Biosynthesis of P700-Chlorophyll a Protein Complex, Plastocyanin, and Cytochrome b$_6$/f Complex

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ABSTRACT

Changes in the amount of P700-chlorophyll a protein complex, plastocyanin, and cytochrome b$_6$/f complex during greening of pea (Pisum sativum L.), wheat (Triticum aestivum L.), and barley (Hordeum vulgare L.) leaves were analyzed by an immunochemical quantification method. Neither subunit I nor II of P700-chlorophyll a protein complex could be detected in the etiolated seedlings of all three plants and the accumulation of these subunits was shown to be light dependent. On the other hand, a small amount of plastocyanin was present in the etiolated seedlings of all three plants and its level increased about 30-fold during the subsequent 72-hour greening period. Furthermore, cytochrome f, cytochrome b$_6$, and Rieske Fe-S center protein in cytochrome b$_6$/f complex were also present in the etiolated seedlings of all three plants. The level of each subunit component increased differently during greening and their induction pattern differed from species to species. The accumulation of cytochrome b$_6$/f complex was most profoundly affected by light in pea leaves, and the levels of cytochrome f, cytochrome b$_6$, and Rieske Fe-S center protein increased during greening about 10–20-, and more than 30-fold, respectively. In comparison to the case of pea seedlings, in wheat and barley leaves the level of each subunit component increased much less markedly. The results suggest that light regulates the accumulation of not only the chlorophyll protein complex but also the components of the electron transport systems.

Dark-grown higher plants are incapable of photosynthesis, primarily because they lack Chl and some polypeptides essential for the photosynthetic reactions (6). Upon illumination of dark-grown plants, the synthesis of Chl and previously missing polypeptides occurs together with the induction of photosynthetic activities.

The expression of both nuclear and chloroplastic genomes is required to achieve light-regulated chloroplast development. The biosynthesis of light-harvesting Chl a/b protein associated with PSII and ribulose 1.5-bisphosphate carboxylase has been extensively studied (2, 5, 20, 26). The increased formation of these proteins occurring upon illumination of dark-grown plants is partly due to the increase in levels of the corresponding mRNAs (2, 5, 20, 26). In contrast, there is a paucity of information concerning the biosynthesis of P700-Chl a$^2$ protein complex, plastocyanin, and Cyt b$_6$/f complex.

The P700-Chl a protein complex in higher plants is composed of several subunit components (4, 16, 17, 23). The subunit I (60–65 kD) is comprised of reaction center Chl (P700) and directly involved in the photochemical reaction (23). Although the function of the small subunits is largely obscure, it has been postulated that they have a role as electron acceptors of P700 and/or structural components of the protein complex (4, 12). Recently, Nechushtai and Nelson (18) have reported that a significant amount of subunit I is present in the etiolated seedlings of oat, bean, and spinach and its amount does not significantly change during greening, whereas the small subunits of P700-Chl a protein complex accumulate only after those plants are illuminated. On the contrary, the absence of subunit I in the etiolated seedlings of barley has been reported by Viering and Alberte (27). The effects of light on the biosynthesis of subunit I reported by these two groups are clearly different. Moreover, relatively few workers have studied the synthesis of the small subunits of P700-Chl a protein complex. We have studied the biosynthesis of large and small subunits of P700-Chl a protein complex during greening using other plant species.

Most biochemical studies on the accumulation of plastocyanin, Cyt f, and Cyt b$_6$ during greening have been performed by spectroscopic methods (3, 7, 8, 10, 19). Previous results have shown that in the dark-grown bean leaves, only a very small amount of Cyt f and b$_6$ is present and a marked accumulation starts upon illumination (7). In contrast, dark-grown barley leaves contain a large amount of Cyt f and b$_6$, and there is little increase upon illumination (3, 8, 10, 19). In addition to Cyt f and b$_6$, it is known that Cyt b$_6$/f complex contains a Rieske Fe-S center protein and a 17 kD protein of unknown function (13). The biosynthesis of Rieske Fe-S center protein and 17 kD polypeptide during greening has not been studied. We have measured the levels of above described proteins during chloroplast development in pea, wheat, and barley by using an immunochemical quantification method (25). For this study, specific antibodies raised against subunits I and II of P700-Chl a protein complex, plastocyanin, Cyt f, Cyt b$_6$, and Rieske Fe-S center protein were used throughout.

MATERIALS AND METHODS

Plant Materials. The pea (Pisum sativum L.), wheat (Triticum aestivum L.), and barley (Hordeum vulgare L.) seeds were soaked in tap water overnight and planted in vermiculite. Pea seedlings were grown in darkness at 20°C, and both wheat and barley seedlings were grown at 28°C. The period of etiolation was 12 d for pea, 10 d for wheat, and 8 d for barley. At the end of these periods, the plants were illuminated with tungsten lamps providing 10 W/m$^2$. After exposure to light for various times, apical buds approximately 2 cm long of pea and the upper 4 to 5 cm of primary leaves of wheat and barley were harvested and used.

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2 Abbreviation: P700, reaction center chlorophyll of photosystem I.

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Biosynthesis of Chloroplast Proteins

Electron paramagnetic resonance spectra were made using a JEOB JES-FE1XG X-band spectrometer with an Oxford Instrument liquid helium cryostat and variable temperature system. Microwave power and temperature for electron paramagnetic resonance spectra were 5 mW and 13 K, respectively. Spectroscopic method to estimate the contents of Cyt f and b₆ in the membranes was carried out using a Hitachi 557 double beam spectrophotometer according to the procedure of Leto and Miles (15).

Electrophoresis was carried out usually on 13% (w/v) acrylamide-SDS gels according to Laemmli (14). Proteins and hemes were stained with Coomassie brilliant blue R-250 and 3',5',5'-tetramethylbenzidine/hydrogen peroxidase (24), respectively. Protein was determined according to the method of Lowry and was determined according to Arnon as previously described.

RESULTS

Protein Isolation and Antibody Preparation. Polypeptides of Cyt b₆ and Rieske Fe-S center protein were isolated from the preparation of native Cyt b₆/f complex as described under "Materials and Methods." The absorption spectrum of the Cyt b₆/f complex preparation was essentially the same as that reported by Hurt and Hauska (13) (data not shown). The specific activity of plastoquinol-plastocyanin oxidoreductase was high (about 10 \( \mu \text{mol-nmol}^{-1} \cdot \text{h}^{-1} \)). The electron paramagnetic resonance spectrum of the purified Cyt b₆/f complex showed the characteristic signal \((g=1.89)\) of Rieske Fe-S center protein (data not shown). The polypeptide composition of the purified Cyt b₆/f complex tested by SDS-PAGE is presented in Figure 1 track 1. The four main bands of 33, 23, 20, and 17 kD can be seen by Coomassie blue staining. Two hemi-associated bands of 33 and 23 kD were identified as Cyt f and \(b₆\), respectively (Fig. 1, track 2). Rieske Fe-S center protein could be separated from the Cyt b₆/f complex by hydroxyapatite column chromatography. The bands for Rieske Fe-S center protein and its depleted Cyt b₆/f complex are shown in Figure 1, tracks 3 and 4, respectively. Identification of the 20 kD polypeptide as the Rieske Fe-S center protein was confirmed by its electron paramagnetic resonance spectra (data not shown).

Immunological specificity was examined for the quantitative analysis of the developmental formation of P700-Chl a protein complex, plastocyanin, and Cyt b₆/f complex. It was of prime importance to obtain specific antisera. The antisera raised against Cyt f, Cyt b₆, and Rieske Fe-S center protein only cross-reacted with 33 (Fig. 1, track 5), 23 (track 6), and 20 kD (track 7) of the purified Cyt b₆/f complex, respectively. Specificity of the antibodies against subunits I and II of P700-Chl a protein complex from spinach is also shown in Figure 1. As can be seen in track 8, P700-Chl a protein complex contains five polypeptides (60–65, 23, 20, 18, and 12 kD). The antibodies against subunits I and II of P700-Chl a protein complex reacted only with a single antigenic protein in the complex (Fig. 1, tracks 9 and 10). Immunological cross-reactivities of each antibodies against the spinach polypeptides such as subunits I and II of P700-Chl a protein complex, plastocyanin, Cyt b₆, and Rieske Fe-S center protein with the corresponding proteins of pea, wheat, and barley was observed. It was also confirmed that antibody against Cyt f from Brassica comosa cross-reacted with Cyt f from all three species. Linearity of staining intensity and the antigen amount was observed.

Formation of P700-Chl a Protein Complex in Pea, Wheat, and Barley Seedlings. In pea, there was a prominent lag of Chl formation about 24 h after exposure of the plant to light. On the other hand, in both wheat and barley Chl formation started instantaneously upon illumination. To measure changes of polypeptide abundance in leaves during chloroplast development, an immunological quantification method was employed. It was

for protein extraction.

Protein Extraction. Seedlings (2 g) were frozen in liquid N₂, powdered by means of pestle and mortar, and further homogenized using 2 ml of 50 mM Tris-HCl (pH 8.0) buffer containing 0.2 M sucrose, 10 mM NaCl, 0.1% (w/v) Na₂SO₄, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through two layers of Miracloth (Calbiochem) and used as the total protein fraction of leaves. The membrane and soluble protein fractions of leaves were prepared by centrifugation of the total fraction for 5 min at 15,800 g. Each fraction was frozen and stored at −80°C until analyzed.

Protein Purification and Antibody Preparation. The P700-Chl a protein complex from spinach chloroplasts was purified as previously described (23). The photochemically active reaction center protein containing only subunit I was prepared from the purified P700-Chl a protein complex as previously described (23). The polypeptides of subunits I and II of P700-Chl a protein complex were prepared by electroelution from polyacrylamide gels after SDS-electrophoresis of the purified reaction center protein and P700-Chl a protein complex, respectively. Cyt b₆/f protein complex from spinach was purified according to the method of Hurt and Hauska (13) after a minor modification. After fractionation by (NH₄)₂SO₄, Cyt b₆/f complex was applied to a Sephacryl S-300 column (90 cm × 3 cm) equilibrated with 10 mM Tris-HCl (pH 8.0) and 0.05% (v/v) Triton X-100. Fractions rich in Cyt b₆/f complex were concentrated by ultrafiltration (Millipore Immersible CMX-10) and applied to the same column once again. Cyt b₆ and Rieske Fe-S center proteins were prepared by the electroelution of a polyacrylamide gel after SDS-electrophoresis of the purified Cyt b₆/f complex. Cyt f and plastocyanin were purified from Brassica komatsuna and spinach, respectively, as previously described (21, 22).

Purified specimens of each individual protein or subunit (0.5 mg) were mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into white female rabbits. Then 0.5 mg proteins mixed with Freund's incomplete adjuvant was injected twice at 2-week intervals. Rabbits were bled 2 to 3 weeks after the last booster injection, and specificity of the antibodies obtained was examined by an immunodiffusion analysis (see below).

Detection of the Relative Amounts of Polypeptides in the Seedlings. To measure the relative amounts of polypeptides in the leaves, samples were electrophoresed on 13% (w/v) acrylamide-SDS gels according to the methods of Laemmli (14). The proteins separated were electrophoretically transferred to the nitrocellulose filters by using a Marysol (Tokyo, Japan) Trans Blot apparatus. The nitrocellulose filters were presoaked for 1 h in 10 mM phosphate buffer (pH 7.5) containing 150 mM NaCl, 0.1% (v/v) Tween 20, 0.1% (w/v) Na₂SO₄, and 5% (w/v) skim milk (PBS-Tween-Milk solution), and then treated with the specific antibodies for 1 h at room temperature. After washing with PBS-Tween-Milk solution for 1 h, alkaline phosphatase-conjugated goat anti-rabbit antibody was incubated with the filter papers for 50 min. The antigen-antibody complex was then detected by immersing the blot sections in 0.15 M barbital sodium (pH 9.0), 4 mM MgCl₂, 0.01% (w/v) Nitroblue tetrazolium, and 0.005% (w/v) 5-bromo-4-chloro-3-indoly-phosphate. The resulting purple bands were scanned by a densitometer (Shimadzu Dual-Wavelength TLC Scanner CS-910).

Assay Methods. Activity of Cyt b₆/f complex was assayed using a Hitachi 557 spectrophotometer by measuring the activity of plastoquinol-plastocyanin oxidoreductase. The wavelength pairs 597 to 540 nm was used. The assay mixture contained in a total volume of 1 ml: Cyt b₆/f complex 10 μM, oxidized plastocyanin 2 μM, 10 mM Tris-HCl (pH 8.0), and 0.05% (v/v) Triton X-100. The reaction was started by the addition of spinach plastoquinone (13).
found that the subunits I and II of P700-Chl a protein complex are not detectable in the dark-grown seedlings of pea, wheat, and barley. Figure 2 shows that subunit I of P700-Chl a protein complex is undetectable in the etiolated seedlings of pea (Fig. 2A) and wheat (Fig. 2B), and its accumulation is light dependent. Moreover, it took 24 h for pea and 12 h for wheat for the appearance of the subunit I after exposure of the plant to light. A similar pattern was observed with the light-induced formation of subunit II of P700-Chl a protein complex in the pea (Fig. 2C) and wheat (Fig. 2D) leaves. The pattern of accumulation of subunits I and II in barley leaves is presented in Figure 5.

Appearance of Cyt b₆/f Complex and Plastocyanin. The Cyt b₆/f complex contains four main polypeptides, of which only a Rieske Fe-S center protein is encoded by the nuclear DNA (1). We attempted to analyze the amount of Cyt f, Cyt b₆, and Rieske Fe-S center protein by the same immunochemical technique as described above. We have found that Cyt f was present in the etiolated seedlings of pea, wheat, and barley, but the relative amounts differed from one species to the other. Etiolated seedlings of pea contain a small amount of Cyt f and its amount increased about 10-fold upon 72 h illumination as shown in Figure 3A. In contrast, etiolated wheat seedlings contained a significant amount of Cyt f and its amount increased only about 2-fold upon illumination (Fig. 3B). The amount of Cyt f in the etiolated seedlings of barley was almost the same as that of greened one (Fig. 5). These results were also confirmed by the heme-staining and the spectroscopic assay (data not shown). We also found that Cyt b₆ was present in the etiolated seedlings of all three plants (Figs. 3 and 5). The presence of large amount of Cyt f and b₆ in the etiolated barley seedlings has been reported by heme-staining and incorporation of [14C]α-aminolevulinic acid (11), which is consistent with the present result (Fig. 5).

Only a small amount of Rieske Fe-S center protein was detected in the etiolated pea leaves and the level increased more than 30-fold during greening (Fig. 3E). In the etiolated seedlings of wheat and barley, a relatively higher amount of the protein existed and the level increased about 5-fold and 1.7-fold, respectively (Figs. 3F and 5).

The formation of plastocyanin was analyzed by using the antibody directed against spinach plastocyanin. This protein has been reported to be encoded by the nuclear DNA (6). Since it is known that plastocyanin is easily released from thylakoid membranes, the total protein fraction from seedlings at various stages were electrophoresed and subjected to the immunoblotting. As shown in Figures 4 and 5, only a small amount of plastocyanin was present in the etiolated seedlings, and its level increased about 30-fold during the 72 h greening period in all three plant species tested.
Fig. 2. Immunological detection of subunits I and II of P700-Chl a protein complex during greening of etiolated pea and wheat seedlings. For experimental details see text. In each case 20 µg of leaf membrane proteins were electrophoresed. A, subunit I of pea seedlings; B, subunit I of wheat seedlings; C, subunit II of pea seedlings; D, subunit II of wheat seedlings. Exposure to light for dark-grown leaves was: 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), 48 h (lane 4), and 72 h (lane 5).
**Fig. 3.** Immunological detection of Cyt f, Cyt b₆, and Rieske Fe-S center protein during greening of etiolated pea and wheat seedlings. The experimental conditions were the same as those of Figure 2. A, Cyt f of pea seedlings; B, Cyt f of wheat seedlings; C, Cyt b₆ of pea seedlings; D, Cyt b₆ of wheat seedlings; E, Rieske Fe-S protein of pea seedlings; F, Rieske Fe-S protein of wheat seedlings. The exposure of light was: 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), 48 h (lane 4), and 72 h (lane 5).

**DISCUSSION**

Numerous investigations have given evidences that etioplasts contain both soluble and insoluble membranous polypeptide components required for photosynthesis, but they lack Chl and organized structure of photosynthetic membrane system (6). Extensive studies have been carried out on the biosynthesis of light-harvesting Chl a/b protein (2, 5). It has been hypothesized that synthesis of this protein is regulated by light and the stabilization of the mRNA and the accumulation of this protein depends on the availability of Chl (2, 5).

Recently, P700-Chl a protein complex has been highly purified from several higher plant species (4, 16, 17, 23) and its biosynthesis has been studied (12, 18, 27). Vierling and Alberte (27) have reported that little or no subunit I accumulates in the etiolated barley seedlings, whereas the accumulation of a significant amount of subunit I in the etiolated oat, bean, and spinach leaves has been reported by Nechushtai and Nelson (18). Heij et al. (9) have also detected subunit I in the dark-grown thylakoid membranes of Spirodela oligorhiza using antiserum directed against *Pisum* subunit I. Our data are consistent with the previous finding by Vierling and Alberte of the light-dependent biosynthesis of subunit I in barley leaves (27). Since we could not detect the subunits I and II of P700-Chl a protein in the dark-grown
other hand, the in and the level increased about...troscopy of Phaseolus that plastocyanin during greening.

Subunit II of P700-Chl a protein complex was also not detectable in the etiolated pea, wheat, and barley seedlings, and a measurable accumulation of the polypeptide became prominent at almost the same time as that of subunit I, supporting a coordinated synthesis of the subunits I and II in the P700-Chl a protein complex. Recently, the absence of two small subunits in the etiolated barley seedlings has also been reported (12). Thus, the synthesis of small subunits of P700-Chl a protein complex is induced by light in all plant species tested hitherto (12, 18).

There have been two reports concerning the biosynthesis of plastocyanin during greening. Haslett and Cammack (8) have reported that plastocyanin was detectable in the etiolated leaves of Phaseolus vulgaris by electron paramagnetic resonance spectroscopy and the level increased about 8-fold 120 h after illumination. They have also suggested that phytochrome is involved in the accumulation of the polypeptide in bean leaves. On the other hand, Plesnicar and Bendall (19) have reported that the abundance of plastocyanin in barley leaves does not change during greening. Results of our investigation are consistent with those previous investigations showing its accumulation in the etiolated seedlings. However, we have found that the level of the protein increased about 30-fold during greening of pea, wheat, and barley leaves. In pea leaves, light-regulated biosynthesis of ribulose 1,5-bisphosphate carboxylase has been extensively studied (20, 26). This protein is present in the etiolated pea leaves and the level increases 15-fold during 72 h greening period (20). In comparison to this case, the light-dependent accumulation of plastocyanin seems quite significant.

We have also found that in pea leaves the levels of Cyt f, Cyt b6, and Rieske Fe-S center protein are regulated by light in much the same way as that of plastocyanin. The light effect on the biosynthesis of these polypeptides is less significant in wheat and barley leaves. In all three plant species, the levels of Cyt f, Cyt b6, and Rieske Fe-S center protein increased in a time sequential manner. The pattern of induction of Cyt f and b6 as presented in Figures 3 is somewhat different even though it is known that both genes are located on the chloroplast genome. Therefore, it is worthwhile to elucidate the differential light effect on the biosynthesis of both cytochromes. It must be also noted that the
Fig. 5. Pattern of the accumulation of chloroplast proteins during greening of etiolated barley seedlings. Basic experimental conditions were the same as those of pea and wheat leaves. A, (●), subunit I of P700-Chl a protein complex; (▲), subunit II of P700-Chl a protein complex; (■), plastocyanin. B, (●), Cyt f; (▲), Cyt b; (■), Rieske Fe-S center protein.

level of Rieske Fe-S center protein which is encoded in the nuclear genome increased the latest. One might postulate that the formation of this protein is a limiting element for the expression of Cyt b6/f complex activity during the greening of etiolated plants. However, further study on the relationship between biosynthesis of apo-protein and heme or iron-sulfur centers in Cyt b6/f complex is needed to establish this. Our data suggest that light regulates biosynthesis of not only Chl protein complex but also electron transport carriers in chloroplasts. Information concerning the levels of the corresponding mRNAs is needed to better understand the biosynthetic mechanisms of those chloroplast proteins.

LITERATURE CITED

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