Organelle-specific Isozymes of Aspartate-α-Ketoglutarate Transaminase in Spinach Leaves

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ABSTRACT

Four distinct isozymes of aspartate-α-ketoglutarate transaminase in a spinach (Spinacia oleracea L.) leaf extract were separated by starch gel electrophoresis. Of the total aspartate-α-ketoglutarate transaminase activity, approximately 45% was represented by the chloroplast isozyme, 26% by the cytosol isozyme, 19% by the mitochondrial isozyme, and 10% by the peroxisomal isozyme. The aspartate-α-ketoglutarate transaminase activity in the four subcellular compartments behaved similarly. It was freely reversible and α-ketoglutarate was preferred to pyruvate or glyoxylate as the amino group acceptor. With glutamate as the amino group donor, oxaloacetate was superior to pyruvate or glyoxylate as the acceptor in chloroplasts, mitochondria, and cytosol, while pyruvate or glyoxylate was preferred to oxaloacetate as the acceptor in peroxisomes.

Aspartate-α-ketoglutarate transaminase has been considered to be an active transaminase with important physiological roles in many plant tissues. Suggestion has been made that the enzyme participates in amino acid metabolism (4), electron shuttle (7), the proposed glycylate pathway (18), and the intercellular transport of metabolites during C4 photosynthesis (5). To carry out these proposed functions, it is necessary for the enzyme to be present in different subcellular compartments. The enzyme was detected in both the soluble and the particulate fraction (6, 7, 12, 19, 20). In addition, chloroplasts (7, 13, 17), mitochondria (13, 17), and peroxisomes (13, 20) from spinach leaves were shown to contain the enzyme. It was demonstrated that in spinach leaves, the peroxisomes contained isozymes that were distinct from the common isozyme shared by chloroplasts and mitochondria (13, 20). No distinct isozyme was reported to be present in the cytosol. Even though the enzyme was always detected in the soluble fraction, it might arise from organellar breakage during the extraction procedure. Thus, the presence of the enzyme in the cytosol has not been firmly established. In a recent report on spinach leaves (17), suggestion was made that even though the enzyme was present in the chloroplasts and mitochondria, it was absent from the peroxisomes. No estimation had been made as to the relative activity of the enzyme present in the various cellular compartments.

In this paper, we report the separation and the estimation of the relative activity of four distinct isozymes, each of which is associated with one subcellular compartment: cytosol, mitochondria, chloroplasts, and peroxisomes. The reversibility and specificity of transamination activity involving aspartate-α-ketoglutarate and glutamate-oxaloacetate in different subcellular compartments are also reported.

MATERIALS AND METHODS

Spinach leaves (Spinacia oleracea L.) were obtained locally. The leaves were deribbed, chopped into small pieces in grinding medium with an onion chopper, and then ground vigorously with a mortar and pestle for 5 min. The grinding medium contained 0.4 m sucrose, 1 m EDTA, 10 mM KC1, 1 m MgCl2, 10 mM dithiothreitol, and 0.15 m Tricine buffer adjusted with KOH to pH 7.5. The homogenate was filtered through Nitex cloth with pore size 102 μm2. The filtrate was layered directly onto a sucrose gradient which was composed of a 30-ml gradient from 30% (w/w) to 60% sucrose over a cushion of 1 ml of 60% sucrose. Four identical gradients were put into a Beckman SW 27 rotor and centrifuged at 10,000 rpm for 10 min in a Beckman L2-65B ultracentrifuge. This short term sucrose gradient centrifugation was a modification of the method described earlier (11) for the separation of uncontaminated intact chloroplasts. Two gradients were removed and fractionated as described (9). The remaining two gradients were recentrifuged at 21,000 rpm for 4 hr and then fractionated. The preparation of mitochondria free of contaminating intact chloroplasts followed the method described earlier (9, 13, 14). The intact chloroplasts and a fraction of the broken chloroplasts in the crude extract were disposed of by centrifugation at 2000g for 10 min (9). The resulting supernatant fraction was recentrifuged at 10,000g for 30 min to yield a particulate fraction which was then subjected to sucrose gradient centrifugation as described (9).

AT2 (EC 2.6.1.1.) and its reverse reaction (glutamate-oxaloacetate) were assayed under identical conditions (2,15) except for changes in the amino acids and α-ketoacids, and in the coupling enzymes and coenzymes. For the forward reaction, the assay mixture contained 0.06 m K-phosphate buffer (pH 7.5), 10 mM aspartate, 0.14 mM NADH, 100 enzyme units of NAD-malate dehydrogenase (Sigma), 0.06 mM pyridoxal-5'-phosphate, and 10 mM α-ketoglutarate. In the assay of the reverse reaction (glutamate-oxaloacetate transaminase), the reaction mixture contained 0.06 m K-phosphate buffer (pH 7.5), 10 mM glutamate, 0.14 mM NADPH, 35 enzyme units of glutamate dehydrogenase (type II, Sigma), 3.1 mM NH4Cl, and 10 mM oxaloacetate. In the assay of specificity on amino group acceptors, 5 or 10 mM of α-ketoacids was used which was in the range of optimal concentrations.

The assays of catalase, Cyt oxidase, Chl (8, 9), and alkaline fructose 1,6-bisphosphatase (21) followed those described earlier.

Horizontal starch gel electrophoresis was performed according to Meisel and Markert (10) using tris-citrate buffer (pH 7). The samples were subjected to electrophoresis for 7.5 hr at 12 v/cm and 4 C. AT activity in the gel was stained by the method of DeLorenzo and Ruddle (3). The gel was sliced into pieces of 2-mm thickness and stained at 30 C for 2.5 hr. After fixation in 50% (v/v) glycerol for 1 hr, the gel was photographed or

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2 Abbreviation: AT: aspartate-α-ketoglutarate transaminase.
scanned spectrophotometrically at 627 nm. Scanning was performed in a quartz cuvette in a Gilford gel scanner (model 2520) attached to a Gilford spectrophotometer and recorder.

RESULTS AND DISCUSSION

Chloroplasts, mitochondria, and peroxisomes were separated intact and essentially free of contamination by methods involving differential centrifugation as well as rate and equilibrium sucrose gradient centrifugation. The organelles were located in sucrose gradients by pigment or marker enzymes: Chl for intact and broken chloroplasts, Cyt oxidase for mitochondria, catalase for peroxisomes, and alkaline fructose 1,6-bisphosphatase for intact chloroplasts. As shown in Figure 1, short term (10-min) centrifugation of the crude extract in a sucrose gradient resulted in a separation of intact chloroplasts at a density of 1.21 g/cm³. This intact chloroplast fraction was free from contamination by mitochondria (1.15 g/cm³), broken chloroplasts (1.16 g/cm³), and...
peroxisomes (1.17 g/cm³). A similar method for the separation of chloroplasts from spinach leaves was reported previously (11). Our finding that the peroxisomes migrated faster, instead of slower, than the mitochondria is in disagreement with that reported (11). A distinct peak of AT was associated with alkaline fructose 1.6-bisphosphatase, a marker enzyme of intact chloroplasts. On further centrifugation of the sucrose gradient to equilibrium, although the intact and broken chloroplasts remained at their respective densities as in short term centrifugation, the peroxisomes migrated to a density of 1.25 g/cm³. The peroxisomal fraction was free of contamination since no other organelle marker enzymes were detected. The mitochondria migrated to a density in the vicinity of 1.18 to 1.22 g/cm³ and thus contaminated the intact chloroplasts. A minor but distinct peak of AT was associated with the peroxisomes in the gradient. The peak of AT associated with intact chloroplasts was enhanced and broadened by the contaminating mitochondria.

In the preparation of mitochondria free of contaminating organelles, the total extract was first centrifuged at 2000g for 10 min to remove intact chloroplasts. The resulting supernatant fraction was recentrifuged at 10,000g for 30 min to yield a particulate fraction which was then subjected to equilibrium sucrose gradient centrifugation. Data of similar preparation had been published earlier (9, 13, 14). In the present preparation (Fig. 1), little activity of alkaline fructose 1.6-bisphosphatase was detected in the gradient since the intact chloroplasts had been removed. A fraction of the mitochondria (Cyt oxidase as a marker) was trapped by the broken chloroplasts at density 1.17 g/cm³, as also reported by others (13, 14). A distinct peak of AT was associated with the major peak of Cyt oxidase in the gradient. Again, there was a minor but distinct peak of AT associated with that of catalase.

The total extract and the various subcellular fractions were subjected to starch gel electrophoresis for the separation and identification of isozymes of AT (Fig. 2). In the total extract, three major bands (bands 2, 3, and 4) and one minor band (band 1) of isozymes were observed. The supernatant fraction obtained after centrifugation of the total extract at 10,000g for 30 min contained isozymes 1, 3, and 4, while the resulting particulate fraction possessed isozymes, 1, 2, and 4. Since isozyme 3 appeared only in the supernatant fraction, we suggest that it represents the cytosol isozyme. Isolated intact chloroplasts contained only isozyme 4 as the chloroplast isozyme. The occurrence of isozyme 4 in the supernatant fraction should represent the enzyme derived from chloroplasts which were broken during the extraction. This suggestion is supported by the fact that in our normal preparation, approximately 70 to 80% of the chloroplasts were broken, as evidenced by the per cent recovery of Chl and alkaline fructose 1.6-bisphosphatase in intact chloroplasts (Fig. 1). Isolated mitochondria contained only isozyme 2 as the mitochondrial isozyme. The presence of isozyme 2 in the supernatant fraction was minimal since we obtained a good recovery of Cyt oxidase in the mitochondrial fraction (Fig. 1). Isolated peroxisomes contained isozyme 1. The presence of isozyme 1 in the supernatant fraction was due to broken peroxisomes since catalase, a peroxisomal marker enzyme, was also present in that fraction. Because the peroxisomes contained low activity of AT, we had to overload the sucrose gradient with leaf extract in order to obtain a high concentration of organelles for enzyme staining in starch gel. In such a preparation, the peroxisomal fraction was slightly contaminated with mitochondria, as evidenced by the presence of a trace amount of Cyt oxidase (Fig. 1) and the mitochondrial isozyme of AT (Fig. 2). In most fractions, there was a very slow migrating band near the origin. This band may represent an aggregation of AT with other cellular components, but since its distribution in the various fractions followed iso-
zyme 1 fairly closely, it may be another peroxisomal isozyme. Such a slow migrating band was also observed previously in spinach leaf peroxisomal fraction (13, 20).

The various isozymes were quite stable during the period of electrophoresis, as judged by the following method. The various subcellular fractions (cytosol, mitochondria, chloroplasts, and peroxisomes) were applied to starch gel, and after electrophoresis, the gels were cut and homogenized gently in a glass homogenizer with 0.05 m tris-citrate buffer (pH 7). After incubation at 4 C for 30 min, the homogenate was centrifuged at 10,000g for 30 min, and the supernatant fraction was retained. The extraction procedure was repeated once with the starch gel pellet. The two supernatant fractions were combined and assayed for AT activity. Approximately 60 to 75% of the enzyme from each fraction was recovered by this method. The rest of the enzyme might have been trapped in the starch gel or lost during the extraction procedure. Even if inactivation did occur, no specific isozyme was selectively inactivated during the period of electrophoresis.

After 2 hr of staining, the intensity of the isozyme bands in the gel remained at their maxima for several hr. Within the limit of the amount of enzyme extract that we applied to the gel, the intensity of an isozyme in one gel was proportional to the amount of enzyme extract applied initially. Such proportionality was checked on the chloroplast, mitochondrial, and cytosol isozymes by successive dilution of the various fractions. The peroxisomal isozyme was not checked because it was difficult to obtain peroxisomal preparation with activity high enough to carry out the test. The intensity of the stain could be measured quantitatively by scanning the starch gel spectrophotometrically at 627 nm and computing the area under each peak. This scanning method was used to estimate the relative amount of each isozyme in the total extract. As shown in Figure 3, roughly 45% of the total activity was represented by the chloroplast isozyme. 26% by the cytosol isozyme, 19% by the mitochondrial isozyme, 3% by the peroxisomal isozyme, and 7% by the unidentified slow migrating band. These estimated percentages are consistent with those calculated from organelle fractionation. The latter calculation was based on the amount of AT activity present in each type of organelles in sucrose gradients and the per cent recovery of the organelles estimated by the retention of marker enzymes.

The transamination activities involving glutamate and aspartate in the isolated chloroplasts, mitochondria, and peroxisomes were studied. The supernatant fraction obtained after centrifugation of the crude extract at 10,000g for 30 min was used as the cytosol fraction, but since it was heavily contaminated with the other AT isozymes due to organelle breakage (Fig. 2), acceptance of the information on this fraction should be reserved. The activity ratios of aspartate-α-ketoglutarate transamination to glutamate-oxyaloacetate transamination were 2.5 in chloroplasts, 1.6 in mitochondria, 1 in peroxisomes, and 3.2 in the supernatant fraction. Thus, the transamination reaction in the four cellular compartments was freely reversible. The transamination in all four cellular compartments preferred α-ketoglutarate to pyruvate or glyoxylate as the amino group acceptor (Table I).

With glutamate as the amino group donor, oxyaloacetate was superior to pyruvate or glyoxylate as the acceptor in chloroplasts, mitochondria, and glyoxysomes, while pyruvate or glyoxylate was a preferred acceptor to oxyaloacetate in peroxisomes. It is likely that the transamination activities observed are the combined activities of several different transaminases having diverse substrate specificities, since these enzymes have been reported to occur in many green leaves (1, 6, 13, 16, 20).

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