Metabolism of Glycolate and Glyoxylate in Intact Spinach Leaf Peroxisomes

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ABSTRACT

Intact and broken (osmotically disrupted) spinach (Spinacia oleracea) leaf peroxisomes were compared for their enzymic activities on various metabolites at 0.25 molar sucrose solution. Both intact and broken peroxisomes had similar glycollate-dependent O2 uptake activity. In the conversion of glycollate to glycine in the presence of serine, intact peroxisomes had twice the activity of broken peroxisomes at low glycollate concentrations, and this difference was largely eliminated at saturating glycollate concentrations. However, when glutamate was used instead of serine as the amino group donor, broken peroxisomes had slightly higher activity than intact peroxisomes. In the conversion of glycine to glycine in the presence of serine, intact peroxisomes had only about 50% of the activity of broken peroxisomes at low glycine concentrations, and this difference was largely overcome at saturating glycine concentrations. In the transamination between alanine and hydroxypyruvate, intact peroxisomes had an activity only slightly lower than that of broken peroxisomes. In the oxidation of NADH in the presence of hydroxypyruvate, intact peroxisomes were largely devoid of activity. These results suggest that the peroxisomal membrane does not impose an entry barrier to glycine, alanine, and O2 for matrix enzyme activity; such a barrier does not allow the study of metabolism of intact peroxisomes as they function in vivo, and we do not have information on the permeability of the peroxisomal membrane to various metabolites or the possible coordination of metabolite flow in intact organelles. This lack of information of leaf peroxisomes also exists in peroxisomes (microbodies, glyoxysomes) of other plant and animal tissues as well as microbes (1, 7, 13).

Recently, we have established a procedure to isolate leaf peroxisomes from spinach in 0.25 m sucrose solution (8). A similar procedure to isolate leaf peroxisomes in 0.5 m sucrose solution was established independently (10). In this report, we present experimental results of our studies on the metabolism of intact leaf peroxisomes in vitro, and broken (osmotically disrupted) peroxisomes were used for comparison. The results show that the peroxisomal membrane imposes an entry barrier to some metabolites and that there is a coordination of metabolite flow within the peroxisomes.

MATERIALS AND METHODS

Isolation of Leaf Peroxisomes. Intact peroxisomes suspended in a solution containing 1 mM K-phosphate (pH 7.5) and 0.25 m sucrose were isolated from spinach leaves in Percoll density gradients after velocity centrifugation as described (8). The combined peroxisomal fraction from four gradients was essentially free of chloroplast or mitochondrial contamination. It was washed and centrifuged three times to remove the Percoll (8). The yield was 0.5 ml containing about 0.3 to 1.0 mg peroxisomal proteins. About 95% of the peroxisomes in the suspension were intact, as judged by three different integrity tests (8). The isolated peroxisomal fraction was used immediately.

Assays of Enzyme Activities in Intact and Broken Peroxisomes. Unless otherwise stated, the assay of enzyme activity using a radioactive substrate was as follows. A 50-μl aliquot of the peroxisomal fraction (diluted with the resuspension buffer to give 3–30 μg protein/50 μl) was mixed with 100 μl of the assay buffer containing either no sucrose (to break the organelles osmotically) or 0.25 m sucrose (to maintain organelle integrity). After 10 min of incubation at room temperature, the remaining assay components were added to make a final volume of 250 μl containing 0.25 m sucrose in both the intact and broken peroxisome assays. An aliquot of 35 μl of reaction mixture was added to 10 μl of 1 M H2SO4 at five time intervals, usually 3 min apart. Immediately after the sampling was completed (usually 30–40 min after mixing the peroxisomal fraction with the assay medium), an aliquot of the remaining reaction mixture was assayed for peroxisome integrity using the hydroxypyruvate reductase method (8). Routinely, the peroxisomal fraction contained about 95% intact peroxisomes before mixing with the reaction medium. Immediately after the assay, this percentage was 85 to 95%. In the reaction mixture containing broken peroxisomes, the percentage of peroxisome integrity immediately after the enzyme assay was always zero. Data on product formation at five time

Leaf peroxisomes play a central role in carbon metabolism during photorespiration (13). Glycolate released from the chloroplasts is oxidized in the peroxisomes to glyoxylate which is transaminated to glycine. Glycine leaves the peroxisomes and is converted to serine and CO2 in the mitochondria. Serine returns to the peroxisomes and is transaminated to hydroxypyruvate which is reduced by NADH to glyceraldehyde. Glyceraldeyde leaves the peroxisomes and is converted to sugars in the chloroplasts.

In studies of leaf peroxisomes, the organelles have been routinely isolated by isopycnic sucrose density gradient centrifugation (1, 7, 13) in which they migrate to an equilibrium density of 1.25 g/cm2 (1.9 M sucrose). Although the peroxisomal fraction is essentially free of other subcellular particles, attempts to dilute the fraction to a physiological concentration of osmotica (0.25 M sucrose) without breaking the peroxisomes have not been successful. Thus, study of peroxisomal metabolism in vitro has been carried out with broken peroxisomes. This approach does not allow the study of metabolism of intact peroxisomes as they function in vivo, and we do not have information on the permeability of the peroxisomal membrane to various metabolites or the possible coordination of metabolite flow in intact organelles. This lack of information of leaf peroxisomes also exists in peroxisomes (microbodies, glyoxysomes) of other plant and animal tissues as well as microbes (1, 7, 13).

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intervals ensured an adequate determination of the initial reaction rate. Enzyme activities presented in various figures are those in 35 l. reaction mixture. Each experiment was repeated one to four times yielding similar results; the representative results from one single experiment are presented. The activity of hydroxypyruvate reductase (8) in osmotically lysed peroxisomes was used to illustrate the amount of peroxisomes used in different experiments (see legends to figures).

Formation of [14C]Glycine from [14C]Glycerate or [14C]Glyoxylate. The assay procedure was similar to that described earlier (3). The reaction mixture, in a final volume of 250 l., contained 50 mM K-phosphate (pH 7.8), 0.25 mM sucrose, peroxisomal preparation containing 3 to 30 mg protein, and amino acid at a concentration specified in "Results." The reaction was initiated by adding 10 l. of 0.25 M sucrose solution containing 0.5 uCi [14C]glycerate or [14C]glyoxylate to give a designated concentration in the final reaction mixture. The reaction was stopped by acidification as described in the preceding paragraph. Each acidified reaction mixture sample of 35 l. was applied onto a column (0.5 x 5.0 cm) of Dowex-50H+. The column was washed with 10 ml H2O to remove the organic acids. The glycine absorbed onto the column was displaced with 8 ml 2 M NH4OH. The recovery and identification of glycine in this chromatography were described earlier (3). The effluent was dried by an air stream at 55 to 60°C. The residue was redissolved with 100 l. H2O, and subjected to scintillation counting.

Formation of [14C]Pyruvate from [14C]Alanine. The reaction mixture, in a final volume of 250 l., contained 50 mM K-phosphate (pH 7.8), 0.25 mM sucrose, peroxisomal preparation containing 3 to 30 mg protein, and hydroxypyruvate at a concentration specified in "Results." The reaction was initiated by adding 10 l. of 0.25 M sucrose solution containing 0.5 uCi [14C]alanine to give a designated concentration in the final reaction mixture. The radioactive pyruvate produced was determined as described (15) with modification. The reaction was stopped at five time intervals of 3 min apart by placing 40 l. of the reaction mixture into a vial containing 100 l. 2 M HCl. A piece of folded 1.5- x 2-cm filter paper soaked with 100 l. 30% KOH solution was hung on the upper part of the vial, and the vial was covered with a rubber cap. A solution of 100 l. 10% H2O2 (w/w) was injected with a syringe into the acidified reaction mixture. Three mol CO2 were produced from 1 mol pyruvate. After an incubation of 3 h at room temperature, the filter paper with the absorbed radioactive CO2 was subjected to scintillation counting.

Glycolate-Dependent O2 Uptake. O2 uptake activity was determined polarographically at 25°C using a Clark-type O2 electrode (Yellow Springs Instrument Co.). A solution of 0.2% KOH was used to arrest the reaction and was available for the direct measurement of O2 uptake. The reaction mixture, in a final volume of 1 ml, contained 50 mM K-phosphate (pH 7.8), 0.25 mM sucrose, and 0 to 40 l. peroxisomal protein. The reaction was initiated with 50 l. glycolate at 0.25 mM sucrose to give a final substrate concentration specified in "Results." Immediately after the assay, an aliquot of the reaction mixture was used to determine the integrity of the peroxisomes by the hydroxypyruvate reductase method (8).

Chemicals. Nonradioactive chemicals of reagent grade were obtained from Sigma. [U-14C]Glycolate was obtained from ICN Pharmaceuticals, Inc., [14C]glyoxylate from Amersham Corp., and [U-14C]alanine from New England Nuclear Corp.

RESULTS

Experimental Design. All the spinach leaf peroxisomal enzymes so far examined are present in the matrix of the organelles and are not associated with the surrounding membrane (6). A series of experiments was performed on the metabolism of various metabolites in intact peroxisomes; and broken peroxisomes derived from osmotic disruption were used for comparison. The measured initial activity of an enzyme in intact peroxisomes is the result of (a) the rate of the substrate entering the organelles and (b) the actual activity of the enzyme per se. If the passage of the substrate through the membrane is much faster than the enzyme activity such that the latter is the limiting step, the enzyme activity measured in intact peroxisomes should be equal to the activity of the enzyme per se. Under this condition, the observed enzyme activity in both intact and broken peroxisomes would be the same. If a membrane barrier exists, intact peroxisomes would have less detectable enzyme activity than broken peroxisomes. This membrane barrier may be detected more readily at nonsaturating substrate concentrations when the substrate available to the enzyme (i.e., the product of the membrane passage) becomes the limiting factor in the measured enzyme activity. With the above considerations in detecting possible membrane barriers, we assayed enzyme activities in intact and broken peroxisomes using nonsaturating and increasing concentrations of substrates.

Glycolate-Dependent O2 Uptake. The glycolate-dependent O2 uptake activities were the same in intact and broken peroxisomes in the presence of increasing concentrations of glycolate (Fig. 1). The apparent Km value was 0.15 mM, which is similar to the reported value of 0.26 mM on purified spinach glycolate oxidase (4). The peroxisomal membrane does not appear to exert a barrier to passage of glycolate from the exterior to the glycolate oxidase inside the organelles. Also, the membrane is highly permeable to O2 in a solution under atmospheric pressure. Conversion of Glycolate to Glycine. Intact and broken peroxisomes were incubated with radioactive glycolate and nonradioactive amino acid, and the production of radioactive glycine was measured. This glycine production is the combined activities of two enzymes in a sequence, glycolate oxidase, and either

![Graph](https://example.com/graph.png)
GLYCOLATE METABOLISM IN INTACT LEAF PEROXISOMES

Fig. 2. Formation of glycine from glycolate by intact (○) and broken (O) peroxisomes at increasing glutamate concentrations. Glycolate of 1 mM was used. Data are the activities in 35 μl reaction mixtures, each containing 2.3 nmol/min hydroxypyruvate reductase activity.

Fig. 3. Formation of glycine from glycolate by intact (○) and broken (O) peroxisomes at increasing serine concentrations. Glycolate of 1 mM was used. Data are the activities in 35 μl reaction mixtures, each containing 12 nmol/min hydroxypyruvate reductase activity.

glutamate-glyoxylate aminotransferase or serine-glyoxylate aminotransferase (13). In isolated peroxisomes assayed under broken conditions, glycolate oxidase activity is about 10 times higher than glutamate-glyoxylate or serine-glyoxylate aminotransferase activity (12). Thus, the aminotransferase activity would be the limiting step in the sequence of the two-enzyme reaction.

In the presence of 1 mM glycolate (close to the saturated concentration for maximal glycolate oxidase activity, see Fig. 1) and increasing concentrations of glutamate, intact peroxisomes converted glycolate to glycine at a lower rate than broken peroxisomes (Fig. 2). The reduction ranged from 10 to 30%, depending on individual experiments, and it was consistently observed. Inasmuch as we established earlier that the membrane does not impose an entry barrier to glycolate and O2 for maximal glycolate oxidase activity, the findings shown in Figure 2 suggest that the membrane imposes a barrier hindering the entry of glutamate. The degree of hindrance cannot be calculated from the available information, since the higher activity in broken peroxisomes than in intact peroxisomes could be greater if the coordinate effect of glycolate oxidase and aminotransferase is taken into consideration (next two paragraphs). When glycolate to glycine conversion was measured in the presence of 1 mM glycolate and 1 mM alanine, intact peroxisomes had about 20% lower activity than broken peroxisomes. Thus, alanine is similar to glutamate in its ability to penetrate through the membrane.

In contrast to glutamate, in the presence of increasing concentrations of serine instead of glutamate as the amino group donor, intact peroxisomes converted glycolate to glycine at rates higher than those of broken peroxisomes (Fig. 3). The enhancement was about 10 to 30%, depending on individual experiments, and an enhancement was consistently observed. The cause of this enhancement resides in the aminotransferase activity rather than the glycolate oxidase activity, since intact and broken peroxisomes had identical glycolate oxidase activity (Fig. 1). The findings (Figs. 2 and 3) also suggest that, relative to glutamate, serine can penetrate the membrane readily. This suggestion is based on the information that the glutamate-glyoxylate aminotransferase and serine-glyoxylate aminotransferase in the peroxisomes have similar activities (12). The \( K_m \) values of serine and glutamate in the two-enzyme assay system in both intact and broken peroxisomes were estimated to be around 0.7 to 0.9 mM. This value is similar to that for glyoxylate aminotransferase purified from kidney bean (11) and slightly lower than that for the enzyme purified from spinach leaves (9).

When glycolate to glycine conversion was assayed in 1 mM serine and increasing concentrations of glycolate, intact peroxisomes showed higher activity than broken peroxisomes (Fig. 4). At glycolate concentrations of 0.5 mM and lower, the activity of intact peroxisomes was about twice that of broken peroxisomes. However, at higher concentrations of glycolate, the activity of broken peroxisomes approached that of intact peroxisomes, as revealed in a double reciprocal plot (Fig. 4). This enhancement of activity in intact peroxisomes is likely the consequence of a coordination of glycolate oxidase and aminotransferase activities by a confinement of glyoxylate in the vicinity of aminotransferase due to the existence of a loosely associated complex of the two enzymes and/or (b) within the peroxisomes due to the
glycolate, in activity the is availability next aminotransferase concentration (Fig. 13). (c) had about involved. (d) Similarly, glyoxylate is glycolate, ferases which had the peroxisomes was some. Glyoxylate gives effect no Serine-glyoxylate aminotransferase 5-phosphate. The presence of glyoxylate concentrations of glycolate coordinate is not enough higher than intact to peroxisomes after aminotransferase activity. The coordinate effect is therefore most prominent at nonsaturated concentrations of glycolate such that the concentration of glyoxylate produced becomes a limiting factor in the aminotransferase activity. In broken peroxisomes at a high concentration of glycolate, glyoxylate is produced rapidly to a concentration that is high enough to yield maximal aminotransferase activity.

The higher activity in intact peroxisomes was due to a greater availability of glyoxylate for aminotransferase in intact peroxisomes and not to a loss of enzyme cofactors to the apoenzyme in broken peroxisomes after solubilization. This statement is made based on the following observations. (a) Intact and broken peroxisomes had the same glycolate oxidase activity (Fig. 1). (b) Serine-glyoxylate aminotransferase activity in broken peroxisomes was the same in the presence or absence of 60 μM pyridoxal 5-phosphate. The addition of pyridoxal phosphate did not affect the activities of several purified plant aminotransferases which had the cofactor tightly bound to the apoenzyme (13). (c) A double reciprocal plot of activity versus glyoxylate concentration (Fig. 4) shows that broken and intact peroxisomes had about the same apparent $V_{max}$ value. This suggests that glycolate, and indirectly glyoxylate, is the only or major factor involved. (d) Similarly, a double reciprocal plot of serine-glyoxylate aminotransferase activity versus glyoxylate concentration (see next section) shows that broken and intact peroxisomes had about the same apparent $V_{max}$ value. This suggests that glyoxylate availability is the only or major factor involved in the difference in activity between intact and broken peroxisomes.

Conversion of Glyoxylate to Glycine. The conversion of glyoxylate to glycine in peroxisomes in the presence of 1 mM serine and increasing concentrations of glyoxylate is shown in Figure 5. Intact peroxisomes had activities lower than those of broken peroxisomes. At concentrations of glyoxylate below 1 mM, the activity of intact peroxisomes was about 50% that of broken peroxisomes. However, at higher concentrations of glyoxylate, the activity of intact peroxisomes approached that of broken

![Figure 5](https://academic.oup.com/plphys/article/73/1/147/6079105)

**Fig. 5.** Formation of glycine from glyoxylate by intact (●) and broken (○) peroxisomes at increasing glyoxylate concentrations. Serine of 1 mM was used as the amino group donor. Data are the activities in 35 μl reaction mixture each containing 21.4 nmol/min hydroxypyruvate reductase activity.

![Figure 6](https://academic.oup.com/plphys/article/73/1/147/6079105)

**Fig. 6.** Transamination between hydroxypyruvate and alanine by intact (●) and broken (○) peroxisomes at increasing concentrations of either hydroxypyruvate (upper figure, using 1 mM alanine) or alanine (lower figure, using 1 mM hydroxypyruvate). Data are the activities in 35 μl reaction mixture, each containing 13.7 (upper figure) or 9.1 (lower figure) nmol/min hydroxypyruvate reductase activity.

![Figure 7](https://academic.oup.com/plphys/article/73/1/147/6079105)

**Fig. 7.** Effect of increasing sucrose concentrations in the assay medium on the glycolate-dependent O$_2$ uptake by intact (●) and broken (○) peroxisomes. Glycolate of 0.5 mM was used. Data are the activities in 1 ml reaction mixture, each containing 57 nmol/min hydroxypyruvate reductase activity.
poxisomes, as revealed in a double reciprocal plot of activity versus substrate concentration (Fig. 5). Apparently, the membrane presents a barrier to glyoxylate (an apparent \( K_m \) of 2.0 mm, compared to the apparent \( K_m \) of 0.73 mm in broken peroxisomes) rather than serine for maximal aminotransferase activity, and this barrier can be overcome by increasing the concentration of glyoxylate.

**Transamination between Alanine and Hydroxypyruvate.** The transamination activity between alanine and hydroxypyruvate in intact and broken peroxisomes is shown in Figure 6. In the presence of 1 mm alanine and increasing concentrations of hydroxypyruvate, intact peroxisomes had activities slightly lower than those of broken peroxisomes. This reduction was also observed in the presence of 1 mm hydroxypyruvate and increasing concentrations of alanine. The findings suggest that the peroxisomal membrane imposes a barrier, slightly hindering the entry of both alanine and hydroxypyruvate.

**Hydroxypyruvate-Dependent Oxidation of NADH.** Intact peroxisomes were largely incapable of oxidizing externally added NADH (0.14 mm) in the presence of 1 mm hydroxypyruvate. A trace amount of activity (about 5% of that in broken peroxisomes) was observed, which could be due to the presence of broken peroxisomes in the preparation (8) and/or the metabolites entering the peroxisomes slowly. This finding is similar to that observed by others on leaf peroxisomes and glyoxysomes in crude particulate fractions (7, 13, 10). The finding alone shows that the membrane imposes an entry barrier to NADH or hydroxypyruvate, or both metabolites, for maximal hydroxypyruvate reductase activity. Since we established earlier (preceding paragraph) that hydroxypyruvate can penetrate the peroxisomal membrane to a certain extent, it is likely that the peroxisomal membrane imposes a strong entry barrier to NADH for maximal hydroxypyruvate reductase activity.

**Conversion of Glycolate to Glycine in Peroxisomes Subjected to Osmotic Stress.** The glycolate oxidase activity assayed with intact and broken peroxisomes in the presence of 0.5 mm glycolate (nsaturating; see Fig. 1) remained about the same in media with increasing concentrations of sucrose from 0.25 to 1.0 M (Fig. 7). In the conversion of glycolate to glycine in the presence of glutamate, broken peroxisomes had slightly higher activity than intact peroxisomes (Fig. 2). When the sucrose concentration in the assay medium was increased from 0.25 to 1.0 M (Fig. 8), broken peroxisomes still maintained activity higher than that of intact peroxisomes. The activities of both peroxisome preparations in higher sucrose concentrations decreased slightly, but in 1.0 M sucrose they retained some 75 to 80% of the activity.

**DISCUSSION**

Our findings establish that intact leaf peroxisomes carry out metabolism in a manner that is different from that observed with broken peroxisomes. Of most physiological significance is that intact peroxisomes have a higher activity than broken peroxisomes in the conversion of glycolate to glycine. This enhancement likely is due to the confinement of glyoxylate within the peroxisomes by the membrane barrier and/or the existence of a loosely associated multi-enzyme complex of glycolate oxidase and glyoxylate aminotransferase. The confinement of metabolic intermediates within the peroxisomes for a highly orchestrated metabolic flow in other metabolic reactions in purified, intact peroxisomes should now be examined. One example is the NADH/NAD reutilization in an electron shuttle system by the coupled reactions involving hydroxypyruvate reductase and malate dehydrogenase in leaf peroxisomes (13). Another example is the production of \( \text{H}_2\text{O}_2 \) by glycolate oxidase and its immediate destruction by catalase in the leaf peroxisomes, so that undesired reactions such as the nonenzymic reaction between \( \text{H}_2\text{O}_2 \) and glyoxylate to produce formate and \( \text{CO}_2 \) would not occur (13).

At metabolite concentrations expected to occur in the cell under physiological conditions, the peroxisomal membrane imposes an entry barrier to some metabolites such that the matrix enzyme activities are reduced. There is no speculation at this time that a pH difference exists across the peroxisomal membrane. We selected pH 7.8 as the pH in the assays because it is around the reported pH values for optimal activity of glycolate oxidase, aminotransferase, and catalase. Therefore, our conclusion on the entry barrier of the peroxisomal membrane to these metabolites for matrix enzyme activities deals with their anionic forms. The peroxisomal membrane does not impose a barrier to glycolate, \( \text{O}_2 \), and serine for matrix enzyme activities; such a barrier does exist to glutamate, alanine, hydroxypyruvate, glycine, and NADH. The possible effect of this membrane barrier on the operation of photosynthetic pathway remains to be seen. A partial reduction in the activity of an enzyme does not necessarily impose a hindrance in the photosynthetic metabolite flow, if the reduced enzyme activity still is not the limiting step in the overall pathway.

Water stress impairs the ability of the chloroplasts to carry out photosynthesis (2). This impairment has been studied with isolated chloroplasts under low osmotic potentials *in vitro*, and the findings indicate that both the electron transport system and some steps of the carbon fixing pathway are affected (2). Our findings show that osmotic stress *in vitro* (up to 1 M sucrose having an osmotic potential of -29 bars) has little effect on the photosynthetic pathway from glycine to glycine in the peroxisomes. Whether or not other portions of the glycine pathway are affected remains to be seen.

**LITERATURE CITED**