Compartmentation Studies on Spinach Leaf Peroxisomes¹

Evidence for Channeling of Photorespiratory Metabolites in Peroxisomes Devoid of Intact Boundary Membrane

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ABSTRACT

In concurrence with earlier results, the following enzymes showed latency in intact spinach (Spinacia oleracea L.) leaf peroxisomes: malate dehydrogenase (85%), hydroxypropyruvate reductase (85%), serine glyoxylate aminotransferase (75%), glutamate glyoxylate aminotransferase (41%), and catalase (70%). In contrast, glycolate oxidase was not latent. Aging of peroxisomes for several hours resulted in a reduction in latency accompanied by a partial solubilization of the above mentioned enzymes. The extent of enzyme solubilization was different, being highest with glutamate glyoxylate aminotransferase and lowest with malate dehydrogenase. Osmotic shock resulted in only a partial reduction of enzyme latency. Electron microscopy revealed that the osmotically shocked peroxisomes remained compact, with smaller particle size and pleomorphic morphology but without a continuous boundary membrane. Neither in intact nor in osmotically shocked peroxisomes was a lag phase observed in the formation of glycinate upon the addition of glycolate, serine, malate, and NAD. Apparently, the intermediates, glycolate, hydroxypropyruvate, and NADH, were confined within the peroxisomal matrix in such a way that they did not readily leak out into the surrounding medium. We conclude that the observed compartmentation of peroxisomal metabolism is not due to the peroxisomal boundary membrane as a permeability barrier, but is a function of the structural arrangement of enzymes in the peroxisomal matrix allowing metabolite channeling.

Peroxisomes are organelles consisting of a homogeneous granular matrix surrounded by a single membrane and having a diameter of 0.2 to 1.7 μm (5, 7). Leaf peroxisomes, a specialized form of these organelles, participate in photorespiratory metabolism (Fig. 1), whereby glycolate is oxidized to glyoxylate, and then transaminated to glycine, these reactions being called glycolate pathway (27). After conversion of glycine to serine in the mitochondria, the serine is transferred to the peroxisomes, where it is transaminated to hydroxypropyruvate followed by reduction to glycine. This reversible sequence is called glycinate pathway (27). The photorespiratory metabolism requires the peroxisomal enzymes GOX², CAT, SGAT, GGAT, HPR, and probably also MDH. The glycolate and glycinate pathway is linked by SGAT. Because the formation of serine requires the conversion of two glyoxylates to two glycines, GGAT as a second glyoxylate aminotransferase is necessary. Most of the peroxisomal enzymes mentioned above have been isolated and characterized (for review see ref 8). Techniques have also been developed to isolate intact leaf peroxisomes of high metabolic competence (13) that are capable of synthesizing glycinate from the substrates serine, malate, and NAD in the presence of glycolate that was used to generate glyoxylate for the transaminase reaction (3, 29, 31).

Tolbert (27) stated in a review that all reactions involving glyoxylate biosynthesis and metabolism are compartmentalized in peroxisomes to prevent undesired side reactions of glyoxylate. This notion is supported by the fact that the enzymes involved in glyoxylate metabolism show latency (7). Usually, the compartmentation of metabolic reactions in a cell organelle is caused by the impermeability of a boundary membrane. Assuming that this also holds for peroxisomes, the latency of the peroxisomal marker enzyme HPR in previous studies has been used as a criterion of peroxisomal intactness (7, 13, 27). On the other hand, intact peroxisomes from various plant tissues have been found to be permeable to metabolites such as glycolate, glycine, glycerate, and serine (12, 27), malate (31), oxaloacetate, aspartate, and α-ketoglutarate (14), and to some extent also to NAD and NADH (14, 31). Whether specific translocators are involved in this permeability could not be resolved. Permeability studies with rat liver peroxisomes suggest, however, that a nonspecific permeability for small molecules up to a mol wt of 800 may be due to a protein forming an unselective pore in the peroxisomal membrane (28).

It is the aim of the present publication to elucidate the cause of metabolic compartmentation in leaf peroxisomes. For this, the effects of structural alterations, as monitored by EM, on the latency of enzymes and on the metabolic functions of spinach (Spinacea oleracea) leaf peroxisomes have been

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² Abbreviations: GOX, glycolate oxidase; CAT, catalase; SGAT, serine-glyoxylate aminotransferase; GGAT, glutamate-glyoxylate aminotransferase; HPR, hydroxypropyruvate reductase; MDH, malate dehydrogenase; CS, citrate synthase.
Assay of Serine-Dependent Formation of Glycerate

Peroxisomes were assayed for their enzymic activity in the conversion of glycolate and serine to glycerate at 20°C in a water bath. Incubation occurred in a 1000 μL reaction mixture containing 50 mM KH₂PO₄, pH 7.5, 0.25 mM sucrose, and the substrates at the concentrations indicated in “Results.” The reaction was initiated by addition of peroxisomes. To determine the activity of osmotically shocked peroxisomes, the organelles were incubated in water for 10 min. In this case, the reaction was initiated with substrate addition in an equal volume of double-concentrated buffer. Likewise, detergent-treated peroxisomes were incubated in the above-mentioned buffer plus 0.1% Triton X-100 for 10 min, the activity being determined after substrate addition in buffer. At appropriate time intervals, the reaction was terminated by transferring a 500 μL aliquot of the reaction mixture to a tube containing 100 μL 10% perchloric acid and 50 mM EDTA.

Each aliquot of the acidified reaction mixture was neutralized with 5 M KOH and 1 M triethanolamine (pH 6.8-7.2). To separate the resulting KClO₃ and the precipitated protein, the tubes were centrifuged for 5 min at 10,000g. In the supernatant, the glycerate formed was analyzed spectrophotometrically as described in ref. 19 using a Sigma double wavelength spectrophotometer. Glycerate kinase was partially purified from osmotically shocked pea chloroplasts (20), except that the precipitation of glycerate kinase and proteins was accomplished by addition of solid (NH₄)₂SO₄ to 55% of saturation and sucrose density-gradient centrifugation was replaced by the application of a 2.5 x 100 cm Sephadex G-75 gel filtration column.

Enzyme Assays

HPR and SGAT were assayed as described in ref. 26. The assay mixtures were modified to contain 50 mM KH₂PO₄ (pH 6.5), 0.4 mM NADH, and 4 mM lithium hydroxyppyruvate for HPR, and 100 mM Hepes-KOH (pH 7.0), 0.2 mM NADH, 5 mM glyoxylate, 10 mM L-serine, 0.1 mM pyridoxal-5-phosphate, and 1 nkat glyoxylate reductase for SGAT assay. MDH was assayed as described previously (30), except that the assay mixture contained 100 mM Hepes-KOH (pH 7.4), 0.4 mM NADH, and 0.4 mM oxaloacetate. HPR, SGAT, and MDH assays were carried out using a 1101 M Eppendorf photometer.

The reaction mixture for GGAT assay was according to Rehfeld and Tolbert (18), but instead of radioactivity measurement the formed α-ketoglutatarate was assayed spectrophotometrically. α-Ketoglutatarate formation was terminated and aliquots were neutralized as described for glycerate formation. The formed α-ketoglutatarate was assayed in a reaction mixture containing 100 mM KH₂PO₄-KOH (pH 7.3), 0.08 mM NADH, and 100 nkat glutamate dehydrogenase (ammonium suspension). The NADH oxidation was monitored at 334 nm using a Sigma double wavelength spectrophotometer. CAT and GOX were assayed at 25°C using an oxygen electrode (2) measuring oxygen evolution or consumption, respectively.

Assay conditions were as described by Stegink et al. (24).

The reaction mixtures of all enzyme assays contained 0.3 mM sucrose. To determine the enzyme activities in osmotically

Figure 1. Reactions of the glycolate pathway located in peroxisomes.

investigated. It appears from these studies that the compartmentation of metabolism in spinach leaf peroxisomes is not caused by the selectivity of the boundary membrane, but may be a consequence of the structure of the protein matrix.

MATERIALS AND METHODS

Plant Materials

Spinach (Spinacea oleracea, U.S. Hybrid 424, Ferry-Morse Seed-Comp., Mountain View, CA) was grown in growth chambers under a period of 19°C/9 h in light in hydroponic culture (15). The illumination was about 350 μE m⁻² s⁻¹ using tungsten and mercury lighting. Deribbed mature leaves of 2-month-old plants were used for isolation of peroxisomes. Peas (Pisum sativum, var “Kleine Rheinländerin”) were grown in the greenhouse with supplementary tungsten lighting. For purification of glycerate kinase, 12- to 16-d-old plants were used.

Isolation of Leaf Peroxisomes

Peroxisomes were isolated using Percoll gradients (13, 31). Intactness was judged by the latency of different peroxisomal enzymes. For analysis of mitochondrial and chloroplastic contamination, the gradient was collected into 1 mL fractions which were analyzed for HPR (peroxisomal marker), citrate synthase (CS, mitochondrial marker) and Chl. Both marker enzymes were assayed in the presence of 0.1% Triton X-100.

Treatment of Peroxisomes

To determine the intactness of peroxisomes, enzyme activities were measured in the absence and presence of 0.1% Triton X-100. For osmotic shock, peroxisomes were incubated in a more than 10-fold volume of water for 10 min and then diluted with an equal volume of double-concentrated homogenizing medium before intactness was determined.
shocked peroxisomes, the organelles were treated as described above.

CS activity was determined as described by Stitt (25) using a Sigma double wavelength spectrophotometer.

Electron Microscopy

Peroxisomes were treated in the same way as for the latency studies and glycerate formation as described above. Samples were fixed with 1% (v/v) glutaraldehyde in 50 mM KH2PO4 (pH 7.5) and 0.25 mM sucrose for 1 h on ice and then loaded onto a two-step sucrose gradient containing 2% OsO4 in 50 mM KH2PO4 (pH 7.5), 0.4 mM sucrose, and 50 mM KH2PO4 (pH 7.5), 0.35 mM sucrose. The peroxisomes were pelleted by centrifugation at 10,000g for 30 min. The pellets were post-fixed in phosphate-buffered 2% OsO4 for 1 h on ice. After being washed in buffer and water, the pellets were stained with 2.5% (w/v) uranyl acetate overnight. The material was dehydrated with acetone and embedded in Spurrs resin (23). Thin sections were stained with uranyl acetate (saturated in methanol) and 0.3% (w/v) lead citrate and examined in a Phillips EM 400 electron microscope operated at 80 kV.

Other Assays

Protein was determined with the Lowry procedure (16). Chl was calculated using the procedure of Arnon (1).

RESULTS

Peroxisomal Preparation

A very pure preparation of peroxisomes from spinach leaves was prepared according to the method of Yu and Huang (31), which involves a Percoll-density gradient purification. Figure 2 shows the distribution of marker enzymes and of Chl in such a gradient separation. The distribution of HPR shows that the peroxisomes are found in the range of 45 to 60% Percoll, whereas mitochondria and chloroplasts, monitored by the marker enzyme CS and by Chl, respectively, remain in the lighter fractions. It may be noted that in order to obtain highly intact peroxisomes it is essential that the peroxisomal preparation be washed immediately after fractionation, because high concentrations of Percoll have a harmful effect on the functional integrity of the peroxisomes. The peroxisomal fraction is practically free of Chl and shows a 15 to 20-fold increase in the specific activity of HPR and the HPR/CS ratio. The homogeneity and high purity of the obtained peroxisomal preparation is apparent from EM (Fig. 3b). The intactness of the peroxisomes used in our experiments, as judged by latency of HPR, was at least 80% and in most cases lay between 85 and 95%.

Latency of Peroxisomal Enzymes

In the experiment shown in Table I, the latency of six peroxisomal enzymes involved in glycolate metabolism was determined in a suspension of intact peroxisomes and after lysis by the detergent Triton X-100. Latency is defined as that percentage of total enzyme activity assayed in the presence of detergent that is not measurable in a suspension of intact peroxisomes. With the exception of GOX, all other enzymes showed latency, although to a different extent, ranging from 40% (GGAT) to about 90% (MDH).

In parallel experiments, the peroxisomes were centrifuged for 20 min at 6000 g followed by assaying of enzyme activities in supernatant and sediment in the presence of detergent. In the control experiment (intact peroxisomes), the values for latency of MDH, HPR, SGAT, and CAT were similar to the percentages of enzyme activities in the peroxisomes, indicating that the nonlatent portion mainly represented the activity of the solubilized enzymes. Thus, the latency of enzymes in intact organelles is even higher than that of enzymes assayed in the suspension. This does not apply to GGAT, in which the latency is lower than the portion of the bound enzyme, nor to GOX, of which almost 100% is found in the particulate fraction although there is no latency found.

Treatment of the peroxisomes with Triton X-100 resulted in almost total solubilization of MDH, HPR, SGAT, and GGAT, whereas a considerable portion of CAT and GOX (19 and 28%, respectively) was retained in the particulate remainder of the peroxisomes (Table I). The detergent used for lysis of the peroxisomes did not have any effect on the peroxisomal enzymes per se. In experiments not shown here, three different concentrations of Triton X-100 (0.01, 0.1, and 0.5%) and the structurally different detergents Thesit (0.01%), Dodecylmaltoside (0.1%), and Tween 20 (0.1%) did not alter the activities of the six enzymes listed above.

The latency of the peroxisomal enzymes decreases to a different extent according to the time elapsed after the end of preparation (Fig. 4). The age of the preparation also affected the maximal activities of the enzymes assayed after lysis with detergents, resulting in a decrease of enzyme activities of 1 to 5%/h (data not shown). For comparative studies of enzyme latency, the changes in latency and maximal activity caused by aging of the peroxisomal preparations create experimental problems, because individual activity measurements are time
Figure 3. Electron micrographs of peroxisomes after different treatments. a, intact peroxisome (in 50 mM KH₂PO₄ [pH 7.5], 0.25 mM sucrose); b, survey of the isolated peroxisomal fraction; c, detergent-treated peroxisomes (10 min in 50 mM KH₂PO₄ [pH 7.5], 0.25 mM sucrose plus 0.1% Triton X-100); d, osmotically treated peroxisomes (10 min in distilled water). For extra details see “Materials and Methods.” a, c, d, magnification × 75,000, bar = 0.25 μm; b, magnification × 10,000, bar = 1 μm.
COMPARTMENTATION OF LEAF PEROXISOMAL METABOLISM

remained that of the intact peroxisomes, whereas GGAT, which exhibited the peroxisomes enzymes, the loss of enzymes, shaking. of a diminsh latency of the enzymes. were not decrease of the enzymes. The soluble enzymes contained in the supernatant after sedimentation of the peroxisomes for 20 min at 6000 g were not increased in their activity by the presence of detergents (Table II), clearly demonstrating that the solubilized enzymes are no longer latent. The only exception was MDH, in which the activity in the supernatant increased by about 30% upon addition of 0.1% Triton X-100.

Effect of Osmotic Shock on Latency of Enzymes

An alternative way to rupture cell organelles, and thus diminish latency of enzymes, is exposure to osmotic shock. For a complete lysis of peroxisomes, Yu and Huang (31) incubated peroxisomes for 10 min at room temperature in 0.125 M sucrose, 1 mM Hepes followed by 2 min of vigorous shaking. In our experiments, in which shaking was omitted, the peroxisomes remained particulate after 10 min incubation in distilled water at room temperature and only a partial release of latency was observed (Table III). For the various enzymes, the loss of latency was very different. In the case of GGAT, which exhibited the lowest latency in intact peroxisomes, the latency decreased to 7% upon osmotic shock, whereas in the case of MDH, with a very high latency in intact peroxisomes, the latency after shock still remained 70%. Centrifugation of the peroxisomes after osmotic shock revealed that between 44 and 60% of the enzyme activities remained bound to the shocked peroxisomes (Table III).

Figure 4. Effect of aging on the latency of enzymes of isolated peroxisomes. Enzymes activities were measured ± 0.1% Triton X-100 to determine enzyme latency at times indicated. A, Latency of MDH, HPR, and CAT; B, latency of SGAT and GGAT.

These results show that treatment of the peroxisomes with distilled water affected the various enzymes differently. In the case of MDH, HPR, and CAT, the decrease of latency mainly reflected the release of enzymes from the peroxisomes, whereas in the case of catalase and even more in the case of GGAT, the solubilization of enzymes was lower than the decrease in latency. Obviously, in these cases the decrease in latency was in part due to the alteration of activities of the sedimentable enzymes.

Morphological Studies

The differences in the changes of enzyme latency observed upon incubation of the peroxisomes in distilled water made

<table>
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<th>Enzyme</th>
<th>Peroxisomes</th>
<th>Latency</th>
<th>Peroxisomes</th>
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<tbody>
<tr>
<td></td>
<td>Intact</td>
<td>+ 0.1% Triton X-100</td>
<td>Intact</td>
</tr>
<tr>
<td></td>
<td>μmol mg prot⁻¹ min⁻¹</td>
<td>%</td>
<td>% activity in pellet</td>
</tr>
<tr>
<td>MDH</td>
<td>6.5 ± 3.7</td>
<td>56.3 ± 16.0</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>HPR</td>
<td>2.1 ± 1.0</td>
<td>13.8 ± 5.0</td>
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</tr>
<tr>
<td>SGAT</td>
<td>0.71 ± 0.47</td>
<td>3.13 ± 1.00</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>CAT</td>
<td>490 ± 290</td>
<td>1580 ± 830</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>GGAT</td>
<td>1.81 ± 0.50</td>
<td>3.01 ± 0.60</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>GOX</td>
<td>1.73 ± 0.50</td>
<td>1.73 ± 0.50</td>
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Table I. Latency of Peroxisomal Enzymes and Percentage of Total Enzyme Activity in Peroxisomes

Activities were measured ± 0.1% Triton X-100 at four different times and extrapolated to time = 10 min after preparation. To determine the percentage of total enzyme activity inside the peroxisomes (columns 4 and 5), a peroxisomal suspension was centrifuged for 20 min at 6000g, 10 min after preparation. In the resulting supernatant and sediment, the enzyme activities were assayed in the presence of 0.1% Triton X-100. The enzyme activities are expressed as percentages in the pellet relative to the original fraction (% activity in pellet). Data are mean values of five experiments ± SD.
it seem unlikely that the latency is merely due to the presence of the peroxisomal boundary membrane. To elucidate the matter, we examined the effects of osmotic shock on the peroxisomal ultrastructure. Figure 3b shows an electron micrograph of peroxisomes suspended in a medium containing 0.25 M sucrose (control). These peroxisomes are roughly spherical and have a clearly visible single boundary membrane (Fig. 3a). Figure 3c shows the particulate remainder of peroxisomes that had been treated with 0.1% Triton X-100. The particles are much smaller than intact peroxisomes, have a dense matrix, and are devoid of any boundary membrane. The matrix probably consists of proteins that are only partially solubilized, e.g. CAT and GOX (see Table I).

Figure 3d shows peroxisomes that had been exposed to distilled water for 10 min, after which double-concentrated medium was added to adjust the sucrose concentration to 0.25 M as in the control (the same procedure as employed in the experiment of Table III). In contrast to control peroxisomes, osmotically shocked peroxisomes are smaller and have pleomorphic morphology and a continuous boundary membrane is not visible. Nevertheless, the peroxisomal matrix still remained compact, although its size had decreased and its shape was altered. These results clearly demonstrate that the latency of enzymes remaining after treatment of peroxisomes with distilled water (Table III) is not a function of the peroxisomal boundary membrane. Apparently, the peroxisomal matrix per se contributes to latency. It is feasible that the dense packing of proteins in the peroxisomal matrix restricts the diffusion of externally added metabolites to the peroxisomal enzymes. The latency of, for example, HPR, commonly employed as a criterion of peroxisomal intactness (7, 12, 13, 27), probably reflects the state of aggregation of the peroxisomal matrix rather than the intactness of the boundary membrane. Therefore, the term latency, which for the sake of simplicity has been used in the present report to designate a well-known measuring phenomenon, cannot be employed as a criterion of the intactness of the peroxisomal membrane, although it may be still of value as some qualitative indicator for the intactness of the structure of the peroxisomal matrix.

### Functional Studies

As a criterion of functional integrity of peroxisomes, the conversion of serine to glycerate was determined. The reaction was started by adding peroxisomes to a medium containing serine, malate, and also NAD at roughly physiological concentrations. Glycolate was used to generate glyoxylate, which is necessary for the transaminase reaction for converting serine to hydroxybutyrate. At the times indicated, samples were withdrawn for assay of the formed glycerate. Glycerate was determined enzymatically by means of a dual wavelength spectrophotometer allowing a very sensitive assay at 50-fold scale expansion. Figure 6 shows two experiments in which the peroxisomal concentration differed by a factor of 6. In both experiments, the initial rate was the same; at higher peroxisomal concentration the rate decreased during the experiment, probably due to the accumulation of oxaloacetate as end product. From the initial rate of glycerate synthesis (0.7 µmol/mg peroxisomal protein min), based on a content of 1.04 mg peroxisomal protein/mg Chl in spinach leaves (as determined from the activity of the peroxisomal marker enzyme HPR in isolated peroxisomes and whole leaves), the peroxisomal capacity to transform serine into glycerate can be evaluated as 43.7 µmol/mg Chl h. The evaluation of several other experiments yielded a mean value of 66.6 µmol/
mg Chl·h. The spinach leaves used for these experiments showed a photosynthesis rate of 80 μmol/mg Chl·h under our growth conditions. Assuming a ratio of carboxylation/oxygenation of 2.5:1 (21), the rate of glycerate formation would be 20 μmol/mg Chl·h. The capacity of our isolated peroxisomes in the conversion of serine to glycerate is high enough to meet this demand. This comparison shows that the peroxisomes used in our experiments had retained their full metabolic competence.

Although the conversion of serine to glycerate in the presence of glycolate and malate involves the formation of glyoxylate, hydroxyxpyruvate, and NADH as intermediates, synthesis of glycerate occurred without any lag at a constant rate right from the beginning. The absence of any lag period is also apparent in similar experiments of Yu and Huang (31). With a peroxisomal concentration of 1.8 μg peroxisomal protein/mL (Fig. 6), the amount of glycerate formed in 1 min is 1.2 nmol. Diluted in a suspension volume of 1 mL, this represents a concentration of as low as 1.2 μM. The fact that the synthesis of such small quantities of glycerate by highly diluted peroxisomal suspensions occurs without any notice-

able lag clearly demonstrates that the internal concentrations of the intermediates of the peroxisomal reactions do not readily equilibrate with the concentrations in the medium. Apparently, these reactions are compartmentalized to prevent leakage of the intermediates. The existence of such a compartmentation of intermediates can be illustrated by a simple calculation. With GOX having a $K_m$ for glycolate in the range of 0.38 mM (32) and 0.25 mM (10) and a $V_{max}$ of 1.7 μmol/mg peroxisomal protein·min (Table I), the addition of 2 mM glycolate to a peroxisomal suspension of 1.8 μg peroxisomal protein/mL (Fig. 6) would in 1 min result in the formation of 3 nmol glyoxylate, equivalent to a concentration of 3 μM when being released into the medium. In the chain of reactions, the newly formed glyoxylate is the substrate of SGAT. The $K_m$ value of SGAT for glyoxylate has been determined in Phaseolus as 0.6 mM (22) and in Pisum as 4.6 mM (9). Assuming the lower value of 0.6 mM, at a maximal rate of 3 μmol/mg peroxisomal protein·min (Table I) in the presence of 3 μM glyoxylate, the rate of hydroxyxpyruvate formation from serine would be 0.015 μmol/mg peroxisomal protein·min, which is only 2% of the rate of glycerate formation by intact peroxisomes shown in the experiment of Fig. 6. This calculation clearly demonstrates that in intact peroxisomes the intermediates of glycerate synthesis are highly compartmentalized.

A disruption of the peroxisomal structure by treatment with detergent largely diminishes the ability to synthesize glycerate. As shown in Figure 6, with Triton X-100 added, in the experiment with the lower peroxisomal concentration the synthesis of glycerate occurs after a long lag period at a rate of only 6% of that in the control experiment. This concurs with earlier results showing that intact peroxisomes convert serine to glycerate at a rate higher than that of lysed peroxisomes (31). At higher peroxisomal concentrations, a higher rate of glycerate formation is obtained, but a long lag period is still observed. Because detergents do not have an inhibitory effect on the single enzymes, these results further support the notion that a compartmentation of peroxisomal reactions is required for effective metabolism.

In the experiment shown in Figure 7, the conversion of serine to glycerate was studied with peroxisomes that had been subjected to incubation in distilled water (Table III). In this case, the kinetics of glycerate formation are linear from the very beginning; no lag period is observed. The rate of

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**Table III. Effect of Osmotic Treatment on the Latency of Enzymes and the Percentage of Total Enzyme Activity in Peroxisomes**

For procedure of latency and activity measurement, see Table I. Osmotically treated peroxisomes were incubated for 10 min in water. Data are mean values of three experiments ± so.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity + 0.1% Triton X-100</th>
<th>Latency</th>
<th>Activity</th>
<th>Activity + 0.1% Triton X-100</th>
<th>Latency</th>
<th>Activity</th>
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<tbody>
<tr>
<td>MDH</td>
<td>99.4 ± 4.6</td>
<td>90 ± 6</td>
<td>91 ± 7</td>
<td>89.7 ± 8.7</td>
<td>70 ± 7</td>
<td>63 ± 0</td>
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<tr>
<td>HPR</td>
<td>19.7 ± 2.8</td>
<td>86 ± 6</td>
<td>85 ± 4</td>
<td>18.7 ± 3.9</td>
<td>58 ± 9</td>
<td>57 ± 1</td>
</tr>
<tr>
<td>SGAT</td>
<td>4.7 ± 2.1</td>
<td>76 ± 1</td>
<td>80 ± 9</td>
<td>4.1 ± 1.6</td>
<td>45 ± 7</td>
<td>52 ± 4</td>
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<tr>
<td>CAT</td>
<td>1760 ± 60</td>
<td>68 ± 6</td>
<td>84 ± 6</td>
<td>1390 ± 200</td>
<td>33 ± 0</td>
<td>51 ± 11</td>
</tr>
<tr>
<td>GGAT</td>
<td>4.3 ± 1.8</td>
<td>52 ± 9</td>
<td>79 ± 7</td>
<td>3.9 ± 1.3</td>
<td>7 ± 5</td>
<td>44 ± 11</td>
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**Figure 6. Time course of glycerate formation by intact and detergent-treated peroxisomes.** Aliquots, containing 1.8 (□, ■) or 10.6 (○, △) μg peroxisomal protein, were incubated in the presence of 15 mM serine, 2 mM glycolate, 20 mM malate, and 1 mM NAD. The glycerate formation was determined at various times, as indicated.
glycerate formation was determined at various times, as indicated.

**DISCUSSION**

Although not proven experimentally, it is generally accepted that peroxisomal compartmentation serves the purpose of confining the enzymes involved in the generation and removal of \( \text{H}_2\text{O}_2 \) in such a way that the toxic \( \text{H}_2\text{O}_2 \) is prevented from having a damaging effect on the cell. From the observation that, at low glycolate concentrations, the conversion of glycolate to glycine occurred two times faster in intact than in ruptured peroxisomes, it was concluded that, in intact peroxisomes, glyoxylate generated by GOX is directly channeled to SGAT (12). Likewise, from measuring the formation of glycerate from serine in intact and lysed peroxisomes, the higher efficiency conferred by intactness was attributed to the coupling of the two reactions catalyzed by MDH and HPR (31). Our results, presented in Figure 6, clearly demonstrate that the intermediates of the conversion of glycolate to glycine and of serine to glycerate are confined to the peroxisomal compartment. This explains that all the enzymes depending on reaction partners generated inside the peroxisomes (SGAT, GGAT, HPR, MDH, CAT) show latency.

Compartmentation of metabolism in mitochondria and in chloroplasts is due to the function of boundary membranes, containing specific translocators for passage of certain metabolites and acting as permeability barriers for metabolic intermediates. In peroxisomes, the function of the boundary membrane has been obscure. Liang and Huang (12) concluded from their findings that the peroxisomal membrane does not impose a barrier to glycolate, \( \text{O}_2 \), serine, and malate (31), whereas such a barrier does exist toward glutamate, alanine, hydroxypropyruvate, glyoxyxlate, and NADH. These authors surmised that glyoxylate may be confined within the peroxisomes due to the membrane barrier and/or the existence of a loosely associated multi-enzyme complex of GOX and SGAT (12). If compartmentation of peroxisomal metabolism were the sole consequence of permeability properties of the peroxisomal boundary membrane, one might have expected at least three different translocators of extremely high specificity, e.g., one translocator transporting glycolate at a very high rate but not transporting glyoxylate at all, and another one transporting glycerate but not hydroxypropyruvate. Although there is no evidence available for the existence of any such highly selective translocators, experimental observations with animal peroxisomes suggest that the peroxisomal boundary membrane may contain unspecific protein channels allowing the passage of metabolites of a molecular mass up to 800 D (28). In view of the similarities between peroxisomes in animal and plant tissues, it is feasible that plant peroxisomes may also contain pores.

Osmotic shock of mitochondria and chloroplasts results in the rupture of the boundary membranes, with the enzymes of the matrix or stroma becoming soluble in the suspension medium. As shown above, this is different with peroxisomes. After treatment in distilled water, the particulate structure of the matrix is maintained, although the boundary membrane has lost its intactness. As these peroxisomal particles still retain a metabolic compartmentation, the confinement of metabolites of the peroxisomal reaction sequences cannot be the result of a selective impermeability of the peroxisomal boundary membrane. Therefore, our results indicate that peroxisomal compartmentation is a function of the peroxisomal matrix. It is proposed that a structural arrangement of the various peroxisomal matrix enzymes leads to metabolite channeling. Any proposed confinement of \( \text{H}_2\text{O}_2 \) formed by GOX could be explained only in this way, because \( \text{H}_2\text{O}_2 \) readily permeates membranes and thus could not be confined by the peroxisomal boundary membrane. A close association of GOX and CAT would be further advantageous in the prevention of nonenzymic oxidation of glyoxylate by \( \text{H}_2\text{O}_2 \), occurring especially at higher temperatures (6). A structural arrangement of the peroxisomal matrix enzymes would facilitate the coupling of the reactions catalyzed by GOX and CAT (mentioned above), GOX and aminotransferases (12, 17), HPR and MDH (31), and, in contrast to confinement by the boundary membrane, would ensure elimination of undesired side reactions of the intermediates that can occur inside the peroxisomes (7, 17, 29).

The physiological function of peroxisomal compartmentation may not only be the confinement of toxic \( \text{H}_2\text{O}_2 \), but also of hydroxypropyruvate and glyoxyxlate, which are both very reactive substances. Glyoxylate at very low concentrations was...
found to strongly inhibit Calvin cycle enzymes (4). Metabolite channeling may ensure that the bulk of the glyoxylate and hydroxypyruvate formed in the photorespiratory cycle is eliminated in the peroxisomes. However, even a very efficient channeling may not prevent the escape of a minor portion of metabolic intermediates from the peroxisomal matrix. Because of the apparent impermeability of the peroxisomal matrix to these substances, and the putative gradient between the concentrations of these metabolites at the reaction site and in the cytosol, it seems impossible that substances like glyoxylate or hydroxypyruvate, which had leaked out from the peroxisomes, could be again taken up by the peroxisomes for metabolic conversion. This may be the reason for the existence of NADPH-dependent glyoxylate and hydroxypyruvate reductases in the cytosol of leaf cells. These enzymes may represent an auxiliary system to eliminate glyoxylate and hydroxypyruvate (11) that have been lost from the peroxisomes due to an imperfection in metabolite channeling.

In summary, our data show that the peroxisomal matrix has a highly organized structure. The complexity of this structure is illustrated by the fact that GOX, which is the most insoluble of the studied enzymes, does not show latency at all. Although all the other peroxisomal enzymes studied here depend in their activity on reaction partners generated inside the peroxisomes, and thus show latency, both substrates of GOX, glycollate and O$_2$, have to enter the peroxisome from outside. It seems that the enzymes in the peroxisomal matrix are arranged in such a way that, on the one hand, the intermediates of peroxisomal metabolism are retained inside and, on the other hand, the reactants from outside have very easy access.

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