flux through this step (19).

MATERIALS AND METHODS

Chemicals

AdoMet (iodide salt, 85–90% pure) and L-aspartic acid-β-hydroxamate were obtained from Sigma Chemical Corp.

Solvents

Solvent A contained 2-propanol:88% HCOOH:H_2O (7:1:2, v/v). Solvent B contained 95% ethanol:7.25 mM NH_4OH (90:10, v/v).

Buffer

Buffer A contained 25 mM K phosphate (pH 7.5), 2 mM EDTA, 2 mM $MgCl_2$, 50 mM KCl, 2.0 M glycerol, and 30 mM 2-mercaptoethanol.

Radioactive Compounds

L-[U-^{14}C]Aspartic acid (ICN Radiochemicals) and L-[2,3-^{3}H]aspartic acid (Amersham) were purified by chromatography on Dowex 1. The radioactive compound was applied in 10 mL of 20 mM pyridine formate (pH 5.9), to a column of Dowex 1 (0.8 × 2 cm) equilibrated with the same buffer. The column was washed with 5 mL of this buffer and labeled aspartate eluted with 20 mL of 0.1 M HCOOH. This solution was lyophilized to dryness and the residue dissolved in water. [^{3}H]Aspartyl hydroxamate was prepared by incubation of [^{3}H]

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[2] Abbreviation: AdoMet, *S*-adenosylmethionine.

[3] As generally recommended (16, 20), the term 'rate-limiting' ('pacemaker,' 'bottle neck,' etc.) is avoided, and is used here only for the sake of accurate reporting of other workers' conclusions.

aspartic acid (585 nmol, 2.39×10^8 dpm) under the standard assay conditions of aspartokinase, modified by inclusion of 0.4 M NH_2OH (K salt) in the reaction, and by increases in reaction time (to 4.3 h), temperature (to 30°C), and enzyme (to 116 μg of protein). [^{3}H]Aspartyl hydroxamate was purified by Dowex 1 and paper chromatography as described for the standard aspartokinase assay. Marker [^{14}C]aspartate was added to the preparation prior to paper chromatography in order to confirm resolution of aspartyl hydroxamate from aspartate. The peak of [^{3}H]aspartyl hydroxamate was eluted from the chromatogram in an overall yield of 20% from [^{3}H] aspartate. The product cochromatographed in solvent A with authentic L-aspartic acid-β-hydroxamate visualized by reaction of the α-amino group with ninhydrin or the hydroxamate group with $FeCl_3$.

Plants

Lemna paucicostata Hegelm. 6746 was grown mixotrophically in medium 4 with 20 μM inorganic sulfate (9).

Preparation of Enzyme Extracts

All procedures were carried out at 0 to 4°C. Extracts were prepared from fresh plants, since approximately one-third of the enzyme activity was lost in plants frozen at −80°C overnight. Fronds (80–300) were homogenized in a glass homogenizer with buffer A (approximately 1 μL/frond) containing 1 mM L-lysine and 1 mM L-threonine. The homogenate was centrifuged at 20,000*g* for 5 min and the supernatant fraction removed with a Pasteur pipette. The pellet was suspended in 100 μL of buffer A, recentrifuged as above, and the supernatant solution combined with that from the original centrifugation. The combined supernatant fraction was gel filtered (28) with Sephadex G-25 (medium) equilibrated with buffer A. Gel filtration was repeated to minimize any transfer of lysine and threonine from the extraction medium to the assay mixture. Gel-filtered extracts were assayed immediately after preparation, since approximately one-half of the activity was lost after storage at −80°C overnight. Protein was determined as described (28).

Assay of Aspartate Kinase

When not specified either as threonine- or lysine-sensitive, 'aspartokinase activity' refers to the total activity in both forms.

Assay in Absence of NH_2OH (Standard Assay)

This assay was developed for specific and sensitive assay of aspartokinase, and was used routinely unless stated otherwise. Plant extract was incubated with [^{14}C]aspartate, resulting in formation of product [^{14}C]aspartyl phosphate. After termination of enzyme activity, NH_2OH was added to convert [^{14}C] aspartyl phosphate to the stable derivative, [^{14}C]aspartyl hydroxamate. Aspartokinase activity was determined from the amount of ^{14}C in this derivative after purification by chromatography on Dowex 1 and by paper chromatography. The assay contained the following components in a final volume of 100 μL; 6 mM L-[U-^{14}C]aspartate (2.3×10^6 dpm), 15 mM ATP, 18 mM $MgCl_2$, 1 mM DTT, 25 mM K phosphate, and enzyme extract containing up to 7 μg of protein. The final pH was 7.5. Incubation was for 5 min at 25°C. Aspartyl phosphate has a half-life of 150 min at 25°C and pH 7.7 to 7.8 (26), so little chemical decomposition occurred during the incubation period. The assay was terminated by addition of 45 μL of a solution containing 44 mM *p*-chloromercuriphenylsulfonic acid and 0.22 M EDTA. Incubation was then continued for 5 min at 15°C to ensure complete inactivation of the enzyme. [^{14}C]Aspartyl phosphate was then converted to [^{14}C]aspartyl hydroxamate by incubation for 20 min at 15°C with 100 μL of 1.7 M NH_2OH (K salt) (pH 7.5). Finally, the reaction was diluted with 1.0 mL of ice-cold H_2O. To an aliquot of 0.4 mL of this solution was added 400 nmol of carrier aspartyl hydroxamate, authentic [^{3}H]aspartyl hydroxamate to serve as an internal marker, 32 μL of 1 N HCOOH, and 20 μL of 1 M pyridine formate (pH 5.9); the final pH was 5.9. The mixture was applied to a column of Dowex 1 (0.8 × 5 cm) equilibrated with pyridine formate (pH 5.9), and the column washed with 5 mL of 25 mM pyridine formate (pH 5.9). Unreacted [^{14}C]aspartate was retained on the column. Aspartyl hydroxamate was contained in the combined flow-through and wash fractions. These were concentrated by evaporation, and chromatographed for 18 h with solvent A. Aspartyl hydroxamate migrated at R_F 0.31, and was well resolved from traces of [^{14}C]aspartate (R_F, 0.47). In rare instances (noted in the text) where resolution was not adequate, radioactive aspartyl hydroxamate was eluted from the chromatogram and again chromatographed with solvent A. The ratio of ^{14}C/^{3}H in the aspartyl hydroxamate peak was used to calculate the amount of aspartyl hydroxamate formed.

Assay in the Presence of NH_2OH

In this widely used assay, aspartokinase is determined by the rate of formation of aspartyl hydroxamate in the continuing presence of NH_2OH. The assay is linear up to much higher concentrations of protein than is the aspartyl phosphate assay described above and was used for measurements of the relatively low activities of aspartokinase in the presence of highly inhibitory concentrations of lysine. The assay contained 0.4 M NH_2OH (K salt) in addition to the components of the standard assay. The reaction was terminated after 5 min incubation at 25°C by addition of the *p*-chloromercuriphenylsulfonic acid-EDTA mixture described above, followed by 1.0 mL of H_2O. An aliquot of 0.07 to 0.40 mL of this mixture, to which was added carrier and marker [^{3}H]aspartyl hydroxamate as described above, was titrated with 1 N HCOOH to approximately pH 5.9 (methyl red indicator). After addition of 20 μl of 1 M pyridine formate (pH 5.9), the mixture was applied to a column of Dowex 1 and [^{14}C]aspartyl hydroxamate determined as described for the standard assay.

RESULTS

Extraction of Aspartokinase

The presence of 1 mM lysine plus 1 mM threonine in the extraction medium increased recovery of total and lysine-

sensitive aspartokinase activity by factors of 1.7- and 1.9-fold, respectively, without significantly affecting threonine-sensitive activity. The complete medium containing lysine plus threonine was therefore used routinely for extraction of plants. Almost all (85%) of the recovered aspartokinase activity was in the supernatant fraction.

Effects of Incubation Time, Enzyme Concentration, and NH_2OH on Aspartokinase Assay

The limits of incubation time and protein concentration specified for the standard assay should be exceeded only with extreme caution. In preliminary experiments in which protein concentrations and incubation times considerably exceeded those specified, the amount of product obtained decreased with increased enzyme and time of incubation. For example, after a 1 h incubation, the amount of aspartyl phosphate obtained with 130 μg of protein was only 15% that obtained after a similar incubation with 35 μg of protein. As a result, the specific activity determined with the higher concentration of protein was only 4% that with the lower. With further decreases in enzyme and incubation time to those specified in the standard assay, the activity increased by another order. Within the limits defined in the standard assay, aspartyl phosphate formation was linear with both time and enzyme concentration. Activities of aspartokinase determined in the presence of NH_2OH were identical to those determined with the standard assay.

Catalytic Properties of Aspartokinase

Aspartokinase activity was completely dependent upon the presence of enzyme and ATP. Omission of Mg^{2+} reduced the rate to less than 3% that of the standard assay. Figure 1 shows an Eadie-Hofstee plot of the results of experiments in which aspartokinase activity was determined at increasing concentrations of aspartate. The linear plot obtained in these limited studies is consistent with a hyperbolic relationship between velocity and aspartate concentration, with an apparent K_m for aspartate of 10 mM (SE, 0.8) and V_{max} of 78 (SE, 5) nmol/min/mg protein.

Presence of Lysine- and Threonine-Sensitive Aspartokinase Activities

Lysine inhibited aspartokinase activity by a maximum of 80 to 94%, indicating the dominance of the lysine-sensitive form of the enzyme. In determinations with extracts from four separate plant cultures, a mean value for maximal inhibition by lysine of 93% (SE = 1.2, range = 89–94) was obtained with the standard aspartokinase assay, while a mean of 82% (n = 4, SE = 0.5, range = 80–83) was obtained for assays performed in the presence of NH_2OH. The former value, indicating that the lysine-sensitive form comprises approximately 90% of the total aspartokinase activity, is regarded as more reliable; the possibility cannot be excluded that enzymes other than aspartokinase may catalyze the formation of small amounts of aspartyl hydroxamate when NH_2OH is included in the assay mixture (see "Discussion"). Percentage inhibitions by lysine remained unchanged over a wide range (0.163, 0.663, 2.063, and 25.0 mM) of aspartate concentrations. Threonine caused approximately 20% maximal inhibition of total aspartokinase activity, consistent with the minor contribution of the threonine-sensitive component.

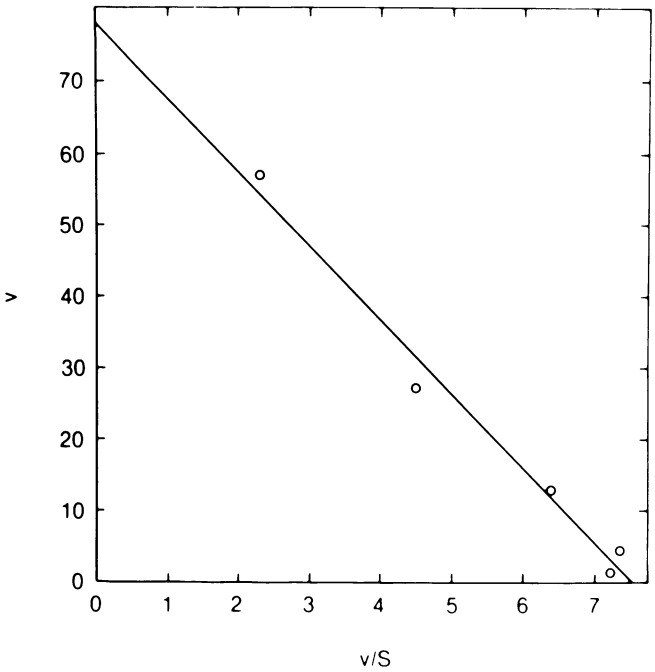

Figure 1. Effects of aspartate concentration on aspartokinase activity—Eadie-Hofstee plot. Velocity (v) is in nmol/min/mg protein. Concentration of aspartate (S) is in mM. The line of best fit was calculated according to the method of Zivin and Waud (34). Reaction mixtures are those described for the standard assay, except for increased amounts of ^{14}C in aspartate, and changes in aspartate concentrations. The additional radioactivity in aspartate required that radioactive aspartyl hydroxamate be subjected to rechromatography in solvent A before determination of the ratio of $^{14}C/^{3}H$.

The effects of increasing concentrations of amino acid inhibitor on each of the aspartokinase components are illustrated in Figure 2. Both lysine- and threonine-sensitive enzymes are strongly inhibited by their respective amino acid inhibitor, with 50% inhibition observed at approximately 40 μM lysine and 60 μM threonine. Lysine-sensitive aspartokinase approached almost complete inhibition at concentrations of lysine above 1 mM; the threonine-sensitive form approached an apparent maximum inhibition of approximately 90% at comparable concentrations.

Inhibitions in the combined presence of lysine and threonine approximated the sum with either inhibitor alone, with no indication of cooperative inhibition by these amino acids.

Cooperative Effect of AdoMet on Lysine Inhibition

Figure 3 demonstrates the cooperative inhibition of aspartokinase by lysine and AdoMet. Concentrations of AdoMet alone up to 200 μM had negligible effects on aspartokinase activity. By contrast, over the same range of concentrations, AdoMet caused progressive increases up to fourfold in the inhibition by 16 μM lysine. No cooperative effects were observed with threonine and AdoMet.

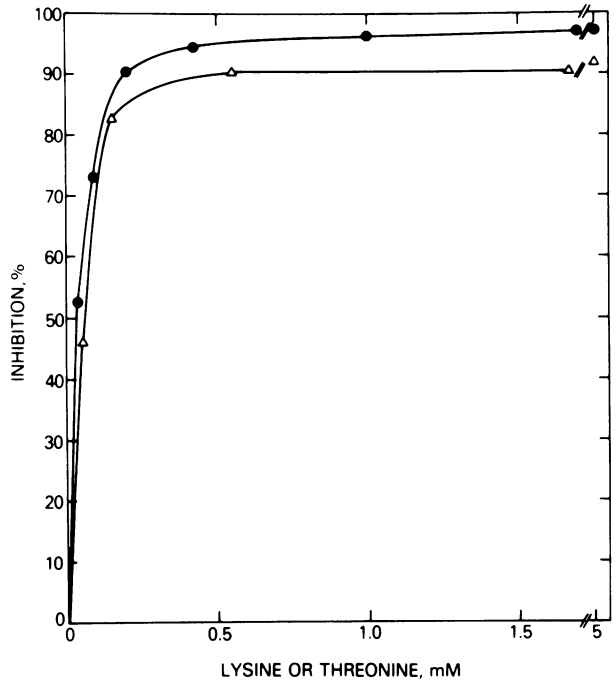

Figure 2. Inhibition of lysine- and threonine-sensitive aspartokinases by the respective amino acids. (●), Inhibition of lysine-sensitive aspartokinase by lysine. Inhibitions were calculated from the activities determined at each specified concentration of lysine (plus 5 mM threonine) compared to that determined with 5 mM threonine alone. (△), Inhibition of threonine-sensitive aspartokinase by threonine. Inhibitions were calculated from the activities determined at each specified concentration of threonine (plus 5 mM lysine) compared to that determined with 5 mM lysine alone. All assays were in the presence of NH_2OH.

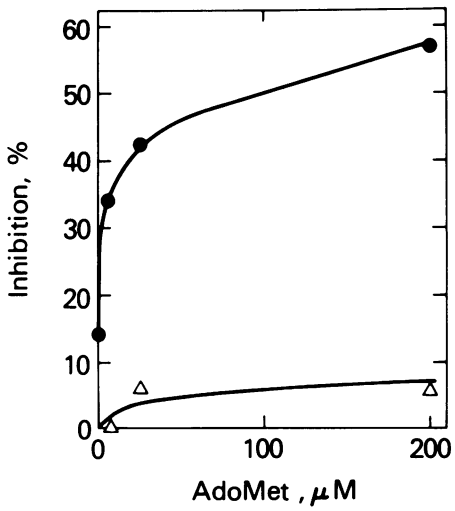

Figure 3. Cooperative inhibition of aspartokinase by AdoMet and lysine. Percentage inhibition is plotted against increasing concentrations of AdoMet (△), or AdoMet plus 16 μM lysine (●).

DISCUSSION

A major problem in studies of aspartokinase in plants has been the lack of a suitable assay of this enzyme in crude extracts (10, 18). Neither of the two types of assays currently in use is entirely satisfactory. In one, aspartyl phosphate production is coupled via aspartic semialdehyde dehydrogenase to NADH oxidation. This assay can be complicated by high background rates of NADH oxidation, and requires isolation of the coupling enzyme and demonstration that it is not affected by the presence of the effectors, etc. under study. In the other type of assay, aspartyl phosphate reacts with NH_2OH present in the reaction mixture to form the stable derivative, aspartyl hydroxamate. This assay suffers from the disadvantages of possible modification of enzyme properties by high concentrations of NH_2OH (10) and in not being specific for aspartokinase. In the constant presence of NH_2OH in the assay, asparagine synthetase and aspartyl-*t*-RNA synthetase each catalyze synthesis of aspartyl hydroxamate via an enzyme-bound aspartyladenylate intermediate. Both disadvantages can be avoided by omission of NH_2OH from the reaction mixture until after termination of enzyme activity, as was done in the present work. This tactic has not been used previously for routine determinations of aspartokinase, although it has occasionally been used for comparison with assays carried out in the presence of NH_2OH (10, 11). This tactic, as well as the use of [^{14}C]aspartate of high specific activity, provided an assay of extreme sensitivity, capable of measuring rates as low as 100 pmol/min. The high sensitivity was a critical factor in permitting the assay to be carried out at the low levels of protein and short incubation times which yielded maximal specific activities.

Both the total aspartokinase activity and the relative proportions sensitive to lysine and threonine were of major interest in these studies. It was therefore important to provide optimal conditions for recovery of these activities. Buffer A has been used successfully by Rognes and coworkers (23, 24) for isolation from a variety of plant tissues of aspartokinase activities sensitive predominantly to either lysine or threonine. In the present work, addition of lysine plus threonine to buffer A approximately doubled recovery of the major, lysine-sensitive aspartokinase activity without affecting recovery of threonine-sensitive aspartokinase. The improved recovery probably results from protection by lysine of lysine-sensitive aspartokinase, as reported for this enzyme from other plant tissues (6, 22, 27).

Aspartokinase activity from *L. paucicostata* resembles that isolated from a number of other plants (4, 19) in that a major fraction (at least 80%) is inhibited by lysine, with most of the remaining activity being inhibited by threonine. The apparent K_m (10 mM) for aspartate is similar to values reported for aspartokinases isolated from other tissues (18). The concentrations of 40 μM lysine or 60 μM threonine required for half-maximal inhibition of the respective lysine- and threonine-sensitive activities of *L. paucicostata* fall near the lower ends of the ranges of 13 to 700 μM and 100 to 570 μM, respectively, reported for aspartokinases isolated from other plants (4, 18). While having little effect alone, AdoMet appreciably increased the inhibition by lysine. No evidence was obtained for cooperative inhibition by lysine and threonine, as has been claimed for aspartokinase from *L. minor* (32).

Based on the determined properties of the enzyme and estimations of its cellular environment, calculations were made of the maximum potential rates of aspartokinase *in*

Table I. *V_{max} Values of Aspartokinase Relative to in vivo Requirements*

Total flux through aspartokinase *in vivo* (13.4 nmol/frond·doubling) was estimated from the combined amounts of protein lysine (4.5 nmol/frond, ref. 14), protein methionine (1.0 nmol/frond, ref. 15), protein threonine (4.5 nmol/frond, Ref. 13), and protein isoleucine (3.4 nmol/frond, ref. 13) in *L. paucicostata*. Aspartokinase activities are reported at saturating concentrations of substrates (aspartate and ATP) and Mg^{2+}. Lysine- or threonine-sensitive forms of aspartokinase are not assigned to any particular branch in the aspartate family, since our studies (13, 14) fail to indicate any channeling of the products of these two activities. Condition 1: Activity in the absence of inhibitors. The mean aspartokinase activity for control cultures was 70 pmol/min/frond (n = 11, SE = 6, range 42–109) determined under standard assay conditions (6 mM aspartate, 15 mM ATP, 18 mM Mg^{2+}). From this value was calculated a V_{max} of 187 pmol/min/frond based on an apparent K_m of 10 mM for aspartate. It was assumed that the concentrations of ATP and Mg^{2+} in the standard assay approached saturation; studies of aspartokinase in other plant tissues show the apparent K_m of ATP ranges between 0.6 and 5 mM (19, 22), with optimal rates obtained at approximately equimolar concentrations of Mg^{2+}. The latter aspartokinase activity was then converted to a value of 565 nmol/frond·doubling by multiplication by 60 × 24 × 2.1. The value of 2.1 is the number of frond·d equivalent to a frond·doubling for control plants with a doubling time of 35 h, and was calculated as described (7). Condition 2: Activity in the presence of the physiological concentrations of 29 μM lysine (14), 190 μM threonine (14), and 15 μM AdoMet (8) present in control cultures of *L. paucicostata*, assuming uniform distributions of these compounds throughout the plant. The value of 242 nmol/frond·doubling was calculated from the combined amounts of threonine-sensitive (6 nmol/frond·doubling) and lysine (plus AdoMet)-sensitive (236 nmol/frond·doubling) activities. Threonine-sensitive activity in the presence of 190 μM threonine was calculated by multiplying the threonine-sensitive aspartokinase activity of control plants (7% of 565 = 40 nmol/frond·doubling) by the fraction (15%) of this activity remaining at this concentration of threonine (Fig. 2). Activity of lysine-sensitive aspartokinase in the presence of 29 μM lysine (315 nmol/frond·doubling) was calculated by multiplying lysine-sensitive activity of control plants (93% of 565 = 525 nmol/frond·doubling) by the percentage (from Fig. 2) of approximately 60% of this activity remaining in the presence of this concentration of lysine. The additional effect of 15 μM AdoMet was estimated from the results of Figure 3, which indicate that activity in the presence of 15 μM AdoMet plus lysine would have been approximately 75% of that with lysine alone. Condition 3: Activity in the presence of estimated chloroplastic concentrations of lysine, threonine, and AdoMet. Chloroplastic concentrations of lysine (70 μM) and threonine (750 μM) were approximated by multiplying the tissue concentrations of these amino acids given in condition 2 by the ratio of the relative concentrations of the respective amino acid in chloroplasts and whole leaves of *Zea mays* (5). A value of 15 μM AdoMet was assumed in the absence of data on the chloroplastic concentration of this compound. Aspartokinase activities were calculated essentially as described in condition 2. Condition 4: Complete inhibition of threonine-sensitive aspartokinase, without inhibition of the lysine-sensitive form. Shown in parentheses are corresponding values that also include inhibition of the lysine-sensitive form by tissue concentrations of 29 μM lysine and 15 μM AdoMet in control plants. Derivation of the resultant activity of 236 nmol/frond·doubling is given under condition 2. Condition 5: Complete inhibition of lysine-sensitive aspartokinase, without inhibition of the threonine-sensitive form. Shown in parentheses are corresponding values that also include inhibition of the threonine-sensitive form by the tissue concentration of 190 μM threonine in control plants. Derivation of the resultant activity of 6 nmol/frond·doubling is given under condition 2. Condition 6: Complete inhibition of lysine- and threonine-sensitive aspartokinase. The value of 4 nmol/frond·doubling is based on the calculation that approximately 0.7% of the total aspartokinase appeared insensitive to inhibition by either lysine or threonine. The value of 0.7% was calculated from the expression: percentage of total activity resistant to inhibition by lysine (100-93 = 7%) × percentage (10%) of lysine-resistant activity resistant to threonine inhibition (Fig. 2). The value of 0.7% sets an upper limit, since it remains to be established conclusively that the extremely low activity in the presence of lysine plus threonine can in fact be attributed to aspartokinase.

Condition No.	Conditions of Measurement	Aspartokinase Activity	
		nmol/frond·doubling	*-fold in vivo flux*
1	No inhibitors	565	42
2	Plus Lys, Thr, AdoMet (tissue concentrations)	242	18
3	Plus Lys, Thr, AdoMet (chloroplast concentrations)	118	9
4	Complete inhibition of Thr-sensitive	525 (236)	40 (18)
5	Complete inhibition of Lys-sensitive	40 (6)	3 (0.45)[a]
6	Complete inhibition of Lys- and Thr-sensitive	4	0.3

[a] The value of 0.45 was calculated as described in the legend on the basis of the estimated combined *in vivo* flux of 13.4 nmol/frond·doubling into protein lysine, methionine, threonine, and isoleucine. Tissue concentrations of lysine resulting in complete inhibition of lysine-sensitive aspartokinase would cause extensive feedback inhibition of lysine biosynthesis (14), thereby reducing the estimated *in vivo* flux through aspartokinase to approximately 13.4–4.5 = 8.9 nmol/frond doubling. Based on this value, threonine-sensitive aspartokinase would provide 6/8.9 = 0.67-fold the *in vivo* flux.

vivo. These estimates (Table I) indicate that the maximum capacity (V_{max}) of aspartokinase far exceeds the *in vivo* requirement for this enzyme. The large potential excess capacity of aspartokinase pertains not only in the absence of inhibitors (condition 1), but also with the concentrations of lysine and threonine (and AdoMet) determined in whole tissues (condition 2), and estimated in chloroplasts (condition 3), the organelle in which aspartokinase is localized (29, 30). The results of Table I further show that the V_{max} for lysine-sensitive aspartokinase acting alone is in large excess of *in vivo* requirements (condition 4). Even the V_{max} of the minor, threonine-sensitive activity approximates the *in vivo* requirements (condition 5). Only as complete inhibition of both lysine- and threonine-sensitive components is approached (condition 6) was the V_{max} of aspartokinase reduced below that required for *in vivo* flux for combined synthesis of the aspartate family amino acids. Together, these finding suggest that although lysine and threonine may normally inhibit aspartokinase activity, such inhibition is not severe enough to make aspartokinase activity limiting, *i.e.* feedback inhibition by lysine and

threonine may not normally be a major factor in regulating flux through the aspartokinase step. Confirmation of these suggestions is provided in the companion paper (14) which presents definitive *in vivo* studies involving measurements of the actual fluxes through the aspartokinase step and into each of the aspartate family of amino acids under a variety of growth conditions.

To what extent are these findings relevant to other higher plants? Aspartokinase activities reported for other plants are typically an order of magnitude less than those observed here for *L. paucicostata*,[4] and less than half the activity of any other enzyme in the aspartate pathway for which assays are available (25). On the basis of the latter observation, Rognes *et al.* (25) tentatively concluded that aspartokinase is the overall 'rate-limiting' step for entry of 4-carbon units from aspartate into the aspartate family of amino acids. We believe that the reported activities of aspartokinase may have appreciably underestimated the maximum *in vivo* capacity of this enzyme,[5] and that the proposed role of aspartokinase in regulating entry of 4-carbon units into the aspartate family of amino acids should be reevaluated. In agreement with this possibility, in spite of the apparently low aspartokinase activity reported for excised barley embryos, the ability of this tissue to grow at a normal rate with a concentration of lysine expected to cause severe inhibition of aspartokinase *in vivo* (25) strongly suggests that this enzyme normally operates below its maximum capacity, and that lysine is not a major regulator *in vivo* of flux through the aspartokinase step. A further example is given by studies of Yamada *et al.* (33) with wheat cell cultures. The aspartokinase activity of 3.6 nmol/min/mg protein reported for this tissue is typical of the low values reported for most plant tissues. However, as pointed out by Yamada *et al.* the ability of cultures supplemented with lysine to grow at a normal rate shows that "considerable inhibition of aspartokinase by lysine does not affect its ability to provide precursors required for the biosynthesis of other aspartate-derived amino acids." An additional problem in assessing the physiological significance of previously reported aspartokinase activities is that quantitative data are rarely available to permit comparison of these activities with the required *in vivo* fluxes. A review of the literature revealed only one example that allowed such direct comparison. Interestingly, this study (2) showed that after 5 d growth of carrot cell suspension cultures the extracted aspartokinase activity was approximately 3-fold the *in vivo* requirement, and increased to more than 60-fold after 18 d growth. These limited observations suggest that higher plants in general may contain excess capacity of aspartokinase, and that this enzyme step is not a major site for feedback regulation of flux.

[4] The mean specific activity of aspartokinase determined with crude extracts of *L. paucicostata* (38 nmol/min/mg protein, $n = 10$; SE = 3, range = 19–53) was compared with reported values determined under similar assay conditions for crude extracts (sometimes subjected to preliminary purification) from other plant tissues. Of a total of 13 different tissues for which values were available (1, 11, 17, 18, 21, 24, 32), only one was reported to have a specific activity of aspartokinase greater than 12% that of *L. paucicostata*: aspartokinase from pea leaves purified to an unspecified extent with DEAE-cellulose (17) had an activity of 24% that of *L. paucicostata*.

[5] A number of reasons may be proposed why previously reported activities of aspartokinase may have underestimated *in vivo* activities. Results presented in this work show that appreciable activity can be lost during isolation (in the absence of lysine plus threonine) and storage of the enzyme. Furthermore, the low and variable activities determined in preliminary experiments (see "Results") emphasize the critical importance of optimization of the particular assay conditions (especially time of incubation and enzyme concentration) being employed.

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