Malate Dehydrogenase Isoenzymes in Division Synchronized Cultures of Euglena

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ABSTRACT
Sucrose density gradient centrifugation of broken cell suspensions of autotrophically grown Euglena gracilis Klebs. has allowed the separation of chloroplasts, mitochondria, and peroxisomes. Chlorophyll was taken as a marker for chloroplasts, fumarase and succinate dehydrogenase for mitochondria, and glycolate oxidoreductase for peroxisomes. Peaks of malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37) activity were found in the mitochondrial and peroxisomal fractions. Acrylamide gel electrophoresis showed specific isoenzymes in the mitochondrial and peroxisomal fractions and a third isoenzyme in the supernatant. The mitochondrial isoenzyme which had a Km (oxaloacetate) of 30 µM was inhibited by oxaloacetate concentrations above 0.17 mM, an inhibition of 50% being given by 0.9 mM oxaloacetate. The peroxisomal isoenzyme had a Km (oxaloacetate) of 24 µM, was inhibited by oxaloacetate concentrations above 0.13 mM, 50% inhibition being given by 0.25 mM oxaloacetate. Malate dehydrogenase activity in the supernatant did not show inhibition by increasing oxaloacetate concentration, the Km (oxaloacetate) being 91 µM.

In division synchronized cultures of Euglena, all three isoenzymes of malate dehydrogenase were synthesized over the light phase of the cycle. Darkening light phase cultures did not affect malate dehydrogenase activity. The addition to cultures of cycloheximide at a concentration previously shown to inhibit protein synthesis on Euglena cytoplasmic ribosomes completely inhibited increase in malate dehydrogenase activity over the cell cycle. Malate dehydrogenase activity was unaffected by the addition of chloramphenicol in amounts known to inhibit preferentially protein synthesis on 70S ribosomes.

In most higher plant and animal tissues, two proteins with malate dehydrogenase activity (L-malate-NAD oxidoreductase, EC 1.1.1.37) can be demonstrated, one associated with the supernatant fraction of the cell and the other is a particulate form localized in mitochondrial fractions (26-28). In addition, recent work has demonstrated a further isoenzyme of malate dehydrogenase localized in plant microbodies (22, 23, 29). Although the isoenzymes of malate dehydrogenase in higher plant tissues have been the subject of many investigations (11, 21-23, 28-30), little is known about the distribution and function of these isoenzymes in the algal cell. When Euglena cell organelles were separated by sucrose density gradient centrifugation, malate dehydrogenase activity was recorded in several fractions (17). Besides malate dehydrogenase activity in the mitochondrial fraction, activity was present in the particulate glycolate oxidoreductase fraction, the supernatant, but not in the chloroplast fraction (17). When grown phototrophically on an appropriate light-dark regime, cultures of Euglena gracilis divide synchronously, an appropriate doubling of cell number occurring in each dark period within certain limits of cell concentration (7). By using division synchronized cultures, the development of malate dehydrogenase activity in relation to other cellular activities can be investigated. In the present paper, we report on the characterization of malate dehydrogenase isoenzymes in cell fractions and their expression over the division cycle in Euglena cultures.

MATERIALS AND METHODS
Growth, Synchronization Regime, and Sampling of Culture. Division synchronized cultures of E. gracilis Klebs strain Z were obtained exactly as described previously (4). As before, samples were removed at t0, t1/2, t1, and t2, this referring to the hour of sampling after commencement of the 24-hr cycle in which the culture was used. Asynchronous cultures were grown at 25°C in the photoautotrophic growth medium of Kramer and Myers (9) at a continuous light intensity of 6000 lux provided by banks of fluorescent tubes (Osram white) and gassed with air at a rate of 7l/hr. Cells were harvested in middle exponential phase of growth by centrifugation at 1000g for 4 min.

Cell Disruption Techniques. Total enzyme activity per cell was determined by harvesting the cells by centrifugation, washing once, resuspending in 0.1 M potassium phosphate buffer, pH 7.0, and disrupting by treatment with three 15-sec bursts of ultrasonic waves (MSE ultrasonic disintegrator 1.5 amp). After centrifugation at 150g for 10 min, the supernatant was used in enzyme assays.

A less drastic technique was required for the isolation of intact cell organelles. Of the various methods tried, shaking with glass Ballotini beads in a Braun MSK rotary cell homogenizer gave the best yield of intact organelles. Cells were harvested by centrifugation, washed once with 20 mM glycylglycine buffer, pH 7.0, then resuspended in 0.4 M sucrose containing 20 mM glycylglycine buffer, pH 7.0, to give a final cell density of 3.2 x 10⁸ cells/ml. The cell suspension was cooled to 4°C in a Duran glass bottle and 1 g of No. 7 Ballotini added per ml of suspension before shaking for 5 sec at 2000 rpm in the homogenizer. The mash was centrifuged at 150g for 10 min, the supernatant was decanted, and the pellet was resuspended carefully in fresh media and treated as before. After centrifugation the supernatants were combined and stored at 0°C. Approximately 80% of the cells were disrupted as a result of the two treatments, and over 85% of glycolate oxidoreductase was particulate.
Separation of Organelles by Sucrose Density Gradient Centrifugation. A broken cell suspension (7 ml) was carefully layered on top of the sucrose density gradient. This gradient was prepared at 4 C by pipetting in succession 4 ml of 2.0 M sucrose, 4 ml of 1.75 M sucrose, 4 ml of 1.50 M sucrose, 4 ml of 1.25 M sucrose, 4 ml of 1.0 M sucrose, and 4 ml of 0.5 M sucrose into a 40-ml MSE polycarbonate centrifuge tube. The 2.0 M sucrose was prepared in 20 mM glycolllyglycine buffer, pH 7.5, and was diluted with this buffer to the other sucrose concentrations. The centrifuge tube was placed in the bucket of a 3 x 40 ml swing out rotor and spun at 20,000 rpm (65,000g average) for 4.5 hr at 4 to 4 C in an MSE Superspeed 60 centrifuge. At the end of the run, the tube was removed, clamped in an MSE tube piercer, and fractions were removed through a needle from the bottom of the tube and enzymes assayed immediately.

Enzyme Assays. Malate dehydrogenase (EC 1.1.1.37) was assayed spectrophotometrically by measuring the decrease in extinction at 340 nm following the oxidation of NADH by oxaloacetate (20). The reaction mixture contained in a final volume of 3.0 ml, 33 mM potassium phosphate buffer, pH 7.5, 0.33 mM NADH, and cell extract. The reaction was started by the addition of 0.5 μ mole of oxaloacetate. Oxaloacetate was adjusted to the required pH with NaOH before use in the assay. Michaelis constants for NADH were determined by spectrophotometric assay at pH 7.5 in 33 mM HEPES buffer at a constant oxaloacetate concentration of 0.17 mM, whereas for oxaloacetate, Michaelis constants were determined in 33 mM HEPES buffer, pH 7.0 and 9.0, at a constant NADH concentration of 0.33 mM.

Glycolate oxidoreductase was assayed as described previously by Lord and Merrett (18).

Succinate dehydrogenase (EC 1.3.99.1) was assayed by a modification of the procedure of Els (13), which used phenazine methosulfate to couple electron transfer between succinate and 2,6-dichlorophenolindophenol. The reaction mixture contained in a final volume of 3.0 ml, 33 mM potassium phosphate buffer, pH 7.6, 0.1 mM 2,6-dichlorophenolindophenol, 3.3 mM KCN, 6.6 mM sodium succinate, and enzyme. The reaction was started by the addition of 0.1 ml of 1% (w/v) phenazine methosulfate and was measured by following the decrease in extinction at 600 nm.

Fumarase (EC 4.2.1.2) was assayed by the method of Massey (19). The reaction mixture contained in a final volume 3.0 ml, 50 mM phosphate buffer, pH 7.3, 50 mM l-malate, and 0.1 ml of cell extract. The reaction was started by the addition of malate and followed by measuring fumarate formation at 300 nm. Protein was determined by the method of Lowry et al. (18) and chlorophyll by the method of Arnon (2).

Polycrylamide Gel Electrophoresis. Polycrylamide gel electrophoresis was carried out at pH 8.1 according to Davis (10). The running gel was 0.7 x 8.0 cm and was polymerized with ammonium persulfate. The electrolyte contained 2.9 g of glycine, 0.6 g of tris, and 0.5 ml of 1 N HCl per liter; 0.5% (w/v) bromophenol blue in 1% acetic acid was used as a tracking dye. Electrophoresis at a current of 5 mA per tube was carried out for 35 min by which time the tracker dye was within 0.5 cm of the end of the gel. Following electrophoresis the gels were loosened with a fine hypodermic needle under water, removed from the glass columns, and malate dehydrogenase activity was detected by incubating at 37 C in a reaction mixture prepared according to Fine and Costello (14). Protein was detected by staining with 0.1% (w/v) naphtholph blue black in 7% (v/v) acetic acid for 1 hr, removing excess stain with 3% (v/v) acetic acid and fixing in 7% (v/v) formalin.

Reagents. NAD+, NADH, and NADPH were purchased from Sigma Chemical Company, London, oxaloacetic acid and cycloheximide from Koch-Light Laboratories, Bucks., U.K., and chloramphenicol from Parke-Davis & Co. Ltd., Middlesex, U.K. All other materials were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest purity commercially available.

RESULTS

Separation of Organelles by Sucrose Density Gradient Centrifugation. The separation of Euglena cell organelles on sucrose density gradients has previously been carried out by Lord and Merrett (17) with extracts obtained by passing cell suspensions through a French pressure cell at 600 to 800 lb/in². In the present work, cells were broken with a MSK rotary cell homogenizer, because of all methods tried this gave the best yield of particulate enzymes, over 85% of glycolate oxidoreductase activity from the cells consistently being particulate. After centrifugation fractions were collected by eye, the first band was located at the interface between 1.5 M and 1.75 M sucrose layers and was assumed to contain mitochondria while the second band formed at the 1.25 M and 1.5 M sucrose interface and consisted of chloroplasts. A third band formed at the interface between the 1.0 M and 1.25 M sucrose layers, and enzyme assays showed that this contained the peroxisomes. Succinate dehydrogenase and fumarase were taken as mitochondrial marker enzymes because attempts to locate mitochondria by cytochrome c oxidase assays were unsuccessful confirming the observation of Krawiec and Eisenstadt (16) that euglenoid flagellates lack a conventional cytochrome c oxidase. Fraction 3 (Fig. 1) gave a peak of activity for both these enzymes, confirming that this fraction contained the bulk of mitochondria. Glycolate oxidoreductase was taken as a marker enzyme for peroxisomes, and a peak of activity was present in fraction 5 (Fig. 1). Electron microphotographs of this fraction showed that it contained single membrane-bound cell organelles similar to those reported in intact Euglena cells by Graves et al. (15). Malate dehydrogenase showed peaks in fractions 3 and 5, the peak in fraction 3 represented mitochondrial malate dehydrogenase, whereas activity in fraction 5 was a specific peroxisomal enzyme. After sucrose density gradient centrifugation of broken cell suspensions of Euglena, 90% of the protein, 85% of glycolate oxidoreductase, 91% of malate dehydrogenase, 70% of fumarase, and 63% of succinate dehydrogenase were recovered in various gradient fractions.

Acrylamide gel electrophoresis of the gradient fractions at pH 8.9 confirmed the existence of different isoenzymes. Fractions 1, 2, and 3 contained mitochondrial malate dehydrogenase of low electrophoretic mobility, fractions 4 and 6 showed no activity, whereas fraction 5 contained malate dehydrogenase of greater electrophoretic mobility showing that peroxisomal malate dehydrogenase was a different isoenzyme from the mitochondrial enzyme. In fraction 7, three distinct bands of malate dehydrogenase activity were present (Fig. 2). One band corresponded to the mitochondrial enzyme, a second to the peroxisomal enzyme, whereas the third band present only in fraction 7 showed the greatest electrophoretic mobility.

pH Dependence. The pH optima for malate dehydrogenase activity in various cell fractions was determined using the standard assay system but varying the pH by means of zwitterionic buffers to minimize changes in ionic strength. The optimum pH for mitochondrial malate dehydrogenase activity was pH 8.0, while for the peroxisomal enzyme it was 8.5 (Fig. 3). The supernatant fraction gave a broad pH optimum for malate
dehydrogenase over the range pH 7.0 to pH 9.0, presumably resulting from the presence of all three isoenzymes.

**Michaelis Constants for Oxaloacetate and NADH.** In order to characterize further the various isoenzymes, the kinetic properties of the malate dehydrogenases of the various cell fractions were compared (Fig. 4). The effect of oxaloacetate concentration on reaction rate for mitochondrial malate dehydrogenase activity is given in Figure 4. At oxaloacetate concentrations greater than 0.17 mM substrate inhibition was observed, whereas at 0.9 mM oxaloacetate, 50% inhibition of activity resulted. From Lineweaver-Burke plots the apparent \( K_m \) (oxaloacetate) at pH 7.5 for the mitochondrial fraction was 30 \( \mu \)M. The peroxisomal malate dehydrogenase in response to increasing oxaloacetate concentration showed a much sharper

![Fig. 1. Distribution of enzymic activities after sucrose density gradient centrifugation of *Euglena* cell extracts. A: Succinate dehydrogenase; B: glycollate oxidoreductase; C: malate dehydrogenase; D: fumarase. Enzyme units (---); specific activity (---). Fractions are numbered in order they were drained from centrifuge tube. Width of column is proportional to volume of fraction collected.](image1)

![Fig. 2. Schematic representation of acrylamide gel electrophoresis patterns of malate dehydrogenases in fractions obtained from *Euglena* by sucrose density gradient centrifugation. a: Mitochondrial malate dehydrogenase; b: peroxisomal malate dehydrogenase; c: supernatant malate dehydrogenase. Dotted line represents faint band appearing only at G stage of cell cycle. Number represents order in which fractions were collected from gradient as in Fig. 1.](image2)

![Fig. 3. Effect of pH on malate dehydrogenase activity in cell fractions. Mitochondrial fraction (○); peroxisomal fraction (●); △—△, supernatant fraction (△). Assay as in "Materials and Methods," except pH varied by using 33 mM TES over pH range 5.0 to 7.5 and 33 mM HEPES over the range 6.5 to 9.5.](image3)

![Fig. 4. Effect of oxaloacetate concentration on malate dehydrogenase activity in cell fractions. Mitochondrial fraction (○); peroxisomal fraction (●); supernatant fraction (△). Assay as in “Materials and Methods,” except oxaloacetate concentration varied as indicated.](image4)
inhibition of activity than the mitochondrial enzyme (Fig. 4). At oxaloacetate concentrations greater than 0.13 mM, substrate inhibition was observed, while 50% inhibition was reached at 0.25 mM oxaloacetate. The apparent Km (oxaloacetate) for this enzyme at pH 7.5 was 24 μM. Malate dehydrogenase activity in the supernatant fraction did not show significant substrate inhibition (Fig. 4), and the apparent Km (oxaloacetate) for this fraction was 91 μM at pH 7.5. The apparent Km values for malate dehydrogenase activity in all three fractions were pH dependent (Table I).

The pyridine nucleotide specificity of all three isoenzymes was tested, but measurable activity was not recorded with NADPH even when the cell fractions were incubated with dithiothreitol (5 mM final concentration) before assay. The Km values for NADH were very similar for malate dehydrogenase activity in the mitochondrial and peroxisomal fractions, but the Km (NADH) was greater for the supernatant fraction (Table I).

**Table I. Apparent Michaelis Constants for Malate Dehydrogenase in Euglena Cell Fractions**

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>pH</th>
<th>Substrate</th>
<th>Km (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial</td>
<td>7.5</td>
<td>OAA1</td>
<td>30</td>
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<tr>
<td></td>
<td>9.0</td>
<td>OAA</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>NADH</td>
<td>107</td>
</tr>
<tr>
<td>Peroxisomal</td>
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<td>OAA</td>
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<td></td>
<td>9.0</td>
<td>OAA</td>
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</tr>
<tr>
<td></td>
<td>7.5</td>
<td>NADH</td>
<td>222</td>
</tr>
<tr>
<td>Supernatant</td>
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<td>OAA</td>
<td>91</td>
</tr>
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</tr>
<tr>
<td></td>
<td>7.5</td>
<td>NADH</td>
<td>95</td>
</tr>
</tbody>
</table>

1 Oxaloacetate.

The development of malate dehydrogenase in different cell fractions, corresponding to different isoenzymes, over the division cycle was followed (Fig. 7). Cells were harvested at various stages over the cycle and disrupted with care, and after sucrose density centrifugation, malate dehydrogenase activity in the various cell fractions was determined (Fig. 7). Mitoc-
chondrial malate dehydrogenase activity increased nearly 2-fold over the light phase of the cycle and had doubled by early dark phase (Fig. 7); peroxisomal malate dehydrogenase activity showed a similar pattern. Acrylamide gel electrophoresis of all extracts over the division cycle shared a similar pattern of activity to that obtained by direct enzyme assay in cell fractions, but without gel-scanning equipment, we cannot present confirmatory quantitative results. One interesting feature of acrylamide gel electrophoresis over the division cycle was the appearance of a faint band of activity corresponding to a fourth isoenzyme with an Rₚ value slightly less than the peroxi-

somal enzyme, but only at the t₀ stage of the cycle.

**DISCUSSION**

When crude cell extracts of *Euglena* were subjected to gel electrophoresis, three isoenzymes of malate dehydrogenase were present. Separation of subcellular fractions by density gradient centrifugation gave two particular peaks of malate dehydrogenase plus activity in the soluble fraction, whereas gel electrophoresis of fractions from the gradient gave Rₚ values for malate dehydrogenase that corresponded with the observed Rₚ values for individual isoenzymes in the crude homogenate. Thus, although in higher plant tissues several malate dehydrogenase isoenzymes have been reported (11, 21, 28–30) in relation to total cellular protein, there appear to be only three major isoenzymes in phototrophically grown *Euglena* cells corresponding to intracellular localizations in the mitochondria, peroxisomes, and cytoplasm. Although a fourth isoenzyme can be detected at the t₀ stage of the cycle, it is present in only trace amounts at one stage of the cycle, so it seems unlikely it can have an important role in cellular metabolism in phototrophically grown *Euglena*.

The expression of malate dehydrogenase activity over the cell cycle in *Euglena* can be compared with enzymes previously investigated, being unlike phosphoglycerate or phosphoglycolate phosphatases, both of which showed increased activity over the dark phase of the cycle (5) or phosphopyruvate carboxylase which was a “peak” enzyme (6). The pattern of activity resembles that for ribulose 1,5-diphosphate carboxylase (4) doubling over the light phase, marginally increasing by the t₁ stage, but decreasing again by the t₀ stage, so that activity has just increased 2-fold over a complete cycle. This is as would be expected for *de novo* synthesis of enzyme activity in increasing activity taking place at the time of maximum protein synthesis (8). Although Codd (3) has shown a flavon mononucleotide-mediated photoinduction of malate dehydrogenase in *Euglena* extracts, there was no direct effect of light on malate dehydrogenase activity over the cell cycle.

Specific inhibitors of protein synthesis show that increased malate dehydrogenase activity results from the synthesis of new enzyme. The increase in activity was effectively inhibited by cycloheximide added to the culture at concentrations known to inhibit protein synthesis of *Euglena* cytoplasmic ribosomes although not affecting chloroplast ribosomes (1, 25). The addition of chloramphenicol in amounts known to inhibit preferentially protein synthesis on organelle ribosomes only slightly inhibited increase in malate dehydrogenase activity (1, 25). Although some doubt exists regarding the specificity of these inhibitors in higher plant systems (12), the protein-synthesizing machinery of *Euglena* differs from the higher plant, in that the cytoplasm contains an unusually large 8S ribosome (24), while these inhibitors have been shown to effectively inhibit protein synthesis on *Euglena* ribosomes in vitro (1).

The temporal expression of mitochondrial and peroxisomal malate dehydrogenase isoenzymes was similar (Fig. 7), both showing a near doubling of activity over the light phase of the cycle. Soluble malate dehydrogenase showed a similar pattern, and much of the activity in this fraction was shown by electrophoresis to have the same Rₚ values as the mitochondrial and peroxisomal isoenzymes. Whether this represented solubilization of isoenzymes during cell organelle isolation or malate dehydrogenase not yet incorporated into cell organelles is difficult to determine. The cell disruption technique finally adopted was selected for consistency in the proportion of particulate to soluble enzyme for specific organelle marker enzymes at a given stage of the cell cycle. Succinate dehydrogenase and glycolate oxidoreductase were always at least 65% and 85% particulate, respectively, suggesting that at least some soluble malate dehydrogenase represents enzyme not yet incorporated into cell organelles. However, succinate dehydrogenase and probably glycolate oxidoreductase being membrane-bound enzymes would be less easily lost from partially damaged organelles than malate dehydrogenase.

**LITERATURE CITED**