Development of glutamate:glyoxylate aminotransferase in the cotyledons of cucumber (*Cucumis sativus*) seedlings

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Glutamate:glyoxylate aminotransferase 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]. Glutamate:glyoxylate aminotransferase activity was already present in the etiolated cotyledons of cucumber (*Cucumis sativus*) seedlings, and increased during greening. The enzyme was present only in the cytosol of the etiolated cotyledons and appeared in the peroxisomes during greening. The enzyme was purified to homogeneity from the cytosol of the etiolated cotyledons and from the peroxisomes of the green cotyledons of cucumber seedlings. The two enzyme preparations had nearly identical enzymic and physical properties. On the basis of these findings, roles of glutamate:glyoxylate aminotransferase in the glycollate pathway in photorespiration, and the mechanism of its appearance in the peroxisomes during greening, are discussed.

A metabolic transition occurs in the cotyledons during early germination of 'fatty' seedlings. During the period of fat mobilization, glyoxysomes are found that are involved in the conversion of stored fat into carbohydrate via the glyoxylate cycle (Beevers, 1971*a,b*). After greening, glyoxysomes disappear, although other forms of microbodies, namely peroxisomes, can be found that are involved in photosynthetic glycollate catabolism (photorespiration); the loss of enzymes of the glyoxylate cycle is accompanied by striking increases in glycollate oxidase and glyoxylate reductase activities (Tolbert, 1971; Kagawa *et al.*, 1973).

Two aminotransferases as well as the two enzymes described above had been reported to be present exclusively in the peroxisomes of green leaves and to participate in the glycollate pathway in leaf photorespiration, namely glutamate:glyoxylate aminotransferase and serine:glyoxylate aminotransferase (EC 2.6.1.45) (Tolbert, 1971).

We have reported that alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2) is mainly located in the peroxisomes of spinach (*Spinacia oleracea*) leaves and is identical with glutamate:glyoxylate aminotransferase, suggesting the need of further detailed studies on the roles of this aminotransferase in the glycollate pathway (Noguchi & Hayashi, 1981).

The present paper describes changes in activity, properties or subcellular distribution of glut-

amate:glyoxylate aminotransferase during development and greening of the etiolated cotyledons of cucumber (*Cucumis sativus*) seedlings.

Experimental

Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus*) were soaked for 30 min, surface-sterilized with 0.01% HgCl₂, rinsed with deionized water and spread on moist cotton wool in Petri dishes. The seeds were germinated in the dark at 27°C. The cotyledon emerges at day 3 from the start of soaking. On day 2 after germination, the dark-grown seedlings were exposed to continuous white light (2000 lx).

Organelle separation

All procedures were performed at 0–4°C. The etiolated or green cotyledons were homogenized for 10 s in a Waring blender with 1.5 vol. of a grinding medium containing 150 mM-Tricine{*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, pH 7.5, 10 mM-KCl, 1 mM-EDTA and 0.4 M-sucrose. The crude homogenate was filtered through six layers of coarse cheesecloth and centrifuged at 300 g for 5 min to sediment nuclei and any whole cells. A portion (4 ml) of the postnuclear homogenate was layered on a 32 ml discontinuous sucrose gradient (0.6–2.3 M) and centrifuged at 132 400 g for 80 min in a Hitachi (Tokyo, Japan) 55P-72 centrifuge with a vertical

rotor (RPV50T-160). The sucrose gradient was prepared by the method of Köller & Kindl (1978). Fractions (2.0 ml) were collected from the bottom of the tube.

Purification of glutamate:glyoxylate aminotransferase

The enzyme was purified from the cytosolic fraction of the etiolated cotyledons and from the peroxisomal fraction of the green cotyledons. All manipulations were performed at 0–4°C. Potassium phosphate buffer, pH 7.5, containing 10% (v/v) glycerol, was used throughout.

The green cotyledons at day 4 after the exposure of 4-day-old dark-grown seedlings to continuous illumination, and the etiolated cotyledons of 5-day-old dark-grown seedlings were separately subjected to organelle separation by sucrose-density-gradient centrifugation as described above. Peroxisomal fractions (fractions 3, 4 and 5 from each sucrose gradient) were prepared from about 330 g of the green cotyledons and the cytosolic fractions (fractions 15 and 16 from each sucrose gradient) from 150 g of the etiolated cotyledons (see Fig. 2 below). Peroxisomal fractions were diluted with 4 vol. of 5 mM-phosphate buffer, sonicated for 20 s at 10 kHz, and centrifuged at 105 000 g for 60 min and the precipitate discarded. The same purification procedures were applied for the peroxisomal and cytosolic enzymes.

The peroxisomal extract and the cytosolic fraction were separately applied to a column (3 cm × 10 cm for the peroxisomal extract and 5 cm × 13 cm for the cytosolic fraction) of DEAE-cellulose, equilibrated with 5 mM-phosphate buffer. After the column had been washed with 250 ml (500 ml for the cytosolic fraction) of the same buffer, the enzyme was eluted with a 500 ml (1500 ml for the cytosolic fraction) linear gradient of 5–250 mM-buffer. Fractions (20 ml) were collected at a flow rate of 65 ml/h. The active fractions with alanine:2-oxoglutarate aminotransferase and glutamate:glyoxylate aminotransferase activities coincident were pooled (see Fig. 3 below) and concentrated by ultrafiltration. The concentrated enzyme solution was applied to a column (2.5 cm × 100 cm) of Sephacryl S-200, equilibrated with 50 mM-potassium phosphate buffer, containing 100 µM-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 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 × 5 cm), equilibrated with 5 mM-phosphate buffer, containing 100 µM-pyridoxal 5'-phosphate buffer and 10% (v/v) glycerol. After the column had been washed with 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 1.5 ml with 50% glycerol, and fractions (100 µl) were applied to each of 14 continuous polyacrylamide [10% (w/v) total acrylamide gels (0.5 cm × 8 cm) containing methylenebisacrylamide]. After electrophoresis for 2 h at a current of 1.5 mA/column at pH 8.9 (0.01 M-Tris/glycine), each gel was cut into 1 mm slices, and these were separately shaken at about 4°C for 16 h in 150 µl of 50 mM-potassium phosphate buffer, pH 7.5, containing 20 µM-pyridoxal 5'-phosphate and 10% glycerol to extract the enzyme. Both glutamate:glyoxylate aminotransferase and alanine:2-oxoglutarate aminotransferase activities were determined for each buffer extract from one gel, and only alanine:2-oxoglutarate aminotransferase activity for other gels. Electrophoresis gave a single symmetrical peak with both enzyme activities coincident for both the peroxisomal and cytosolic enzymes. A fraction with the highest activity was collected from each gel, homogenized and centrifuged at 105 000 g for 60 min. The resulting supernatant was concentrated by ultrafiltration to about 1 ml.

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 otherwise specified, 20 mM-L-amino acids, 10 mM-2-oxo acid, 40 µM-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) (Lück, 1965), isocitrate lyase (EC 4.1.3.1) (Cooper & Beevers, 1969) and glutamate dehydrogenase (EC 1.4.1.2) (Beaufay *et al.*, 1959) were assayed by the methods given in the cited references. 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 µmol/min at 37°C.

Other methods

Protein determination, polyacrylamide-gel disc electrophoresis in the presence and absence of sodium dodecyl sulphate, and sucrose-density-gradient centrifugation for the determination of approximate molecular weight were performed as previously described (Noguchi *et al.*, 1976, 1978).

Results and discussion

Fig. 1 shows changes in serine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase activities during development and greening of the etiolated cotyledons of cucumber seedlings.

Fig. 1. Developmental changes of serine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase in the cotyledons of dark- and light-grown cucumber seedlings

(a) shows developmental profiles of serine:glyoxylate aminotransferase (SGT) and (b) those of glutamate:glyoxylate aminotransferase (GGT). ●, Seedlings grown in darkness; ○, seedlings transferred to continuous illumination at day 2 and day 4 after germination as indicated by arrows.

Serine:glyoxylate aminotransferase was not present in the etiolated cotyledons of dark-grown seedlings and appeared during greening by their exposure to continuous illumination. In contrast, glutamate:glyoxylate aminotransferase activity was already present in the etiolated cotyledons and its activity increased during greening. The absence of serine:glyoxylate aminotransferase in the etiolated cotyledons, its appearance during greening, and the increase in glutamate:glyoxylate aminotransferase activity during greening may be explained by the initiation of photosynthetic glycolate catabolism (photorespiration); the two enzymes had been postulated to participate in the glycolate pathway in green-leaf photorespiration. However, the physiological significance of the presence of glutamate:glyoxylate aminotransferase in the etiolated cotyledons is unclear.

Representative sedimentations in sucrose density gradients of homogenates of the etiolated and green

Fig. 2. Subcellular distribution of glutamate:glyoxylate aminotransferase in the etiolated and green cotyledons

The postnuclear supernatants from the etiolated cotyledons (a) and the green cotyledons (b) were separately subjected to sucrose-density-gradient centrifugation as described in the text. ○, Glutamate:glyoxylate aminotransferase (GGT); ●, catalase; △, glutamate dehydrogenase (GDH).

cotyledons are presented in Fig. 2. Isocitrate lyase was used as the glyoxysomal marker, catalase as the glyoxysomal or peroxisomal marker and glutamate dehydrogenase as the mitochondrial marker. In each case the glyoxysomes or the peroxisomes and the mitochondria were separated; both etiolated cotyledon glyoxysomes and green-cotyledon peroxisomes were at a density of 1.24 g/ml, and the mitochondria at a density of 1.19 g/ml. In the green cotyledons, 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 green-cotyledon homogenates at 300g for 5 min before sucrose-density-gradient centrifugation. In the etiolated cotyledons, glutamate:glyoxylate aminotransferase was detected only in the soluble fraction but not in intracellular organelle, showing this enzyme is present only in the cytosol. In contrast, in the green cotyledons, glutamate:glyoxylate aminotransferase appeared in the peroxisomes; about 30% of the homogenate activity was present in the peroxisomes and the remainder in the soluble fraction.

Both in the etiolated cotyledons and in the green cotyledons, glutamate:glyoxylate aminotransferase

showed subcellular-distribution profiles nearly identical with those for alanine:2-oxoglutarate aminotransferase activity, suggesting the identity of the two enzymes (not shown in Fig. 2). Fig. 3 shows elution profiles of glutamate:glyoxylate amino-

Fig. 3. Chromatography of the peroxisomal extract from the green cotyledons and the cytosolic fraction from the etiolated cotyledons on DEAE-cellulose

The peroxisomal extract (a) from the green cotyledons and the cytosolic fraction (b) from the etiolated cotyledons were separated on DEAE-cellulose columns and eluted by the method described in the text. ○, Glutamate:glyoxylate aminotransferase (GGT); ●, alanine:2-oxoglutarate aminotransferase (GPT); ----, concentration of potassium phosphate buffer, pH 7.5.

transferase and alanine:2-oxoglutarate aminotransferase activities from a DEAE-cellulose column with the cytosolic fraction from the etiolated cotyledons or the peroxisomal extract from the green cotyledons (see the purification procedures for glutamate:glyoxylate aminotransferase in the Experimental section). An activity peak with glutamate:glyoxylate aminotransferase and alanine:2-oxoglutarate aminotransferase coincident was obtained in each case. An additional minor activity peak of alanine:2-oxoglutarate aminotransferase (about 8% of the cytosolic activity) without glutamate:glyoxylate aminotransferase was obtained with the cytosolic fraction; owing to its low activity and instability, the enzyme was not characterized.

The results of the purification of glutamate:glyoxylate aminotransferase and alanine:2-oxoglutarate aminotransferase from the cytosolic fraction of the etiolated cotyledons and the peroxisomal extract of the green cotyledons are given in Table 1. The activity ratio alanine:2-oxoglutarate aminotransferase/glutamate:glyoxylate aminotransferase remained constant throughout the purification from both the peroxisomal and cytosolic fractions. Alanine:2-oxoglutarate aminotransferase without glutamate:glyoxylate aminotransferase in the cytosolic fraction (see Fig. 3 below) did not change the activity ratio of the two enzymes, presumably owing to a minor activity (about 8% of the cytosolic activity).

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 Pharmalyte gradient and Sephacryl S-200 gel filtration. These results show that the two enzyme activities are derived from the same protein.

The cytosolic enzyme from the etiolated cotyledons and the peroxisomal enzyme from the green

Table 1. Purification of glutamate:glyoxylate aminotransferase from the cytosolic fraction of the etiolated cotyledons and from the peroxisomal fraction of the green cotyledons

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

	Cytosolic enzyme from the etiolated cotyledons				Peroxisomal enzyme from the green cotyledons			
	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Activity ratio	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Activity ratio
Crude homogenate	533.5	0.11	1	0.77	5100	0.27	1	0.78
Peroxisomal extract					54.2	6.8	24.8	0.81
Cytosolic fraction	201.1	0.27	2.5	0.80				
DEAE-cellulose	63.6	0.71	6.5	0.83	15.6	13.5	50.0	0.82
Sephacryl S-200	9.5	4.10	37.3	0.80	5.3	25.7	95.2	0.80
Hydroxyapatite	1.1	14.2	129	0.81	2.5	34.3	127	0.81
Polyacrylamide-gel electrophoresis	0.14	41.3	375	0.83	0.24	43.8	162	0.83

cotyledons had nearly identical physical and enzymic properties. On polyacrylamide-disc-gel electrophoresis at pH 8.9 in 10% acrylamide gel, both enzyme preparations migrated to the anode as a single protein band with a mobility of 0.51 (relative to that of a Bromophenol Blue marker) (Fig. 4). Both enzymes were specific for L-glutamate (sp. activity 43.8 units/mg for the peroxisomal enzyme and 41.3 units/mg for the cytosolic enzyme) and L-alanine (70.1 units/mg for the peroxisomal enzyme and 66.1 for the cytosolic enzyme) with glyoxylate as amino acceptor and for L-alanine (49.6 units/mg for the peroxisomal enzyme and 52.6 units/mg for the cytosolic enzyme) with 2-oxoglutarate. Other L-amino acids (serine, asparagine, leucine, isoleucine, glutamine, methionine, valine, aspartic acid, phenylalanine, tyrosine, histidine and tryptophan) were all inactive with each amino acceptor. With each enzyme preparation, the proportion (1:1.2:1.6 for both the peroxisomal and cytosolic enzymes) of these three aminotransferase activities (glutamate:glyoxylate, alanine:2-oxoglutarate and alanine:glyoxylate aminotransferase) were not changed after isoelectric focusing on a pH 3.0–10 Pharmalyte gradient, polyacrylamide-gel-disc electro-

phoresis in the absence of sodium dodecyl sulphate, Sephacryl S-200 gel filtration or sucrose-density-gradient centrifugation. These results suggest that 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 value for L-glutamate with glyoxylate (10 mM) and that for L-alanine with 2-oxoglutarate (10 mM) were 3.8 and 3.1 mM for both enzyme preparations respectively. The apparent K_m value for glyoxylate with L-glutamate (20 mM) and that for 2-oxoglutarate with L-alanine (20 mM) were 2.4 and 2.6 for both enzymes. Over the pH range 6.0–9.5, glutamate:glyoxylate aminotransferase and alanine:2-oxoglutarate 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 9.0, and pyrophosphate buffer (0.1 M) was used between pH 8.0 and 9.5. Isoelectric focusing on a pH 3.0–10.0 Pharmalyte gradient yielded a pI value of 5.0 for both enzymes. The molecular weight of the enzyme was estimated as approx. 100 000 for both enzyme preparations by sucrose-density-gradient centrifugation. Polyacrylamide-disc-gel electrophoresis in sodium dodecyl sulphate gave a mol.wt. of approx. 48 000 for both enzyme preparations.

Two aminotransferases had been postulated to participate in glycollate metabolism in leaf photorespiration, namely serine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase (Tolbert, 1971). As demonstrated in the present study, glutamate:glyoxylate aminotransferase, which is associated with photorespiration, had already been present in the etiolated cotyledons of dark-grown seedlings before the initiation of photorespiration by greening, whereas serine:glyoxylate aminotransferase was not present in the etiolated cotyledons and appeared during greening. We have purified and characterized tryptophan:glyoxylate (hydroxypyruvate) aminotransferase (Noguchi & Hayashi, 1980) and glutamate:glyoxylate aminotransferase (Noguchi & Hayashi, 1981) from spinach leaves; the former enzyme catalyses transamination between serine, alanine, tryptophan, asparagine and 5-hydroxytryptophan and glyoxylate or hydroxypyruvate, and has been suggested to be identical with serine:glyoxylate aminotransferase in the peroxisomes. The latter enzyme also showed broad substrate specificity; it had alanine:2-oxoglutarate aminotransferase, glutamate:glyoxylate aminotransferase and alanine:glyoxylate aminotransferase activities. In the present study glutamate:glyoxylate aminotransferase and alanine:2-oxoglutarate aminotransferase were co-purified from the cytosol of the etiolated cotyledons

Fig. 4. Polyacrylamide-disc-gel electrophoresis of peroxisomal glutamate:glyoxylate aminotransferase from the green cotyledons and the cytosolic enzyme from the etiolated cotyledons

Electrophoresis was performed in Tris/glycine, pH 8.9, in the absence of sodium dodecyl sulphate. (a) Purified cytosolic enzyme; (b) purified peroxisomal enzyme.

and from the peroxisomes of the green cotyledons. The activity ratio alanine:2-oxoglutarate aminotransferase/glutamate:glyoxylate aminotransferase maintained a constant value during all purification steps, 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 cucumber cotyledons. Difference of the two aminotransferases in developmental profiles, and their broad substrate specificity suggest the need of the further studies on the roles of the two enzymes in photorespiration.

The mechanism of the appearance of glutamate:glyoxylate aminotransferase in the peroxisomes during greening is unclear. However, it is suggested that glutamate:glyoxylate aminotransferase is directly transported from the cytosol to the peroxisomes in the complete form, because cytosol glutamate:glyoxylate aminotransferase from the etiolated cotyledons and peroxisomal one from the green cotyledons have nearly identical physical and enzymic properties.

Further detailed studies on the roles of glutamate:glyoxylate aminotransferase in the glycolate pathway in photorespiration and on the mechanism of its appearance in the peroxisomes during greening are required.

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References

- Beaufay, H., Bendall, B. S., Baundhuin, P. & de Duve, C. (1959) *Biochem. J.* **73**, 623–628
- Beevers, H. (1971a) *Ann. N.Y. Acad. Sci.* **168**, 313–324
- Beevers, H. (1971b) in *Photosynthesis and Photorespiration* (Hatch, M. D., Osmond, C. B. & Slatyer, R. O., eds.), pp. 483–493, Wiley-Interscience, New York
- Cooper, T. G. & Beevers, H. (1969) *J. Biol. Chem.* **244**, 3507–3513
- Kagawa, T., McGregor, D. I. & Beevers, H. (1973) *Plant Physiol.* **51**, 66–71
- Köller, W. & Kindl, H. (1978) *Z. Naturforsch.* **33C**, 962–968
- Lück, H. (1965) *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 886, Academic Press, New York
- Noguchi, T. & Hayashi, S. (1980) *J. Biol. Chem.* **255**, 2267–2269
- Noguchi, T. & Hayashi, S. (1981) *Biochem. J.* **195**, 235–239
- Noguchi, T., Okuno, E. & Kido, R. (1976) *Biochem. J.* **159**, 607–613
- Noguchi, T., Okuno, E., Takada, Y., Minatogawa, Y., Okai, K. & Kido, R. (1978) *Biochem. J.* **169**, 113–122
- Tolbert, N. E. (1971) *Annu. Rev. Plant Physiol.* **22**, 45–74