The Intracellular Localization of Glycollate Oxidoreductase in *Euglena gracilis*

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(Received 25 February 1971)

1. Lowering of the concentration of carbon dioxide in air available to phototrophically growing *Euglena* cultures from 5% to the normal value (0.03%) resulted in an increased specific activity of glycollate oxidoreductase. 2. The effects of chloramphenicol and cycloheximide suggested that this increase in activity was due to enzyme synthesis *de novo* on cytoplasmic ribosomes. 3. The K_m for glycollate oxidation by the enzyme in crude cell extracts was 3.0×10^{-3} M. 4. Differential centrifugation established that glycollate oxidoreductase present in phototrophically grown *Euglena* is a particulate enzyme. The enzyme was partially solubilized by the non-ionic detergent Triton X-100. 5. Sucrose-density-gradient centrifugation achieved the separation of the particulate glycollate oxidoreductase from chloroplasts and mitochondria. 6. Glutamate-glyoxylate aminotransferase was associated with particulate glycollate oxidoreductase.

Recent investigations have established the presence of the glycollate pathway of metabolism in unicellular green algae (Lord & Merrett, 1970a; Bruin, Nelson & Tolbert, 1970). Glycollate formed from carbon dioxide during photosynthesis is oxidized to glyoxylate and subsequently converted via glycine and serine into glycerate. This pathway is well documented in higher plants (Tolbert, 1963), and is a feature of all green plant tissues. The nature of the enzyme catalysing the oxidation of glycollate to glyoxylate constitutes a fundamental biochemical difference between green algae and higher plants. Higher plants contain the FMN-flavoprotein glycollate oxidase (glycollate-oxygen oxidoreductase, EC 1.1.3.1), but the algal enzyme differs in that oxygen is not the terminal acceptor for glycollate electrons in several species (Nelson & Tolbert, 1969; Codd, Lord & Merrett, 1969).

Studies on the intracellular localization of glycollate oxidase in higher plants have established that this enzyme is present in the peroxisome, a cytoplasmic organelle characterized by the association of one or more flavin oxidase enzymes with an excess of catalase (Tolbert, Oeser, Kiseki, Hageman & Yamazaki, 1968). These microbodies also contain several other glycollate-pathway enzymes, including a glutamate-glyoxylate aminotransferase, a serine-pyruvate aminotransferase and an NADH-hydroxypyruvate reductase (Yamazaki & Tolbert, 1970).

The intracellular localization of glycollate oxido-

reductase in algae has not been investigated. The present paper reports on the intracellular localization of this enzyme in *Euglena gracilis*. This organism was selected because of the high specific activity of glycollate oxidoreductase in cells grown phototrophically on air (Codd *et al.* 1969; Codd & Merrett, 1971).

MATERIALS AND METHODS

Growth of alga. *Euglena gracilis* Klebs strain Z was grown phototrophically at 25°C and 6000 lx light-intensity, gassed with air, in the medium of Cramer & Myers (1952). For experiments on the effect of antibiotics on enzyme synthesis, the cells were gassed with 5% CO₂ in air. Bleached cells were obtained by growing *Euglena* at 25°C in the medium of Hutner, Bach & Ross (1956) in continuous darkness for 4 days; the major carbon source in this medium is glucose.

Cells were harvested by centrifugation at 500 g for 5 min.

For the preparation of cell-free extracts, cells were washed once with, and then were resuspended in the appropriate buffer to give a 20–40% (v/v) cell suspension, cell volume being determined by centrifuging at 500 g for 5 min in a graduated centrifuge tube.

In differential-centrifugation experiments, cells were resuspended in 0.4 M-sucrose in 50 mM-potassium phosphate buffer, pH 7.0. When appropriate, this buffer contained 1% (v/v) of Triton X-100. Extracts used for isopycnic sucrose-density-gradient centrifugation were prepared in 0.4 M-sucrose in 20 mM-glycylglycine buffer, pH 7.5.

Preparation of cell extracts. For differential and sucrose-density-gradient centrifugation, the cells were broken by passing suspensions through a cold French pressure cell at

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600–800 lb/in² (Milner, Lawrence & French, 1950). Intact cells were removed by centrifugation at 250g for 5 min and the extract was used immediately.

Differential centrifugation of *Euglena* extracts. A broken-cell suspension was centrifuged at 3000g for 2 min to spin down intact chloroplasts (Ellyard & San Pietro, 1969), then at 10000g for 10 min to spin down mitochondria and chloroplast fragments (Buetow & Buchanan, 1964) and finally at 100000g_{av.} for 1 h to sediment the remaining particulate matter. The various pellets, resuspended in buffer, and the corresponding supernatant solutions, were assayed for glycollate oxidoreductase activity.

Separation of *Euglena* organelles by sucrose-density-gradient centrifugation. A broken-cell suspension (5 ml) was carefully layered on top of the sucrose density gradient. This gradient was prepared at 4°C by pipetting in succession 5 ml of 2.0M-sucrose (68%, w/v), 5 ml of 1.75M-sucrose (60%, w/v), 5 ml of 1.5M-sucrose (51%, w/v), 5 ml of 1.25M-sucrose (43%, w/v), 5 ml of 1.0M-sucrose (34%, w/v) and 5 ml of 0.5M-sucrose (17%, w/v) into a 40 ml MSE polycarbonate centrifuge tube. The 2.0M-sucrose was prepared in 20 mM-glycylglycine buffer, pH 7.5, and was diluted with this buffer to the other sucrose concentrations. The centrifuge tube was carefully placed in the bucket of a 3 × 40 ml swing-out rotor and spun at 20000 rev./min (65000g_{av.}) for 4½ h at 0–4°C in an MSE Superspeed 40 centrifuge. At the end of the run the tube was removed, clamped in an MSE tube piercer, and fractions for enzyme assay, protein and chlorophyll determination were removed through a needle from the bottom of the tube.

Enzyme assays. Spectrophotometric assays were carried out in silica cuvettes (3.0 ml volume, 1 cm light-path) with a Unicam SP.500 spectrophotometer.

Glycollate oxidoreductase, glutamate-glyoxylate aminotransferase (EC 2.6.1.4), malate dehydrogenase (EC 1.1.1.37), D-glycerate dehydrogenase (EC 1.1.1.29) and glyoxylate reductase (EC 1.1.1.26) were assayed as described previously (Lord & Merrett, 1970a).

Succinate dehydrogenase (EC 1.3.99.1) was assayed by a modification of the procedure of Ells (1959), which uses phenazine methosulphate to couple electron transfer between succinate and 2,6-dichlorophenol-indophenol. The reaction mixture contained, in a final volume of 3.0 ml: 100 µmol of potassium phosphate buffer, pH 7.6, 0.3 µmol of 2,6-dichlorophenol-indophenol, 10 µmol of KCN, 20 µmol of sodium succinate and enzyme. The reaction was started by the addition of 0.1 ml of 1% (w/v) phenazine methosulphate and was measured by following the decrease in extinction at 600 nm.

Cytochrome *c* oxidase (EC 1.9.3.1) was assayed by the method of Simon (1958). The reaction mixture contained, in a final volume of 3.0 ml: 105 µmol of potassium phosphate buffer, pH 7.0, and 1 µmol of cytochrome *c* reduced with dithionite. The reaction was started by the addition of enzyme and was measured as the decrease in extinction at 550 nm.

Protein determination. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), by using a calibration curve prepared for crystalline bovine serum albumin.

Chlorophyll determination. Chlorophyll was determined by the method of Arnon (1949), except that in samples of

high sucrose concentration the acetone extracted so much water that the chlorophyll and sucrose formed a second phase. Sucrose is more soluble in cold acetone and it was necessary to keep these samples at –20°C overnight to obtain effective chlorophyll extractions. Samples were centrifuged before the extinction was read at room temperature.

Effect of antibiotics on glycollate oxidoreductase synthesis. *Euglena* cells growing phototrophically on 5% CO₂ in air were harvested during the exponential phase of growth and a portion was retained to determine glycollate oxidoreductase activity. The remaining cells were resuspended in fresh growth medium or growth medium containing either 15 µg of cycloheximide/ml or 1 mg of chloramphenicol/ml. The cultures were gassed with air, portions were removed at intervals and glycollate oxidoreductase activity in cell-free extracts was determined.

Materials. Sodium [1,2-¹⁴C]glyoxylate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; nicotinamide nucleotides, pyridoxal phosphate and cytochrome *c* were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; phenazine methosulphate was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other materials were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

RESULTS

Effect of antibiotics on glycollate oxidoreductase synthesis. Alteration of the gas phase of phototrophically growing *Euglena* cultures from 5% CO₂ in air to air (containing 0.03% CO₂) resulted in an increased specific activity of glycollate oxidoreductase (Fig. 1). The increase in specific activity was completely inhibited by cycloheximide added at a concentration known to inhibit protein synthesis on *Euglena* cytoplasmic (80S) ribosomes although not affecting chloroplast (70S) ribosomes (Smillie, Graham, Dwyer, Grieve & Tobin, 1967). The addition of chloramphenicol in amounts known to inhibit preferentially protein synthesis on chloroplast ribosomes only slightly inhibited this increase in enzyme activity (Fig. 1).

Differential centrifugation of *Euglena* extracts. *Euglena* cells disrupted in osmotically buffered medium were fractionated by differential centrifugation (Table 1). After 2 min centrifugation at 3000g all the glycollate oxidoreductase remained in the supernatant. When the 3000g pellet, resuspended in osmotically buffered solution, was viewed through a phase-contrast microscope, it was seen to consist almost entirely of intact chloroplasts, of which 60–70% appeared to be 'phase-bright', indicating that the outer chloroplast membrane was intact. When this pellet was resuspended in hypo-osmotic buffer (50 mM-potassium phosphate buffer, pH 7.0) and subjected to the maximum output of an MSE ultrasonic disintegrator for 30 s, glycollate oxidoreductase

activity was still not released. The pellet obtained by centrifuging for 10 min at 10000g, which mainly contained chloroplast fragments and mitochondria, contained negligible glycollate oxidoreductase

activity. However, all the glycollate oxidoreductase activity was sedimented by centrifuging at 100000g_{av.} for 1 h (Table 1), indicating that the enzyme was present in *Euglena* as a particulate enzyme not associated with chloroplasts or mitochondria.

The addition of 1% (v/v) of Triton X-100 to the suspending medium for the cells before disruption and differential centrifugation partially solubilized glycollate oxidoreductase, but provided no evidence for latent glycollate oxidoreductase activity. After centrifugation at 100000g_{av.} for 1 h the supernatant solution contained appreciable glycollate oxidoreductase activity (Table 2). Most of the activity, however, was still sedimented, indicating that the particulate glycollate oxidoreductase was not easily solubilized.

Separation of Euglena organelles by sucrose-density-gradient centrifugation. After sucrose-density-gradient centrifugation of broken-cell suspensions of *Euglena*, 87% of the protein and 95% of the glycollate oxidoreductase activity were recovered in the various gradient fractions. Most of the recovered glycollate oxidoreductase activity (63%) was found in the 0.5M- and 1.0M-sucrose layers (Fig. 2a). Glycollate oxidoreductase activity was distinctly separated from whole chloroplasts, which formed a band at the interface between the 1.25M- and 1.5M-sucrose layers.

Two other bands were visible after centrifugation. The first was located at the interface between the 1.0M- and 1.25M-sucrose layers and consisted mainly of chloroplast fragments. The second band formed at the 1.5M-/1.75M-sucrose interface and contained NAD-malate dehydrogenase activity, suggesting that the band consisted of mitochondria. However, malate dehydrogenase was also present in the 0.5M-sucrose layer and, to a smaller extent, in the soluble (0.4M-sucrose) fraction.

In an attempt to establish further the location of

Fig. 1. Effect of antibiotics on the change in the specific activity of glycollate oxidoreductase resulting from changing the gas phase of phototrophically growing *Euglena* cultures from 5% CO₂ in air to air. ○, Control; △, 1 mg of chloramphenicol/ml; □, 15 μg of cycloheximide/ml. For experimental details see the text. Specific activities were calculated as μmol of substrate oxidized/h per mg of protein.

Table 1. *Differential centrifugation of Euglena extracts*

Cells were grown phototrophically in air, resuspended in 0.4M-sucrose in 50 mM-potassium phosphate buffer, pH 7.0, disrupted by passage through a French pressure cell at 600–800 lb/in² and centrifuged. Glycollate oxidoreductase activity was assayed as described by Lord & Merrett (1970a).

Fraction	Sp. activity (μmol of glycollate oxidized/h per mg of protein)	% of total activity
3000g × 2 min pellet	0.00	0
3000g × 2 min supernatant	0.91	100
10000g × 10 min pellet	0.02	4
10000g × 10 min supernatant	0.80	71
100000g × 60 min pellet	1.05	63
100000g × 60 min supernatant	0.01	2
100000g × 60 min washed pellet	0.79	55
100000g × 60 min washings	0.00	0

Table 2. *Effect of Triton X-100 on particulate glycollate oxidoreductase from Euglena*

Cells were grown phototrophically in air, resuspended in 0.4M-sucrose in 50mM-potassium phosphate buffer, pH7.0, containing when appropriate 1% (v/v) of Triton X-100, disrupted by passage through a French pressure cell at 600–800lb/in² and centrifuged. Glycollate oxidoreductase activity was assayed as described by Lord & Merrett (1970a).

Fraction	Sp. activity (μ mol of glycollate oxidized/h per mg of protein)	
	Control	+Triton X-100
10000g $\times$ 10 min pellet	0.00	0.01
10000g $\times$ 10 min supernatant	2.00	1.85
100000g $\times$ 60 min pellet	4.00	1.50
100000g $\times$ 60 min supernatant	0.02	0.40

mitochondria after gradient centrifugation, cytochrome c oxidase was assayed, but activity could not be detected in any gradient fraction or in the crude broken-cell suspension initially layered on the gradient. Succinate dehydrogenase activity was sought over the gradient by using phenazine methosulphate to couple electron transfer from succinate to 2,6-dichlorophenol-indophenol. Slight activity was observed in the 1.5M-sucrose layer. This assay was not very satisfactory because a high rate of dye reduction was observed in controls without succinate.

The location of mitochondria on the sucrose gradient was more convincingly established by repeating these assays after centrifugation of a broken-cell suspension of dark-grown bleached *Euglena* (Fig. 2b). The number of mitochondria per *Euglena* cell is greatly increased under conditions resulting in a decrease in chloroplast number, although the general shape, size and organizational pattern of mitochondria (and, it is assumed here, the density) remain the same (Wolken & Palade, 1953). Again, a colourless band was observed at the 1.5M-/1.75M-sucrose interface, which corresponded to the peak of succinate dehydrogenase activity (Fig. 2b).

Malate dehydrogenase activity was again found in the 0.5M-sucrose layer with bleached cells, but the greatest specific activity was in the 1.75M-sucrose layer. The peak for NADH oxidase was also in the 1.75M-sucrose layer (Fig. 2b).

Thus the gradient employed resulted in the separation of particulate glycollate oxidoreductase from both chloroplasts and mitochondria, and confirmed that the enzyme was associated with a cytoplasmic particulate fraction.

The location of other glycollate-pathway enzymes in sucrose gradients was investigated. Glutamate-glyoxylate aminotransferase had a distribution almost identical with that of glycollate oxidoreductase over the less dense part of the gradient, with an additional peak corresponding to the fraction containing mitochondria (Fig. 2a). D-

Glycerate dehydrogenase activity was present in the 0.5M-sucrose fraction and corresponded to the distribution of the non-mitochondrial isoenzyme of malate dehydrogenase (Fig. 2a). NAD- and NADP-glyoxylate reductase activities were present in all gradient fractions with possible peaks in the chloroplast (1.25M-sucrose) fraction (results not shown). Although the presence of serine hydroxymethyltransferase (EC 2.1.2.1) was recorded in crude cell homogenates (Codd & Merrett, 1971), we were unable to detect activity in any fraction after sucrose-density-gradient centrifugation.

K_m and substrate specificity of Euglena glycollate oxidoreductase. The apparent *K_m* for glycollate oxidation by crude cell homogenates of *Euglena* was determined to be 3.0×10^{-3} M. *Euglena* extracts, in common with those of other green algae, will oxidize D-lactate, but not L-lactate, in addition to glycollate (Nelson & Tolbert, 1970). In many algae one enzyme will oxidize both substrates, but in *Euglena* there appear to be two enzymes, as rates of oxidation are additive when D-lactate and glycollate are used in mixed-substrate assays. The oxidation of these two substrates after sucrose-density-gradient centrifugation was also consistent with this conclusion. The peak for glycollate oxidation was in the 0.5M-sucrose layer whereas that for D-lactate was in the 1.0M-sucrose layer (Fig. 3). The apparent *K_m* values for D-lactate oxidation were identical in both fractions (6.66×10^{-3} M); similarly, little difference was observed in the apparent *K_m* values for glycollate oxidation, the *K_m* being slightly lower in the 1.0M-sucrose layer (1.3×10^{-3} M) than that in the 0.5M-sucrose layer (1.6×10^{-3} M).

DISCUSSION

The effects of antibiotics on the synthesis of glycollate oxidoreductase in *Euglena gracilis* (Fig. 1) and the non-aqueous fractionation of such cells (Codd & Merrett, 1970) showed that the enzyme was synthesized and located in the cytoplasm rather

Fig. 2. Distribution of enzymic activities after sucrose-density-gradient centrifugation of crude cell extracts of (a) phototrophically grown *Euglena* cells and (b) heterotrophically grown bleached *Euglena* cells. Fractions were numbered in the order they were drained from the centrifuge tube. The width of the column is proportional to the volume of the fraction removed for assay. Enzymes were assayed as described in the text. Specific activities were calculated as μmol of substrate transformed/h per mg of protein. (i) and (ii) Content of protein (—) and chlorophyll (-----); (iii) glycollate oxidoreductase; (iv) succinate dehydrogenase; (v) glutamate-glyoxylate aminotransferase; (vi) NADH oxidase; (vii) and (viii), malate dehydrogenase (—) and D-glycerate dehydrogenase (-----); (ix) and (x), concn. of sucrose.

Fig. 3. Distribution of enzymic activity catalysing the oxidation of glycollate (○) and D-lactate (△) among fractions after sucrose-density-gradient centrifugation of crude extracts of phototrophically grown *Euglena* cells. Enzymes were assayed as described in the text. Specific activities were calculated as μmol of substrate oxidized per mg of protein.

than the chloroplasts. Differential and sucrose-density-gradient centrifugation (Tables 1 and 2 and Fig. 2a) showed the enzyme to be particulate and present in the cytoplasm distinct from mitochondria and chloroplasts. This particulate activity was dispersed throughout the 0.5M- and 1.0M-sucrose fractions after centrifugation, and this distribution was not affected by centrifuging for 3, 4½ or 5 h, or by including an additional 0.75M-sucrose layer in the gradient. One possible explanation for this result is that the enzyme is present in a macromolecular complex with a range of densities corresponding to 0.5–1.0M-sucrose occurring either naturally in *Euglena* cells or as a result of shearing forces during the cell disruption or centrifugation processes. However, the results are also equally consistent with the possibility that glycollate oxidoreductase is part of a multiprotein complex that, because of its buoyant density, would not reach equilibrium in the density gradient used, but is of such a size that during the course of the experiment it has sedimented some distance through the density gradient as a zone. Our results do not allow us to distinguish between these possible explanations, so we use the term 'particulate glycollate oxidoreductase' when referring to glycollate oxidoreductase activity from *Euglena* that can be sedimented by centrifugation.

Attempts to locate mitochondria by cytochrome *c* oxidase assays were unsuccessful, confirming the observation made by Krawiec & Eisenstadt (1970). Similar findings in *Aetasia* extracts have been reported (Webster & Hackett, 1965), and it is concluded that euglenoids lack a conventional

cytochrome *c* oxidase. Maximum succinate dehydrogenase activity was recorded in the 1.5M-sucrose fraction and maximum malate dehydrogenase activity was in the 1.75M-sucrose fraction after centrifugation of extract from bleached *Euglena* cells (Fig. 2b). Krawiec & Eisenstadt (1970) separated mitochondria from bleached *Euglena* on linear sucrose density gradients and recovered particulate fractions with respiratory activity at 1.4–1.5M-sucrose and also at 1.7M-sucrose. The less dense fraction was concluded to be damaged mitochondria and the 1.7M-sucrose fraction was a distinct naturally occurring population of mitochondria. It is possible that in the present investigation the 1.5M-sucrose fraction contains damaged mitochondria that retain their membrane-bound succinate dehydrogenase although losing other enzymes, including malate dehydrogenase, to the soluble fraction. The 1.75M-sucrose fraction, containing the greatest malate dehydrogenase activity, might represent intact mitochondria.

Other enzymes were associated with glycollate oxidoreductase; glutamate-glyoxylate aminotransferase, besides being present in mitochondria, is associated with glycollate oxidoreductase in *Euglena* (Fig. 2a). Thus this alga contains particulate enzymes that compartmentalize certain enzymic steps of the glycollate pathway, including those responsible for the formation and further metabolism of glyoxylate. Glyoxylate has been shown to inhibit tricarboxylic acid-cycle activity in isolated mitochondria by directly inhibiting pyruvate oxidase and probably α -oxoglutarate dehydrogenase and succinate dehydrogenase (Davies & Ribereau-Gayon, 1969), and the addition products of glyoxylate with oxaloacetate, i.e. oxalomalate and γ -hydroxy- α -oxoglutarate, also inhibited three tricarboxylic acid-cycle enzymes (Ruffo, Testa, Adinolfi, Pelizza & Moratti, 1967). Earlier work has shown a functional role for the tricarboxylic acid cycle during glycollate metabolism by *Chlorella* (Lord & Merrett, 1970a) and exogenous glycollate stimulates respiration in the dark by *Scenedesmus* (Nelson, Tolbert & Hess, 1969), further indicating that the products of glycollate metabolism are ultimately catabolized via the tricarboxylic acid cycle. Thus there is no evidence that glyoxylate inhibits tricarboxylic acid-cycle activity during glycollate metabolism in algae, which could be explained by an intracellular isolation of glyoxylate from the mitochondrial respiratory sites.

The absence of glutamate-glyoxylate aminotransferase from the chloroplast-containing fraction (Fig. 2a) eliminates the possibility of glycine synthesis from glyoxylate in the chloroplast, and, although this enzyme has been reported to be

present in pea chloroplasts (Shah & Cossins, 1970), the isolation of chloroplasts by a non-aqueous technique may have resulted in contamination of the chloroplast fraction by cytoplasmic proteins.

Although the primary carbon compound metabolized by this particulate glycollate oxidoreductase from *Euglena* is probably glycollate, this fraction is not biochemically identical with the peroxisomes isolated from higher-plant leaves (Tolbert *et al.* 1968). The glycollate-oxidizing enzyme of *Euglena*, unlike the higher-plant oxidase, does not link to oxygen (Codd *et al.* 1969) and is therefore not involved in hydrogen peroxide production. In addition, catalase, a key enzyme of plant peroxisomes, could not be detected in *Euglena*, although this enzyme is readily demonstrated in many algae (results not shown). However, in common with peroxisomes, it is probable that this particulate enzyme complex is mainly concerned with the further metabolism of recently formed products of photosynthetic CO₂ fixation. The inhibition of glycollate oxidoreductase by α -hydroxypyridin-2-yl methanesulphonate in photosynthesizing *Euglena* results in the accumulation of glycollate (Codd & Merrett, 1971). Similarly the greening of bleached *Euglena* cells (Codd & Merrett, 1970) or the de-repression of ribulose 1,5-diphosphate carboxylase in acetate-grown *Chlamydomonas mundana* (Lord & Merrett, 1970b) are accompanied by striking increases in glycollate oxidoreductase activity. In addition to the compartmentalization of glyoxylate this enzyme complex could also function in the formation of C₂ and C₃ intermediates outside the chloroplasts from photosynthetically fixed carbon independent of the reactions of the glycolytic pathway.

J. M. L. thanks the Science Research Council for a research studentship during this work. This work was supported by Science Research Council Grant B/SR/4327.

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