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Isolation and Enzymic Characterization of *Euglena* Proplastids

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ABSTRACT

Organelles were isolated from dark-grown *Euglena gracilis* Klebs by sucrose density gradient centrifugation. Plastids, identified by triosephosphate isomerase and NADP glyoxylate reductase were present at an equilibrium density of 1.24 grams per cubic centimeter clearly separated from mitochondria at an equilibrium density of 1.22 grams per cubic centimeter. Assay for choline phosphotransferase and glucose-6-phosphatase showed that endoplasmic reticulum membranes were present at a density of 1.12 grams per cubic centimeter. The plastid fraction contained phosphofructokinase, pyruvate kinase, triosephosphate isomerase and aldolase indicating the operation of a glycolytic pathway. During regreening pyruvate kinase and phosphofructokinase in the developing proplastid decreased, neither enzyme being present in the mature chloroplast. However, plastids were present in the photosynthetic cell as shown by a peak of glycolysis enzymes at an equilibrium density of 1.24 grams per cubic centimeter.

The integrity of isolated plastids was demonstrated by their capacity for protein synthesis. Plastids isolated from dark-grown cells rapidly incorporated [^{35}S]methionine into protein with an absolute dependence on added ATP. The large subunit of ribulose diphosphate carboxylase was the major polypeptide synthesized by these isolated plastids.

The euglenoid flagellates are unique in being the only algal group to contain proplastids. Those in dark-grown cells of *Euglena* are 1 to 2 μm in size, roughly spheroidal (30, 31) bounded by a double membrane, and contain ribosomes (19). On illumination the proplastids in dark-grown *Euglena* cells differentiate into chloroplasts, providing a model system for investigating the proplastid to chloroplast transition. Proplastids from *Euglena* have not been enzymically characterized nor has the effect of differentiation on enzyme activity been determined. In this paper we report on the enzyme complement of intact proplastids from dark-grown cells and changes in enzyme activity during regreening.

MATERIALS AND METHODS

Growth of Alga. Dark-grown cells were obtained by growing *Euglena gracilis* Klebs strain z Pringsheim at 25 C, with stirring, in the medium of Hutner *et al.* (20) in the dark for 4 days. The cells were harvested by centrifugation, transferred to mannitol resting medium (35), maintained in the dark for 24 h to deplete reserve carbohydrates, then regreened by exposure to continuous white light at 1,500 lux. Asynchronous photosynthetic cultures were grown at 25 C in the phototrophic growth medium of Cramer and Myers (14) at a continuous light intensity of 6,000 lux. The initial inoculum added to 6 liters of medium was 150 ml of an exponential phase culture containing approximately 10^5 cells/ml. Cells were harvested in midexponential phase of growth by centrifugation at 1,000*g* 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 Tris-HCl (pH 7.5) and disrupting by three 15-s bursts of ultrasonic waves (MSE Ultrasonic disintegrator 1.5A). After centrifugation at 250*g* for 5 min to remove cell debris the supernatant was used immediately for enzyme assays.

For isolation of intact organelles cells were harvested by continuous flow centrifugation (MSE continuous action rotor, 8,000 rpm), washed once and resuspended in a medium containing 150 mM Tricine (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA (pH 7.5), and 400 mM sucrose to give a 50% (v/v) suspension. This suspension was added to an equal volume of glass ballotini (No. 10) in a prechilled mortar, and gently ground for 5 min at 2 C. The beads were washed three times with half-volumes of isolation medium, the combined washings centrifuged at 250*g* for 5 min and the supernatant used for separation of organelles.

Separation of *Euglena* Organelles. Organelles were separated by sucrose density gradient centrifugation. Portions (5 ml) of cell extract were layered onto sucrose gradients, which consisted of 25 ml sucrose solution increasing linearly in concentration from 30 to 60% (w/w) over a 2-ml cushion of 60% sucrose and topped with a 5-ml layer of 20% (w/w) sucrose. All sucrose solutions contained 1 mM EDTA (pH 7.5). Gradients were centrifuged for 2.5 h at 65,000*g* (average) in a SW 27 rotor on a Beckman L5.40 centrifuge. After centrifugation 1-ml fractions were collected from the bottom of the gradient using a Beckman density gradient fractionator.

Enzyme Assays. Spectrophotometric assays were carried out in silica cuvettes (3.0-ml volume, 1-cm light path) with a Gilford series 2000 recording spectrophotometer. Activities of the following enzymes were determined using the assays cited: fumarase (13), malate dehydrogenase (13), citrate synthase (13), succinate dehydrogenase (13), fructose diP aldolase (18), triose-P isomerase (5), triose-P dehydrogenase (7), pyruvate kinase (11), phosphofructokinase (23), glucose-6-phosphatase (27), fructose 1-6 diphosphatase (1), glyoxylate reductase (36), ribulose-1,5-biP carboxylase (25), phosphorylcholine-glyceride transferase (8). Protein was determined by the method of Lowry *et al.* (26), Chl was determined by the method of Arnon (2) and Pi by the method of Atkinson *et al.* (3).

Protein Synthesis by Isolated Plastids. Fractions from sucrose gradients showing maximum triose-P isomerase activity were pooled and diluted to 0.4 M sucrose by the slow addition of isolation medium lacking sucrose. The diluted proplastic fraction was centrifuged at 20,000*g* for 15 min and the proplastids gently resuspended in the KCl resuspension medium of Siddell and Ellis (33) containing 100 μmol KCl, 33 μmol Tricine-KOH (pH 8.3), 3.3 μmol $MgCl_2$ in a final volume of 0.5 ml. The proplastid suspension was preincubated at 25 C with 1 μmol ATP, 3 μmol creatine phosphate, and 50 μg creatine phosphokinase before adding 5 μCi L-[^{35}S]methionine (480 Ci/mmol). Aliquots were removed at intervals and the reaction stopped by the addition of 1 ml 20% (w/v) trichloroacetic acid and 100 μl of a saturated aqueous solution of L-[^{32}S]methionine and left overnight at room temperature. The precipitate was filtered onto a glass fiber disc, washed with 100 ml ice-cold 5% (w/v) trichloroacetic acid, 60 ml absolute ethanol, and 10 ml diethyl ether. The discs were dried at 60 C, transferred to vials containing Bray's solvent/scintillator,

and counted at 50% efficiency in a Packard Tri-Carb spectrometer. All solutions used in proplastid isolation and *in vitro* protein synthesis were sterilized before use.

Determination of Polypeptides Synthesized by Isolated Plastids. After incubation of plastids in the complete reaction medium for 1 h the reaction was stopped by the addition of 100 μl of aqueous L-[^{32}S]methionine and the plastids pelleted by centrifugation at 20,000*g* for 15 min. The supernatant fraction and the pellet were separated and the pellet resuspended in 1 ml of a hypotonic buffer containing 2.5 mM Tris-glycine (pH 8.5) and 4 mM 2-mercaptoethanol. The supernatant was added to the lysed plastid and the suspension centrifuged at 100,000*g* for 30 min. The clear supernatant was removed, 240 μl of an aqueous solution of 20% (w/v) SDS added to it, and the sample boiled for 3 min. Soluble proteins were separated by electrophoresis in buffers containing 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol on gels polymerized from 10% (w/v) acrylamide as described previously (9).

Gels were stained for protein with Coomassie blue, scanned at 600 nm with a Gilford gel scanner, frozen with powdered solid CO_2, and cut into 1-mm-thick slices with a Mickle gel slicer. Gel slices were dissolved in 200 μl of H_2O_2 (30%) by heating at 70 C for 2 h, then added to 5 ml of Bray's solvent/scintillator and counted at 50% efficiency in a Packard Tri-Carb spectrometer.

Purification of Ribulose DiP Carboxylase. The enzyme was purified as described previously from *E. gracilis* (9).

RESULTS

Separation of Organelles by Sucrose Density Gradient Centrifugation. After centrifugation of a broken-cell suspension of *Euglena* on a sucrose gradient the distribution of specific marker enzymes for organelles was determined (Fig. 1). Fumarase was assayed as a marker enzyme for mitochondria and gave a sharp peak of activity in fraction 20 corresponding to an equilibrium density of 1.22 g/cm^3 (Fig. 1). Triose-P isomerase and NADP glyoxylate reductase were taken as marker enzymes for plastids and these enzymes showed sharp peaks of activity in fraction 25 corresponding to an equilibrium density of 1.24 g/cm^3, but considerable triose-P isomerase activity was also present in the soluble fraction of the gradient. Neither NADPH triose-P dehydrogenase nor ribulose diP carboxylase was detected after gradient centrifugation but this was as expected because both enzymes are labile and present in only low amounts in dark-grown *Euglena*.

Choline phosphotransferase was selected as a marker enzyme for the ER and assay for this enzyme over a gradient gave a clear-cut peak of activity in fraction 10, corresponding to an equilibrium

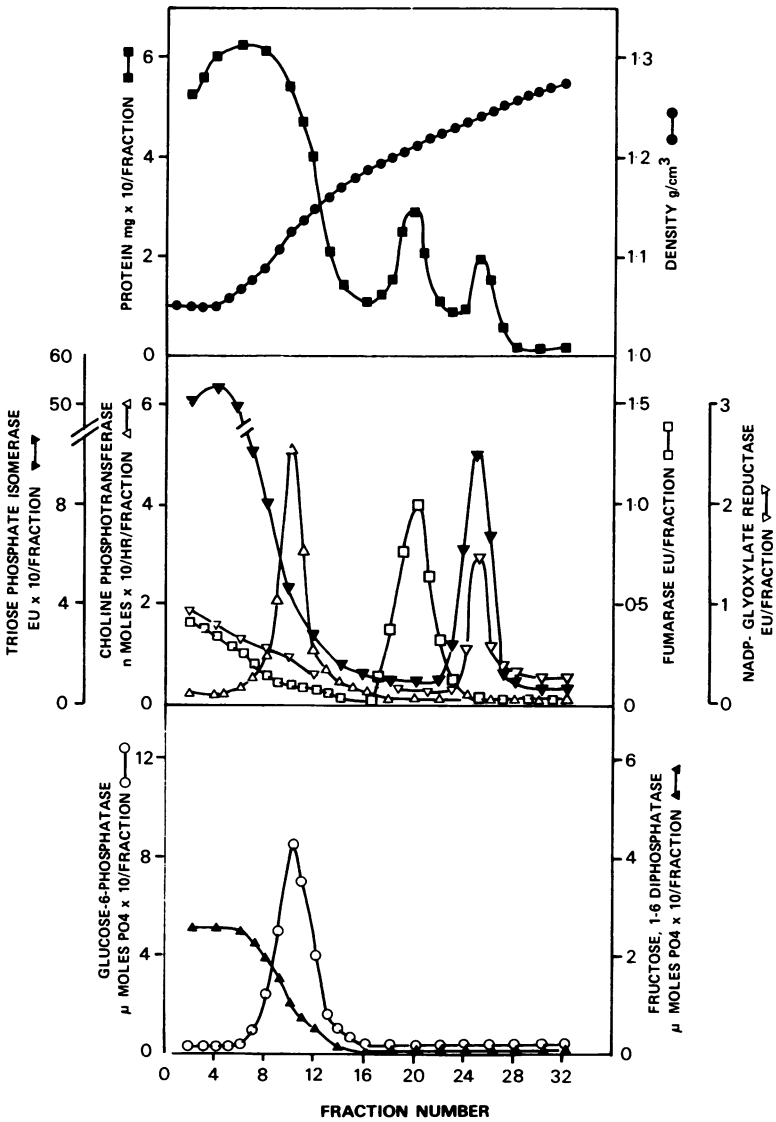

FIG. 1. Distribution of organelles on a sucrose gradient from bleached cells of *Euglena*.

density of 1.12 g/cm^3. A second marker enzyme for the ER, glucose-6-phosphatase, was assayed and a peak of activity confirmed the presence of ER in fraction 10 (Fig. 1).

The proximity of the mitochondrial fraction to the proplastids on the gradient prompted the assay of several mitochondrial enzymes over the gradient. Citrate synthase, malate dehydrogenase, and succinic dehydrogenase all showed peaks of activity in fraction 20, with no activity in fraction 25, confirming the conclusions derived from results with fumarase that the proplastid fraction was free from contamination by mitochondria.

Activity and Localization of Glycolysis Enzymes during Regreening. The transition from organotrophic to phototrophic growth requires a shift in metabolism from the breakdown of preformed carbon compounds by glycolysis to a dependence on carbon compounds derived from the Calvin cycle. Phosphofructokinase, pyruvate kinase, and triose-P isomerase all showed a major decrease in specific activity during regreening so that in fully regreened cells the activity had fallen to only 10% of that in

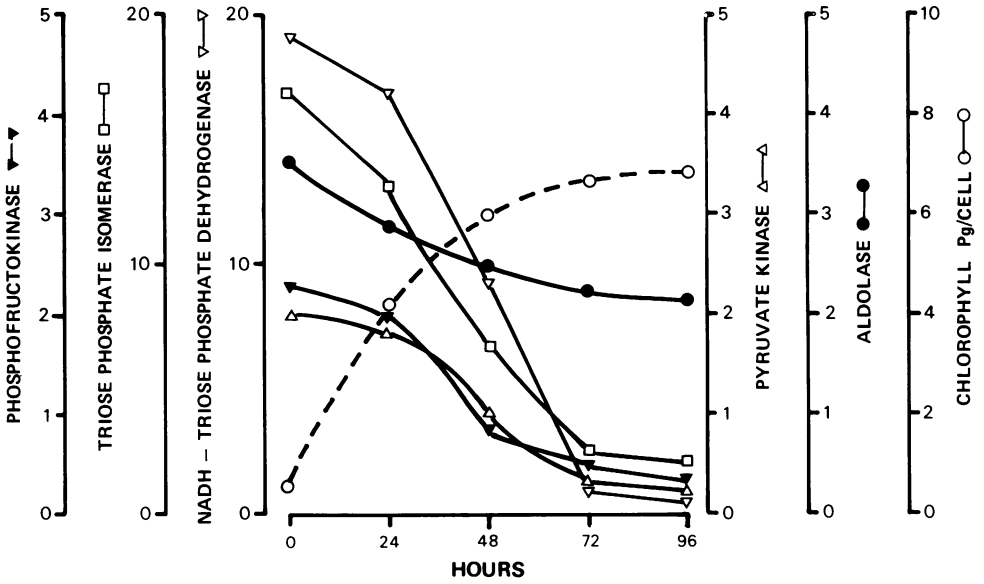

FIG. 2. Changes in enzyme activity during regreening of bleached *Euglena* cells. All activities expressed as μmol/h·mg protein.

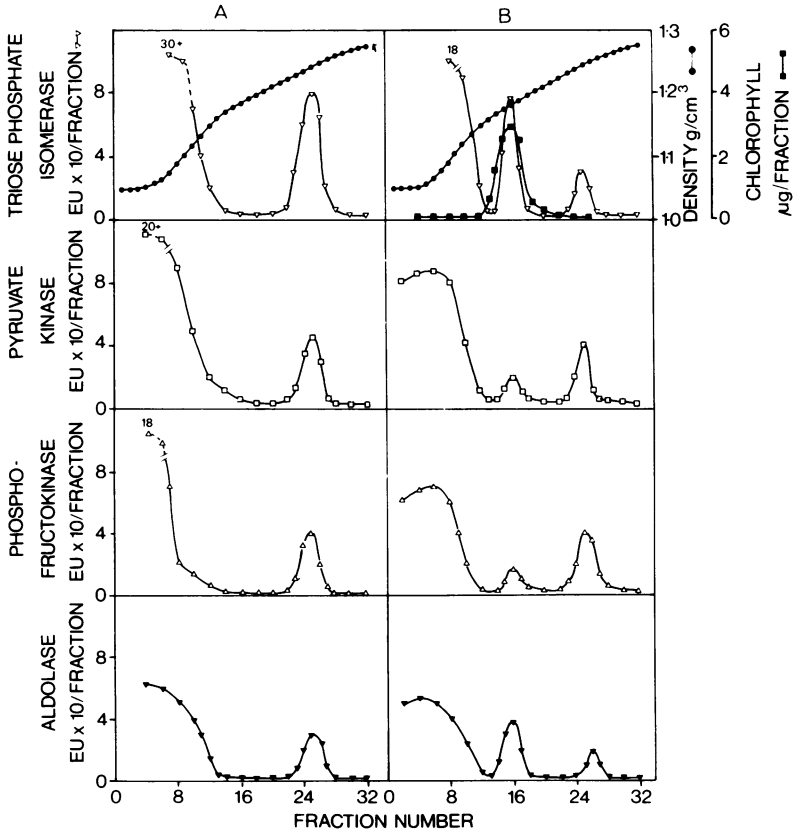

FIG. 3. Effect of regreening on distribution of *Euglena* enzymes on sucrose gradients. A: gradient from bleached cells; B: gradient from cells after regreening for 15 h.

dark-grown cells. Over the same period the decrease in aldolase was far less, so that fully regreened cells still showed appreciable activity (Fig. 2). Regreening cells in resting medium show no net synthesis of protein so a decrease in specific activity does not result from an increase in total protein with enzyme activity per cell remaining constant. When expressed on a per cell basis, phosphofructokinase activity decreased from 3 μmol/min·10^7 dark-grown cells to 0.5 μmol/min·10^7 cells after 96 h regreening.

In dark-grown cells both phosphofructokinase and pyruvate kinase showed peaks of activity in fraction 25 on sucrose gradients, corresponding to the marker enzymes for proplastids (Fig. 3). Aldolase also gave a peak of activity in fraction 25 (Fig. 3) as did hexokinase (results not shown). In contrast, the key enzymes of gluconeogenesis, fructose 1,6-diphosphatase, and glucose-6-phosphatase were not detected in the proplastid fraction (Fig. 3). Fructose 1,6-diphosphatase was present only in the soluble fractions of the gradient and glucose-6-phosphatase in the ER fraction (Fig. 3).

The buoyant density of the plastids altered during development from 1.24 g/cm^3 in dark-grown cells to 1.18 g/cm^3 after regreening for 15 h and 1.17 g/cm^3 for the mature chloroplast. The change in buoyant density from 1.24 g/cm^3 to 1.17 g/cm^3 coincided with Chl formation. Organelles which equilibrated at a buoyant density of 1.24 g/cm^3 always lacked Chl. After 15-h illumination, lower levels of phosphofructokinase and pyruvate kinase were detected in the developing proplastid fraction than in fraction 25, the plastid fraction of dark-grown cells. Aldolase and triose-P isomerase gave peaks of activity in fractions 16 and 25 (Fig. 3) corresponding to both the developing proplastid and the original plastid fractions. Fumarase gave a peak of activity in fraction 20 throughout regreening showing that the buoyant density of mitochondria was unaltered during development.

The percentage of the total enzyme activity present in the developing proplastid was unchanged for aldolase and increased for triose-P isomerase during regreening (Fig. 4) but there was a dramatic decrease in aldolase activity in the original plastid fraction (Fig. 4). The presence of these "nondeveloping" plastids may have resulted from a proportion of the cell population failing to regreen so giving a mixture of dark-grown cell plastids and developing chloroplasts. This possibility was investigated by growing cells phototrophically on inorganic medium for many generations to eliminate nonphotosynthetic cells. Separation of organelles from these cultures still resulted in phosphofructokinase and pyruvate kinase activity at an equilibrium density of 1.24 g/cm^3 (Fig. 5). Ribulose diP carboxylase gave a peak of activity in fraction 14 corresponding to the chloroplasts, with a smaller peak in fraction 25, corresponding to the plastids in dark-grown cells. Thus, an organelle fraction, equilibrating at the same density as plastids from dark-grown cells, and containing a significant proportion of the total activity of glycolysis enzymes, was still present in chloroplast-containing cells.

Protein Synthesis by Isolated Plastids. Plastids from dark-grown *Euglena* were able to incorporate L-[^{35}S]methionine into trichloroacetic acid-insoluble material with a linear time course for the first 10 to 20 min (data not shown). The incorporation was completely dependent on added ATP, increased by an ATP-regenerating system, and the ATP requirement could not be replaced by light. Protein synthesis was unaffected by cycloheximide at 15 μg/ml but was inhibited by 90% D-*threo*-chloramphenicol at 1 mg/ml.

The nonmembrane polypeptides synthesized by isolated plastids were analyzed by SDS-polyacrylamide gel electrophoresis. A

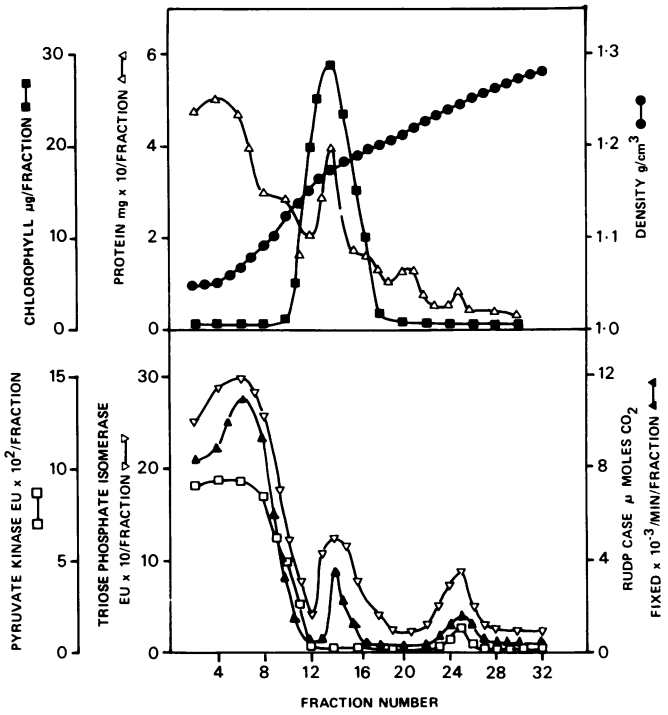

FIG. 5. Distribution of organelles on a sucrose gradient from photosynthetic *Euglena* cells.

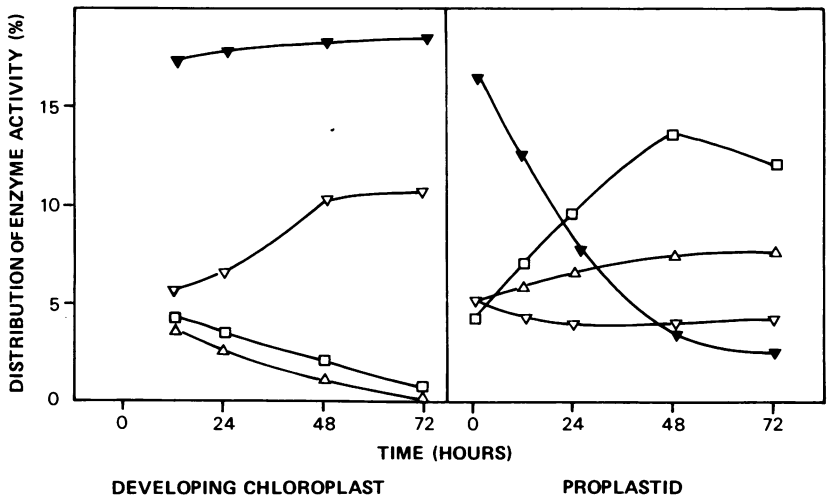

FIG. 4. Distribution of glycolysis enzymes between plastid and developing proplastid during regreening of *Euglena* cells. (▼——▼): Aldolase; (▽—▽): triose-P isomerase; (□——□) pyruvate kinase; (△——△): phosphofructokinase.

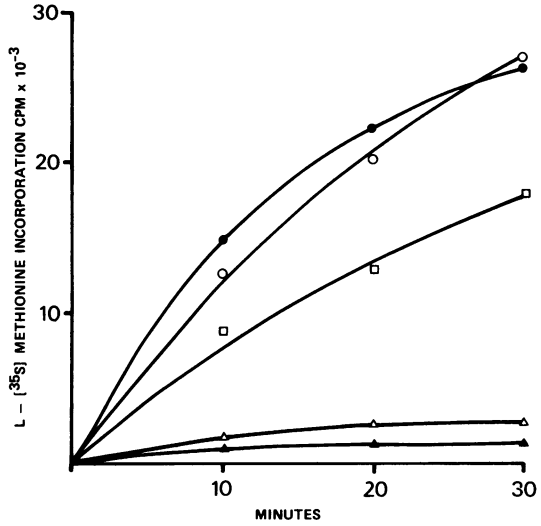

FIG. 6. Protein synthesis by plastids isolated from dark-grown bleached *Euglena* cells. (○——○): Complete reaction mixture with creatine phosphate/creatine phosphokinase; (●——●): complete reaction mixture plus 15 μg/ml cycloheximide; (△——△): complete reaction mixture plus 1 mg/ml D-*threo*-chloramphenicol; (▲——▲): reaction mixture minus ATP and creatine phosphate/creatine phosphokinase; (□——□): reaction mixture with ATP but no creatine phosphate/creatine phosphokinase. Each reaction mixture contained 100 μg protein.

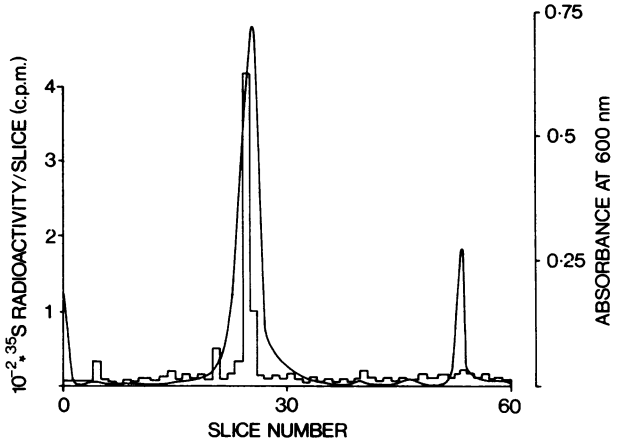

FIG. 7. Comparison of polypeptides synthesized by isolated plastids with purified ribulase diP carboxylase. Polypeptides were extracted from plastids as described under "Materials and Methods" and separated by SDS-polyacrylamide gel electrophoresis.

small amount of purified ribulose diP carboxylase was added to the extract before electrophoresis for comparison. The bulk of the [^{35}S]methionine was incorporated into one major polypeptide that corresponded to the large subunit of ribulose diP carboxylase (Fig. 6). Moreover, the incorporation of [^{35}S]methionine into this polypeptide was completely inhibited by chloramphenicol.

DISCUSSION

Proplastids have been isolated from several higher plant tissues (16, 29, 32, 34) but separation of *Euglena* proplastids from mitochondria was not achieved by zonal centrifugation (22) while urografin gradients were required to obtain an enriched proplastid fraction for ultrastructural studies (28). Minor modification of the method previously used to isolate functional *Euglena* organelles (12, 13) gave a plastid fraction, uncontaminated by mitochondria at an equilibrium density of 1.24 g/cm^3, similar to the value obtained for higher plant proplastids (10, 16). NADP glyoxylate reductase, a presumptive proplastid enzyme (36), was taken as a marker for plastid integrity (32) and following centrifugation 50% of the activity was recovered from the soluble fraction showing a 50% plastid breakage. Identification of the plastid nature of the fraction was reinforced by studies of its ability to accomplish protein synthesis. This activity was inhibited by D-*threo*-chloramphenicol, an effective inhibitor of organelle ribosomes in *Euglena* (4) as elsewhere, but not by the inhibitor of cytoplasmic ribosomes. Analysis of the products of protein synthesis by SDS-polyacrylamide gel electrophoresis demonstrated the synthesis of one major polypeptide by isolated plastids. (Fig. 7). This polypeptide had the same R_F value as the large subunit of ribulose diP carboxylase, a characteristic polypeptide of proplastids (33).

The presence of phosphofructokinase, pyruvate kinase, triose-P isomerase and aldolase shows the operation of a glycolytic pathway in the *Euglena* plastid, as in the plastids of castor bean endosperm (15, 34). The peak of glycolysis enzymes in photosynthetic cells remaining at an equilibrium density of 1.24 g/cm^3 suggests the presence of two types of organelle in dark-grown cells both equilibrating at this density. These would be plastids that contain glycolysis enzymes but do not develop into chloroplasts, and proplastids that on illumination develop into chloroplasts. After 15-h regreening the developing proplastids, now clearly separated from the plastid fraction, also contain glycolysis enzymes showing that these enzymes were present in the true proplastids in dark-grown cells. The activities of phosphofructokinase and pyruvate kinase decreased in proplastids as they developed so that neither enzyme was detected in the mature chloroplast, in contrast with the presence of both enzymes in higher plant chloroplasts (6, 21). Nongreening plastids in photosynthetic cells may be necessary for the mobilization of the reserve carbohydrate, paramylon, which is always formed in the cytoplasmic matrix of the cell never inside the chloroplast.

The introduction of a 20% (w/w) sucrose cushion on the gradient gave a clear-cut peak of activity for the ER enzymes choline phosphotransferase (24) and glucose-6-phosphatase at a density of 1.12 g/cm^3. The degree of contamination of the ER membranes by other membranes such as Golgi body fragments could not be determined because potential marker enzymes for Golgi, such as latent IDPase, were not detected after gradient centrifugation. The isolation of distinct ER membrane and proplastid fractions from a regreening cell provides a useful technique for investigating the role of the ER in the synthesis of organelle components such as chloroplast phospholipids (17).

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