Intracellular Localization of Glycolate Dehydrogenase in a Blue-Green Alga

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ABSTRACT

Glycolate dehydrogenase activity was detected in cell-free extracts of Oscillatoria sp. prepared by osmotic lysis of spheroplasts in 0.05 M potassium phosphate buffer, pH 7.5, containing 0.3 M mannitol. Most of the enzyme activity was found in a particulate fraction and localized in the photosynthetic lamellae after centrifugation in a discontinuous sucrose density gradient. Enzyme activity was detected in this fraction both in the presence and absence of the artificial electron acceptor 2,6-dichlorophenolindophenol (DPIP) and a low rate of O2 uptake was detected in this lamellar fraction. Activity was lost from the lamellar fraction by repeated washing or by treatment with 0.005% Triton X-100 and the solubilized enzyme activity was DPIP-dependent. The data indicate that both glycolate dehydrogenase and its natural electron acceptor are bound to the photosynthetic lamellae in vivo. In contrast, catalase activity was found in the soluble cytoplasmic fraction.

The enzyme catalyzing the oxidation of glycolate to glyoxylate in the leaves of higher plants, glycolate oxidase, is localized in peroxisomes together with catalase (23). A number of unicellular eucaryotic algae have been found to have a glycolate pathway similar to that of higher plants, but there is no clear evidence for the association of the glycolate-oxidizing enzyme in these cells, glycolate dehydrogenase, with a microbody of the peroxisomal type (18). An enzyme catalyzing glycolate oxidation, similar to that in green algae, has been detected in several blue-green algae (11, 12), and glycolate is readily oxidized to CO2 by these cells (5, 13, 19). The spatial location of this enzyme within the blue-green algal cell is not known.

The object of the present study was to attempt to relate glycolate dehydrogenase activity to an identifiable subcellular fraction obtained from cells of a blue-green alga by osmotically disrupting spheroplasts of the alga. A preliminary account of this work has been presented previously (7).

MATERIALS AND METHODS

Culture of Alga. A bacteria-free culture of Oscillatoria sp. was a gift of Dr. S. R. Brown, Queen’s University, Kingston, Ontario. The alga was grown axenically and harvested as described previously (11).

Preparation of Cell-free Extracts. Cell-free preparations were obtained by lysing spheroplasts of the alga prepared in a manner similar to that described by Biggins (1). Washed cells were resuspended in 0.55 M mannitol-0.03 M K-phosphate buffer, pH 6.8, containing 0.06% (w/v) lysozyme (eggwhite muramidase, grade 1, Sigma Chemical Co.) and incubated at 32 C with gentle agitation at a light intensity of 8 klux for about 2 hr. Spheroplasts prepared in this manner lysed more readily and uniformly than those prepared in the dark (9). After incubation any intact filaments were removed by filtration through loosely packed prechilled glass wool, the spheroplasts collected by centrifugation, washed twice with mannitol-phosphate buffer, pH 6.8, and lysed either in 0.05 M K-phosphate buffer, pH 7.5, containing 0.3 mm magnesium chloride, or in the same medium with 0.3 M mannitol. The viscosity of the resulting suspension was reduced by the addition of about 0.1 mg deoxyribonuclease/mg dry weight of cells. The suspension was centrifuged at 5000g for 40 min at 0 to 4 C and the resulting supernatant fluid, referred to as the crude lysate, was used for enzyme assay.

Fractionation of Cell-free Preparations. Particulate fractions were obtained from the crude lysates either by centrifugation at 100,000g for 90 min or by centrifugation at 45,000g for 4 hr in a discontinuous gradient of sucrose comprised of layers of 0.3, 0.5, 0.67, 1.133, 1.67, and 1.9 M sucrose (Fig. 1) containing 0.05 M K-phosphate buffer, pH 7.5, and 0.3 mm magnesium chloride in a 40-ml polyallomer tube, at 0 to 4 C in an IEC B-60 ultracentrifuge equipped with a swinging bucket rotor. Samples were removed from the top of the gradient by pumping a 2.1 M sucrose solution through the bottom of the tube using an ISCO model 82 density gradient fractionator.

Enzyme Assays. Glycolate dehydrogenase was assayed by determination of glyoxylate phenylhydrazone formation in the presence or absence of DPIP (11) or by monitoring O2 uptake manometrically (24). Succinic dehydrogenase was assayed by following DPIP reduction at 600 nm (8) and cataolase was determined by monitoring H2O2 disappearance (17). Protein was determined by the procedure of Lowry et al. (16), and photosynthetic pigments by the methods of Myers and Kratz (20).

Electron Microscopy. Samples of the gradient fractions were centrifuged for 3 hr at 180,000g at 4 C in an International B-60 ultracentrifuge. The resulting pellets were fixed for 2 hr at 20 to 25 C in 2-ml Kellenberger buffer, pH 6.8 (15), containing 0.55 M mannitol and 2% glutaraldehyde. The samples were centrifuged as indicated above, resuspended in 1% agar, rinsed with buffer, and postfixed in 1% OsO4 overnight. The fixed samples were dehydrated with a series of increasing concentrations of ethanol and propylene oxide, then embedded in Epon and sections cut and stained with uranyl acetate and lead citrate (21). Sections were examined with a Phillips EM 201 electron microscope.

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ABBREVIATIONS:
DPIP: 2,6-dichlorophenolindophenol; FMN: flavin mononucleotide; HPMS: α-hydroxyphenyldimethane sulfonate; FAD: flavin adenine dipropid oxidoreductase.
RESULTS

Glycolate dehydrogenase activity was detected in crude lysates obtained by lysing spheroplasts in K-phosphate buffer. Most of the enzyme activity was found in the supernatant fraction after centrifugation of the lysate at 100,000g for 2 hr (Table I). However, when spheroplasts were lysed in the same buffer containing 0.3 M mannitol a larger proportion of enzyme activity was associated with the 100,000g pellet fraction (Table I). Resuspension of this 100,000g pellet fraction in mannitol-phosphate buffer normally resulted in the recovery of about 70% of this activity in a second pellet fraction after a repeated centrifugation at 100,000g for 60 min. In contrast only 10 to 15% of the enzyme activity was recovered in the second pellet fraction after resuspension of the original 100,000g pellet fraction in K buffer without mannitol (Table I). Enzyme activity was almost completely solubilized after treatment with 0.005% Triton X-100 (Table I).

Glycolate dehydrogenase activity was measured in the presence of DPIP but some activity was detected in the absence of this acceptor (Table I) and as indicated below O₂ uptake was also detected (Table II). This endogenous activity was markedly higher in the particulate fraction but was lost on treatment of the pellet with detergent (Tables I and II).

Density Gradient Fractionation. A number of continuous and discontinuous gradients were used in the course of these studies and the discontinuous gradient described above was finally chosen since it repeatedly gave the highest concentrations of glycolate dehydrogenase activity.

After centrifugation of the crude lysate in a discontinuous sucrose gradient a number of pigmented and clear zones were visible. Two blue zones, fractions I and II, contained 80 to 85% of the phycocyanin pigments and no Chl or carotenoid pigments (Fig. 1). In contrast, fraction VI, which was dark green in color, contained most of the Chl and carotenoid pigments, but only a trace of phycocyanin (Fig. 1). The only fraction which contained large amounts of all three types of pigment was fraction VIII which was blue-green in color. The Chl to carotenoid ratios of fractions VI and VIII were close to that expected for whole cells (2:1). Fraction VIII contained slightly more Chl and carotenoid with respect to phycocyanin than would be expected for whole cells. Only trace amounts of pigments were detected in fractions other than fractions I, II, VI, and VIII.

The bulk of glycolate dehydrogenase activity of the crude lysate was recovered in the four pigment-containing fractions of the density gradient (Fig. 1) with the highest activities in the two chlorophyllous fractions (VI and VIII). Of these two fractions, fraction VI demonstrated a higher specific activity of glycolate dehydrogenase both in the presence and absence of DPIP (Fig. 1). When aliquots of fractions VI and VIII were layered on a density gradient, none of the enzyme activity was lost from these

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Table I. Recovery of Glycolate Dehydrogenase Activity after Centrifugation of Cell-free Preparations of Oscillatoria

<table>
<thead>
<tr>
<th>Spheroplast lysis</th>
<th>% activity of crude lysate</th>
<th>Glycolate dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>100.0</td>
<td>99.0</td>
</tr>
<tr>
<td>100,000 g supernatant</td>
<td>68.2</td>
<td>93.3</td>
</tr>
<tr>
<td>100,000 g pellet</td>
<td>27.7</td>
<td>71.9</td>
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</table>

<table>
<thead>
<tr>
<th>Recentrifugation of 100,000 g pellet</th>
<th>% activity of crude lysate</th>
<th>Glycolate dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000 g supernatant</td>
<td>25.0</td>
<td>12.8</td>
</tr>
<tr>
<td>100,000 g pellet</td>
<td>1.6</td>
<td>49.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recentrifugation of 100,000 g pellet after treatment with X-100% Triton X-100</th>
<th>% activity of crude lysate</th>
<th>Glycolate dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000 g supernatant</td>
<td>28.6</td>
<td>56.2</td>
</tr>
<tr>
<td>100,000 g pellet</td>
<td>0.0</td>
<td>2.2</td>
</tr>
</tbody>
</table>

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Table II. Oxygen Uptake and Glycolate Formation by Fraction VI of Sucrose Density Gradient

<table>
<thead>
<tr>
<th>Additions</th>
<th>-DPIP</th>
<th>-DPIP</th>
<th>+DPIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3,1</td>
<td>9,8</td>
<td>13,6</td>
</tr>
<tr>
<td>0.1% DPIP</td>
<td>4,0</td>
<td>10,7</td>
<td>14,3</td>
</tr>
<tr>
<td>3% 1-min-1,2,4-triacetate</td>
<td>3,6</td>
<td>9,5</td>
<td>12,8</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>0.6</td>
<td>0.8</td>
<td>1.9</td>
</tr>
<tr>
<td>0.005% Triton X-100</td>
<td>0.2</td>
<td>1.9</td>
<td>10.8</td>
</tr>
</tbody>
</table>

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Fig. 1. Distribution of Chl, phycocyanin, and carotenoid pigments, and glycolate dehydrogenase activity, assayed in the presence and absence of DPIP, in a discontinuous sucrose density gradient after centrifugation of a crude lysate of Oscillatoria at 45,000g for 6 hr.

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fractions and an increasing amount of DPIP-dependent enzyme activity was detected at the top of the gradient (i.e. fraction I). Treatment of the lamellar fractions with 0.005% Triton X-100 resulted in a loss of lamella-bound activity and an increase in activity in fraction I. Detergent treatment reduced the percentage recovery and endogenous enzyme activity (Table II) and although some Chl and carotenoid appeared as new minor bands at interfaces in the gradient (i.e. 0.67 mM and 1 mM and 1.33 mM) no enzyme-rich fractions appeared to be associated with these new pigment bands.

Fractionation on a density gradient of the pellet obtained after centrifugation of the crude lysate at 100,000g, gave distributions of glycolate dehydrogenase and Chl and carotenoid pigments similar to those in Figure 1.

The glycolate dehydrogenase associated with the lamellar fraction (fraction VI) displayed many of the same properties as those previously reported for this enzyme isolated from blue-green algae (11, 12). Activities of both the bound and the soluble enzymes were not stimulated by the addition of FMN, FAD, NAD, or NADP; was markedly inhibited by 0.1 mM HPMS and by 1 mM KCN and were found to oxidize D-lactate faster than L-lactate. However, unlike the soluble enzyme previously reported (11), the activity of the lamella-bound enzyme was not completely DPIP-dependent. Since glyoxylate-phenylhydrazone formation was detected in the absence of DPIP, an attempt was made to determine whether the glycolate-oxidizing enzyme recovered in this fraction could utilize molecular O2 as does the glycolate oxidase of higher plants. Although some O2 uptake was detected manometrically, the amount of O2 taken up was always much less than the amount of glyoxylate phenylhydrazone formed (Table II) and when the enzyme was removed from the lamellae by detergent treatment, its activity was DPIP-dependent and O2 uptake was not detected. O2 uptake by the lamellar fraction was negligible in the absence of glycolate. The low rate of O2 uptake of the lamellar fraction in the presence of glycolate might be due to the presence of catalase activity which would diminish the observed rate of O2 uptake by catalyzing the breakdown of H2O2 to produce molecular O2. However, the rate of O2 uptake did not increase significantly in the presence of sufficient 3-amino-1,2,4-triazole (20 mM) to inhibit any catalase activity in this fraction (Table II). Furthermore, the catalase activity detected in this alga was found not to be associated with glycolate dehydrogenase in the lamellar fraction but in the soluble fractions (Fig. 2). In comparison, the distribution of succinic dehydrogenase in the gradient was similar to that of glycolate dehydrogenase (Fig. 2).

**Electron Microscopy of Gradient Fractions.** The four major pigment- and enzyme-containing zones (i.e. I and II, VI and VIII) were examined by electron microscopy. Fractions I and II contained no visible lamellar structures nor did they contain any visible cellular inclusions. The electron-dense material in fraction II had a uniform amorphous appearance and may consist of phycobiliprotein aggregates. However, these structures were not discernible after negative staining of either of these preparations.

Fraction VI, which contained most of the Chl and carotenoid as well as most of the glycolate dehydrogenase activity, consisted almost entirely of discrete lamellae which were arranged in concentric circles, similar to the arrangement of the photosynthetic lamellae in unbroken cells and spheroplasts of this alga (Fig. 3).

The second major Chl and carotenoid-containing fraction, VIII, contained many photosynthetic lamellae similar to that described above. Remnants of cell walls and plasma membrane were also observed in this fraction. In addition, this fraction contained a number of unbroken spheroplasts and considerably more debris than fraction VI. Fraction X was comprised of a considerable amount of cellular debris.

**DISCUSSION**

Glycolate dehydrogenase activity in *Oscillatoria* sp. was originally detected in soluble fractions after vigorous homogenization of these cells (11). Similarly, in the present study, most of the activity in crude lysates was detected in a soluble fraction containing little Chl and carotenoid (Table I). It appears that even after applying more gentle rupture techniques in the absence of an osmoticum such as 0.3 mM mannitol most of the enzyme activity is still found in the soluble fraction. However, when spheroplasts were lysed in an osmoticum a significant portion of the enzyme activity was recovered in a pellet fraction after centrifugation at 100,000g and in the photosynthetic lamellae fraction (i.e. VI) after separation in a discontinuous sucrose gradient (Fig. 1). Enzymic activity in fraction VIII also appears to be bound to the photosynthetic lamellae, except that this preparation contains cellular debris and some unbroken spheroplasts. Thus glycolate dehydrogenase appears to be lamella-bound but easily removed in the presence of detergent or the absence of a suitable stabilizing agent (Table I). An electron microscopic study of fraction VI from *Oscillatoria* confirmed that this fraction was composed of photosynthetic lamellae. It is noteworthy that succinic dehydrogenase was also recovered in the same Chl-carotenoid-rich lamellar fraction as the glycolate dehydrogenase (Fig. 2). These data are consistent with the observation made by Bisalputra et al. (3) by electron microscopy that after incubation of *Nostoc* *sphaerium* in potassium tellurite and tetraniotroblue tetrazolium in different respiratory substrates including succinate in either light or dark, a deposit of reduction products were observed associated with thylakoids. They suggested that the photosynthetic lamellae of *Nostoc* also serve as mitochondrial equivalents, a view which is not unreasonable since the lamellae of blue-green algae appear to contain the major components of the electron transport chain (2).

The glycolate dehydrogenase from blue-green algae is similar to that isolated from a number of unicellular green algae (6, 11, 12) and like the enzyme from eucaryotic algae (18) does not appear to utilize O2 readily as a final electron acceptor. The natural electron acceptor of glycolate dehydrogenase in algae is unknown (18) and the highest estimation of enzyme activity (i.e. glyoxylate formation) was always obtained in the presence of an artificial electron acceptor such as DPIP (Tables I and II).
Glyoxylate phenylhydrazone formation and O₂ uptake was detected in the absence of any artificial acceptor in the lamellae fractions. These activities were negligible after treatment with detergent (Table I). It would appear that in vivo both glycolate dehydrogenase and its natural cofactor or electron acceptor system are bound to the lamellae of the alga. A similar conclusion was drawn by Sallal and Codd (22) who reported the finding of a glycolate oxidase enzyme associated with an un-specific Chl-containing particulate fraction of Anabaena cylindrica.

In the leaves of higher plants, glycolate oxidation and H₂O₂ breakdown are localized in peroxisomes (23), thereby minimizing but not eliminating the possibility of non-enzymatic oxidation of glycolate to CO₂ (10, 14). If the glycolate-oxidizing enzyme of blue-green algae can function as an oxidase (6, 22) then it is possible that peroxide may be generated in vivo during glycolate oxidation. Although the lamella-bound glycolate-oxidizing activity in Oscillatoria may be linked via respiratory electron carriers to O₂, the enzyme cannot be considered to be a glycolate oxidase since there is no clear stoichiometry between O₂ consumption and glyoxylate formation even in the presence of an inhibitor of catalase activity (Table II). Furthermore, while catalase activity has been detected in a number of blue-green algae (4), in the present study catalase was not recovered with glycolate dehydrogenase in the lamellar fractions but in a soluble cytoplasmic fraction (Figs. 1 and 2). While it is possible that catalase was removed from the lamellae during cell breakage, the evidence from this study suggests that there is no discrete particle or lamellar component which might function as a primitive peroxisome in this procarcyotic alga. The apparent association of glycolate dehydrogenase with the photosynthetic lamellar fraction appears to be related to its natural cofactor or the electron transport system which is required for its operation in vivo.

LITERATURE CITED