Polypeptides of a Light-Harvesting Complex of the Diatom Phaeodactylum tricornutum Are Synthesized in the Cytoplasm of the Cell as Precursors\textsuperscript{1}

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ABSTRACT

A light-harvesting fucoxanthin-chlorophyll a/c-protein complex has been isolated from the diatom Phaeodactylum tricornutum by detergent extraction of thylakoid membranes coupled with sucrose density gradient centrifugation. The isolated complex was devoid of photochemical activity and displayed spectral characteristics consistent with light harvesting function. It has three major polypeptides of apparent molecular weights 18,000, 19,000, and 19,500 as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Using protein synthesis inhibitors, these polypeptides were shown to be synthesized on 80S cytoplasmic ribosomes. Antibodies raised to a mixture of the 19,000 and 19,500 dalton components of the complex were used to demonstrate structural similarity among the three polypeptide components. Immunoprecipitation from primary translation products synthesized in a reticulocyte lysate system primed with \textit{P. tricornutum} poly(A) RNA, indicates that the polypeptide components are synthesized as precursors 3,000 to 5,000 daltons larger than the mature polypeptides.

In higher plants and green algae, the light-harvesting Chl-protein complex contains as many as 5 polypeptides plus both Chl \textit{a} and \textit{b} (8). Polypeptides of LHCP\textsuperscript{2} are synthesized as precursors on cytoplasmic ribosomes from nuclear encoded mRNA (29). These precursor polypeptides are translocated across the chloroplast envelope, the transit sequences cleaved, and the mature polypeptides assembled into LHCP in the thylakoid membrane (28). In contrast, several polypeptide components of the light-harvesting phycobilisomes of red algae are synthesized on 70S chloroplast ribosomes (9) and encoded by the plastid genome (19, 20).

Other algae possess light-harvesting complexes rich in photosynthetically active carotenoids. Only a few of these complexes have been isolated, including a peridinin-Chl \textit{a} complex from dinoflagellates (14, 25, 26), a fucoxanthin-Chl \textit{a/c} complex from brown algae (1), and a fucoxanthin-Chl \textit{a/c} complex from a diatom (11). Some of these organisms have very large antennae of light-harvesting pigment-protein complexes, with a Chl:P700 ratio as high as 1300 (10, 24). Furthermore, Chl \textit{c}-containing organisms display a plastid structure and thylakoid arrangement different from that of green algae and higher plants. In the latter, the photosynthetic membranes are separated into stromal lamellae and granal stacks. This distinction does not occur in the plastids of Chl-\textit{c}-containing algae. Higher plant, green and red algal plastids are bounded by a double membrane, while many Chl-\textit{c}-containing algae have an additional membrane, the chloroplast ER, which is continuous with the outer nuclear envelope (30). The effects of these structural differences on the organization of the photosynthetic apparatus and the biosynthesis of these complexes are unknown.

The fucoxanthin-Chl \textit{a/c}-protein complex is the major light-harvesting complex of diatoms (11). We have isolated this complex from the diatom \textit{Phaeodactylum tricornutum} and describe here some of the events involved in its biosynthesis.

MATERIALS AND METHODS

Growth Conditions. \textit{Phaeodactylum tricornutum} Bohlin (University of Texas Culture Collection of Algae, strain 646) was grown at 20°C with continuous illumination of 200 \(\mu\text{E m}^{-2} \text{s}^{-1}\). Cultures were bubbled with air in ESAW artificial seawater medium (13) enriched with 10 times the normal levels of nitrate \((\text{NaNO}_3)\) and phosphate \((\text{K}_2\text{HPO}_4)\) and buffered at pH 7.7 with 10 mm Tris-HCl. Vitamins and silicate were omitted from the medium.

Isolation of the Light-Harvesting Complex. Harvesting of the cells and all subsequent steps were performed at 0 to 4°C. All buffers contained the protease inhibitors benzamidine-HCl (5 mm) and \(\epsilon\)-amino-n-caproic acid (1 mm). Cells from two 10 L cultures (density approximately \(2 \times 10^6\) cells ml\(^{-1}\)) were harvested by centrifugation at 6,000 \(g\) for 10 min, washed with 300 mm sucrose containing 125 mm K-phosphate (pH 7.2) and 10 mm KCl, resuspended in the same buffer, and passed through a chilled French pressure cell at 6,250 p.s.i. The lysed cell suspension was diluted 4-fold with 50 mm Tricine-KOH (pH 7.5) (buffer A) and NaCl was added to 100 mm final concentration. The membranes were pelleted by centrifugation at 40,000 \(g\) for 20 min and the lightly pigmented supernatant was discarded. The pellet was washed once with 40 ml of 2 mm EDTA, 10 mm NaCl, 50 mm Tricine-KOH (pH 7.5), and the final membranous pellet resuspended with a ground glass homogenizer in buffer A to 300 \(\mu\text{g}\) ml\(^{-1}\) Chl \textit{a} + \textit{c} prior to solubilization with 0.4% (w/v) TDOC. Incubation with the detergent was for 30 min on ice. TDOC-extracted membranes were centrifuged for 2.5 min in an Eppendorf model 5414 microfuge. Approximately 90% of the Chl remained in the supernatant after the microfugation. The supernatant was layered onto linear 10 to 60% (w/v) sucrose gradients containing 50 mm Tricine-KOH (pH 7.5), and centrifuged for

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\textsuperscript{2} Abbreviations: LHCP, light harvesting chlorophyll protein; TDOC, sodium taurodeoxycholate; FCPc, fucoxanthin-Chl \textit{a}, \textit{c} protein complex.
15 h at 38,000 rpm in a Sorvall TST-41.14 rotor. Three pigmented bands were resolved on the sucrose gradient: a light green band at the top of the gradient, a diffuse green band at about 30% sucrose, and a dense brown band at 38 to 40% sucrose. The brown band was removed, diluted 4-fold with buffer A plus 100 mM NaCl, and centrifuged for 1 h at 45,000 rpm in a Beckman Ti50 rotor. The dark brown pellet was resuspended by sonication to 300 µg ml⁻¹ Chl a + c in buffer A and Triton X-100 was added to a final concentration of 0.5% (v/v). After a 10 min incubation on ice, 1.0 ml or less of solubilized membranes was loaded onto a 10 to 60% sucrose gradient containing buffer A, and centrifuged for 20 h at 38,000 rpm using a Sorvall TST-41.14 rotor. Two bands were resolved: a dark brown band near the top of the gradient and a green band at 40% sucrose. Both fractions were diluted 4-fold with 50 mM Tricine-KOH (pH 7.5) containing 100 mM NaCl, 5 mM MgCl₂, 50 mM KCl. The fractions were centrifuged at 45,000 rpm in a Beckman 50Ti rotor for 4 h. The addition of MgCl₂ and KCl were required for effective pelleting of the brown membranous material. Pellets were resuspended in either a small volume of 100 mM Na₂CO₃ containing 100 mM DTT, for electrophoretic analysis of the polypeptides, or buffer A for spectral analyses and photochemical assays.

Photochemical Assays and Spectral Analyses. Chl concentrations were determined by using the equations of Jeffrey and Humphrey (17). PSI activity was measured by the light-induced change in Δ at 699 nm due to P700 photooxidation (16) and PSI electron transport was measured by monitoring the reduction of 2,6-dichlorophenol indophenol using 1,5-diphenylcarbohydrazide as an electron donor (27). A Cary model 17 spectrophotometer was used to measure transmission spectra at various N₂ temperatures and a Perkin-Elmer 652 fluorescence spectrophotometer was used for fluorescence spectroscopy at 77 K as previously described (2). For fluorescence emission spectra, the emission slit width was 2 nm and the excitation slit width 8 nm. For fluorescence excitation spectra, the excitation slit width was 2 nm and the emission slit width 8 nm.

Polyacrylamide Gel Electrophoresis. The polypeptide composition of membrane fractions was determined by gel electrophoresis using the Laemmli buffer system (18) and linear gradients of 12 to 18% (w/v) acrylamide containing 8 μM urea. Samples to be analyzed by crossed immunoelectrophoresis and double immunodiffusion were resolved on 7.5 to 15% polyacrylamide gradient gels (6) containing no urea and electrophoresis was performed at 4°C. Membrane samples were suspended in 100 mM DTT containing 100 mM Na₂CO₃ and solubilized by adding one-half sample volume of 5% NaDODSO₄ in 30% sucrose and 0.1% bromophenol blue, and dissociated by boiling for 2 min. Electrophoresis was carried out overnight at a constant current of 7.5 µamp and the polypeptides stained with Coomasie brilliant blue G-250. Mol wt markers were lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), BSA (66,200), and phosphorylase B (92,500).

In Vivo Labeling. Cultures were harvested, washed in sterile ES62 medium minus sulfate, and resuspended in the same medium to about 2 x 10⁸ cells ml⁻¹. After 10 min of illumination at 200 µE m⁻² s⁻¹, [³⁵S]Sulfate (10 µCi ml⁻¹ final concentration) was added to the cultures and the incubation continued for 1 h. Incorporation of radioactive sulfur into TCA precipitates was monitored during the course of the incubation. When appropriate, cycloheximide (1.0 µg ml⁻¹) and chloramphenicol (200 µg ml⁻¹) were included in the medium during the labeling. These levels of inhibition were optimal for the inhibition of protein synthesis on 80S and 70S ribosomes, respectively. Inhibitors were added to the cultures 10 min prior to the addition of labeled sulfate. The brown thylakoid fraction from sucrose gradients following TDOC treatment of total cellular membranes was prepared as described above. Samples were prepared for electrophoresis as described above. Polypeptides were separated by electrophoresis on 7.5 to 15% polyacrylamide gels, and visualized by staining with Coomasie brilliant blue G-250 and autoradiography.

Isolation and Translation of RNA. RNA isolations were performed with the buffers used by Cashmore et al. (5). Mid-log phase cultures of P. tricornutum were pelleted and resuspended in 50 mM Tris-HCl (pH 9.0) containing 15 mM EDTA, 1.0% NaDODSO₄, and 5 mM DTT. The cells lysed under these conditions. The lysate was extracted with phenol, phenol:chloroform (1:1), then twice with chloroform alone. Following extraction, RNA (in the aqueous phase) was sequentially precipitated with 0.8 volumes of isopropanol, 2 ml LiCl, and then twice with two volumes of ethanol. Poly(U)-derivatized Sepharose 4B was used to select for poly(A) RNA (21). After sequential precipitation with isopropanol and ethanol, the poly(A) RNA was vacuum dried, dissolved in sterile, distilled H₂O, and stored at −80°C. Poly(A)RNA was translated in vitro in a reticulocyte lysate system (Bethesda Research Laboratories), used according to the specifications of the manufacturer. Translation mixtures were brought to 3% NaDODSO₄, 15% sucrose, 75 mM DTT, and 2 mM EDTA prior to electrophoresis on a 12 to 18% polyacrylamide gel containing 8 μM urea.

Antibody Preparation and Characterization. Antibodies were prepared against a mixture of the 19,000 and 19,500 D polypeptides associated with the fucoxanthin-Chl a/c-protein complex of P. tricornutum. These polypeptides were isolated by preparative electrophoresis (7.5–15% polyacrylamide gels) of both thylakoid membranes recovered from sucrose gradients following TDOC extraction of cellular membranes, and the isolated light-harvesting complex (see above). Polypeptide bands were excised from the gels, electroluted, combined with Freund's adjuvant, and injected into rabbits following an immunization schedule described by Chua and Blomberg (6). Immunoglobulin G (IgG) was purified from serum by (NH₄)₂SO₄ precipitation followed by DEAE-Sephadex G-50 chromatography (6). Crossed immunoelectrophoresis (6) and double immunodiffusion assays (23) were used to show that the antibodies were specific for the three polypeptide constituents of the light-harvesting complex. Immunoprecipitations from primary translation products were performed according to the protocol of Cashmore et al. (29), except that protein A-Sepharose was used to precipitate the antibody instead of formalin fixed cells of Staphylococcus aureus.

RESULTS

The characteristics of the brown pigmented fraction generated on sucrose gradients following Triton X-100 treatment of P. tricornutum membranes indicate that it is a FCPC involved in light-harvesting. No attempt was made to distinguish between Chl c₁ and c₂. The low temperature absorption spectrum, presented in Figure 1 (dotted line), shows that Chl c and fucoxanthin are present in the complex as demonstrated by A at 470 and 510 to 540 nm, respectively. The Chl c/a ratio of thylakoid membranes from P. tricornutum is about 7:1, while that of the isolated light-harvesting complex is about 3:1. In contrast, the green band isolated on the same sucrose gradients shows less prominent A at 470 and 510 to 540 nm (Fig. 1, solid line), although some is still present.

Fluorescence emission of the complex is maximum at 685 nm when excitation is at 438 nm (Fig. 2, broken line), appropriate for energy transfer to long wavelength absorbing reaction center Chl species. The emission maximum remains at 685 nm when the excitation wavelength is changed to 660 nm (Chl c absorbance) or 540 nm (fucoxanthin absorbance) (not shown), indicating that these pigments are effectively coupled to Chl a. The
Finally, fluorescence excitation spectra (emission: 685 nm) demonstrated the transfer of energy within FCPC from both Chl c and fucoxanthin to Chl a, with excitation (Fig. 3, dotted line) closely following absorbance (Fig. 3, solid line) except in the region near 490 nm. Pigment data indicate that another carotenoid is present in FCPC in addition to fucoxanthin, unpublished data). The additional carotenoid may not be capable of energy transfer to Chl a, which would result in the inefficiency near 490 nm. Energy transfer was completely uncoupled by the addition of 2% NaDodSO4 (Fig. 3, broken line), with a concurrent appearance of Chl c fluorescence emission at about 640 nm (data not shown).

Denaturing PAGE of the thylakoid fraction from sucrose gradients after TDOC extraction of total cellular membranes resolves approximately 25 polypeptides (Fig. 4, lane 3). However, only three polypeptides of mol wt 18,000, 19,000, and 19,500 are present in FCPC (Fig. 4, lane 1). These three polypeptides are depleted from the green band resolved on sucrose gradients after Triton X-100 treatment of thylakoids (Fig. 4, lane 1).

Polyclonal antibodies were raised to a mixture of the 19,000 and 19,500 D polypeptides of the FCPC and were shown to be specific for all three polypeptides of the complex by crossed immunoelectrophoresis against total thylakoid membranes (Fig. 5) as well as against the polypeptides from the isolated complex (not shown). Structural similarity between these three polypeptides was demonstrated by double immunodiffusion (Fig. 6). The confluence of the precipitin lines indicate that they have similar immunochemical properties. The inner ring of precipitin represents an artifact in this preparation.

In vivo labeling studies in the presence of translation inhibitors demonstrated that all three polypeptides of the FCPC are synthesized on 80S cytoplasmic ribosomes. *P. tricornutum* takes up [35S]sulfate and effectively incorporates it into protein. In the absence of protein synthesis inhibitors, essentially all thylakoid membrane polypeptides become labeled (Fig. 7, lane 2). Chloramphenicol, an inhibitor of translation on 70S ribosomes, inhibits the synthesis of most thylakoid polypeptides; however, the FCPC polypeptides continue to be synthesized (Fig. 7, lane 4). Cycloheximide, an inhibitor of translation on 80S cytoplasmic ribosomes, inhibits the synthesis of the FCPC polypeptides while the synthesis of most other thylakoid polypeptides is not affected.
Fig. 4. Analysis of thylakoid membrane polypeptides from *P. tricornutum* by NaDodSO₄ PAGE using a linear 12 to 18% polyacrylamide gradient containing 8 M urea. Electrophoresis and sample preparation are described in "Materials and Methods." Lane 1, green fraction from sucrose gradients after Triton X-100 treatment of thylakoids; lane 2, brown fraction from sucrose gradients after Triton X-100 treatment of thylakoids; lane 3, brown fraction from sucrose gradients, following TDOC treatment of total cellular membranes.

Fig. 5. Crossed immuneelectrophoresis of thylakoid membrane polypeptides using antibodies raised to the 19,000 and 19,500 D polypeptides of FCPC. Polypeptides of the thylakoid band (brown fraction) purified by sucrose gradient centrifugation of TDOC extracted membranes were separated on a 7.5 to 15% NaDodSO₄ polyacrylamide gel (first dimension) and electrophoresed (at right angles to the first dimension) into an agarose bed containing 0.45 mg IgG cm⁻². The antibody preparation was raised to a mixture of the 19,000 and 19,500 D polypeptides. Second dimension electrophoresis was for 16 h at 100 V. Following electrophoresis the agarose was washed extensively with 0.15 M NaCl, 50 mM Tris-HCl (pH 8.0), and then with distilled H₂O prior to staining with Coomassie brilliant blue. 1986 American Society of Plant Biologists. All rights reserved.
Fig. 6. Double immunodiffusion analysis of the polypeptide constituents of FCPC from *P. tricornutum*. Polypeptides of the thylakoid fraction of TDOC extracted cellular membranes were separated on 7.5 to 15% NaDodSO₄ polyacrylamide gels and individual polypeptides were electroeluted from the gels. Isolated polypeptides were placed in the outer wells as indicated, and antibodies raised to a mixture of the 19,000 and 19,500 D polypeptides from FCPC of *P. tricornutum* were placed in the center well. Diffusion proceeded for 24 h at room temperature in an agarose gel. The agarose was washed extensively with 0.15 M NaCl and then with distilled H₂O prior to staining with Coomassie brilliant blue R-250.

(Fig. 7, lane 3). The presence of both inhibitors prevents the synthesis of essentially all thylakoid polypeptides (Fig. 7, lane 5). Synthesis of FCPC polypeptides in the presence of chloramphenicol but not in the presence of cycloheximide indicates that these polypeptides are synthesized on cytoplasmic ribosomes.

In *vitro* translation of poly(A) RNA isolated from *P. tricornutum* generated translation products ranging in mol wt from over 100,000 to approximately 5,000 (Fig. 8, lane 2). No major products were synthesized with mol wt similar to the FCPC polypeptides, and almost no polypeptide synthesis was observed in RNA-minus controls (Fig. 8, lane 5). Two polypeptides were immunoprecipitated from the translation mixture with antibodies raised to the 19,000 and 19,500 D polypeptides of FCPC. These polypeptides had mol wt of 22,500 and 23,000 (Fig. 8, lane 3), between 3,000 and 5,000 larger than the mature FCPC polypeptides (Fig. 8, lane 1). No translation products were precipitated by preimmune serum (Fig. 8, lane 4).

**DISCUSSION**

The FCPC isolated by the procedure presented here is similar in many respects to the complex described by Friedman and Alberte (11) from *P. tricornutum*. However, our characterizations do reveal additional information as well as some differences. While an absorption spectrum of the complex isolated by our procedure is in close agreement with a spectrum previously reported (11), the low temperature (77 K) fluorescence emission spectrum of FCPC displays a maximum at 685 nm. This can be compared to the 675 nm maximum reported at room temperature (11), which looks similar to emission spectra of whole cell...
translated mixture with primed stained electrophoresis and products precipitated from products synthesized (pFCPC); translation ing 154 Plant FIG. 8. products synthesized 31000 X500> C1 O- Immunoprecipitation poly(A) RNA 4, of 19,000 from preimmune and serum; 19,500 products precipitated D, precursors to lytes and Methods.” Lane 1, stained profile of polypeptides from FCPC; lane 2, autoradiogram showing translation products synthesized in a reticulocyte lysate system primed with poly(A) RNA from P. tricornutum; lane 3, primary translation products precipitated from the translation mixture with antibodies prepared against the 19,000 and 19,500 D polypeptides of FCPC (denoted pFCPC); lane 4, primary translation products precipitated from the translation mixture with preimmune serum; lane 5, autoradiogram showing translation products synthesized in a reticulocyte lysate system in the absence of exogenous RNA.

Fig. 8. Immunoprecipitation of precursors to constituent polypeptides of FCPC. Immunoprecipitation was from poly(A) RNA translation products synthesized in a reticulocyte lysate system primed with RNA from P. tricornutum. RNA isolation, translation, immunoprecipitation, and electrophoresis are described in “Materials and Methods.” Lane 1, stained profile of polypeptides from FCPC; lane 2, autoradiogram showing translation products synthesized in a reticulocyte lysate system primed with poly(A) RNA from P. tricornutum; lane 3, primary translation products precipitated from the translation mixture with antibodies prepared against the 19,000 and 19,500 D polypeptides of FCPC (denoted pFCPC); lane 4, primary translation products precipitated from the translation mixture with preimmune serum; lane 5, autoradiogram showing translation products synthesized in a reticulocyte lysate system in the absence of exogenous RNA.

Thylakoid membranes. The difference in maxima may well be caused by temperature differences. Furthermore, we resolve three polypeptides from FCPC of mol wt 18,000, 19,000, and 19,500, in contrast to the single, broad polypeptide band (thought to represent a doublet) previously reported (11). Differences in the polypeptide composition of the complex may be due to differences in the isolation procedures or the resolving ability of the polyacrylamide gel system used to analyze the complex. Since all three polypeptides are present at all stages of the purification procedure, and since protease inhibitors were included in all the buffers during the isolation of FCPC, it seems unlikely that the 18,000 or 19,000 D polypeptide is a proteolytic degradation product of the 19,500 D polypeptide. Immunologically, all three of the polypeptide components of FCPC appear to share at least some determinants, as indicated by the confluence of precipitin lines in double diffusion assays.

In green algae and higher plants, the polypeptides of the light-harvesting complex are synthesized on cytoplasmic ribosomes (28, 29), while in red algae, the light-harvesting phycobiliproteins are synthesized on chloroplast ribosomes (9) and encoded by the plastid DNA (19, 20). Our data clearly demonstrate that the polypeptide of FCPC are synthesized on cytoplasmic ribosomes like the polypeptide components of LHCP. Cycloheximide, an inhibitor of translation on cytoplasmic ribosomes, completely blocks the synthesis of the three polypeptides associated with FCPC, while chloramphenicol, an inhibitor of translation on 70S plastid ribosomes, has little effect on their synthesis. In contrast, the synthesis of almost all other thylakoid polypeptides is blocked by chloramphenicol, indicating their translation on chloroplast ribosomes.

Two primary translation products with mol wt of approximately 22,500 and 23,000 were precipitated from translation mixtures with antibodies raised to a mixture of the 19,000 and 19,500 species. These polypeptides are between 3,000 and 5,000 D larger than the mature FCPC polypeptides. It is of interest that only two translation products were precipitated by these antibodies which react with three polypeptides of the thylakoid membranes. There may be co-migration of two preproteins or two of the three mature polypeptides could arise from different posttranslational modifications of the same precursor.

Generally, nuclear encoded chloroplast proteins are synthesized in the cytoplasm of the cell as precursors (7, 12, 15). Polypeptides of the light-harvesting complex of green algae and higher plants are translated from poly(A) RNA as precursors 4,000 to 5,000 D larger than the mature polypeptides (29). These preproteins pass through the chloroplast envelope and the presequence (transit sequence) is removed. The transit sequence may be important for the transport of cytoplasmically synthesized polypeptides into the chloroplast, but may also serve other functions (i.e., targeting the protein following transport into the organelle). In at least some cases two proteolytic events may be involved in the maturation of transported polypeptides (22). The polypeptides of the diatom FCPC are also synthesized in the cytoplasm as precursors larger than the mature polypeptides. The additional sequences present on these precursors probably are analogous to those of the presequences of light-harvesting complex polypeptides of green plants and function in the transport of these species across the chloroplast envelope. However, in diatoms an additional barrier to the translocation of these polypeptides from the cytoplasm to the thylakoid membranes may exist. A chloroplast ER is present, in addition to the double membrane of the chloroplast envelope. The role of a presequence in the passage of these polypeptides into the chloroplast, and the nature of the barrier presented by the chloroplast ER, remain to be analyzed.
BIOSYNTHESIS OF DIATOM LIGHT-HARVESTING

LITERATURE CITED


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