Immunogold Localization of Ribulose-1,5-Bisphosphate Carboxylase with Reference to Pyrenoid Morphology in Chloroplasts of Synchronized Euglena gracilis Cells

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ABSTRACT

Euglena gracilis strain (Z) cells were synchronized under phototrophic conditions using a 14 hour light:10 hour dark regimen. The cells grew during the light period (growth phase) and divided during the following 10 hour period either in the dark or in the light (division phase). Changes in morphology of the pyrenoid and in the distribution of ribulose-1,5-bisphosphate carboxylase:oxygenase (Rubisco) within the chloroplasts were followed by immunoelectron microscopy during the growth and division phases of Euglena cells. Epon-embedded sections were labeled with an antibody to the stroma, and less densely distributed throughout the stroma. Toward the end of the division phase, the pyrenoid began to form in the center of a chloroplast, and the immunoreactive proteins started to concentrate over that rudimentary pyrenoid. During the growth phase, small areas rich in gold particles, called 'satellite pyrenoids,' were observed, in addition to the main pyrenoid. From a comparison of photosynthetic CO₂-fixation with the total carboxylase activity of Rubisco extracted from Euglena cells in the growth phase, it is suggested that the carboxylase in the pyrenoid functions in CO₂-fixation in photosynthesis.

The pyrenoid is a specialized region of the chloroplast of many algae, taking the form of a proteinaceous mass of material (5, 7, 8). Holdsworth (9) showed that pyrenoids isolated from the green alga Eremosphaera viridis had high specific activity of ribulose-1,5-bisphosphate carboxylase, and that these two polypeptides of the same size as the two subunits of the carboxylase made up 90% of the total pyrenoid protein. Similar observations were made for pyrenoids isolated from the prasinophycean alga Micromonas squamata (26) and the brown alga (Pilayella littoralis) (11, 12). Satoh et al. (27) suggested that much of the Rubisco of the green alga Bryopsis maxima is localized in the pyrenoid, and that 10 minor polypeptides comprised about 15% of the total pyrenoid protein. Recently, nitrate reductase has been found in the pyrenoids of several green algae (16).

Vladimirova et al. (28) studied the distribution of Rubisco in the chloroplasts of two green algae Chlamydomonas reinhardtii and Dunaliella salina by the immunofluorescence method and observed an intense fluorescence over the pyrenoid. The distribution of Rubisco in the pyrenoidal thylakoid region of the chloroplast of C. reinhardtii was also studied by Lacoste-Royal and Gibbs (15) by immunoelectron microscopy. They reported that about 40% of the total small subunit in the plastid and 30% of the large subunit are localized in the thylakoid region, presumably in the stroma, the remaining portions existing in the pyrenoid. In a recent immunoelectron microscope study of Chlorella pyrenoidosa by McKay and Gibbs (21), it was shown that in either light-limited or light-saturated cells, the pyrenoid was heavily labeled by antibody to each subunit of Rubisco, whereas chloroplast stromal labeling was not above background levels, suggesting that the pyrenoid Rubisco is functional in vivo. Kuchitsu et al. (14) characterized polypeptides of the pyrenoid isolated from C. reinhardtii, and found that substantial amount of Rubisco protein is present as a particulate form in the pyrenoid.

Recent studies in our laboratory (25) showed that the pyrenoid is absent in proplastids of dark-grown cells of Euglena gracilis, and when these cells are transferred to an inorganic medium, rudimentary pyrenoids are formed in the dark in developing plastids, with fully developed pyrenoids only appearing after exposure of these cells to light. Cook et al. (4) reported that the pyrenoid is recognized in the chloroplasts of synchronized cells of E. gracilis only in the first half of the light period. Recently, Kiss et al. (13) showed by immunofluorescence method that the pyrenoid of E. gracilis is labeled by a Rubisco antibody, and that the presence and disappearance of the pyrenoid occurring under different nutritional conditions are related to the distribution of Rubisco in the chloroplast.

In the present study, we followed changes in morphology of the pyrenoid and distribution of Rubisco in the chloroplasts of synchronized cells of E. gracilis during the cell cycle by immunoelectron microscopy, and found that the appearance and disappearance of the pyrenoid is accompanied by concentration of Rubisco in the pyrenoid and its dispersion through-
measurements of photosynthetic CO₂-fixation in Euglena cells were taken at appropriate times were mixed with 2 mL of 100 mm (Hepes)-KOH buffer (pH 6.8) in a 5 mL vial and then aerated for 10 min. Photosynthetic CO₂ fixation was assayed in the presence of added 300 mm NaH¹⁴CO₃ (520 dpm/nmol) under the same light and temperature conditions as in the cultivation of the cells. Part of the reaction mixture (1 mL) was withdrawn at appropriate times, and the reaction was stopped by adding 0.5 mL of 4 m formic acid. The acidified mixture was dried in an oven at 70°C. Acid, heat-stable ¹⁴C was counted with a scintillation counter.

Preparation of Antibody Against Rubisco

Euglena Rubisco was purified as described previously (30). Possible contaminants in the purified Rubisco preparation were removed by DEAE-cellulose chromatography by the method of McCurry et al. (20), and stored as ice beads of the polyethylene glycol-undissolved enzyme (30). Three mg of purified Rubisco was dissolved in 1 mL of 10 mm potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl, and emulsified with 1 mL of a complete Freund's adjuvant. A male albino rabbit (about 2 kg weight) was intradermally injected with the emulsion at multiple sites. The rabbit was again immunized with 3 mg of Rubisco in a similar way 3 weeks later, and a booster injection containing 1 mg of Rubisco and an incomplete Freund's adjuvant was given 3 weeks after the second immunization. Two days after the booster injection, the rabbit was bled, and the serum was prepared by standard methods. The γ-globulin fraction was isolated by ammonium sulfate fractionation, and dialyzed thoroughly against 10 mm sodium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl.

Protein was determined by the method of Bradford (1) with bovine serum albumin as the standard.

Immunogold Electron Microscopy

Euglena cells were fixed in glutaraldehyde (final concentration 2%; w/v) in 0.01 m phosphate-buffer (pH 7.2) for 60 min at 4°C, rinsed in the same buffer and blocked in agar, followed by dehydration in ethanol series and then acetone. Samples were embedded in Epon-812 resin (Tabb Lab.). Thin sections were placed on nickel slit grids. These were floated section-
Figures 2-7. Figure 2. A cell at the beginning of the light period (0-h cell) labeled with anti-Rubisco followed by protein A-gold (x20,000). The arrow indicates the pyrenoid region, where gold particles are denser than in the rest of the chloroplast. C: chloroplast. In control cells treated with preimmune serum, the labeling with gold was insignificant in all immunogold experiments.
side down on drops of 0.01 M PBS with 0.5% BSA for 30 min at room temperature, and then incubated in PBS with 0.5% BSA containing antibody to Rubisco or in preimmune rabbit serum at 37°C for 10 min. The sections were washed twice in PBS, and incubated in PBS containing protein A-gold for 20 min. Sections were subsequently stained with uranyl acetate and viewed in a JEOL 100B electron microscope at 80 kV. In control experiments, cells were treated with preimmune serum prior to protein A-gold labeling.

RESULTS

Synchronized *Euglena* cells grew during the 14 h light period ('growth phase') and divided during the following 10 h period either in the dark or in the light ('division phase'), as seen from Figure 1 (upper panel). The cell division in the light was delayed by a few hours. The carboxylase activity of Rubisco extracted from these synchronized cells increased nearly twofold during the growth phase and decreased, in terms of the activity per cells, during the division phase (Fig. 1, middle panel). As seen in Figure 1 (lower panel), the rate of photosynthetic CO₂-fixation by the cells increased during the initial several hours in the light, attaining the maximum level around 10 h and gradually decreased thereafter even in the light.

As seen from Figures 2 and 3, the chloroplasts of the cells at the beginning of the light period (0-h cells) contained rudimentary pyrenoids, and when sections were labeled with anti-Rubisco followed by protein A-gold (see "Materials and Methods"), gold particles were more concentrated in the pyrenoid region than in other areas of the chloroplasts. These rudimentary pyrenoids rapidly changed into typical pyrenoids with adjacent paramylum granules, and the gold particles also became markedly concentrated over the pyrenoid, as seen from Figures 4 and 5. Chloroplast division began soon after the beginning of the cell division (6). Shortly before the start of the chloroplast division, the pyrenoid began to disappear concurrently with the dispersion of gold particles from the pyrenoid region into the stroma, and became totally undetectable in the chloroplasts during the division phase of the cell cycle either in the dark (Figs. 6 and 7) or in the light (Figs. 10 and 11). Gold particles in these chloroplasts were completely dispersed throughout the stroma. Toward the end of the division phase, gold particles in the chloroplasts appeared to be arranged in short lines in the narrow area of stroma between thylakoids, either in the dark (Fig. 8) or in the light (Fig. 12). Subsequently, the gold particles were again seen to be clustered in the center of chloroplast (Fig. 9), concurrently with the reappearance of the rudimentary pyrenoid (Fig. 13).

When immunoelectron micrographs of serial sections through the chloroplasts in actively growing cells were examined in further details, some small areas distinctly rich in gold particles were observed, besides the main pyrenoid region. These areas, called 'satellite pyrenoids,' could not be detected in the cells of the division phase. As seen from Figure 14, some larger satellite pyrenoids were in association with the paramylum granule as was the main pyrenoid. Table I shows the relative distribution of gold particles among the main and satellite pyrenoids and the stroma in four chloroplasts of a serially sectioned 10-h cell.

DISCUSSION

The pyrenoid may be regarded as a device which has become established in the algae for the storage of Rubisco, the vital enzyme of the photosynthetic carbon-fixation cycle (8). It is not known, however, whether Rubisco in the pyrenoid is functional or is simply stored there. Recent studies (14, 21) suggested that pyrenoid Rubisco can be functional in vivo. The data in Figure 1 show that the maximum CO₂-fixation observed in cells (10-h cell) grown under 6000 lux was nearly the same as the total activity of Rubisco extracted from the cells at the same stage. The enzyme activity was measured at 30°C, and the activity at 25°C, the temperature at which the CO₂-fixation of cells was determined, can be estimated as 75% of the measured value according to the result obtained by Yokota et al. (30). When photosynthetic CO₂-fixation was measured at higher light intensities (10,000–20,000 lux), the maximum fixation reached more than 100% of the total enzyme activity of cells (our unpublished data). It was confirmed that at these higher light intensities, the pyrenoid was present in the chloroplasts of growing cells. This might imply that Rubisco in the pyrenoid can function in photosynthetic CO₂-fixation, although kinetic characteristics of the enzyme existing in the pyrenoid remain to be worked out.

Considering a possible relationship between the algal pyrenoid and certain inclusions of higher plant chloroplasts, Griffiths (8) described that a gradual progression can be traced through the plant kingdom, starting with the massive, single pyrenoid, then to smaller multiple pyrenoids, and finally to very small proteinaceous areas distributed throughout the chloroplast. He pointed out that the pyrenoid of the liverwort *Anthoceros* has particular significance in this connection. The liverwort pyrenoid is reported to consist of small units (25 to many hundreds) which may form an aggregated central mass within the chloroplast, or may be distributed throughout the chloroplast as small fragments (17–19). Kaja (10) and Burr (2) described the possible evolutionary steps in pyrenoid disintegration in a comparison of different species of *Anthoceros*, which show a range of forms from a homogenous pyrenoid to complete dispersal throughout the chloroplast. Different morphologies of the pyrenoid, including satellite pyrenoids, in *Euglena* chloroplasts observed during the cell cycle appears

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Figure 3. A transverse section of a chloroplast in a 0-h cell (×28,000). The arrow indicates the pyrenoid region.

Figure 4. A chloroplast in a 13-h cell (×23,000). The arrow shows the pyrenoid, where gold particles are densely localized. Note that paramylum (PA) is present surrounding the chloroplast at the pyrenoid region. V: vacuole.

Figure 5. A transverse section of a chloroplast in a 13-h cell (×15,000). The arrow indicates the pyrenoid.

Figure 6. A section through chloroplasts in a 18-h cell (4 h in the dark) (×11,500). Serial sections showed that no pyrenoid was detectable in these chloroplasts. Note that gold particles are dispersed throughout the chloroplast.

Figure 7. A transverse section of a chloroplast in an 18-h cell (4 h in the dark) (×19,000). No pyrenoid is seen.
Figures 8–13. Figure 8. A section through chloroplasts in a 20-h cell (6 h in the dark) (×17,000). Gold particles are localized in rows in the spaces between the thylakoids.
Table I. Relative Distribution of Rubisco-Gold Particles Among the Stroma and Pyrenoid Regions in Chloroplasts of a Cell from the Growth Phase Culture

The cell sample was taken from a synchronized culture at the 10th h from the beginning of the light period (10-h cell). Gold particles were counted in each of 41 serial sections of the cell and were summed.

<table>
<thead>
<tr>
<th>Chloroplast</th>
<th>No. of Gold Particles in the Stroma</th>
<th>No. of Gold Particles in Pyrenoids</th>
<th>Sum Total</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Main</td>
<td>Satellite</td>
</tr>
<tr>
<td>1</td>
<td>339</td>
<td>1451</td>
<td>300</td>
</tr>
<tr>
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<td>307</td>
<td>1780</td>
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<tr>
<td>4</td>
<td>316</td>
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</tbody>
</table>

Figure 14. A section through a chloroplast in a 10-h cell (x22,400). The upper arrow indicates the main pyrenoid and the lower one a satellite pyrenoid. Note that both pyrenoids have a paramylum granule associated with them.

Figure 9. A section of a chloroplast in a 23-h cell (9 h in the dark) (x22,000). Gold particles are again concentrated in the center of the chloroplast (the pyrenoid region).

Figure 10. A section of a chloroplast in an 18-h cell (18 h in continuous light) (x 8,800). Euglena cells synchronized under the 14 h light: 10 h dark regimen were placed in continuous light. No pyrenoid was detected in the chloroplasts, and gold particles were dispersed throughout the chloroplast, as in the cases in Figures 6 and 7.

Figure 11. A transverse section through a chloroplast in an 18-h cell (18 h in continuous light) (x 22,000). No pyrenoid is seen.

Figure 12. A section through chloroplasts in a 21-h cell (21 h in light) (x18,000). Gold particles appear to be gathering in a row, as in Figure 8.

Figure 13. A section through chloroplasts in a 24-h cell (24 h in light) (x13,000). The arrows indicate pyrenoids. Gold particles are concentrated over the pyrenoid. Note that the paramylum granule is present near the pyrenoid.
to be analogous to the different morphologies of the pyrenoid in the liverwort that might represent evolutionary steps.

The dispersion of pyrenoid in *Euglena* chloroplasts was observed to begin shortly before the start of the chloroplast division in the *Euglena* cell cycle. Chloroplast division begins shortly after cell division (6). The dispersion of the pyrenoid might facilitate distribution of Rubisco between daughter chloroplasts during chloroplast division. According to Cook et al. (4), the pyrenoid was present only in the first half of the light period in the light-dark synchronized *Euglena* cells. Cook (3) reported that chloroplast division took place slightly before cell division. Presumably, the pyrenoid had dispersed and become undetectable long before the chloroplast division under the conditions used by Cook.

Kiss et al. (13) showed by transmission electron microscopy and immunofluorescence microscopy that the appearance and disappearance of pyrenoids concomitant with concentration and dispersion of Rubisco in chloroplasts of *Euglena gracilis* are controlled by nutritional conditions. Low nutrient levels are related to the presence of pyrenoid, while high nutrient levels with the absence of the pyrenoid. Orcaival-Lafont and Calvayrac (23) also found a similar correlation between nutrient levels and pyrenoid presence. Miyachi et al. (22) reported that the pyrenoid is affected by environmental CO₂ concentration. The pyrenoid and the starch sheaths around the pyrenoids develop much more in low-CO₂ cells than in high CO₂-cells of three unicellular algae, *Chlorella* and *Scenedesmus*.

We have observed that the pyrenoid is newly formed, concurrently with accumulation of Rubisco over it, in the dark in the proplastids of dark-organotrophically grown *E. gracilis* cells transferred to an inorganic medium (25).

**LITERATURE CITED**