Plant leaf alanine:2-oxoglutarate aminotransferase

Peroxisomal localization and identity with glutamate:glyoxylate aminotransferase

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The distribution of alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2) in spinach (*Spinacia oleracea*) leaf homogenates was examined by centrifugation in a sucrose density gradient. About 55% of the total homogenate activity was localized in the peroxisomes and the remainder in the soluble fraction. The peroxisomes contained a single form of alanine:2-oxoglutarate aminotransferase, and the soluble fraction contained two forms of the enzyme. Both the peroxisomal enzyme and the soluble predominant form (about 90% of the total soluble activity) were co-purified with glutamate:glyoxylate aminotransferase to homogeneity; it had been reported to be present exclusively in the peroxisomes of plant leaves and to participate in the glycollate pathway in leaf photorespiration [Tolbert (1971) *Annu. Rev. Plant Physiol.* 22, 45–74]. The evidence indicates that alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities are associated with the same protein. The peroxisomal and soluble enzyme preparations had nearly identical properties, suggesting that the soluble predominant alanine aminotransferase activity is from broken peroxisomes and about 96% of the total homogenate activity is located in the peroxisomes.

The most active transaminations have been reported to occur between 2-oxoglutarate and aspartate or alanine in seedling leaf-tissue extracts (Wilson *et al.*, 1954). In spinach (*Spinacia oleracea*) leaves, aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1) is located in the peroxisomes, mitochondria and chloroplasts; the peroxisomal malate dehydrogenase (EC 1.1.1.82) and aspartate:2-oxoglutarate aminotransferase have been postulated to shuttle cytoplasmic reducing equivalents into the peroxisomes to complete the photorespiratory process (Rehfeld & Tolbert, 1972). Two forms of the aspartate aminotransferase have been purified and characterized from seedling oat (*Avena sativa*) leaves (Reed & Hess, 1975).

In contrast, there is no report on the subcellular distribution and detailed properties of the alanine aminotransferase in plant leaves. In the present study, alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2) was found to be mainly located in the peroxisomes of plant leaves and to be identical with glutamate:glyoxylate aminotransferase, a peroxisomal enzyme postulated to participate in photorespiratory carbon metabolism (Tolbert, 1971).

Experimental

Materials

Sephacryl S-200 and Pharmalyte for electro-

focusing were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Spinach was purchased locally, and other materials used were obtained as stated previously (Noguchi *et al.*, 1976a,b, 1978).

Methods

Organelle separation. Homogenization and sucrose-density-gradient centrifugation of spinach leaves were performed by the method of Rehfeld & Tolbert (1972). Spinach leaf tissue (about 100g) without mid-veins was homogenized for 10s in a blender with an equal weight of grinding medium [30% (w/w) sucrose in 20mM-glycylglycine, pH 7.5]. The crude homogenate was squeezed through Miracloth. The filtrate, after centrifugation at 300g for 5min, was applied at the core over a 560ml discontinuous sucrose gradient and centrifuged for 3h at 30000rev./min in a RPZ 48T zonal rotor in a Hitachi (Tokyo, Japan) 55P centrifuge. At the end of centrifugation, the rotor was unloaded by pumping water into the core at the rate of 10ml/min and collecting 13ml fractions from the edge of the rotor. The gradient was prepared with the following sucrose solutions in 20mM-glycylglycine, pH 7.5; 50ml of 2.3M, 85ml of 2.1M, 85ml of 1.8M, 100ml of 1.6M, 35ml of 1.4M, 70ml of 1.2M, 50ml of 1.0M

and 85 ml of 0.6 M, by the method of Köller & Kindl (1978).

Intact chloroplasts were prepared from spinach leaf homogenates as described by Rehfeld & Tolbert (1972). Chlorophyll was determined by the method of Barr & Crane (1971).

Determination of enzyme activity. Transaminations between various L-amino acids and 2-oxo acids were assayed as previously described (Noguchi *et al.*, 1978). The assay mixture contained, unless specified otherwise, 40 mM-L-amino acid, 5 mM-2-oxo acid, 40 μ M-pyridoxal 5'-phosphate, 0.1 M-potassium phosphate buffer, pH 8.0, and enzyme preparation in a total volume of 0.4 ml. Catalase (EC 1.11.1.6) and cytochrome *c* oxidase (EC 1.9.3.1) were assayed by the methods of Lück (1965) and Wharton & Tzagoloff (1967) respectively. A unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of product or a decrease in substrate of 1 μ mol/min at 37°C.

Purification of alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase from the peroxisomal and soluble fractions. The peroxisomal and soluble fractions with catalase and the two coincident aminotransferase activities were pooled separately after sucrose-density-gradient centrifugation of the homogenate from spinach leaves (100 g wet wt.) (see Fig. 1 in the Results and discussion section). Subsequent procedures were performed at 0–4°C. The peroxisomal fraction was dialysed against 5 mM-potassium phosphate buffer, pH 7.5, containing 100 μ M-pyridoxal 5'-phosphate, sonicated for 15 s at 10 kHz, centrifuged at 105 000 *g* for 45 min, and then concentrated to about 10 ml by Diaflo ultrafiltration with a G10T filter (Bioengineering Co., Tokyo, Japan). The same purification procedure was applied with the peroxisomal extract and the soluble fraction. Potassium phosphate buffer, pH 7.5, was used throughout.

The peroxisomal extract and the soluble fraction were applied separately to a column (3 cm $\times$ 10 cm for the peroxisomal extract and 5 cm $\times$ 13 cm for the soluble fraction) of DEAE-cellulose, equilibrated with 5 mM buffer. After the column had been washed with 300 ml (600 ml for the soluble fraction) of the same buffer, the enzyme was eluted with a 600 ml (1500 ml for the soluble fraction) linear gradient of 5–250 mM buffer. Fractions (20 ml) were collected at a flow rate of 70 ml/h. The active fractions with alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities coincident were pooled (see Fig. 2 in the Results and discussion section) and concentrated by ultrafiltration. The concentrated enzyme solution was subjected to isoelectric focusing on a pH 3–10 Pharmalyte gradient as described by Vesterberg & Swenson (1966). Fractions (2 ml) were collected; activity and pH determinations were performed immediately. The

focusing resulted in the detection of a single peak (pI 5.8) with alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities coincident for both the peroxisomal and soluble enzymes. The active fractions were pooled, concentrated by ultrafiltration, and applied to a column of Sephacryl S-200 (2.5 cm $\times$ 100 cm), equilibrated with 50 mM buffer, containing 100 μ M-pyridoxal 5'-phosphate and 10% (v/v) glycerol. The column was eluted with the same buffer at a flow rate of 25 ml/h. The effluent was collected in 3 ml fractions. The active fractions were pooled, concentrated by ultrafiltration, and diluted with water to adjust the buffer concentration to 5 mM. The concentrated enzyme solution was applied to a hydroxyapatite column (2 cm $\times$ 5 cm), equilibrated with 5 mM buffer, containing 100 μ M-pyridoxal 5'-phosphate buffer and 10% (v/v) glycerol. After the column had been washed with 20 ml of the same buffer, the enzyme was eluted with the same buffer. Active fractions were pooled and concentrated by ultrafiltration to about 0.8 ml.

The concentrated enzyme solution was made up to about 1.2 ml with 50% sucrose, and fractions (100 μ l) were applied to each of eleven continuous polyacrylamide [10% (w/v) total] gels (0.5 cm $\times$ 8 cm) containing methylenebisacrylamide. After electrophoresis for 3 h at a current of 1.5 mA/column at pH 8.9 (0.1 M-Tris/glycine), each gel was cut into 2 mm slices, and these were separately shaken at about 4°C for 16 h in 150 μ l of 50 mM buffer to extract the enzyme. Both alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities were determined for each buffer extract from one gel, and only alanine:2-oxoglutarate aminotransferase activity for other gels. The electrophoresis gave a single symmetrical peak, with both enzyme activities coincident for both the peroxisomal and soluble enzymes. A fraction with the highest activity was collected from each gel, homogenized and centrifuged at 10 000 *g* for 30 min. The resulting supernatant was concentrated by ultrafiltration to about 1 ml.

Other methods. Protein determination, polyacrylamide-gel disc electrophoresis, isoelectric focusing and sucrose-density-gradient centrifugation for the determination of approximate molecular weight were performed as previously described (Noguchi *et al.*, 1976b, 1978).

Results and discussion

A representative sedimentation in a sucrose density gradient of spinach leaf homogenate is presented in Fig. 1. The peroxisomes and mitochondria were separated; the peroxisomal fraction, marked by catalase, was at a density of 1.245 g/ml, and the mitochondria, marked by cytochrome *c*

Fig. 1. Subcellular distribution of alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase in spinach leaves

The postnuclear supernatant was prepared from spinach leaves and subjected to sucrose-density-gradient centrifugation as described in the text. (a) ●, Catalase; ○, cytochrome *c* oxidase; (b): ●, alanine:2-oxoglutarate aminotransferase (GPT); ○, glutamate:glyoxylate aminotransferase (GGT).

oxidase, were at a density of 1.185 g/ml. Alanine:2-oxoglutarate aminotransferase activity showed distribution profiles nearly identical with those for catalase and glutamate:glyoxylate aminotransferase activities; about 55% of the homogenate activity was present in the peroxisomes and the remainder in the soluble top fraction. Cytochrome *c* oxidase as the mitochondrial marker showed a different distribution profile from these three enzyme activities; most of the homogenate activity was found in the mitochondrial fraction, with little or no activity in the soluble top fraction. Broken chloroplasts, marked by chlorophyll, were at a density of 1.16 g/ml and were well separated from the mitochondria and peroxisomes (not shown in Fig. 1). The intact chloroplasts were removed by the initial centrifugation of spinach leaf homogenates at 300 g for 5 min before sucrose-density-gradient centrifugation. Little or no activity of alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase was detected in the intact chloroplasts prepared as described in the Experimental section. These results suggest that the two enzymes were not present in chloroplasts.

Fig. 2 shows elution profiles of alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities from a DEAE-

Fig. 2. Chromatography of the peroxisomal extract and the soluble fraction on DEAE-cellulose

The peroxisomal extract (a) and the soluble fraction (b) were prepared from spinach leaves, applied to separate DEAE-cellulose columns and eluted by the method described in the text. ●, Alanine:2-oxoglutarate aminotransferase; ○, glutamate:glyoxylate aminotransferase; ----, concentration of potassium phosphate buffer, pH 7.5.

cellulose column with the peroxisomal extract or the soluble fraction. An activity peak with alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities coincident was obtained in each case. An additional minor activity peak of alanine:2-oxoglutarate aminotransferase (about 10% of the soluble activity) without glutamate:glyoxylate aminotransferase activity was obtained with the soluble fraction; owing to its low activity, the enzyme was not characterized.

The results of the purifications of alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase from the peroxisomal extract and the soluble fraction of spinach leaves are given in Table 1. The activity ratio alanine:2-oxoglutarate aminotransferase/glutamate:glyoxylate aminotransferase remained constant throughout the purification from both the peroxisomal extract and the soluble fraction; alanine:2-oxoglutarate aminotransferase without glutamate:glyoxylate aminotransferase activity in the soluble fraction did not change the activity ratio of the two enzymes, presumably owing to a minor activity (about 10% of the soluble enzyme activity).

On polyacrylamide-disc-gel electrophoresis at

Table 1. Purification of alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase from spinach leaves

Details of purification and assay methods are described in the text. Activity ratio is the ratio of alanine:2-oxoglutarate aminotransferase to glutamate:glyoxylate aminotransferase activity.

	Peroxisomal alanine:2-oxoglutarate aminotransferase				Soluble alanine:2-oxoglutarate aminotransferase			
	Protein (mg)	Sp. activity (units/mg)	Purification (fold)	Activity ratio	Protein (mg)	Sp. activity (units/mg)	Purification (fold)	Activity ratio
Crude homogenate	4720	0.04	1	2.15	4720	0.04	1	2.15
Peroxisomal extract	32.2	1.45	36.3	2.06				
Soluble fraction					1756	0.05	1.3	2.07
DEAE-cellulose	6.0	2.98	74.5	1.98	150	0.32	8.0	1.98
Isoelectric focusing and Sephacryl S-200	1.2	4.14	104	1.92	3.2	3.74	93.5	1.94
Hydroxyapatite	0.6	5.20	130	2.00	1.5	4.02	101	1.90
Polyacrylamide-gel electrophoresis	0.12	7.50	188	1.95	0.42	7.13	178	2.00

pH 8.9 in 10%-acrylamide gel, both enzyme preparations migrated to the anode as a single protein band with a mobility of 0.54 (relative to that of a Bromophenol Blue marker), which coincided with both alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities. The ratio of the two activities was not changed after polyacrylamide-disc-gel electrophoresis in the absence of sodium dodecyl sulphate, isoelectric focusing on a pH 3.0–10.0 Pharmalyte gradient and Sephacryl S-200 gel filtration. These results show that the two enzyme activities are derived from the same protein. The two enzymes are hereafter designated alanine (glutamate) aminotransferase.

The peroxisomal and soluble alanine (glutamate) aminotransferases had nearly identical physical and enzymic properties. Both enzymes were specific for L-glutamate (sp. activity 3.85 units/mg for the peroxisomal enzyme and 3.57 units/mg for the soluble enzyme) and L-alanine (12.3 units/mg for the peroxisomal enzyme and 11.4 units/mg for the soluble enzyme) with glyoxylate as amino acceptor, and for L-alanine (7.50 units/mg for the peroxisomal enzyme and 7.13 units/mg for the soluble enzyme) with 2-oxoglutarate. Other L-amino acids (serine, asparagine, leucine, isoleucine, glutamine, methionine, valine, aspartic acid, phenylalanine, tyrosine, histidine, tryptophan and 5-hydroxytryptophan) were all inactive with each amino acceptor. With each enzyme preparation, the proportions (1:1.95:3.1 for the peroxisomal enzyme and 1:2.00:3.2 for the soluble enzyme) of these three aminotransferase activities (glutamate:glyoxylate, alanine:2-oxoglutarate and alanine:glyoxylate aminotransferases) were not changed after isoelectric focusing of a pH 3–10 Pharmalyte gradient, polyacrylamide-gel-disc electrophoresis in the absence of sodium dodecyl sulphate, Sephacryl S-200 gel filtration or sucrose-density-gradient centrifuga-

tion. These results suggest that these three aminotransferase activities were all manifestations of the same enzyme. The apparent K_m values were determined from double-reciprocal plots of initial velocity and substrate concentration. The apparent K_m values for L-alanine with 2-oxoglutarate (5 mM) and those for L-glutamate with glyoxylate (5 mM) were 8.0 and 5.6 mM for the peroxisomal enzyme respectively, and 8.1 and 5.5 mM for the soluble enzyme respectively. The apparent K_m values for 2-oxoglutarate with L-alanine (40 mM) and those for glyoxylate with L-glutamate were 0.88 and 1.2 mM for the peroxisomal enzyme respectively, and 0.86 and 1.1 mM for the soluble enzyme respectively. Over the pH range 9.0–9.5, alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities showed nearly identical pH profiles, with pH optima between 8.0 and 8.5 with both enzyme preparations; potassium phosphate buffer (0.1 M) was used between pH 6.0 and 8.0, and pyrophosphate buffer (0.1 M) was used between pH 8.0 and 9.5. The molecular weight was estimated as $98\,000 \pm 4\,500$ for both enzyme preparations by sucrose-density-gradient centrifugation.

Alanine:2-oxoglutarate aminotransferase is located in the mitochondria or in the cytosol in animal tissues as described above (Fellman *et al.*, 1969; Miller & Litwack, 1969*a,b*; De Rosa & Swick, 1975). In the present study, the subcellular distribution of alanine:2-oxoglutarate aminotransferase in spinach leaves was found to differ from that in animal tissues; about 55% of the spinach leaf homogenate activity was present in the peroxisomes and the remainder in the soluble fraction. However, most of the activity in the soluble fraction may be from broken peroxisomes, on the basis of the following evidence: (1) catalase is present only in the peroxisomes (Lazarow & de Duve, 1973; Leighton *et al.*, 1975); (2) catalase and alanine:2-oxoglutarate

aminotransferase are similarly distributed in the peroxisomes and in the soluble fraction (Fig. 1); (3) the soluble fraction contained two forms of alanine:2-oxoglutarate aminotransferase, and the predominant form is about 90% of the soluble activity; (4) the soluble predominant enzyme is identical with the peroxisomal enzyme with respect to enzymic and physical properties. In conclusion, about 96% [about 55% in the peroxisomes and about 41% ($45 \times 0.9\%$) in the soluble fraction] of the homogenate activity is located in the peroxisomes.

Two aminotransferases have been postulated to participate in glycolate metabolism in leaf photorespiration, namely serine:glyoxylate aminotransferase (EC 2.6.1.45) and glutamate:glyoxylate aminotransferase (Tolbert, 1971). We have purified and characterized tryptophan:glyoxylate (hydroxypyruvate) aminotransferase from spinach leaves; it catalyses transaminations between alanine, serine, asparagine, tryptophan and 5-hydroxytryptophan and glyoxylate or hydroxypyruvate, and has been suggested to be identical with serine:glyoxylate aminotransferase (Noguchi & Hayashi, 1980). Previous studies of an additional photorespiratory enzyme, glutamate:glyoxylate aminotransferase, from plant leaves have not involved either extensive purification or detailed analysis of physical and enzymic properties, including substrate specificity (Rehfeld & Tolbert, 1972).

In the present study, glutamate:glyoxylate aminotransferase and alanine:2-oxoglutarate aminotransferase were co-purified to homogeneity from the peroxisomal and soluble fractions of spinach leaves. The activity ratio alanine:2-oxoglutarate aminotransferase/glutamate:glyoxylate aminotransferase maintained a constant value during all purification steps in each case, which was unchanged by a variety of treatments of each purified enzyme preparation. These data show that glutamate:glyoxylate aminotransferase is identical with alanine:2-oxoglutarate aminotransferase in spinach leaves. The enzyme had also alanine:glyoxylate aminotransferase activity. The broad specificity of

the present enzyme preparations is curious from the structural point of view if a single catalytic site is responsible. Further detailed studies on the substrate specificity of the enzyme and its roles in the glycolate pathway in photorespiration are required.

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