Association of glycolate oxidation with photosynthetic electron transport in plant and algal chloroplasts
(carbon concentration mechanism/dissolved inorganic carbon pump/photospiration/quinone oxidoreductase)

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ABSTRACT Photosynthetic carbon metabolism is initiated by ribulose-bisphosphate carboxylase/oxygenase (Rubisco), which uses both CO₂ and O₂ as substrates. One 2-phosphoglycolate (P-glycolate) molecule is produced for each O₂ molecule fixed. P-glycolate has been considered to be metabolized exclusively via the oxidative photosynthetic carbon cycle. This paper reports an additional pathway for P-glycolate and glycolate metabolism in the chloroplasts. Light-dependent glycolate or P-glycolate oxidation by osmotically shocked chloroplasts from the algae Dunaliella or spinach leaves was measured by three electron acceptors, methyl viologen (MV), potassium ferricyanide, or dichloroindophenol. Glycolate oxidation was assayed with 3-(3,4-dichloro-phenyl)-1,1-dimethyleurea (DCMU) as oxygen uptake in the presence of MV at a rate of 9 μmol per mg of chlorophyll per h. Washed thylakoids from spinach leaves oxidized glycolate at a rate of 22 μmol per mg of chlorophyll per h. This light-dependent oxidation was inhibited completely by SHAM, an inhibitor of quinone oxidoreductase, and 75% by 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), which inhibits electron transfer from plastoquinone to the cytochrome b₆f complex. SHAM stimulated severalfold glycolate excretion by algal cells, Dunaliella or Chlamydomonas, and by isolated Dunaliella chloroplasts. Glycolate and P-glycolate were oxidized about equally well to glyoxylate and phosphate. On the basis of results of inhibitor action, the possible site which accepts electrons from glycolate or P-glycolate is a quinone after the DCMU site but before the DBMIB site. This glycolate oxidation is a light-dependent, SHAM-sensitive, glycolate-quinone oxidoreductase system that is associated with photosynthetic electron transport in the chloroplasts.

All plants and algae have the same bifunctional enzyme, ribulose bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39) for photosynthetic carbon metabolism. The carboxylation reaction initiates CO₂ fixation and reduction by the C₃ reductive photosynthetic cycle, and the oxygenase reaction initiates the C₃ oxidative photosynthetic carbon cycle. The two cycles coexist as competitive reactions for the two gaseous substrates CO₂ and O₂, and together they represent photosynthetic carbon metabolism (1). 2-Phosphoglycolate (P-glycolate) synthesis in the chloroplasts by the oxygenase activity of Rubisco and its metabolism via the C₃ oxidative photosynthetic carbon cycle in the peroxisomes and mitochondria is equated with photosynthetic carbon metabolism. In higher plants and many multicellular algae, glycolate is oxidized by glycolate oxidase in the peroxisomes. However, in unicellular green algae, glycolate oxidase and leaf-type peroxisomes are absent, and instead these algae contain a low level of a mitochondrial glycolate dehydrogenase [~1 μmol per mg of chlorophyll (mg⁻¹ Chl)·h⁻¹] (1–4). The glycolate-dependent dichloroindophenol (DCIP, also known as DCPIP)-reduction activity of unicellular algae has been termed a dehydrogenase, because the electron acceptor from the partially purified enzyme is not directly linked to oxygen (4). The flux of carbon via glycolate, which is about 10–15 μmol·mg⁻¹·Chl·h⁻¹, is considered to be equal to its excretion in the presence of the aminotransferase inhibitor aminoxy-acetate (AOA), which blocks its metabolism (5). Since the flux of excreted glycolate is substantially higher than the measurable capacity of the dehydrogenase, it is possible that the glycolate dehydrogenase cannot be isolated or measured accurately or that glycolate may also be metabolized by another system. The later hypothesis is consistent with high activity of Rubisco and P-glycolate phosphatase for glycolate synthesis in the chloroplasts (4, 6).

Previous investigators had studied glycolate formation and excretion by chloroplasts, but a system for glycolate metabolism in the chloroplasts has not been described (7–10). The blue green alga Anabaena variabilis has been reported to exhibit photosystem I (PS I)-dependent oxidation of glycolate (11). We find that salicylhydroxamic acid (SHAM) and AOA greatly stimulate glycolate excretion by algal cells or isolated algal chloroplasts (refs. 12 and 13; see Fig. 1). This suggests inhibition of an active glycolate metabolism system in the chloroplasts, because in the absence of inhibitors glycolate did not accumulate in large amounts. SHAM or AOA does not inhibit or change the activity of Rubisco or P-glycolate phosphatase for glycolate synthesis in the chloroplasts. In this paper evidence is presented for a light-dependent glycolate oxidation which is associated with the photosynthetic electron transport system from algae (Dunaliella) or a higher plant (spinach). Preliminary data were reported in an abstract (13).

MATERIALS AND METHODS

Organism, Plant Material, Preparation of Chloroplasts and Thylakoids, and Electron Transport Assay. Axenic cultures of Dunaliella tertiolecta were grown photoautotrophically and concentrated as described elsewhere (14). Photosynthetically active chloroplasts were isolated from either high-CO₂-grown or air-adapted cells, which must be in the logarithmic phase of growth (15). The chloroplasts had to be used within 2 h after preparation, because activity for the Hill reaction and the glycolate-dependent reduction of ferricyanide decreased rapidly (data not shown). Spinach (Spinacia oleracea L. cv. Savoy Hybrid 612; Harris Seed Company, Rochester, NY) was grown in a greenhouse for 4 weeks, and young leaves were picked in the morning. Washed spinach chloroplasts were obtained by...

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Abbreviations: AOA, aminooxyacetate; CCCP, carbonylcyanide 3-chlorophenylhydrazone; Chl, chlorophyll; Cyt, cytochrome; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCIP (also known as DCPIP), dichloroindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyleurea; DIC, dissolved inorganic carbon; E, einstein; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; MV, methyl viologen; PS I or II, photosystem I or II; Rubisco, ribulose bisphosphate carboxylase/oxygenase; SHAM, salicylhydroxamic acid.
the method of Cerovic and Plessnicar (16), and chloroplasts were further purified on a Percoll gradient (15) and resuspended to a concentration of 1 mg of Chl/ml. Thylakoid membranes were prepared from fresh deinned spinach leaves. Leaves (125 g) were homogenized in a Waring blender for 2 periods of 5 sec in 125 ml of isolation medium containing 40 mM Tricine, 400 mM NaCl, 2 mM MgCl₂, and 0.2% bovine serum albumin (BSA), pH adjusted to 8. The homogenate was filtered through four layers of cheesecloth and centrifuged at 300 × g for 1 min to remove broken cells and debris. The resultant supernatant was centrifuged at 4000 × g for 5 min and the pellet was resuspended in 50 ml of "high-salt" medium containing 20 mM Tricine, 150 mM NaCl, 50 mM MgCl₂, and 0.2% BSA, pH adjusted to 8.0. This suspension was centrifuged at 4000 × g for 5 min and the pellet was resuspended in the same manner in a "low-salt" medium containing 20 mM Tricine, 15 mM NaCl, 400 mM sucrose, and 0.2% BSA, pH adjusted to 8.0. The resuspended thylakoids (1 mg Chl/ml⁻¹) were packaged in 0.5-ml aliquots and stored at −80°C until use. Thylakoids exhibited rates of gramicidin-Final concentration 5 μg/ml⁻¹) uncoupled electron transport of 340 μmol of O₂·mg⁻¹ Chl·h⁻¹.

Electron transport was measured by changes in oxygen concentration at 25°C in a temperature-regulated cuvette of a Rank Brothers oxygen electrode. Light intensity at the surface of the cuvette was 1200 μE·m⁻²·s⁻¹ [1 einstein (E) = 1 mol of photons]. The assay mixture contained, in 1.5 ml: 50 μg of Chl, 50 mM Tricine, 50 mM NaCl, 3 mM MgCl₂, and 5 mM NaH₂PO₄, and the pH was adjusted to 8.

**Glycolate Excretion by Whole Cells or by Isolated Chloroplasts.** Five milliliters of high-CHO₂-grown Dunaliella cell suspensions (containing 100 μg of Chl) in 10 ml of sodium phosphate buffer with 170 mM NaCl (pH 7) was stirred and illuminated (800 μE·m⁻²·s⁻¹). One millimolar NaHCO₃ was added at zero time and every 10 min thereafter. For chloroplasts, 5 ml of chloroplast suspension [40 μg Chl·ml⁻¹ in KCl/Hepes buffer (15) in which sorbitol was replaced with 175 mM KCl] with 3-phosphoglyceric acid (1 mM) was placed in a 25-ml beaker in a water bath at 25°C and illuminated (400 μE·m⁻²·s⁻¹) with an incandescent 300-W reflector lamp. After 10 min of preillumination a 0.5-ml sample was withdrawn as zero time and 0.5 mM NaHCO₃ was added; this procedure was repeated at every 10 min thereafter. Aliquots (1 ml) of cell or chloroplast suspensions were withdrawn every 15 min and, after centrifugation, glycolate was measured in the supernatant by the Calkins method (17).

**Methylviologen (MV)- and Light-Dependent O₂ Uptake.** Intact chloroplasts containing 50 μg of Chl were resuspended in 2 ml of assay buffer (50 mM Hepes/7 mM MgCl₂/7 mM sodium phosphate, pH 7.5) in the O₂ electrode, so that they were immediately broken by osmotic shock. To inhibit catalase, 1 mM KCN was added. Because it was not possible to prepare high-quality washed thylakoids from Dunaliella, osmotically shocked chloroplasts were used. MV (0.2 mM) was added to the buffer after the addition of chloroplasts. After incubation in the dark for 2 min, the light was turned on and the MV-dependent O₂ uptake was measured for 2-3 min, before 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU) was added, which immediately inhibited O₂ uptake due to inhibition of electron flow from PS II to the plastoquinone pool. Two minutes after DCMU addition, 3 mM glycolate or other organic acid was added. O₂ uptake resumed immediately (see Fig. 1). The substrate-dependent oxygen uptake was followed for at least 5 min, during which time inhibitors could be used to block electron transfer to MV. SHAM (2.5–5.0 mM) was used to inhibit a proposed "glycolate-quinone oxidoreductase reaction"; 2.5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMB) (10–20 μM), to inhibit flow of electrons from plastoquinone to the cytochrome (Cyt) b₅f complex; and 2 μM carboxylcyanide 4-trifluoromethoxyphenylhydrazide (FCCP) or 20 μM carbon-

**Light- and Glycolate-Dependent Reduction of DCIP or Ferricyanide.** Glycolate-dependent reduction of DCIP was assayed anaerobically (18). The reaction mixture (2.5 ml) contained 100 mM potassium phosphate buffer (pH 8.3), 150 μM DCIP, chloroplasts containing 100 μg of Chl, and appropriate inhibitors in a Thunberg cuvette with 5 mM glycolate in the side arm. The reaction was incubated for 30 min and a control was run in parallel without added glycolate. Afterwards, a 1-ml aliquot was centrifuged to remove thylakoids and A₇₅₀ of the supernatant was measured for the remaining DCIP (ε of 21.9 M⁻¹·cm⁻¹ at pH 8.3). The ΔA₄₅₀ was multiplied by a factor of 3.95 for maximum activity, if a saturating dye concentration could have been used (18). SHAM (2.5 mM) was used to inhibit light-dependent glycolate oxidation, but higher concentrations of SHAM caused nonenzymatic reduction of DCIP.

Potassium ferricyanide reduction by glycolate in the light was assayed aerobically in a 2.5-ml mixture, containing 1.5 mM K₂Fe(CN)₆ in 50 mM sodium phosphate buffer (pH 8.3), chloroplasts containing 50 or 100 μg of Chl, inhibitors, and 5 mM glycolate. After 10 min, a 1-ml aliquot was centrifuged to remove thylakoids, and A₄₅₀ of the supernatant was measured. Potassium ferricyanide (1.5 mM) gave an A₄₅₀ of 1.5, which dropped to 1.4 in a complete reaction mixture. An A₄₅₀ increase of 1.0 would be equivalent to the reduction of 1 μmol of ferricyanide. Uncouplers (FCCP and CCCP) caused increased reduction of ferricyanide (results not shown).

**Determination of Inorganic Phosphate and Glyoxylate.** These were measured after the oxidation of glycolate or P-glycolate by osmotically shocked chloroplasts or thylakoids from algae or spinach. The 2.1-ml reaction mixture contained 50 mM Tricine (pH 8.0), 5 mM MgCl₂, 5 mM NaCl, chloroplasts or thylakoids containing 100–150 μg of Chl, and 5 mM substrate. To prevent nonenzymatic decarboxylation of glyoxy-

**RESULTS**

Glycolate excretion from the whole cells or chloroplasts of a unicellular green alga in the light was increased severalfold by the presence of 2.5 mM SHAM (Fig. 1). SHAM has been used as an inhibitor of an alternative respiration/quinone oxidoreductase reaction in plant mitochondria (21). Consequently our working hypothesis has been that chloroplastic glycolate oxidation may be linked with the electron transport system by a glycolate-quinone oxidoreductase system that is also SHAM inhibited (see Fig. 3). To further test this hypothesis for glycolate metabolism in the chloroplasts, MV, DCIP, and potassium ferricyanide were used as electron acceptors, along with DCMU, to block electron transport from PS II to PS I.

**Effect of Glycolate on the MV-Coupled O₂ Uptake and Inhibition by SHAM and DBMB.** The flow of electrons originating from the oxidation of glycolate or other organic acids was coupled to MV, which accepts the electrons from PS II.
glycolate phosphorylation

DCIP has been demonstrated for noncyclic electron flow originating from a substrate such as glycolate via PSI to MV. Donated electrons from ascorbate through DCIP has been demonstrated for noncyclic photophosphorylation by PSI (22). In the absence of organic acids, the light-induced endogenous O₂ uptake was 12–20 μmol of O₂ mg⁻¹ Chl h⁻¹ (Table 1). Chloroplasts isolated from air-adapted algae with a dissolved inorganic carbon (DIC) pump had about 25% higher rates of endogenous electron transport activity compared with chloroplasts from cells without DIC pump from growth on high-CO₂. Light-induced endogenous O₂ uptake was inhibited almost completely by DCMU, 75% by DBMIB, but only 10% by SHAM (Fig. 2; Table 1). The DCMU-inhibited O₂ uptake was resumed at a reduced level by the addition of 3 mM glycolate.

Osmotically shocked chloroplasts from air-adapted algae had glycolate-dependent MV-linked O₂ uptake rates of up to 9 μmol of O₂ mg⁻¹ Chl h⁻¹, whereas chloroplasts from cells grown with high-CO₂ exhibited rates of O₂ uptake with glycolate of about 50% less (Table 1). The apparent Kₘ (glycolate) for both sources of chloroplasts was 0.5 mM. This glycolate-dependent O₂ uptake was almost completely inhibited by 2.5 mM SHAM (Fig. 2; Table 1). When DBMIB was used to block electron transport between plastoquinone and the Cyt b₅f complex, the glycolate-dependent O₂ uptake was inhibited by 75%, as if DBMIB did not completely inhibit electron flow from plastoquinone to Cyt b₅f (Table 1). These results were consistent with the photoreduction of MV (as an electron acceptor) with the electrons from glycolate oxidation. DCMU inhibited the flow of electrons from the reducing side of PS II, and glycolate oxidation was inhibited by SHAM and DBMIB. These results suggest that the electrons

Table 1. SHAM and DBMIB inhibition of glycolate-dependent MV-coupled O₂ uptake in the light by osmotically shocked chloroplasts from Dunaliella

<table>
<thead>
<tr>
<th>Additions</th>
<th>Uptake, μmol O₂ mg⁻¹ Chl h⁻¹</th>
<th>Air-adapted cells</th>
<th>CO₂-grown cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>17–20</td>
<td>12–15</td>
<td></td>
</tr>
<tr>
<td>+ DCMU</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>+ DBMIB</td>
<td>3–5</td>
<td>4–6</td>
<td></td>
</tr>
<tr>
<td>+ SHAM</td>
<td>15–18</td>
<td>10–12</td>
<td></td>
</tr>
<tr>
<td>+ DCMU + glycolate</td>
<td>6.8 (100%)</td>
<td>3.7 (100%)</td>
<td></td>
</tr>
<tr>
<td>+ DCMU + glycolate + SHAM</td>
<td>0.7 (11%)</td>
<td>0.3 (8%)</td>
<td></td>
</tr>
<tr>
<td>+ DCMU + glycolate + DBMIB</td>
<td>2.0 (30%)</td>
<td>0.9 (25%)</td>
<td></td>
</tr>
<tr>
<td>+ DCMU + glycolate + SHAM + DBMIB</td>
<td>0.5 (8%)</td>
<td>0.3 (8%)</td>
<td></td>
</tr>
</tbody>
</table>

DCMU-blocked electron transport activity was partially (about 40%) restored by 2.5 mM glycolate, and this glycolate-dependent rate was equated to 100% in parentheses.
from glycolate oxidation were donated to the electron transport chain at a site between the primary acceptor of PS II and Cyt b6f. Since SHAM did not inhibit electron transport between PS II and PS I, electrons from glycolate oxidation apparently did not go directly through the plastoquinone pool. We propose that the possible site which accepts electrons from glycolate is a "SHAM-sensitive glycolate-quinone oxidoreductase system" that is linked with the electron transport chain as shown in Fig. 3. In boiled chloroplast preparations, no glycolate-dependent O2 uptake was observed, as if electrons from glycolate oxidation were linked to the electron transport chain by a heat-inactivable enzyme.

Eight other organic acids (acetate, citrate, formate, D- and l-lactate, malate, oxaloacetate, and succinate) were much less effective as electron donors to the noncyclic electron transport. Similar low levels of succinate stimulation of chlororespiration have been reported (23).

**Light- and Glycolate-Dependent Reduction of DCIP and Ferricyanide.** Osmotically shocked Dunaliella chloroplasts reduced DCIP anaerobically in the presence of glycolate and light at a rate of about 6 μmol·mg⁻¹ Chl h⁻¹ (Table 2). Because this reaction was light dependent, the DCIP should have been acting as a Hill acceptor from PS I during glycolate oxidation. DCMU did not inhibit glycolate- and light-dependent reduction of DCIP, suggesting that PS II was not involved and that electrons from glycolate oxidation entered the electron transport chain after the DCMU inhibition site at the quencher. "Q." SHAM (2.5 mM) inhibited glycolate-dependent reduction of DCIP. SHAM alone at this concentration did not inhibit electron transport. SHAM inhibition of a light-dependent glycolate oxidation by thylakoids suggests that it is likely that a quinone-oxidoreductase type of oxidation system is present which can feed electrons to PS I.

Using the DCIP assay for glycolate-oxidizing enzymes, our group and others have reported an FMN-linked glycolate oxidase in the peroxisomes (24, 25) and an alternate form that was thought to be linked to a quinone (26) or to ferridoxin (27). The latter form had been purified from etiolated tissue which contained little activity of the flavin-linked form (28). The quinone form of glycolate oxidase seems to have properties more like the chloroplastic glycolate-oxidizing system that is described in this paper.

In osmotically shocked chloroplasts, ferricyanide as a Hill reagent can accept electrons in the vicinity of plastoquinone as well as on the reducing side of PS I. The light- and glycolatedependent rate of ferricyanide reduction was 27 ± 8 μmol·mg⁻¹ Chl h⁻¹ (Table 2), and this rate was inhibited (90%) by DCMU. The rate of ferricyanide reduction with glycolate was about 4-fold higher than the measured rate of DCIP reduction. An increase of 2-fold could be expected because two electrons are required per DPIP, compared with one electron in the case of ferricyanide. Possibly the DCIP assay is an underestimation from reoxidation of the reduced DCIP by the electron transport chain. Similar to Hill activity,

![Fig. 3. Proposed scheme for the light-dependent glycolate oxidation by the chloroplast thylakoids. "Q." primary electron acceptor (quincher); Q, quinone; PO, plastoquinone; PC, plastocyanin.](image)

Table 2. Anaerobic DCIP reduction and aerobic ferricyanide reduction by osmotically shocked chloroplasts with glycolate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Additions</th>
<th>Acceptor</th>
<th>μmol·mg⁻¹ Chl h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>Glycolate</td>
<td>DCIP</td>
<td>5.6 ± 1.3</td>
</tr>
<tr>
<td>Dark</td>
<td>Glycolate</td>
<td>DCIP</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>Light</td>
<td>Glycolate + DCMU</td>
<td>DCIP</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>Dark</td>
<td>Glycolate + DCMU</td>
<td>DCIP</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td>Light</td>
<td>Glycolate + SHAM</td>
<td>DCIP</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>Dark</td>
<td>Glycolate + SHAM</td>
<td>DCIP</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>Light</td>
<td>None</td>
<td>K3Fe(CN)6</td>
<td>83 ± 5</td>
</tr>
<tr>
<td>Light</td>
<td>Glycolate</td>
<td>K3Fe(CN)6</td>
<td>110 ± 8</td>
</tr>
</tbody>
</table>

Values are mean of at least six independent assays. DCIP experiments could not be run aerobically because reduced DCIP was rapidly oxidized.

...the glycolate-dependent reduction of ferricyanide decreased as the age of isolated chloroplasts increased (data not shown). The substrate-dependent O2 uptake in the light by osmotically shocked chloroplasts or thylakoids from spinach leaves. These chloroplasts without MV had little or no O2 uptake. Upon addition of MV and 3 mM glycolate or P-glycolate, in the light, spinach chloroplasts had rates of O2 uptake of about 22 μmol·mg⁻¹ Chl h⁻¹. The apparent Kₘ (glycolate or P-glycolate) was 0.5 mM (data not shown). The substrate-dependent O2 uptake ceased in the dark. Glyoxylate (1 mM) exhibited only one-half the rate of O2 uptake, and higher concentrations promoted less O2 uptake. l-Lactate (3 mM), a substrate analog of glycolate, also resulted in O2 uptake at about half the rate as with glycolate. Other organic acids (acetate, citrate, formate, D-lactate, oxaloacetate, and succinate) were not effective (results not shown). When catalase was added to the reaction after a few min of O2 uptake with glycolate, half the amount of O2 uptake was released, and the renewed rate of O2 uptake was half of that in the absence of catalase. These results suggest that the O2 uptake was due to the formation of H₂O₂ as in a Mehler reaction.

Electron transport uncouplers (CCCP, FCCP, and NH₄Cl) did not increase the rate of glycolate-dependent O2 uptake. AOA, hydroxylamine, SHAM, glyoxylate, glycolate, and P-glycolate do not uncouple electron transport as do NH₄Cl and gramicidin. Hydroxypyridinemethanesulfonate (HPMS), an inhibitor of peroxisomal glycolate oxidase in higher plants, did not inhibit glycolate or P-glycolate metabolism by chloroplasts or thylakoids. These results indicate that it is unlikely that the specific light- and glycolate-dependent O2 uptake by spinach thylakoids was due to peroxisomal glycolate oxidase or algal mitochondrial glycolate dehydrogenase. The latter are not light dependent and are inhibited by the sulfonate.

Because thylakoids from spinach had a very high rate of O2 uptake in the presence of light, MV, and gramicidin, it was difficult to measure accurately increases in O2 uptake due to glycolate or P-glycolate, within the limitation of the O2 electrode and chart recorder. To observe substrate-dependent oxidation, O2 uptake was measured without gramicidin. Thylakoids metabolized glycolate at a rate of about 22 μmol of O2·mg⁻¹ Chl h⁻¹, and the rate of P-glycolate oxidation was about half the rate with glycolate. Two washings of the thylakoids should have removed nearly all the P-glycolate phosphatase of the stroma, yet P-glycolate was oxidized rapidly without a time lag to hydrolyze it to glycolate, whose Kₘ was 0.5 mM. Glyoxylate (1 mM) inhibited the endogenous rate of O2 uptake by 30%. Manometric observations (N.E.T., unpublished results) have also revealed high rates of oxygen uptake in the light by isolated chloroplasts from spinach leaves with

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either P-glycolate or glycolate without a lag for possible hydrolysis of P-glycolate.

**Products of Glycolate and P-Glycolate Oxidation.** Osmotically shocked chloroplasts from both spinach and Dunaliella formed glyoxylate from either glycolate or P-glycolate in the light, but the rate of glyoxylate formation from P-glycolate was about 40% less than from the glycolate (Table 3). In the dark these chloroplasts formed 10–15% as much glyoxylate as in the light. Glycolate and P-glycolate oxidation was light dependent; therefore their oxidation should not have been by contaminating peroxisomal glyoxylate oxidase or the mitochondrial dehydrogenase. With P-glycolate as a substrate, phosphate was released rapidly in the dark and the light (Table 4), but the rate of phosphate release was higher in the light. Broken spinach chloroplasts converted P-glycolate to phosphate and glyoxylate at twice the rate as Dunaliella chloroplasts. The phosphate release from P-glycolate in the light may have been due to an oxidative dephosphorylation process.

**DISCUSSION**

Earlier work on glycolate metabolism in isolated chloroplasts was summarized by Gibbs (7), who showed an increase in 14C found in glycolate during 14CO2 fixation with increasing O2 and/or decreasing CO2. Such data are consistent with glycolate synthesis by the oxygenase activity of Rubisco (4) and with O2 inhibition of CO2 fixation (Warburg effect) and photorepression (1, 6). The earlier reports probably underestimated glycolate production, since they did not measure total glycolate formation or the formation of unlabelled glycolate from unlabelled carbohydrate reserves, especially in the presence of low levels of 14CO2 and high levels of O2. Gibbs (7) proposed a scheme for chloroplastic synthesis of glycolate, but his report did not consider the oxidation of glycolate by the chloroplasts. Rather, all the glycolate was thought to have been excreted by the chloroplasts and oxidized in the peroxisomes (4). Our paper proposes that some of the glycolate is oxidized in the chloroplasts and that only excess glycolate may be excreted for oxidation in the peroxisomes (4) or mitochondria (29).

This paper demonstrates the existence of a SHAM-inhibited, light-dependent glycolate-quinone oxidoreductase system that is associated with thylakoid membranes of the chloroplasts (Fig. 3). DBMB, which is a plastoquinone antagonist preventing transfer of electrons from plastoquinone to Cyt b6f, also inhibited glycolate oxidation. Because chloroplasts were able to oxidize glycolate in the presence of DCMU, but not in the presence of DBMB, electron donation is proposed to be in the plastoquinone region of the photosynthetic electron transport chain. Minor oxygen uptake in the presence of DBMB may be due to its photoreduction, as it can be reduced by chloroplasts to produce H2O2 (30). SHAM is an inhibitor of alternative respiration or a quinone-oxidoreductase reaction in the plant mitochondria (21). SHAM also specifically increases the excretion of glycolate by the cells and chloroplasts (Fig. 1; ref. 13). Thus stimulated glycolate excretion by chloroplasts would seem to be due to SHAM inhibition of this chloroplastic glycolate metabolism. This appears to be a side-reaction from the complete photosynthetic electron transport system, because SHAM did not inhibit O2 evolution or CO2 fixation by algae or isolated chloroplasts. Heat treatment of thylakoids diminished this activity, suggesting a system involving a proteinaceous component. This component seems to be tightly bound to the thylakoids, as activity for glycolate or P-glycolate oxidation remained after two washings. The glycolate-oxidizing system is designated as a “SHAM-sensitive glycolate-quinone oxidoreductase” in the thylakoids between PS II and PS I. This site is likely to be a special quinone passing electrons in the region of plastoquinone.

In the last decade, growing evidence has been presented for chlororespiration (23, 31), although its terminal oxidase has not been identified. By using chlorophyll fluorescence kinetics, Bennoun (31) also demonstrated SHAM inhibition of chlororespiration. Though chlororespiration is considered to be a process distinct from photosynthesis or the Mehler reaction, it is conceptually similar to oxidation of glycolate by the thylakoid membranes. Treatment of a chloroplast suspension with 5 μM antimycin a, an inhibitor of mitochondrial respira-
tion, had no effect on light-induced O2 uptake with glycolate. Although succinate may donate electrons to the photosynthetic electron transport chain of Chlamydomonas during chlororespiration, light inhibited this oxidation (23). However, the glycolate-oxidizing system in the chloroplast is dependent on light. Therefore the proposed SHAM-sensitive glycolate-quinone oxidoreductase system is different from chlororespiration. In addition, neither mitochondrial glycolate dehydrogenase nor chlororespiration is inhibited by SHAM.

In the complete photosynthetic electron transport process, electrons come from the quencher of PS II after the photolysis of water. During noncyclic photophosphorylation in vivo, ferredoxin and NADP should be reduced by PS I with electrons coming from the oxidation of substrates such as glycolate. If PS I is coupled to oxygen uptake in a Mehler reaction or some other Hill acceptor such as ferric salts, it could generate ATP but not reduce NADPH. Noncyclic phosphorylation by PS I should generate two ATP molecules per glycolate oxidized. This type of noncyclic phosphorylation occurs in anaerobic photosynthetic bacteria or algae without PS II (32). In this concept, electrons from the reduced end of PS I are cycled back through Cyt f. This cyclization has been postulated to involve a NADPH-ascorbate reductase (22). If glycolate oxidation were the electron donor instead, the glyoxylate product could be reduced back to glycolate by the NADPH-glyoxylate reductase in the chloroplast (33). The combination of both reactions creates a cyclic photophosphorylation system for ATP synthesis from glycolate oxidation without NADPH accumulation. Since CO2 fixation requires more ATP (18 molecules per 6 CO2 molecules) than NADPH (12 molecules per 6 CO2 molecules), abundant glycolate and an active PS I oxidation system for it would be an essential part of photosynthetic carbon metabolism. This would justify the oxygenase activity of Rubisco. These anaerobic reactions may have evolved early in photosynthesis, as did Rubisco, but the process

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### Table 3. Glyoxylate production from glycolate or P-glycolate by isolated osmotically shocked chloroplasts from spinach and Dunaliella

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Spinach</th>
<th>Dunaliella</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolate</td>
<td>Light</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>P-glycolate</td>
<td>Light</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Glycolate</td>
<td>Dark</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>P-glycolate</td>
<td>Dark</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

The assay mixture contained 50 mM Tricine (pH 8.0), 5 mM MgCl2, 100 units of catalase, and chloroplasts equivalent to 100 μg of Chl/ml-1.

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### Table 4. Release of inorganic phosphate from 5 mM P-glycolate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spinach chloroplasts</th>
<th>Spinach thylakoids</th>
<th>Dunaliella chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>54</td>
<td>49</td>
<td>18</td>
</tr>
<tr>
<td>Dark</td>
<td>36</td>
<td>47</td>
<td>11</td>
</tr>
</tbody>
</table>

The chloroplasts from spinach or Dunaliella were osmotically shocked. Thylakoids from spinach were washed twice, but thylakoids from Dunaliella could not be prepared.
would have required internal O₂ from PS II for P-glycolate synthesis. The proposed scheme in Fig. 3 explains how glycolate oxidation by the thylakoids may be a regulatory process for cyclic photophosphorylation linked to photosynthetic carbon metabolism. The $K_m$(glycolate) for the glycolate-quinone oxidoreductase system in the chloroplast is 0.5 mM. If this occurred in plant chloroplasts, it should promote more cyclic phosphorylation for the extra ATP for fixing CO₂. Glycolate metabolism by PS I for cyclic photophosphorylation could also provide ATP for the DIC-algal pump (14, 34) and explain why the pump is inhibited by SHAM or darkness (14). SHAM, a quinone inhibitor, blocks glycolate metabolism by isolated chloroplasts and stimulates glycolate excretion (Fig. 1; ref. 13), and thus SHAM seems to reduce the total ATP synthesis for the DIC pump. When the external CO₂ concentration is low, increased oxygenase activity of Rubisco would form more glycolate and, to restore the internal CO₂ level, the CO₂ pump would become more active with ATP generated by glycolate oxidation by PS I.

The presence of a glycolate-oxidizing system associated with the photosynthetic electron transport in the chloroplast may be a way nature can efficiently recycle carbon from glycolate while regenerating energy under extreme environments. It makes use of the glycolate, which is inevitably synthesized, by generating ATP for CO₂ fixation. Under low-CO₂ conditions it may also result in survival of the organism by maintaining some basal rates of metabolism from the utilization of sugar reserves for the regeneration of products of the C₃ cycle.

Although this research was started with Dunaliella chloroplasts because these cells do not contain a peroxisomal glycolate oxidase, it was extended to spinach chloroplasts, which can also metabolize glycolate and phosphoglycolate in the light by using the electron transport system. The oxidation of glycolate by chloroplasts or thylakoids from spinach leaves cannot be due to contaminating peroxisomal glycolate oxidase, which is not light dependent. Thus we have presented evidence for a light-dependent glycolate oxidation, measured as O₂ uptake by the Mehler reaction, via the PS I electron transport chain of chloroplasts from either Dunaliella or spinach leaves.

We thank John Hosler and Charles F. Yocum for discussions during this investigation.

32. Lascelles, J. (1973) Microbial Photosynthesis (Dowden, Hutchinson, & Ross, Stroudsburg, PA).