Metabolism of Glycolate in Isolated Spinach Leaf Peroxisomes

KINETICS OF GLYOXYLATE, OXALATE, CARBON DIOXIDE, AND GLYCINE FORMATION

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ABSTRACT

The flow of glycolate derived from glycolate into various metabolic routes in the peroxisomes during photorespiration was assessed. Isolated spinach leaf peroxisomes were fed [14C]glycolate in the absence or presence of exogenous glutamate, and the formation of radioactive glyoxylate, CO2, glycine, oxalate, and formate was monitored at time intervals. In the absence of glutamate, 80% of the glycolate was consumed within 2 hours and concomitantly glyoxylate accumulated; CO2, oxalate, and formate each accounted for less than 5% of the consumed glycolate. In the presence of equal concentration of glutamate, glycolate was metabolized at a similar rate, and glycine together with some glyoxylate accumulated; CO2, oxalate, and formate each accounted for an even lesser percentage of the consumed glycolate. CO2 and oxalate were not produced in significant amounts even in the absence of glutamate, unless glycolate had been consumed completely and glyoxylate had accumulated for a prolonged period. These in vitro findings are discussed in relation to the extent of CO2 and oxalate generated in leaf peroxisomes during photorespiration.

Photorespiration reduces the efficiency of photosynthesis and thus the growth of a plant. Its operation is attributed to the release of CO2 in the oxidation and subsequent metabolism of glycolate generated in photosynthesis (11, 13, 15). It is now clear that glycolate is first oxidized to glyoxylate catalyzed by glycolate oxidase in the leaf peroxisomes. However, the subsequent fate of glyoxylate is not completely understood. There are at least four different metabolic routes that glyoxylate can follow (Fig. 1). (a) Glyoxylate can go through the glycolate pathway to produce, in successive reactions, glycine, serine, hydroxyproprionate, and glyceraldehyde-3-phosphate (13). (b) Glyoxylate can react with H2O2 nonenzymatically to produce CO2 and formate; H2O2 is produced by the glycolate oxidase reaction and some of the H2O2 is supposed to escape destruction by catalase within the peroxisomes (5, 6). (c) Being a substrate analog of glycolate, glyoxylate can be oxidized to oxalate catalyzed by glycolate oxidase (12). (d) Glyoxylate can move to the chloroplast where it is either decarboxylated (15) or reduced to glycogelse (13). Besides the above four routes, glyoxylate, as a substrate analog of hydroxyproprionate, also can be reduced to glyoxylate by a peroxisomal NADPH-hydroxyproprionate reductase; however, this reaction was considered physiologically unimportant due to the high Km value of glyoxylate (10-3 M) (13).

The relative percentage of glycolate entering each of the above routes is still unknown. The flow is presumably controlled by many factors, an important one being the availability of amino groups for the transamination reaction converting glyoxylate to glycine. Theoretically, the amino groups in the glyoxylate pathway are self-regenerated. However, the NH3 and serine generated in the mitochondrial oxidation of glycine may be required for other metabolic events and thus would not be available directly for the glycolate pathway. Since glyoxylate metabolism leads eventually to the CO2 generated in photorespiration, information on the relative percentage of glyoxylate flowing into each of the various routes is valuable to our understanding of photorespiration.

To obtain such information, we have used isolated peroxisomes to perform time-course experiments on the subsequent metabolism of glyoxylate generated from glycolate. The presence or absence of glutamate as an amino group donor in controlling the flow of glycolate into other metabolites has been assessed. Emphasis has been placed on elucidating the significance of CO2 and oxalate production in the peroxisomes.

MATERIALS AND METHODS

Plant Materials and Preparation of Leaf Peroxisomes. Market spinach (Spinacia oleracea L.) was obtained locally. The preparation of leaf peroxisomes followed essentially the method described earlier (8). Briefly, the particulate fraction obtained between centrifugation forces of 1,000g for 10 min and 10,000g for 10 min was subjected to equilibrium linear sucrose gradient (30–60% w/w) centrifugation. The sucrose gradient contained 1 mm K-phosphate (pH 7.5) instead of EDTA.

Glycolate Metabolism in Peroxisomes. The reaction was allowed to proceed at room temperature in a plastic well attached to a handle (Kontes Corp., Vineland, NJ). The well was hung in a glass vial (19 × 48 mm) above a piece of folded 2 × 2-cm filter paper soaked with 50 μl of 2 N KOH solution, and the vial was covered with a rubber cap (Kontes Corp.). The reaction mixture contained, in a final volume of 250 μl, 50 mm K-phosphate (pH 7.8), 0.2 mm flavin mononucleotide, 5 mm [14C]glycolate (0.25 μCi; either C-1 labeled from Amersham-Searle, or both C-1 and C-2 labeled from ICN Pharmaceuticals, Inc.), and 25 μl (in 2.0 mm sucrose) of peroxisomes (5.5 μg protein). The peroxisomes were broken readily by osmotic shock (7). Glutamate at 5 mm was added where indicated. In some experiments, 60 μM pyridoxal-5-P was added together with 15 mm glutamate. At time intervals, the reaction was stopped by injecting 8 μl of 1 N H2SO4. After acidification, the reaction mixture was incubated for 1.25 h to expel the CO2. This acidification method released more than 92% of the [14C]CO2, as judged from control preparations using [14C]NaHCO3 instead of [14C]glycolate in the reaction mixture. The glass vial, together with the filter paper and absorbed CO2, was subjected to scintillation counting. The reaction mixture, now at about pH 3.0, was layered onto a column (1 × 6 cm) of Dowex-50 H+, and the column was washed with 50 ml H2O. The effluent (organic acid fraction) was neutralized with 2 N NaOH using a drop of phenol red solution (100 mg dissolved in 5.7 ml 0.05 N NaOH and made to 100 ml with water) as an indicator, and then it was evaporated to dryness at 40 C with a rotary evaporator. The

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amino acids absorbed to the Dowex column were displaced with 100 ml of 2 N NH4OH, and the effluent was evaporated to dryness.

Silica Gel Chromatography of Organic Acids. The method of Bullen et al. (2) was employed using a modified proportion of eluting solvents. The organic acid fraction was redissolved in 250 μl of 0.5 N H2SO4 containing 10 μeq of formic acid, 35 μeq of oxalic acid, 5 μeq of glyoxylic acid, and 15 μeq of glycolic acid. The mixture was stirred with 1 g of silica gel, and layered onto a prepacked column (1 × 24 cm) of 10 g silica gel in chloroform. The organic acids were eluted with 70 ml of 10% 1-butanol in chloroform followed by 170 ml of 1-butanol, chloroform, and ether (1:4:2). Three 1-ml fractions were collected with an Isco fraction collector (model 820) using an Isco volumeter (model 405). Each fraction was titrated with 0.02 N NaOH using a drop of phenol red solution as an indicator, and evaporated to dryness at 50°C in an air stream. The residues were dissolved in 0.2 ml of H2O and the solution was transferred to a scintillation vial. Radioactivity counting was performed in a Beckman scintillation counter model LS-3133P.

The pattern of organic acids eluted from the column was predetermined by running each individual organic acid alone and in combination, and by identifying them chemically (3).

Identification of Amino Acids. The amino acid fractions from selected samples were redissolved in water and analyzed for amino acid composition and radioactivity in a Beckman model 120C amino acid analyzer attached to a 314E Packard Tri-Carb scintillation counter. More than 99% of the radioactivity was associated with glycine. For practical purposes, the total radioactivity associated with the amino acid fraction was designated as that in glycine.

RESULTS

Separation of Radioactive Organic Acids. Using a silica gel column with a well defined proportion of eluting solvents, formate, glyoxylate, oxalate, and glycolate in the organic acid fraction were separated from one another. Figure 2 shows the separation of these acids in such a column from the organic acid fraction of a reaction mixture in which isolated peroxisomes had been incubated with [1,2-14C]glycolate for 2 h. Whereas distinct and sharp peaks of formate, oxalate, and glycolate were obtained, a broad, trailing peak of glyoxylate was eluted. The proportion of eluting solvents was designed such that the trailing glyoxylate peak was well separated from the other organic acids. The achievement of separating the major organic acids derived from glycolate in one column chromatography has not been previously reported. It enabled us to monitor the changes of these metabolites accurately in our subsequent experiments.

Peroxisomal Metabolism of [1,2-14C]Glycolate. When isolated peroxisomes were fed with [1,2-14C]glycolate in the absence of glutamate, glyoxylate accumulated and no glycine was formed (Fig. 3). After 2 to 3 h, most of the glycolate had been consumed. During the 3-h incubation, insignificant amounts of CO2, oxalate, and formate were found. This occurred even though glyoxylate had already accumulated and represented 77% of the total radioactivity after 2 h.

In the presence of glutamate, glycine accumulated and glyoxylate remained at a relatively low level (Fig. 3). With compara-
3-h incubation of isolated leaf peroxisomes with radioactive glycolate, less than 5% of the metabolized glycolate was converted to oxalate. In each of the four experimental conditions (i.e., using either [1,2,14C]glycolate or [1,14C]glycolate in the absence or presence of glutamate), there was an increasing accumulation of oxalate during the incubation periods (Figs. 3 and 4). This increase may be due to the decreasing amount of glycolate and the increasing amount of oxalate in the reaction medium, two conditions that would favor the competition at the active site of glycolate oxidase by glycolate to form oxalate (11). To investigate the above possibility, the experimental period was prolonged to 15 h (Table I). In the absence of glutamate after a 2-h incubation, 22% of glycolate remained, and glycolate and oxalate accounted for 72% and less than 1%, respectively, of the total radioactivity. After 15 h, essentially all of the remaining glycolate and the accumulated glycolate had been converted to oxalate (67%) and CO2 (25%). Presumably, the increased CO2 release was due to oxidation of the accumulated glycolate by H2O2 produced in oxalate formation (Fig. 1). In the presence of glutamate, a similar trend of increased production of oxalate and CO2 was observed on prolonged incubation (Table I), but the increase was much less dramatic since most of the glycolate carbon had been converted to glycine.

In the fungus Sclerotium rolfsii (10), oxalate is produced in the peroxisomes by a glycolate dehydrogenase (glycolate:NAD oxidoreductase). Using isolated peroxisomes or commercially purified hydroxypyruvate reductase (Boehringer-Mannheim) from spinach leaves, we failed to detect the activity of the above enzyme at pH 7.5 and 8.7.

**DISCUSSION**

Our findings indicate that in isolated leaf peroxisomes supplied with adequate amino acid, most of the glycolate is metabolized to glycine in the peroxisomes. Only a few percent of the glycolate is decarboxylated to CO2 in the organelle. In the absence of an adequate supply of amino acid, the amount of peroxisomal CO2 released is still relatively small. It was reported that some glyoxylate generated from glycolate is converted to CO2 directly in the peroxisomes (5, 6). In these reports, little information was provided on the kinetics and percentage of CO2 formed in relation to other metabolites. Such detailed analyses of the related metabolites are important. For example, when leaf peroxisomes were incubated with radioactive glycolate in the absence of glutamate (Table I), if only CO2 formation was monitored, its value could be from 5% in 2 h to 26% in 15 h. The variation depended on the duration of the incubation period or the amount of peroxisomes used. On prolonged incubation, the decarboxylation of glyoxylate was enhanced (Table I), presumably by some of the H2O2 generated during oxalate formation. It seems unlikely, however, that glyoxylate as a very reactive metabolite, can accumulate to a high amount in vivo without being consumed in other metabolic reactions. These other metabolic reactions include light dependent decarboxylation (15) or reduction to glycolate catalyzed by NADP-glyoxylate reductase (13), both of which occur at high activities in the chloroplast. It should be valid that the peroxisomal decarboxylation of glyoxylate is affected by environmental factors (5, 6); nevertheless, the magnitude of the peroxisomal decarboxylation would represent only a small percentage of the glycolate carbon passing through the glycolate pathway.

Several plant species, including spinach, accumulate oxalate to more than 10% of the total leaf dry weight (1, 4). Since oxalate is supposed to serve no metabolic role once it is formed (1), the drainage of organic carbon into oxalate represents a severe waste. There are several metabolic pathways of oxalate biosynthesis (4, 12, 14), and one of them is by the route of glycolate and glyoxylate. Purified glycolate oxidase can catalyze the oxidation of glycolate to oxalate in vitro (12). Green leaves in vivo convert externally

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**Table I. Metabolism of Glycolate in Isolated Spinach Leaf Peroxisomes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of total 14C in each compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolate</td>
<td>22.4</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>72.0</td>
</tr>
<tr>
<td>Oxalate</td>
<td>0.8</td>
</tr>
<tr>
<td>CO2</td>
<td>4.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.0</td>
</tr>
</tbody>
</table>

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**FIG. 4.** Time course of metabolism of 5 mM [1-14C]glycolate (0.25 μCi) in isolated spinach leaf peroxisomes at 25 C in the absence or presence of 5 mM glutamate. The percentage of CO2, oxalate, and formate are magnified in the lower part of the figure. Notice the difference in scales between the bottom parts of Figures 3 and 4.
supplied glycolate or glyoxylate to oxalate (4). Our data indicate that such formation of oxalate does occur in isolated peroxisomes, but is only minimal even in the absence of glutamate. A substantial amount of oxalate is formed only after glycolate has been consumed and glyoxylate has accumulated for a prolonged period, an apparent necessity given the $K_m$ values of glyoxylate ($10^{-3}$ M) and glycolate ($10^{-4}$ M) for glycolate oxidase (12). Again, in accord with the argument raised in the preceding paragraph, such an active synthesis of glyoxylate in vivo is not likely to occur. Whether or not a slow oxalate synthesis in peroxisomes in vivo still contributes significantly to oxalate accumulation over a long period of time requires further investigation.

LITERATURE CITED

12. Richardson KE, NE Tolbert 1961 Oxidation of glyoxylic acid to oxalic acid by glycolic acid oxidase. J Biol Chem 231: 1280-1284