INTRACELLULAR LOCALIZATION OF β-ASPARTATE KINASE IN SPINACH (*SPINACEA OLERACEA*)

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1. Introduction

The aspartate-derived amino acid pathway leads to the synthesis of lysine, threonine and methionine, essential in the nutrition of nonruminant animals, including man. The location of the biosynthetic machinery for this pathway in the chloroplasts of higher plants is indicated by the demonstration of the activity of: β-aspartate kinase in pea chloroplasts and root plastids (Burdge et al., in preparation), a lysine- and threonine-sensitive β-aspartate kinase from pea leaf chloroplasts isolated by differential centrifugation [1], diaminopimelate decarboxylase from *Vicia faba* [2], and homoserine dehydrogenase from maize [3]. Isolated pea chloroplasts are also capable of light-dependent incorporation of radiolabeled aspartate and sulfate into soluble and protein-bound amino acids of the aspartate family [4]. These findings suggest the importance of the chloroplast in nitrogen metabolism.

β-Aspartate kinase is a key regulatory enzyme catalyzing the first step in the aspartate pathway leading to the synthesis of lysine, threonine and methionine. As such, its presence in a particular cellular compartment or organelle would suggest that control of the aspartate pathway resides in that organelle. The present work reports results of initial efforts to demonstrate the presence of β-aspartate kinase (ATP:L-aspartate 4 phosphotransferase, EC 2.7.2.4) in organelle preparations from spinach leaf tissues.

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2. Materials and methods

2.1. *Tissue homogenization*

Deribbed tissue of spinach (cv. Bloomsdale Longstanding) leaves from one-month-old plants grown at 22°C with 250 $\mu\epsilon \cdot M^{-2}$ mixed incandescent and fluorescent light for 8 h daily was ground in a 66 mM Tricine buffer (pH 8.4, 1:4 w/v) containing 0.33 M sorbitol, 2 mM $MgCl_2$, 1 mM EDTA, 4 mM 2-mercaptoethanol, and 0.1% bovine serum albumin. A Sorvall omni mixer was used in sucrose gradient preparations, and a polytron homogenizer was employed for differential centrifugation. The crude homogenate was filtered through several layers of cheesecloth and miracloth.

2.2. *Organelle preparation by sucrose density gradient centrifugation*

A combined continuous and discontinuous sucrose gradient was used to isolate intact chloroplasts according to Miflin and Beevers [5] (0–4°C). The crude homogenate was centrifuged at 1500 × *g* for 1 min. The resulting pellet was resuspended in extraction buffer minus mercaptoethanol and centrifuged at 1500 × *g* for 1 min. This pellet was resuspended in extraction buffer minus mercaptoethanol, and 10 ml of the resuspended pellet were layered on top of the sucrose gradient, consisting of a 4-ml cushion of 60%

sucrose, a 6-ml linear gradient of 60–42% sucrose, 5 ml of 42% sucrose, a 10-ml linear gradient of 42–30% sucrose, and a final 3 ml of 30% sucrose. After addition of the homogenate, the gradients were placed in a SW-27 rotor in a Beckman L.5-65 centrifuge and centrifuged at 2500 × *g* for 5 min and then 18 000 × *g* for an additional 10 min. The gradients were fractionated into 1.5-ml samples using an ISCO Model 328 density gradient fractionator. Sucrose concentrations were determined by refractometry. Protein concentrations for each fraction were determined, following protein precipitation with 10% TCA, by a microbiuret assay, using bovine serum albumin (Sigma) as a standard [6]. Chlorophyll was determined on each fraction by the method of Arnon [7].

2.3. *Chloroplast preparation by differential centrifugation*

The spinach leaf crude homogenate was centrifuged at 2000 × *g* for 50 s (0–4°C). The resulting pellet (P_1) was resuspended in the extfaction buffer and centrifuged twice at 2000 × *g* for 50 s. The resulting washed pellet (P_3) was then resuspended in extraction buffer + 25% glycerol and sonicated (Ultrasonics, Inc.) with 2–3-s bursts at 60 W power. This solution was then centrifuged at 2000 × *g* for 50 s to obtain a supernatant (FPS) and the pellet (FPP). The pellet (FPP) was resuspended in extraction buffer + 25% glycerol. The crude, supernatant and pellet fractions were analyzed for protein (by a modified Lowry [8]), chlorophyll, β-aspartate kinase and nitrite reductase activities. This pellet (P_3) was also analyzed for chloroplast integrity by ferricyanide-dependent O_2 evolution and CO_2 fixation.

2.4. *Ferricyanide-dependent O_2 evolution*

The ferricyanide reaction was used as a criterion for intactness of the chloroplast envelope [9]. The degree of intactness of the chloroplasts was estimated from rates of ferricyanide-dependent O_2 evolution before and after osmotic rupture. The oxygen concentration was measured in a YSI model 5331 Oxygen Monitor in a solution containing 50 mM HEPES (pH 7.6), 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. Six μl of $K_3Fe(CN)_6$ (0.1 M), 18 μl of 0.1 M NH_4Cl, and chloroplast suspension corresponding to ~10 μg of chlorophyll were added to a final volume of 2 ml. Oxygen evolution from ruptured chloroplasts was measured in the same HEPES buffer minus sorbitol.

2.5. *CO_2 fixation*

Light-dependent CO_2 fixation was measured according to Walker [10]. The following reagents plus $NaH^{14}CO_3$ (243 dpm/nmole, New England Nuclear) and 50 μg/ml chlorophyll were added to a final volume of 2 ml: 50 mM HEPES (pH 7.6, adjusted with NaOH), 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM Na-isoascorbate, 0.5 mM K_2HPO_4, and 5.0 mM $Na_4P_2O_7$. At 2-min intervals for 10 min, 200 μl of the assay mixture were removed and acidified with 160 μl of 5 N HCl. The samples were oven-dried, and $^{14}CO_2$ fixation, measured as acid-stable product (in 2:1 toluene:Triton containing PPO and POPOP), was determined in a Mark III Searle scintillation counter. Activity is reported as nmol CO_2 fixed/mg chl · h.

2.6. *Enzyme assays*

All enzymes were measured spectrophotometrically at 25°C using a Beckman Model 25 spectrophotometer. The following marker enzymes were assayed on each sucrose density gradient fraction: catalase (EC 1.11.1.6) [11], cytochrome *c* oxidase (EC 1.9.3.1) [12], and triosephosphate isomerase (EC 5.2.1.1) [13]. Nitrite reductase [14] and β-aspartate kinase by the method of Black and Wright [15] as previously described [16] were also assayed.

3. Results and discussion

The distribution of β-aspartate kinase activity in the sucrose density gradients indicates that a sharp peak of β-aspartate kinase coincides with the chlorophyll and triosephosphate isomerase activity peaks in the intact chloroplast (fraction no. 7) (fig.1A–C). This chloroplast peak is essentially free of cytochrome *c* oxidase and catalase activities, and thus mitochondria and microbodies. A substantial portion of the β-aspartate kinase activity occurs in the region of the gradient corresponding to the major chlorophyll-containing fractions (fraction no. 10–20). These fractions represent damaged chloroplasts which are devoid of stroma. In addition, a substantial portion of

the β-aspartate kinase activity appears to coincide with the cytosolic form of triosephosphate isomerase, at the top of the gradient. The pattern of distribution obtained for β-aspartate kinase suggests that a chloroplast- or thylakoid-localized form of β-aspartate kinase is present, or that β-aspartate kinase binds tightly to the outer membrane of the chloroplast.

Representation of these data in tabular form (table 1) substantiates the apparent enrichment of

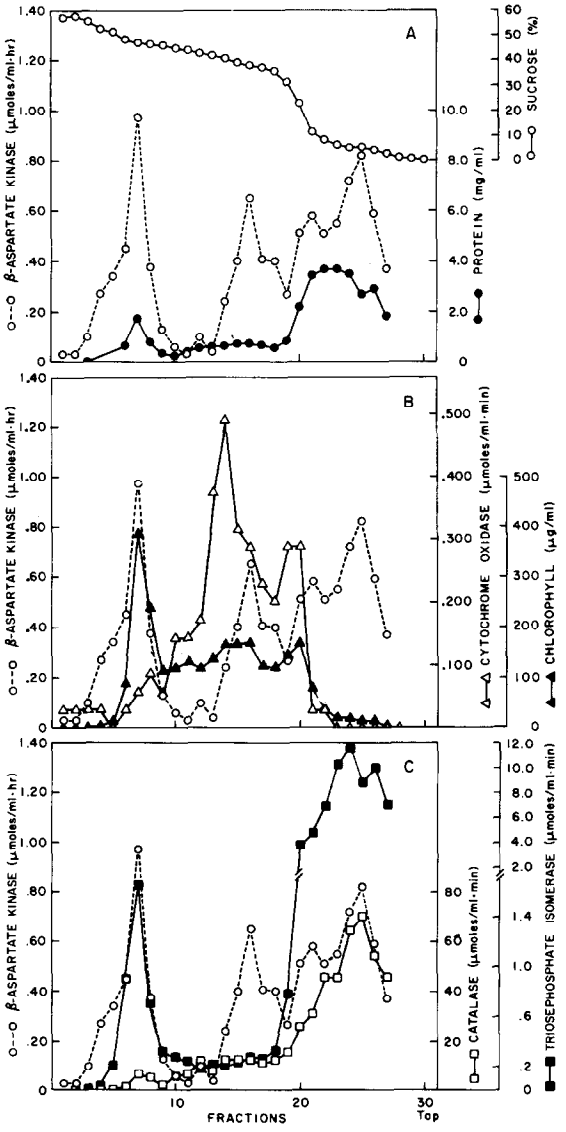

Fig.1A–C. Results of sucrose density gradient centrifugation of young, deribbed spinach leaves.

β-aspartate kinase in the intact chloroplast fractions, although it does seem to be distributed also in the soluble and broken chloroplast fractions. The activity associated with these fractions may have resulted from damage to the chloroplasts by the fractionation procedures; however, there is approximately 30% enrichment of the β-aspartate kinase activity in the intact chloroplast fraction compared to the other fractions. The appearance of β-aspartate kinase activity in the chloroplast peak suggests that β-aspartate kinase is partially localized within the chloroplast.

To further substantiate the localization of β-aspartate kinase within the chloroplasts, a second method (described in 2.3) was employed to isolate intact chloroplasts. Results of a representative differential centrifugation are shown in table 2. The distribution of nitrite reductase and the rate of CO_2 fixation indicate that a substantial portion of the chloroplasts have leaked their contents. The chloroplast pellet contained approximately 40–50% intact chloroplasts as determined by ferricyanide-dependent O_2 evolution and fixed low rates of CO_2 – 10 μmol CO_2/mg chl · h; the ferricyanide assay, however, does not distinguish between intact chloroplasts and those that have opened and resealed. (Subsequent experiments have yielded chloroplast preparations which are approximately 70% intact.) Six to seven percent of the total nitrite reductase activity was found in the chloroplast pellet. Assuming the pellet contains approximately 50% intact chloroplasts, approximately 12–14% of the nitrite reductase total activity can be attributed to the chloroplast. This finding agrees with that of Miflin; i.e., approximately 13% of the nitrite reductase is associated with the intact chloroplast fraction from sucrose density gradient preparations [17], which indicates substantial breakage of the chloroplasts inherent in grinding and centrifugation procedures.

β-Aspartate kinase also shows a substantial portion of activity in the supernatant, with approximately 11% associated with the chloroplast-enriched pellet. If the chloroplasts were 40–50% intact, the most activity that could be attributed to the chloroplast fraction would be approximately 20–25% of the total, roughly equivalent to what was observed in the sucrose density gradient intact chloroplast peak. However, quantitative determination of the estimates of in vivo enzyme content in the chloroplasts by a

Table 1
Enzyme and chlorophyll distribution in sucrose density gradient fractions

Component	Intact chloroplasts	Mitochondria and broken chloroplasts	Microbodies and soluble fraction
Chlorophyll μg/ml	1296.3	894.2	140.1
% of total	(48)	(40)	(5.7)
Catalase[a]	56.3	81.3	409.2
% of total	(9.9)	(14)	(72)
Triosephosphate isomerase[a]	3.92	4.41	65.66
% of total	(4.6)	(5.2)	(78)
Cytochrome oxidase[a]	0.411	1.586	0.084
% of total	(18)	(70)	(3.7)
β-Aspartate kinase[b]	4.44	3.04	3.22
% of total	(39)	(27)	(28)

[a] μmol/ml · min
[b] μmol/ml · h

calculation of recovered enzyme activity in relation to recovered chlorophyll in the intact chloroplast, used by Bryan et al. [3] for homoserine dehydrogenase, indicates that approximately 60% of the total β-aspartate kinase activity is associated with the intact chloroplast.

Analysis of the FPS and FPP fractions shows that the only detectable nitrite reductase activity was associated with the soluble fraction and that the distribution of β-aspartate kinase is roughly equivalent in the two fractions; therefore, we do not yet know if β-aspartate kinase is a soluble stromal or membrane-bound protein in the chloroplast. The data suggest that either two populations exist or β-aspartate kinase sticks to the membrane.

The intracellular distribution of β-aspartate kinase in a leaf homogenate was also determined by another procedure. This involved centrifugation of a crude

Table 2
Distribution of nitrite reductase and β-aspartate kinase activities among differential centrifugation fractions

Fraction	Chlorophyll		Protein		Nitrite reductase		β-Aspartate kinase	
	μg/ml	% total	mg/ml	% total	Total activity[a]	% total	Total activity[b]	% total
Crude	45	100	1.7	100	35.8	100	310	100
$Super_1$	10	20	1.2	62	22.4	62	258	83
$Super_2$	7.6	1.5	0.6	3	ND[c]	–	21.8	7.0
$Super_3$	13	2.7	0.5	2.7	ND	–	4.6	1.4
Pellet[d]	166	56	2.3	20	2.39	6.7	34.3	11
% of pellet								
FPS	67	43	1.2	42	0.57	26	12.3	36
FPP	90	57	1.6	58	ND	–	13.6	40

[a] Units = mmol NO_2^- red/min
[b] Units = μmol AspH/min
[c] ND = not detectable
[d] Characteristics of chloroplasts ~50% intact, fix 10 μmol CO_2/mg chl · h

Table 3
Solubilization of β-aspartate kinase from isolated chloroplasts

	Specific activity[a] (units/mg protein)	Chlorophyll	−BSA[b]			+BSA (0.1%)[b]		
		% recovered	Total units	% total	% protein	Total units	% total	% protein
Crude suspension	2.17	100	2490	100	100	1142	100	100
Supernatant solution minus whole chloroplasts	1.85	42	1296	52	46	832	70	71
Extract from ruptured chloroplasts	3.06	62	95	4	6	56.7	5	13
Supernatant/pellet enzyme ratio			18.84			14.67		

Under standard assay conditions, 60 min, 30°C, [a] and [b] are representative samples of separate experiments

homogenate at 500 × *g* for 1 min to sediment whole cells and debris. The resulting supernatant was centrifuged at 1500 × *g* for 5 min to sediment whole chloroplasts. Analysis of these fractions for β-aspartate kinase activity yielded the following results (table 3). β-Aspartate kinase specific activity in units/mg protein increased from 2.17 to 3.06 in the ruptured chloroplast fractions and was therefore enriched in the chloroplast pellet.

The enrichment of the β-aspartate kinase activity in the chloroplast pellet may be due to nonspecific binding of the protein to the membrane sites on the chloroplasts. To determine if β-aspartate kinase was binding nonspecifically, 0.1% bovine serum albumin was added to the extraction buffer, with the following results (table 3). β-Aspartate kinase was approximately 5% of the crude preparation in both plus and minus bovine serum albumin preparations, showing no difference in the distribution of the enzyme activity. The supernatant/pellet ratios were also approximately similar. The addition of bovine serum albumin to the extraction buffer also seemed to protect the enzyme activity, as noted in the increase in the total percentage recovered.

Although some non-specific binding of β-aspartate kinase may occur, the distribution of β-aspartate kinase in sucrose gradients and differential centrifugation indicates that there is a chloroplast-localized β-aspartate kinase. Experiments are underway to characterize the chloroplast-localized β-aspartate kinase and the cytosolic β-aspartate kinase, and to determine if these are the same enzyme or isoenzyme forms by studying biochemical, kinetic and regulatory properties of the enzyme(s).

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