Microbody-marker Enzymes during Transition from Phototrophic to Organotrophic Growth in Euglena

Received for publication December 24, 1974 and in revised form February 19, 1975

NEVILLE COLLINS AND MICHAEL J. MERRETT
Postgraduate School of Studies in Biological Sciences, University of Bradford, Bradford, Yorkshire BD7 1DP, England

ABSTRACT

Transfer of Euglena gracilis Klebs Z cells from phototrophic to organotrophic growth on acetate results in derepression of the key enzymes of the glyoxylate cycle, malate synthase and isocitrate lyase, which appear coordinately regulated. The derepression of malate synthase and isocitrate lyase was accompanied by increased specific activities of succinate dehydrogenase, fumarase, and malate dehydrogenase, but hydroxyxypyruvate reductase activity was unaltered.

Isolation of organelles from broken cell suspensions of cells grown heterotrophically on acetate was achieved by isopycnic centrifugation on sucrose gradients. Peaks of mitochondrial enzymes were obtained at equilibrium densities of 1.22 g cm−3 and 1.16 g cm−3, and although significant differences in the distribution of tricarboxylic acid cycle enzymes between these two peaks were not recorded adenosine triphosphatase activity was detected only in the less dense fraction (1.16 g cm−3) showing this contained damaged mitochondria. The peak of particulate glyoxylate cycle enzymes was at an equilibrium density of 1.25 g cm−3, this being the same as that for glycolate pathway enzymes from phototrophic cells. Citrate synthase, isocitrate lyase, malate synthase, and malate dehydrogenase were all present in this fraction so it was concluded that Euglena glyoxysomes contain a complete glyoxylate cycle.

Microbodies, organelles with a single limiting membrane, and a granular matrix (21) are widely distributed in higher plants (15) and are of at least two types characterized by different enzyme complements but of similar morphology. The microbodies of leaves (peroxisomes) contain enzymes of the glycolate pathway (32, 33), whereas microbodies present in fatty seeds (glyoxysomes) contain the enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase (5, 6). Electron micrographs of algae have revealed organelles of similar morphology to microbodies in Chlorella (4, 10, 16, 27) Euglena (8, 18), Chlamydomonas (17), Microstretia (34), Nitella (30), and Klebsormidium (31), but because of difficulties of isolation only limited information is available on enzyme complement in relation to nutrition of algae.

The definitive studies of Kornberg (22) have shown that the growth of many microorganisms, including algae (20), on C3 compounds to be dependent on the anaerobic reactions of the glyoxylate cycle. A peroxisome fraction containing the enzymes of the glycolate pathway was isolated by Collins and Merrett (11) from phototrophic Euglena cells, so it was of interest to follow changes in microbody enzymes and their intracellular localization during transition from phototrophic growth to conditions resulting in derepression of glyoxylate cycle enzymes such as heterotrophic growth on acetate.

MATERIALS AND METHODS

Growth of Alga. Asynchronous cultures of Euglena gracilis Klebs strain Pringsheim were grown phototrophically at 25 C as described previously (24). Cultures were grown organotrophically with the same medium as for phototrophic cultures but with the addition of 33 mm acetate or other carbon source. Cells were bleached and grown as described by Lord and Merrett (24).

Preparation of Cell Extracts. Cells were harvested by centrifugation at 500g for 5 min, were washed once with and resuspended in 0.4 M sucrose in 1 M EDTA, pH 7.5, to give a 50% (v/v) cell suspension, cell volume being determined by centrifuging at 500g for 5 min in a graduated centrifuge tube. The cell suspension was added to an equal volume of 5% 7 glass ballotini beads in a mortar and gently ground in the cold for 5 min. The beads were washed three times with half volumes of buffer, the combined washings centrifuged at 250g for 5 min, and the supernatants combined. After centrifuging at 250g for 5 min to remove any remaining cell debris, the crude extract was used for the separation of organelles. The crude-cell extract was layered on top of a linear sucrose gradient anad centrifuged at 65,000g average for 4.5 hr at 0 to 4 C in an MSE superspeed 40 centrifuge. Total protein on gradients never exceeded 15 to 20 mg; for full discussion of method see Collins and Merrett (11).

Enzyme Assays. Spectrophotometric assays were carried out in silica cuvettes (3.0 volume, 1-cm light path) with a Gilford series 2000 recording spectrophotometer. Activities of the following enzymes were determined using the assays cited: glycolate dehydrogenase (24), malate dehydrogenase (11), fumarase (11), succinate dehydrogenase (11), hydroxyxypyrurate reductase (11), malate synthase (12), isocitrate lyase (14), citrate synthase (11), serine-glyoxylate aminotransferase (11), aspartate α-ketoglutarate aminotransferase (11), ATPase (28). Protein was determined by the method of Lowry et al. (26). Chlorophyll was determined by the method of Arnon (2), and inorganic Pi by the method of Atkinson et al. (3).

RESULTS

Enzyme Level during Transition from Phototrophic to Organotrophic Growth. When Euglena cultures growing photo-
Phyll was a major Fumarase enzyme and pensions (Fig. 4). Citrate synthase and malate dehydrogenase gave characteristic peaks of activity in the glyoxysomal fraction, and also peaks in fractions 13 and 16 corresponding to the other mitochondrial enzymes. On heterotrophic gradients, all mitochondrial enzymes tested gave characteristic peaks in fractions 13 and 16 but only one peak in fraction 13 on phototrophic gradients.

Heterotrophic cells contain more mitochondria than phototrophic cells and it was possible their homogenization gave a greater yield of mitochondrial protein resulting in the detection of a secondary peak. Increasing the amount of protein on trophically on air were darkened and acetate added, the transition to organotrophic growth was accompanied by significant changes in enzyme activity. Malate dehydrogenase showed an approximately 3-fold increase in specific activity over 24 hr (Fig. 1), while smaller increases were observed for the mitochondrial markers fumarase and succinate dehydrogenase (Fig. 1). Although the addition of acetate resulted in the derepression of malate synthase the specific activity of the peroxisomal marker enzyme, hydroxypropruvate reductase was altered little under heterotrophic growth (Fig. 1). The specific activity of the peroxisomal enzyme serine-glyoxylate aminotransferase was also unaffacted during transition from phototrophic to organotrophic growth.

The increase in malate synthase and isocitrate lyase activities resulting from the addition of acetate to cultures was inhibited by cyloheximide and p-fluorophenylalanine (Woodward and Merrett, unpublished observations) suggesting that the increase in enzyme activity resulted from de novo protein synthesis rather than enzyme activation.

Relation of Levels of Isocitrate Lyase and Malate Synthase.
A plot of the specific activity of malate synthase against that for isocitrate lyase under different growth conditions is shown in Figure 2. The synthesis of these enzymes in Euglena appears to be coordinately regulated.

Separation of Euglena Organelles by Sucrose Density Gradient Centrifugation. After centrifugation of broken cell suspensions of Euglena on a linear sucrose gradient, the distribution of specific marker enzymes for organelles was determined (Fig. 3). Malate synthase was selected as a glyoxysomal marker enzyme and a major peak of activity was recorded in fraction 9 (Fig. 3) corresponding to an equilibrium density of 1.25 g cm⁻³. Fumarase was assayed as a marker for mitochondria, a major peak of activity was present in fraction 13 at an equilibrium density of 1.22 g cm⁻³, and in addition a second peak of activity was present in fractions 16 and 17 corresponding to an approximate equilibrium density of 1.16 g cm⁻³. Chlorophyll was determined to record the distribution of chloroplasts and chloroplast fragments, a peak of Chl was present in fraction 15 at an equilibrium density 1.19 g cm⁻³ and a second peak in fraction 19 at an equilibrium density of 1.15 g cm⁻³.

Major peaks of protein were present in fractions 9, 13, and 17 corresponding with the peak of malate synthase activity and the two peaks of fumarase activity.

A major difference in enzyme distribution on heterotrophic and phototrophic gradients was that in addition to the peak of fumarase activity in fraction 13 corresponding with the usual mitochondrial fraction in phototrophic cells, a second peak of activity was present in fraction 16 on heterotrophic gradients. When other mitochondrial enzymes were assayed over the heterotrophic gradient succinate dehydrogenase and D-lactate dehydrogenase activity corresponded with fumarase activity (Fig. 4). Citrate synthase and malate dehydrogenase gave characteristic peaks of activity in the glyoxysomal fraction, and also peaks in fractions 13 and 16 corresponding to the other mitochondrial enzymes. On heterotrophic gradients, all mitochondrial enzymes tested gave characteristic peaks in fractions 13 and 16 but only one peak in fraction 13 on phototrophic gradients.

Heterotrophic cells contain more mitochondria than phototrophic cells and it was possible their homogenization gave a greater yield of mitochondrial protein resulting in the detection of a secondary peak. Increasing the amount of protein on
phototrophic gradients failed to give a second mitochondrial peak, while decreasing the amount of protein on heterotrophic gradients still gave two peaks.

The second peak of mitochondrial enzymes was below the major Chl peak on the gradient, so it might arise as a result of some mitochondria being hindered by the chloroplast fraction in their movement on the gradient. When dark-grown Euglena cells were grown on acetate and subjected to the same procedure as chloroplast containing cells, the distribution of mitochondrial enzymes was unaffected by the absence of chloroplasts, and peaks of activity were still present in fractions 13 and 16 (Fig. 5). In agreement with the results obtained with green cells grown on acetate, bleached cells gave characteristic peaks of glyoxysomal enzymes in fraction 9 (Fig. 5).

These experiments had not eliminated the possibility that one peak of mitochondrial enzymes was intact mitochondria and the other damaged mitochondria of altered buoyant density. Adenosine triphosphatase was taken as an indicator of mitochondrial damage, and assay over a heterotrophic gradient revealed only one major peak of activity in fraction 16 (Fig. 6), suggesting this fraction contained damaged mitochondria.

Several other possible microbody enzymes were assayed, although a complete enzyme profile of microbodies from acetate-grown Euglena was not attempted. Serine-glyoxylate aminotransferase was located exclusively in the microbody fraction, while glycolate dehydrogenase was present in the microbody fraction and in the two mitochondrial enzyme fractions (Fig. 5). Aspartate-α-ketoglutarate was located in the microbody fraction and in fractions 13 and 17 (Fig. 5), and although activity in fraction 13 clearly represents a mitochondrial enzyme, the activity in fraction 17 could be due to either a chloroplast or mitochondrial enzyme. However, with bleached cells grown on acetate, aspartate-α-ketoglutarate aminotransferase activity was only present in fraction 9 containing microbody enzymes and fraction 13 containing intact mitochondria, showing that the other peak of activity (fraction 17) recorded for green cells represents a chloroplast enzyme (Fig. 6).

The recoveries for enzyme activities after sucrose density gradient centrifugation were the same as for phototrophic gradients (11), while the recovery for malate synthase was 62%.

**Fig. 4.** Distribution of some tricarboxylic acid cycle enzymes on a continuous sucrose gradient from dark acetate-grown Euglena. All results expressed as μmoles substrate/hr-fraction. Activity in fraction 27 for malate dehydrogenase (~).~

**Fig. 5.** Distribution of some peroxisomal enzymes on a continuous sucrose gradient from dark acetate-grown Euglena. All results expressed as μmoles substrate/hr-fraction.

**Fig. 6.** Distribution of mitochondrial and microbody enzymes from acetate-grown bleached Euglena on a continuous sucrose gradient. All results expressed as μmoles substrate/hr-fraction.
DISCUSSION

The transfer of cells from phototrophic conditions to heterotrophic growth on acetate was accompanied by significant changes in enzyme levels (Fig. 1). The presence of acetate resulted in the derepression of the key enzymes of the glyoxylate cycle, malate synthase, and isocitrate lyase (Fig. 1). These enzymes appear to be coordinately regulated (Fig. 2) as occurs in germinating fatty seeds (35), Aspergillus (1), and Tetrahymena (25), and as might be expected in Euglena if cells were dependent on the glyoxylate cycle for a net synthesis of C compounds during growth on acetate in the dark. The derepression of these enzymes was accompanied by increased levels of the mitochondrial enzymes, succinate dehydrogenase, and fumarase. The derepression of glyoxylate cycle enzymes and resultant growth in the dark on acetate did not result in the repression of specifically peroxisomal enzymes, as hydroxypyruvate reductase and serine-glyoxylate aminotransferase activities were unaltered. The presence of both glyoxysomal and peroxisomal enzymes may be advantageous to cells in a natural environment rich in organic compounds and subjected to diurnal light/dark periods.

The characterization of major organelle fractions from heterotrophic Euglena cells gave equilibrium density values in close agreement with those previously reported for phototrophic cells (11). The microbody marker enzymes from both phototrophic and heterotrophic cells banded together at an equilibrium density of 1.25 g cm⁻³, with a major peak of mitochondrial enzymes at an equilibrium density of 1.22 g cm⁻³. A difference was the presence of an additional peak of mitochondrial enzymes at an equilibrium density of 1.16 g cm⁻³ with heterotrophic cells (Fig. 3). Significant differences in the distribution of tricarboxylic acid cycle enzymes between the peaks of equilibrium density 1.16 g cm⁻³ and 1.22 g cm⁻³ from heterotrophic cells were not detected. Mitochondrial ATPase, located on the inner surface of the inner mitochondrial membrane (23) was assayed to determine mitochondrial integrity. Only one peak of activity in fraction 16 (Fig. 6), corresponding to the second peak of mitochondrial enzymes of equilibrium density 1.16 g cm⁻³ was present on the heterotrophic gradient. Activity was detected only in the lower mitochondrial fraction of equilibrium density 1.22 g cm⁻³ after sonication. While the lower peak of enzyme activity represents intact mitochondria, the additional peak of mitochondrial enzymes at an equilibrium density of 1.16 g cm⁻³ from heterotrophic cells was due to damaged mitochondria. Mitochondrial morphology in Euglena, as revealed by electron microscopy, varies over the cell cycle (9) and with growth conditions. Change in morphology from small discrete mitochondria to a mitochondrial network could result on homogenization in the production of more mitochondrial fragments, thereby giving two peaks of mitochondrial enzymes on gradients.

Fumarase and succinate dehydrogenase were not detected in the microbody fraction, nor were hydroxypyruvate reductase and serine-glyoxylate aminotransferase detected in the mitochondrial fraction of phototropic cells (11). With heterotrophic cells a complete separation of microbody and mitochondrial enzymes was not achieved; although mitochondrial enzymes were not detected in the microbody fraction, malate synthase was always present in the lower mitochondrial fraction (Fig. 3), and this was particularly so with bleached cells (Fig. 6). Total malate synthase activity in this fraction was low compared to the microbody fraction, suggesting that it arose from microbody fragments in the mitochondrial fraction. The presence of some malate synthase activity in the mitochondrial fraction of equilibrium density 1.22 g cm⁻³ may explain the conclusion of Graves et al. (19) that Euglena microbodies have an equilibrium density of 1.20 g cm⁻³, while their value of 1.17 g cm⁻³ for mitochondria corresponds closely to the equilibrium density value obtained for damaged mitochondria (Figs. 3, 4, and 5).

Although Brody and White (8) reported catalase activity in aerated, acetate-grown Euglena cells, in our studies (7, 24) we have consistently failed to detect significant levels of this enzyme in cells under a wide variety of growth conditions. The enzymes catalyzing glycolate oxidation in both the peroxisomal and mitochondrial fractions were dehydrogenases (11) so H₂O₂ would not be generated during glycolate oxidation. The distribution of glycolate dehydrogenase on heterotrophic gradients (Fig. 5) was in agreement with the dual location of this enzyme in microbodies and mitochondria in phototropic Euglena cells (11). The major peak of activity was in the microbody fraction, a second peak in fraction 13, the intact mitochondria fraction with a minor peak in fraction 17 containing damaged mitochondria (Fig. 5). Rates of glycolate and D-lactate oxidation were additive in the mitochondrial fraction, with glycolate oxidation being cyanide-sensitive and D-lactate oxidation -cytochrome-c-resisistant as in phototropic cells (11). Glycolate oxidation was linked to O₂ uptake in the intact mitochondria fraction, as occurs with mitochondria from phototropic cells (11).

The transfer of cells to heterotrophic growth on acetate results in the derepression of malate synthase and isocitrate lyase (Fig. 1), and the particulate peaks of these enzymes occurs with the peroxisomal enzymes hydroxypyruvate reductase and serine-glyoxylate aminotransferase, at an equilibrium density of 1.25 g cm⁻³. However, although glyoxylate cycle and peroxisomal enzymes occur at the same equilibrium density this does not resolve the question as to whether glyoxysomes arise by modification of pre-existing peroxisomes or whether they are a distinct population of microbodies synthesized de novo. The enzyme complement points to a complete glyoxylate cycle in the microbody fraction from Euglena cells grown heterotrophically on acetate. Except for aconitate, all the enzymes required to achieve a net synthesis of one C, molecule from two molecules of acetyl-CoA were detected in the microbody fraction of equilibrium density 1.25 g cm⁻³. Cooper and Beevers (13) found aconitate of castor bean endosperm glyoxysomes to be very unstable on sucrose gradients and this could well be the case in Euglena where activity readily detectable in crude cell extracts was lost after sucrose gradient centrifugation. Citrate synthase was only present in the mitochondrial fraction from phototropic cells (11), whereas in heterotrophic cells the enzyme was present in both mitochondrial and glyoxysomal fractions as required for operation of both tricarboxylic and glyoxylate cycles. Although Stabenau and Beevers (30) concluded that microbodies from Chlorogonium were of the non-specialized type found in many nongreen plant tissues, the enzymic composition of microbodies in Euglena is determined by growth conditions; in phototropic cells microbodies contain glycolate pathway enzymes (11), whereas in cells grown heterotrophically on acetate they contain the enzymes of the glyoxylate cycle.

LITERATURE CITED

4. Atkinson, A. W., P. C. L. John, and B. E. S. Gunning. 1974. The growth and division of the single mitochondrion and other organelles during the...
cell cycle of *Chlorella*, studied by quantitative stereology and three-dimensional reconstruction. *Protoplasma* 81: 77-109.


