REGULATION OF THE ASPARTATE PATHWAY IN ISOLATED PEA CHLOROPLASTS

Effects of *S*-2-aminoethylcysteine and threonine on the metabolism of labeled aspartate

Ernest L. BURDGE* and Kenneth G. WILSON
Department of Botany, Miami University, Oxford, OH 45056, USA

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1. Introduction

S-2-Aminoethylcysteine, a structural analogue of Lys, can inhibit the activity of 2 enzymes involved in Lys biosynthesis, namely aspartate kinase and dihydrodipicolinate synthetase [1–3]. Aspartate kinase catalyzes the first reaction of the Asp pathway and dihydrodipicolinate synthetase catalyzes the first enzyme of the branch to Lys (fig.1).

A majority of the cellular aspartate kinase activity and all of the cellular dihydrodipicolinate synthetase activity is located in the chloroplast [4–7]. Chloroplasts have the ability to synthesize all of the Asp-derived amino acids [8,9] and, recently, it has been suggested that all of the Lys in the leaf is synthesized in the chloroplast [6].

Based on this evidence it is likely that the metabolism of Asp in the chloroplast is particularly sensitive to AEC treatment. To examine this possibility more closely, we have studied the effects of AEC and Thr on the synthesis of the Asp-derived amino acids in isolated pea chloroplasts. These results show that AEC inhibits early reactions of the Asp pathway.

2. Materials and methods

2.1. *Chemicals*

L-[U^{14}-C]Aspartic acid (225 μCi/μmol) was pur-

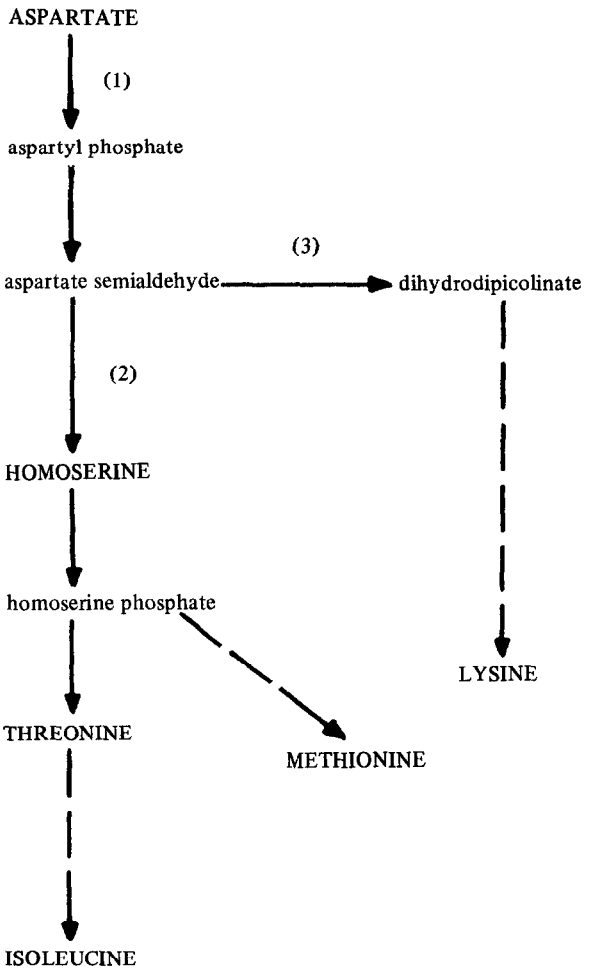

Fig.1. Diagram of the biosynthetic pathway of the aspartate family of amino acids. The numbers in parentheses refer to enzymes catalyzing the reactions: (1) aspartate kinase; (2) homoserine dehydrogenase; (3) dihydrodipicolinate synthetase.

Abbreviations: AEC, *S*-2-aminoethylcysteine; Asp, aspartate; HSer, homoserine; Ile, isoleucine; Lys, lysine; Met, methionine; Thr, threonine

* A portion of this work was included in the Doctoral Dissertation of E. L. B. Present address: USDA, US Plant, Soil and Nutrition Laboratory, Ithaca, NY 14850, USA

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chased from New England Nuclear Corp. (Boston MA). The purity of the [^{14}C]aspartic acid was determined by two-dimensional thin-layer chromatography. All other chemicals were purchased from Sigma Chemical Co. (St Louis MO).

2.2. *Plant material*

Seeds of pea (*Pisum sativum* L. cv. Alaska) were germinated and grown in vermiculite for 10 days as in [10]. Prior to harvest the plants received a 14–16 h dark treatment followed by a 30 min light treatment.

2.3. *Isolation and incubation of chloroplasts*

Chloroplasts were isolated from 6 g pea apices and incubated with [^{14}C]Asp as in [10]. Chloroplast intactness was determined according to [11]. Chlorophyll was determined according to [12] and cytochrome-*c* oxidase was assayed according to [13].

2.4. *Analysis of products of Asp metabolism*

The incubations were stopped by adding an equal volume of 20% trichloroacetic acid containing 1 mM Asp, HSer, Ile, Lys, Met and Thr as carrier-standards. After at least 1 h the precipitated protein was collected by centrifugation and the pellet was washed once with 10% trichloroacetic acid. The supernatants were pooled and the amino acids were desalted using Dowex 50 ion-exchange resin (H^+) as in [14]. The 3 N NH_4OH eluate containing the amino acids was taken to dryness under vacuum and resuspended in water. The samples were spotted onto cellulose-coated 20 × 20 cm thin-layer plates and developed in two dimensions (twice in each dimension) using the solvent system in [15]. The first-dimension solvent was butanol–acetone–ammonia–water (10:10:5:2) and the second-dimension solvent was isopropanol–formic acid–water (20:1:5). The amino acids were detected by lightly spraying the plates with 0.2% ninhydrin in ethanol. The radioactivity in the individual spots was determined according to [16].

3. Results and discussion

The chloroplast preparations contained 70–80% intact chloroplasts (phase-contrast microscopy) and were essentially free of mitochondria (<0.1% total cytochrome-*c* oxidase activity) and bacteria (no colonies after 24 h incubation on nutrient agar).

When isolated chloroplasts were incubated with [^{14}C]Asp in the light for 15 min, the majority of the radioactivity remained in the free pool of amino acids (as Asp) while a small fraction of the radioactivity was detected in protein. The synthesis of the Asp-derived amino acids is shown in table 1. Of the total [^{14}C]Asp converted to the Asp family of amino acids, 85% was recovered in HSer, 6% in Lys, 5% in Thr, 2% in Ile, and 2% in Met. Most of the radioactivity accumulated in Hser while little accumulated in Thr, Ile or Met. This result is attributed to the presence of a large pool of HSer which effectively 'traps' the entering ^{14}C. HSer in pea chloroplasts is 1–5 mM [17].

Table 1
The effects of AEC and THR on the conversion of [^{14}C] aspartate to lysine, homoserine, methionine, threonine, and isoleucine in isolated chloroplasts[a]

	Control cpm per sample (×10^{-2})	AEC cpm per sample (×10^{-2})	THR cpm per sample (×10^{-2})	AEC + THR cpm per sample (×10^{-2})
Lys	42.8	19.7 (46)[b]	92.3 (216)	23.5 (55)
HSer	656.9	433.5 (66)	321.8 (49)	52.6 (8)
Thr	42.0	39.8 (95)	28.6 (68)	26.0 (62)
Ile	12.8	13.2 (103)	11.1 (87)	11.7 (91)
Met	13.8	8.6 (62)	9.5 (69)	6.8 (49)

[a] Seven separate tubes each containing 0.5 μCi [^{14}C]aspartate, 4 μg chl, 200 mM KCl, 66 mM Tricine (pH 8.3) and 6.6 mM $MgCl_2$ in a final volume of 0.5 ml were incubated in the light for 15 min. The tubes were then pooled and treated as described in the text. Where added, AEC and Thr were 5 mM

[b] The values in parentheses are percent of control

The metabolism of [^{14}C]Asp was altered when AEC and/or Thr was included in the incubation mixture (table 1). In each treatment there was a pronounced effect on HSer biosynthesis. The accumulation of radioactivity in HSer was reduced 34% by treatment with AEC, 51% by Thr, and 92% by the combination AEC + Thr.

Lys biosynthesis was also altered by treatment with AEC and/or Thr (table 1). AEC alone, or in the presence of Thr, reduced Lys biosynthesis by ~50%. Treatment with Thr alone, however, increased the accumulation of radioactivity in Lys 2-fold. These data indicate that the block in the Asp pathway created by Thr is before HSer but after the branch to Lys. The block created by AEC is before the branch to Lys.

Aspartate kinase and dihydrodipicolinate synthe-

tase are 2 enzymes early in the Asp pathway that are likely sites for the inhibitory action of AEC. Both enzymes are sensitive to inhibition by Lys or AEC [1–3] and both enzymes are located in the chloroplast [4–7]. Inhibition of dihydrodipicolinate synthetase by AEC would inhibit Lys synthesis but should not reduce the biosynthesis of HSer. However, inhibition of aspartate kinase by AEC would inhibit the synthesis of both Lys and HSer. Our results showing a reduction in both Lys and HSer biosynthesis are compatible with AEC inhibition of aspartate kinase. An additional block by AEC at dihydrodipicolinate synthetase is not excluded.

Aspartate kinase is also a possible site for the inhibitory action of Thr. In pea and in several other organisms, Lys-sensitive and Thr-sensitive forms of aspartate kinase exist and the relative proportion of the 2 isoenzymes changes during development [18–20]. The results in [19] indicate that chloroplasts isolated from 9–11-day-old pea shoots contain both isoenzymes of aspartate kinase, however the Lys-sensitive isoenzyme is the predominant form. Homoserine dehydrogenase (fig.1) is also a likely site for Thr inhibition. This enzyme is found in pea chloroplasts [4] and is sensitive to inhibition by Thr [7,21]. The labeling pattern that we observed with Thr treatment suggests that homoserine dehydrogenase rather than aspartate kinase is the site of Thr inhibition. Inhibition at the level of aspartate kinase would reduce both HSer and Lys biosynthesis yet we found an increase of Lys biosynthesis and an inhibition of HSer biosynthesis. This apparent stimulation of Lys biosynthesis is probably due to an increased availability of aspartate semialdehyde, a common substrate for both homoserine dehydrogenase and dihydrodipicolinate synthetase. A block at homoserine dehydrogenase would then divert aspartate semialdehyde to Lys. The fact that our data are not consistent with the presence of a Thr-sensitive aspartate kinase might be the result of differences in the developmental stages of the plants. It is evident that a detailed developmental study of the aspartate kinase isoenzymes in pea chloroplasts is necessary to resolve this matter.

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