Regulation of Synthesis of the Photosystem I Reaction Center

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ABSTRACT The in vivo biosynthesis of the P700 chlorophyll *a*-apoprotein was examined to determine whether this process is light regulated and to determine its relationship to chlorophyll accumulation during light-induced chloroplast development in barley (*Hordeum vulgare* L.). Rabbit antibodies to the 58,000–62,000-mol-wt apoprotein were used to measure relative synthesis rates by immunoprecipitation of in vivo labeled leaf proteins and to detect apoprotein accumulation on nitrocellulose protein blots. 5-d-old, dark-grown barley seedlings did not contain, or show net synthesis of, the 58,000–62,000-mol-wt polypeptide. When dark-grown barley seedlings were illuminated, net synthesis of the apoprotein was observed within the first 15 min of illumination and accumulated apoprotein was measurable after 1 h. After 4 h, P700 chlorophyll *a*-apoprotein biosynthesis accounted for up to 10% of the total cellular membrane protein synthesis. Changes in the rate of synthesis during chloroplast development suggest coordination between production of the 58,000–62,000-mol-wt polypeptide and the accumulation of chlorophyll. However, when plants were returned to darkness after a period of illumination (4 h) P700 chlorophyll *a*-apoprotein synthesis continued for a period of hours though at a reduced rate. Thus we found that neither illumination nor the rate of chlorophyll synthesis directly control the rate of apoprotein synthesis. The rapidity of the light-induced change in net synthesis of the apoprotein indicates that this response is tightly coupled to the primary events of light-induced chloroplast development. The data also demonstrate that *de novo* synthesis of the apoprotein is required for the onset of photosystem I activity in greening seedlings.

The etioplasts present in leaves of dark-grown plants are predifferentiated organelles which contain many of the soluble and membrane protein components required for photosynthesis, but which are devoid of chlorophyll and organized photosynthetic membranes (1). When dark-grown seedlings are illuminated, photosynthetic activity appears early and increases rapidly as a result of the rapid production and assembly of the thylakoid membrane components. The chlorophyll-binding proteins, the chlorophyll *a/b*-protein and the P700 chlorophyll *a*-protein, account for the majority of thylakoid membrane protein (2). Therefore, the control of biosynthesis of these proteins is fundamental to both photosynthetic membrane development and chloroplast biogenesis.

Recently a number of control points in the regulation of chlorophyll *a/b*-protein synthesis has been investigated in several species of higher plants (3–9) and in the green alga *Chlamydomonas* (10, 11). The apoprotein of this complex is composed of one or more subunits of ~28,000 mol wt which are the products of nuclear encoded genes (12, 13). Evidence from in vitro translation experiments suggests that synthesis of the chlorophyll *a/b*-protein is controlled by the light-regulated production and/or stabilization of the apoprotein mRNA (3–5, 7, 8, 10). Similar experiments have shown that in higher plants phytochrome may mediate this light effect (4, 9). A second control appears to operate at the level of apoprotein stability. It has been observed that the chlorophyll *a/b*-protein mRNA is present in the dark under certain experimental conditions such as high temperature or after a period of illumination (4, 6, 10). However, in vivo accumulation of the apoprotein is not observed under these conditions. From these results it has been hypothesized that apoprotein accumulation depends on the availability of chlorophyll (4, 6, 10), which is synthesized only during illumination (1).

In contrast, there is a paucity of information concerning the control of biosynthesis of the P700 chlorophyll *a*-protein.

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The P700 chlorophyll *a*-protein complex is composed of a single polypeptide species which exhibits molecular weight heterogeneity on SDS gel electrophoresis migrating as two components of 58,000 and 62,000 mol wt (14, 15). This polypeptide is considered to be a chloroplast gene product (16, 17), but has not yet been mapped to the chloroplast genome. It is clear that extensive accumulation of the chlorophyll-binding form of the P700 chlorophyll *a*-protein roughly parallels the increase in chlorophyll content of developing chloroplasts (18–20). Electrophoretic analyses of etioplast and chloroplast membrane polypeptides also suggest that the apoprotein is absent from etioplasts, or present at much lower levels than in mature chloroplasts (21–23). However, the relationship between synthesis of the apoprotein and accumulation of the chlorophyll-protein has not been studied.

To investigate the regulation of production of the P700 chlorophyll *a*-protein we have examined the in vivo biosynthesis of the 58,000–62,000-mol-wt apoprotein in both dark-grown barley seedlings and during the first 12 h of light-induced chloroplast development. In addition, experiments were performed to examine biosynthesis in the dark after a period of illumination. Our results demonstrate that light rapidly and specifically increases the net rate of synthesis of the apoprotein, but that light is not strictly required for either the continued synthesis or accumulation of the apoprotein once development has been initiated.

MATERIALS AND METHODS

Plant Growth: Barley (*Hordeum vulgare* L. cv. Himalaya) seedlings were grown and maintained for all experiments in controlled environment chambers at 23°C and a relative humidity of 70–80%. Seeds were planted in vermiculite and watered with half-strength Hydrosol (Peters Fertilizer, Cambridge, MA). For developmental studies seeds were germinated and grown in a dark chamber for 120 h (5 d). At this stage of development, seedlings were 8–12 cm high and the primary leaf had emerged 3–6 cm from the coleoptile. Seedlings were then transferred to an illuminated chamber with a light intensity of 300 $\mu E\ m^{-2}\ sec^{-1}$ (400–700 nm). All manipulations of dark-grown plants were performed when possible in complete darkness. However, when required, light was provided by a 15-W green safelight. No photoconversion of protochlorophyll(ide) was observed under these conditions. The average leaf fresh weight was determined by weighing 4–10 3-cm segments of primary leaves. The fresh weight was 8.8 ($\pm$ 0.4) $\times\ 10^{-3}$ g cm^{-1} and remained unchanged during the first 24 h of development.

Chlorophyll Measurements: Leaf chlorophyll was extracted from the upper 3.0 cm of 4–10 primary leaves in 2.5 ml 90% acetone, 0.01 N NH_4OH. The concentrations of chlorophyll *a* and *b* were determined according to Arnon (24) and that of protochlorophyll(ide) according to Anderson and Boardman (25).

Determination of P700 Concentration: The upper 3.0 cm from primary leaves were homogenized in 0.5 M sucrose, 0.1 M NaCl, 1 mM dithiothreitol (DTT),[1] 20 mM sodium ascorbate, 50 mM Tris-HCl (pH 8.0) and filtered through 2 layers of Miracloth (Calbiochem-Behring Corp., La Jolla, CA). Membranes were pelleted at 100,000 *g* for 30 min. Pellets were washed in 0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 20 mM sodium ascorbate, 50 mM Tris-HCl (pH 8.0) and recentrifuged. The pellet was solubilized in 1.0% Triton X-100, 10 mM Tris-glycine (pH 8.3) and insoluble material was removed by brief centrifugation in a microfuge (15,000 *g*). The P700 concentration of the supernatants was estimated from the light-oxidized and dark-reduced difference signal as described (26).

Protein Isolation and Antibody Preparation: Purification of the chlorophyll-binding form of the P700 chlorophyll *a*-protein was performed by preparative electrophoresis as described previously (14, 15). Chloroplast membranes solubilized in 1% SDS, 10 mM Tris-glycine (pH 8.3) were electrophoresed on modified Laemmli (27) SDS slab gels (500 μg chlorophyll/gel) at 200 V for 4 h at 15°C. Directly after electrophoresis, the region of the gel containing the chlorophyll-protein was excised from the gel and macerated and the protein was passively eluted into 0.1% Triton X-100, 10 mM Tris-glycine (pH 8.3). The protein was adsorbed to alum (28) and subsequently used to raise antibodies in New Zealand White female rabbits following standard procedures (29). Specificity of the antibodies was assayed by immunoprecipitation (30) and Western blotting (31) analyses (see below). The antibodies were observed to react more strongly with the apoprotein of the P700 chlorophyll *a*-protein than with the chlorophyll-protein complex (14, 15). Furthermore, although the antibodies recognized both the 58,000 and 62,000-mol-wt electrophoretic forms of the apoprotein, preferential recognition of the 58,000-mol-wt form was observed in both immunoprecipitation and Western blot assays (14, 15).

In Vivo Protein Labeling: Primary leaves were excised 3 cm from the tip and placed in individual tubes containing 30 μCi of [^{3}H]leu (>110 Ci/mmole; New England Nuclear, Boston, MA) in 10 μl H_2O. [^{3}H]leu incorporation into cold trichloroacetic acid–precipitable material was linear for at least 60 min beginning after a lag period of 10 min.

Analytical Electrophoresis: Analytical electrophoresis was performed according to Laemmli (27). Samples were prepared for electrophoresis as described. Molecular weight standards used were phosphorylase A (94,000 mol wt), BSA (66,000 mol wt), alcohol dehydrogenase (37,000 mol wt), carbonic anhydrase (29,000 mol wt), β-lactoglobulin (18,400 mol wt) and lysozyme (14,000 mol wt). Gels were stained with Coomassie Brilliant Blue R (Sigma Chemical Corp., St. Louis, MO) and processed for fluorography (32).

Preparation of Leaf Protein Extracts: Leaf proteins were prepared for electrophoresis or immunoprecipitation as follows. For analysis of total leaf proteins, two 3.0-cm segments of primary leaves were homogenized in 2.4 ml of 1.0–2.0% SDS, 50 mM DTT, 10 mM Tris-glycine (pH 8.0) using a Duall homogenizer (Kontes Co., Vineland, NJ). The homogenate was heated to 50°C for 15–20 min and insoluble material was removed by centrifugation at 15,000 *g* for 1 min. No additional protein could be released by resolubilization of the pelleted material. The supernatant was analyzed by electrophoresis after the addition of sucrose (to 10%). For immunoprecipitation, samples were diluted into 10 vol of 1% Nonidet P-40 (NP-40) buffer as described below.

For analysis of soluble and membrane protein fractions, leaf tissue (2 × 3.0 cm from primary leaves) was ground on ice in 5.0 ml of 0.5 M sucrose, 0.1 M NaCl, 1 mM DTT, 20 mM sodium ascorbate, 50 mM Tris-HCl (pH 8.0) using a Duall homogenizer. The homogenate was filtered through Miracloth, brought to a total volume of 7.5 ml with the homogenization buffer, and centrifuged at 100,000 *g* for 50 min. The resulting supernatant is referred to as the "sucrose supernatant". The pellet was resuspended by homogenization in 7.5 ml of 0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 20 mM sodium ascorbate, 50 mM Tris-HCl (pH 8.0) and recentrifuged at 100,000 *g* for 50 min. The supernatant from this step is referred to as the "wash supernatant". The 100,000 *g* supernatants (the soluble protein fractions) were made to 1% NP-40 for immunoprecipitation, or were trichloroacetic acid–precipitated for electrophoretic analysis. The membrane protein pellet was solubilized in 1% SDS, 50 mM DTT, 10 mM Tris-glycine (pH 8.3) and analyzed by electrophoresis or diluted 10-fold into 1% NP-40 buffer for immunoprecipitation.

Staphylococcus aureus Protein A–coupled Immunoprecipitation: Indirect immunoprecipitation using heat-killed *S. aureus* cells ("Pansorbin", Calbiochem-Behring Corp.) was performed essentially according to Kessler (30). Immediately before use, *S. aureus* cells were washed two times in 1% NP-40, 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5) containing 0.1% carbonic anhydrase, and resuspended to a 10% (wt/vol) solution of cells. Leaf protein samples prepared as described were diluted into a 10-fold excess of the same buffer without carbonic anhydrase. For the assay, 1–10 μl of whole serum was added to 0.1–1.0 ml of sample and incubated 1–4 h at 4°C. Washed *S. aureus* cells, 100–200 μl, were added and incubation continued for 2–12 h at 4°C. The *S. aureus* antigen-antibody complexes were pelleted for 1 min at 15,000 *g* and washed three times in 1 ml of 1% NP-40, 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5). Final pellets were dissociated in 50 μl of 1.0–2.0% SDS, 50 mM DTT, 10 mM Tris-glycine (pH 8.3) by being heated at 70°C for 15 min. *S. aureus* cells were removed by centrifugation and 30 μl of the supernatant was analyzed by electrophoresis.

In all experiments duplicate samples were reacted with immune or preimmune serum. Total [^{3}H]Leu cpms assayed were determined from cold trichloroacetic acid precipitates prepared from aliquots of each sample. [^{3}H]Leu incorporation into the P700 chlorophyll *a*-apoprotein was determined by counting 10-μl aliquots of the immunoprecipitated material. Counting efficiency was measured using ^3H_2O as an internal standard. No significant differences in counting efficiency were observed and therefore all values are reported in cpms.

Nitrocellulose Protein Blots: Proteins were separated on SDS Laemmli slab gels and transferred passively to nitrocellulose paper (0.45 μm, Schleicher & Schuell, Inc., Keene, NH), as described (15), by blotting the gels for 18–24 h in 0.22 M sodium phosphate, 0.02% NaN_3 (pH 7.0). Approximately

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; NP-40, Nonidet P-40.

20% of the protein from the gel was transferred to the nitrocellulose filters by this procedure.

The nitrocellulose-bound proteins were reacted with antiserum and ^{125}I-Protein A (New England Nuclear) according to Towbin et al. (31). Additional protein-binding sites on the nitrocellulose were blocked by incubation in 1% gelatin, 0.05% NP-40, 0.005 M EDTA, 0.15 M NaCl, 0.05 M Tris-HCl, (pH 7.4). Filters were then incubated with preimmune or immune serum at 3–30 μl/ml in the same buffer for 2 h at room temperature. Filters were washed with three changes of buffer and then incubated with 1 μl ^{125}I-Protein A (0.1 μCi, 80 μCi/μg Protein A) per milliliter as for the antiserum step. Filters were washed three times, air-dried, and exposed to preflashed Kodak X-Omat AR5 film with an intensifying screen at −70°C.

RESULTS

Accumulation of Chlorophyll and P700 Activity

To investigate the relationship of chlorophyll synthesis to P700 chlorophyll *a*-apoprotein synthesis, we initially characterized the kinetics of chlorophyll accumulation. Protochlorophyll(ide) content of dark-grown leaf tissue was 8.8 (± 0.6) μg/gm fresh wt. After 15 min of illumination, protochlorophyll(ide) was undetectable and was replaced by chlorophyll *a* representing up to 90% (μg/μg) photoconversion of the protochlorophyll(ide). A significant increase in leaf chlorophyll *a* content, above that resulting from photoconversion of protochlorophyllide, was measurable only after 40 min of illumination. The time course of accumulation of total chlorophyll (*a* + *b*) in the upper 3 cm of primary leaf is shown in Fig. 1. Chlorophyll accumulation occurred in three distinct phases: an initial slow or lag phase from 0 to 4 h, a rapid linear phase from 4 to ~12 h, and a final declining phase after 12 h (Fig. 1, inset). The same three phases have been reported in developing barley by Henningsen and Boardman (33). During the initial lag phase the rate of chlorophyll accumulation as determined from four experiments is 10.4 (± 1.1 SD) μg/gm leaf fresh wt × h. This rate increases more than threefold after 4 h to 37.3 (± 6.6 SD) μg/gm × h. Despite variation between experiments in the absolute rate of chlorophyll accumulation, this sharp increase was always observed to occur between 4 and 5 h of illumination.

The chlorophyll *a*/*b* ratios are also presented in Fig. 1. Chlorophyll *b* can be detected as early as 2 h after the start of illumination, though before 5 h chlorophyll *b* represents less than 10% of the total chlorophyll. Chlorophyll *b* increases as a proportion of the total chlorophyll between 5 and 10 h to produce a stable chlorophyll *a*/chlorophyll *b* ratio of 3.75.

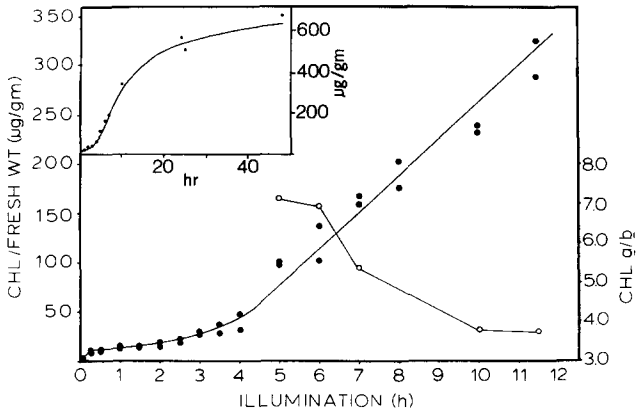

FIGURE 1 Typical time course of total chlorophyll (*CHL*) (*a* + *b*) accumulation during the first 12 h of illumination (solid circles); each point represents a single determination. Chlorophyll *a*/*b* ratios (open circles) represent averages determined from the chlorophyll samples in this experiment. Chlorophyll *b* was detected, but not quantified, between 2 and 5 h of illumination. *Inset*: Time course extended to 48 h.

In cell free, Triton X-100–solubilized membrane extracts, no P700 activity was detected earlier than 1 h after the onset of illumination (Table I). From 1 to 12 h of illumination the concentration of photochemically active P700 increased in parallel with the increase in membrane chlorophyll content. This is evidenced by the approximately constant chlorophyll/P700 ratio during this time period (Table 1). The ratio of 569/1 determined after 1 h of illumination is not significantly higher than the later time points because of a large error in measurement at low P700 concentrations. Alberte et al. (18, 19) reported detection of P700 activity in SDS-solubilized membrane extracts only after 4–6 h of light-induced development. Failure to observe activity at earlier time points in their experiments most likely resulted from inactivation of P700 by SDS. Hiller et al. (20) obtained results with barley similar to those reported here. Henningsen and Boardman (33) have reported Photosystem activity, measured in isolated barley chloroplasts by the light-dependent uptake of O_2, as early as 15 min after the onset of illumination. We conclude that although functional P700 reaction centers are most likely present before 1 h of illumination, they are unstable after the detergent extraction at these early time periods.

Protein Synthesis Patterns during Chloroplast Development

Examination of total soluble or total membrane protein fractions of developing leaves during the first 12 h of illumination indicates that major light-induced changes in polypeptide composition are confined to the membrane fraction. In particular, the membrane protein fraction of etiolated barley seedlings is missing major polypeptide components of ~62,000, 42,000, 32,000, and 28,000 mol wt when compared with membrane protein profiles of 12-h illuminated seedlings (14, 21–23). Based on their molecular weights, the 62,000 and 28,000-mol-wt polypeptides have been indentified as the P700 chlorophyll *a* and chlorophyll *a*/*b* apoproteins, respectively. Membrane protein profiles at time points between 0 to 12 show that significant accumulation of these polypeptides is detectable only after 6–8 h of illumination (14, 21, 22).

TABLE I

Increase in P700 Photochemical Activity during Chloroplast Development in Barley

Illumination	Chl	P700	Chl/P700
h	*μM*	*μM × 10³*	
0.0	0.7	0.00	—
0.5	2.8	0.00	—
1.0	2.7	4.69	569
1.5	6.3	14.06	450
2.0	2.0	4.70	417
3.0	4.7	9.48	498
4.0	9.5	18.78	506
6.0	26.7	55.91	477
8.0	33.3	65.74	507
10.0	32.0	71.91	445
12.0	43.3	95.44	454

Chlorophyll (*Chl*) and P700 values are presented as the concentration of the cell-free detergent extract. For time points between 0 and 4 h illumination, chlorophyll and P700 measurements were determined on undiluted samples. For 6–12 h, samples were diluted fourfold before measurement.

Because of the lag period between the onset of illumination and the observed changes in polypeptide composition, it is not possible from these data to conclude that light directly affects the biosynthesis of these proteins.

When the pattern of in vivo protein synthesis was examined in barley during the first 7–12 h of illumination, qualitative and quantitative changes were also observed primarily in the membrane protein fraction. From these findings, we discerned a more direct relationship between illumination and the production of the 62,000 and 28,000-mol-wt polypeptides (Fig. 2). In these experiments leaves were labeled for 1 h with [^{3}H]Leu either before illumination or during successive hourly intervals after the start of illumination. Total soluble and total membrane protein fractions were prepared, and equal cpms were electrophoresed and fluorographed. As seen in Fig. 2*a*, qualitative changes in the relative synthesis of major soluble polypeptides is restricted to the appearance of an unidentified polypeptide at 27,000–28,000 mol wt after 5 h of illumination. Both the large and small subunits of ribulose bisphosphate carboxylase (52,000 and 14,500 mol wt) are vigorously synthesized in the dark and throughout development. In contrast, changes in the pattern of membrane protein synthesis are observed within the first hour of illumination (Fig. 2 *b*). Both the 62,000 and the 28,000-mol-wt polypeptides were consistently shown to increase in synthesis within this time period. Furthermore, a sharp additional increase in the synthesis of the 28,000-mol-wt polypeptide occurred after 4–5 h illumination. A similar though less dramatic increase in the 62,000-mol-wt polypeptide was observed also between 4 to 5 h. The pattern of proteins synthesized during 8–12 h of illumination was identical to that observed at 7 h (data not shown).

Rate of P700 Chlorophyll a-Apoprotein Synthesis and Membrane Incorporation

Although the examination of total membrane protein profiles suggests that light induces a rapid change in synthesis of the P700 chlorophyll *a*-apoprotein, immunoprecipitation experiments conclusively demonstrate this light effect and enable us to quantitatively evaluate the rate of synthesis (Fig. 3). Soluble and membrane protein extracts prepared from leaves labeled for 1 h with [^{3}H]Leu during the interval indicated were reacted with preimmune or P700 chlorophyll *a*-protein antiserum, and the immunoprecipitates were examined by electrophoresis. As seen in Fig. 3 there is no evidence of newly synthesized P700 chlorophyll *a*-apoprotein in the membrane or sucrose supernatant fractions of plants labeled before illumination (lane *0*). During the second hour of illumination, apoprotein is vigorously synthesized and found associated primarily with the membrane protein fraction. The small amount of protein seen associated with the soluble protein fraction presumably represents contamination with membrane fragments rather than a "soluble" form of the apoprotein. Prominent bands at 120,000 and 180,000 mol wt represent dimer and trimer aggregates of the P700 chlorophyll *a*-apoprotein. These aggregates are also absent in leaves labeled in the dark. No apoprotein was detected in the wash supernatant fractions (data not shown).

The total radioactivity of the membrane fraction immunoprecipitates was used to estimate the rate of P700 chlorophyll *a*-apoprotein synthesis relative to the rate of synthesis of other membrane proteins. Leaves were labeled for 1 h with [^{3}H]Leu at various times during the first 12 h of illumination.

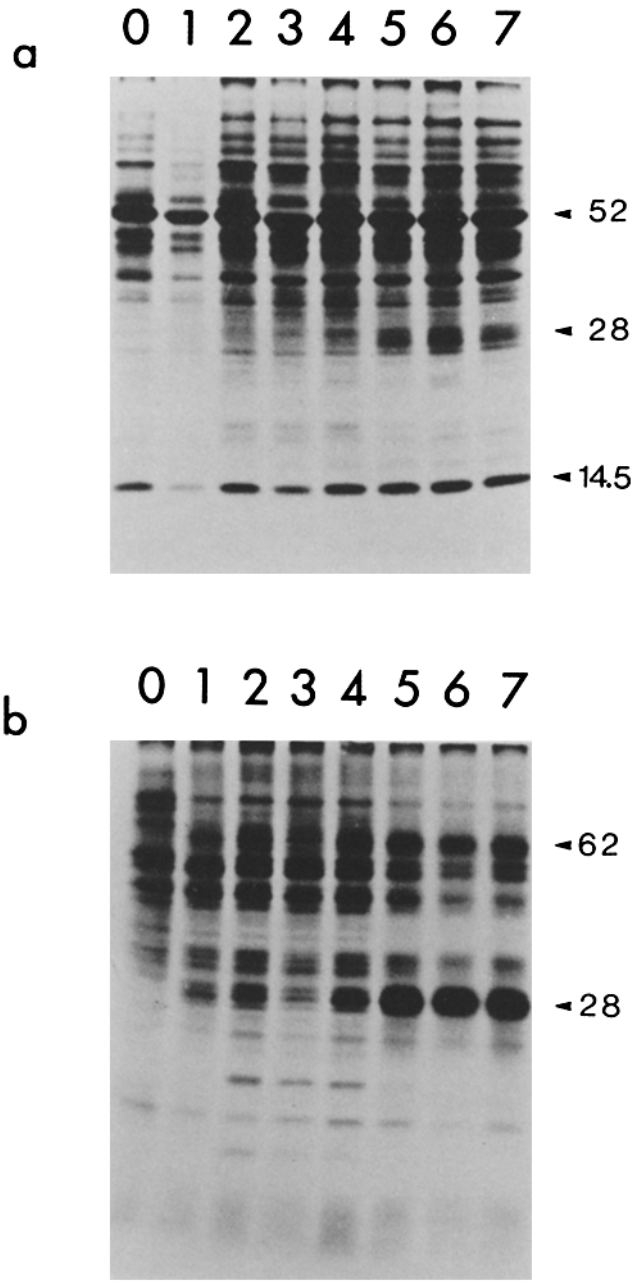

FIGURE 2 Developmental changes in the pattern of leaf protein synthesis. (a) Fluorogram of total soluble proteins (sucrose supernatant) synthesized in the dark (lane *0*) or during 1–7 h of illumination. Approximately 50,000 cpm were loaded per gel lane. Arrows (molecular weight × 10^{-3}): Large (52,000 mol wt) and small (14,500 mol wt) subunits of ribulose bisphosphate carboxylase; unidentified polypeptide (28,000 mol wt). (*b*) Fluorogram of total membrane proteins synthesized during development. Gel lanes marked as for panel *a*. Approximately 20,000 cpm were loaded per gel lane. Arrows indicate P700 chlorophyll *a*-apoprotein (62,000 mol wt) and chlorophyll a/b-protein (28,000 mol wt). All samples were prepared after labeling with [^{3}H]Leu for 60 min, and were separated on 12.5% acrylamide SDS gels. Lanes marked *0* were labeled for 1 h in the dark. Lanes labeled *1–7* were harvested after 1–7 h illumination, respectively.

Total cellular membrane fractions were prepared and reacted with excess antibody. Parallel samples were precipitated with immune or preimmune serum. Specific incorporation into the P700 chlorophyll *a*-protein was quantified as cpms in the

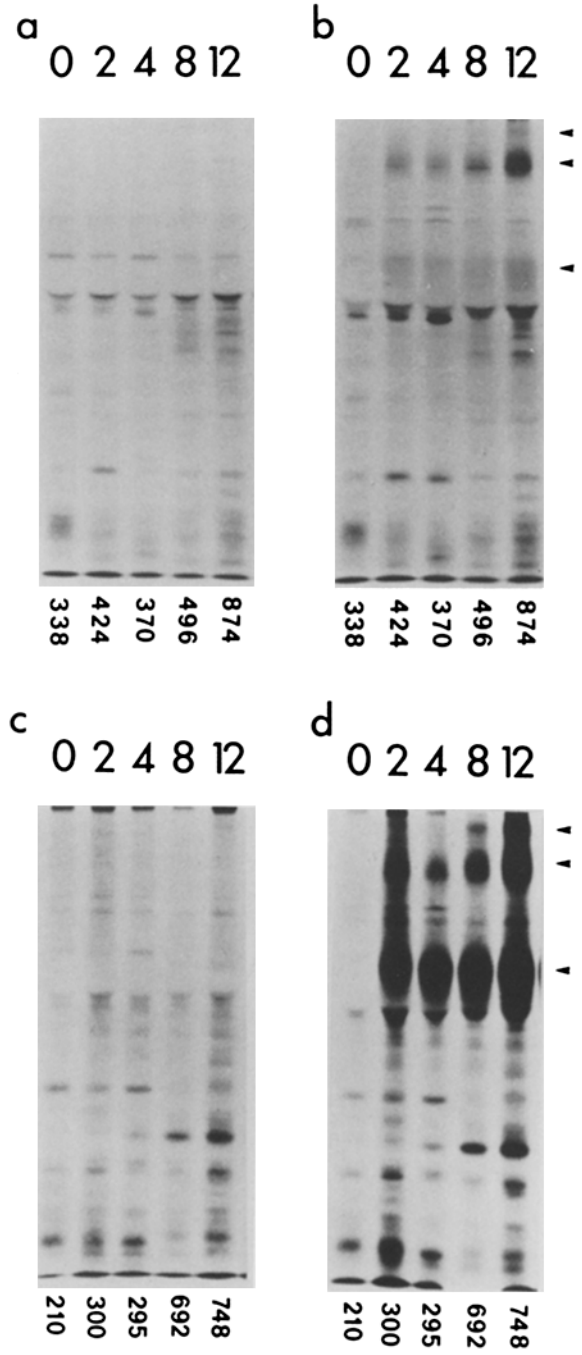

FIGURE 3 Fluorogram of immune and preimmune precipitates of the soluble and membrane fractions from developing barley leaves. Samples immunoprecipitated represented proteins prepared from equivalent leaf fresh weights. Total hours illumination are indicated above each gel lane and approximate amount of protein assayed (cpms $\times 10^{-3}$) is indicated below each gel lane. (*a* and *b*) Precipitates prepared from the soluble, sucrose supernatant fractions. (*c* and *d*) Precipitates prepared from the membrane protein fractions. *a* and *c* are preimmune precipitates, *b* and *d* are immune precipitates. All samples were prepared after the indicated time period of illumination and were labeled with [^{3}H]Leu for 60 min before harvesting. Total [^{3}H]Leu incorporation varied between samples owing to differences in efficiency of label uptake. Incorporation was typically higher after 5 h illumination as a result of increased total protein synthesis. Separation was on 10% acrylamide gels. Arrows indicate the P700 chlorophyll *a*-apoprotein and two higher molecular weight aggregates as discussed in the text. Gels have been overexposed to emphasize the lack of immunoprecipitable protein at time 0.

immunoprecipitates (after subtraction of the preimmune background) and expressed as a percentage of the total cpms assayed. In all experiments aliquots were also used for electrophoresis and fluorography to confirm that the radioactivity measured represented P700 chlorophyll *a*-apoprotein. Results from several experiments show that P700 chlorophyll *a*-apoprotein synthesis and association with the membrane protein fraction increases during the first 5 to 6 h of development and then remains approximately constant through 12 h of illumination (Fig. 4). Qualitative examination of fluorograms of immunoprecipitates prepared between 12 and 24 h illumination indicate that synthesis begins to decline between 13 and 16 h.

Although in two cases, as shown, positive values were calculated for the dark synthesis rates (0.9 and 0.3%), no apoprotein was detectable in the fluorograms prepared from these samples. These values are a useful measure of the error in the measurement as performed, reflecting technical limitations in processing parallel immune and preimmune samples. However, when immunoprecipitates were examined by fluorography the assay was sufficiently sensitive to detect levels of protein synthesis representing as little as 0.1% of the total protein cpms. Because we have never seen evidence of P700 chlorophyll *a*-protein synthesis before illumination, we conclude that the increase to 10% of the total membrane protein synthesis represents as much as a 100-fold increase in synthesis of the protein.

It is important to note that in these and in the following experiments, the polypeptide detected in the immunoprecipitations (or on protein blots) represents primarily the 58,000 mol wt form of the P700 chlorophyll *a*-apoprotein. We have been unable to consistently detect the 62,000-mol-wt polypeptide species in either immunoprecipitation or blotting experiments (15). However, we have occasionally observed synthesis of the 62,000-mol-wt polypeptide during illumination in experiments comparing synthesis in the dark with synthesis at a single time point in the light (6 or 8 h illumination). Based on these data, and because it is not clear exactly how the 62,000 and 58,000-mol-wt polypeptides are related to each other (that is, whether they represent in vivo or in vitro generated heterogeneity), we have assumed that our

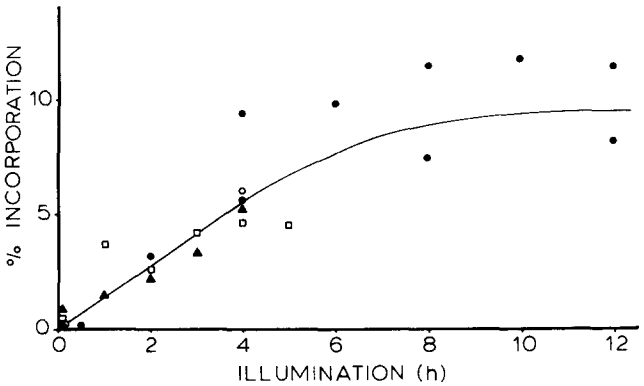

FIGURE 4 Rates of P700 chlorophyll *a*-apoprotein synthesis as a proportion of total membrane protein synthesis during the first 12 h of light-induced development. Values were calculated as the percentage of total radioactivity (cpms) recovered in the immune precipitate after subtraction of preimmune background (cpms). Different symbols indicate separate time course experiments. Total cpms assayed were 50–70,000, solid circles; 64–80,000, open circles; 30–40,000, squares; 30–40,000, triangles.

experiments accurately reflect the total amount of P700 chlorophyll *a*-apoprotein.

Rapidity of the Light Response

To further test when newly synthesized P700 chlorophyll *a*-apoprotein first appears in the membrane fraction after the onset of illumination, plants were labeled with [^{3}H]Leu for 45 min in the dark and then exposed to light for 15 min or allowed to remain in the dark for 15 min. A fluorogram of the total membrane extracts and the immune and preimmune serum precipitates is presented in Figure 5. In agreement with the observations of the previous experiments, there is no detectable P700 chlorophyll *a*-apoprotein present in the membrane fraction of plants labeled in the dark although there is significant synthesis of other membrane proteins. Based on the results shown in Fig. 3, we can conclude that the apoprotein found associated with the membrane fraction after 15 min of illumination was not synthesized in a "soluble" form during the 45 min dark preincubation, but rather resulted solely from synthesis during the light period. We have also performed 15-min labeling experiments, both in the light and in the dark, and have examined immunoprecipitates prepared from total leaf proteins. Despite very low [^{3}H]Leu incorporation, results from these types of experiments also demonstrate rapid light induction of P700 chlorophyll *a*-apoprotein synthesis (14).

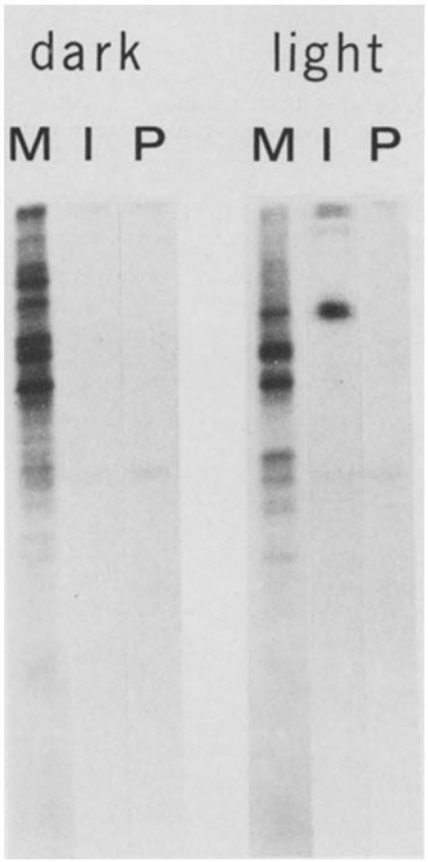

FIGURE 5 Fluorogram of total membranes (*M*), preimmune (*P*), and immune precipitates (*I*) prepared from plants labeled for 60 min in the dark (*dark*) or 45 min in the dark followed by 15 min in the light (*light*). P700 chlorophyll *a*-apoprotein is obvious only in the immune precipitate from the sample labeled in the light.

Control of Synthesis: Relationship to Other Chloroplast Proteins

A specific light-regulated increase in P700 chlorophyll *a*-apoprotein synthesis has been demonstrated in the experiments described above. It is possible that this increase results primarily from a general light-regulated increase in total chloroplast protein synthesis relative to cytoplasmic protein synthesis. Examination of two additional products of chloroplast protein synthesis, the large subunit of ribulose bisphosphate carboxylase and the α- and β-subunits of chloroplast ATPase (CF_1) (17), demonstrates that this is not the case, but rather that differential chloroplast gene expression is involved.

Vigorous synthesis of the large subunit of ribulose bisphosphate carboxylase occurs in the dark and continues relatively unchanged upon illumination as seen from the results already presented (Fig. 2). Synthesis of CF_1 was detected in immunoprecipitation experiments with antibodies provided by Dr. A. Jagendorf (Cornell University) (Fig. 6). The soluble or membrane protein fractions were reacted with CF_1 serum in an experiment similar to that in Fig. 3. The majority of immunoprecipitable CF_1 was found in the sucrose supernatant fraction. We estimate that no more than a twofold increase in synthesis of the α- and β-subunits occurs upon illumination. This small increase most likely reflects an increase in total protein synthetic activity of the chloroplast and is qualitatively different from the increase in P700 chlorophyll *a*-protein synthesis.

Control of Synthesis: Synthesis in the Dark after Illumination

To study further the nature of the light control of P700

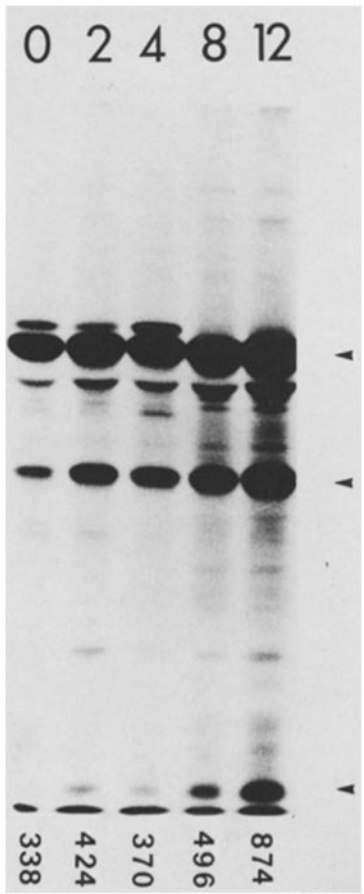

FIGURE 6 Immunoprecipitation of chloroplast ATPase. Fluorogram of immune precipitates prepared from soluble leaf proteins using antibodies prepared against spinach chloroplast ATPase. Upper arrow, α- and β-subunits (56,000 and 53,000 mol wt); middle arrow, γ-subunit (35,000 mol wt); lower arrow, δ-subunit (17,000 mol wt). Total amount of protein assayed (cpms $\times 10^{-3}$) is indicated below each gel lane.

chlorophyll *a*-apoprotein synthesis, plants were returned to darkness at different times during development and the rate of P700 chlorophyll *a*-apoprotein synthesis was monitored. After 4, 8, and 10 h of illumination, leaves were labeled for 1 h in the dark or 1 h in the light. Scintillation counting of membrane fraction immunoprecipitates was performed, and the presence of the P700 chlorophyll *a*-apoprotein was confirmed by electrophoresis and fluorography. The rate of P700 chlorophyll *a*-protein synthesis as a percentage of total membrane protein synthesis for the light and dark sample at each time point was as follows; 4 h, 8.4% light vs. 4.8% dark; 8 h, 11.4% light vs. 12.2% dark; and 10 h, 11.7% light vs. 10.4% dark. These results demonstrate that continuous illumination is not required for P700 chlorophyll *a*-apoprotein synthesis.

The effect of continued dark incubation was also examined. Plants illuminated for 4 h were returned to darkness and leaves were labeled during the first, second, or fourth hour of dark incubation. Leaf samples were also labeled before receiving any illumination or during the fourth hour of illumination. Results from immunoprecipitation of the labeled membrane fractions are presented in Fig. 7. The total cpms assayed at each time point are indicated in the figure legend. Levels of [^{3}H]Leu incorporation into the P700 chlorophyll *a*-apoprotein as a percentage of the light control were 80% after 1 h, 50% after 2 h, and 40% after 4 h of dark incubation.

Accumulation of the P700 Chlorophyll a-Protein

The experiments presented above concern relative rates of P700 chlorophyll *a*-apoprotein synthesis. Protein blotting experiments were also performed to examine apoprotein accumulation in whole tissue. With this type of indirect labeling assay it is possible to determine whether or not significant quantities of apoprotein accumulate in the dark and to examine the rate of stable membrane incorporation relative to the observed rate of synthesis.

Fig. 8 shows the increase in membrane-associated apoprotein during the first 4 h of development. Membranes were isolated from equivalent fresh weights of tissue after 0, 1, 2, or 4 h of illumination, and equivalent aliquots were electrophoresed and blotted to nitrocellulose. Apoprotein is not detectable in dark grown leaves, but shows a measurable increase during successive hours of illumination. Based on the analysis of a dilution series of isolated P700 chlorophyll *a*-protein on the same blot, it was possible to detect a minimum of 16 ng of protein per gel lane by this procedure. Calculating from the known amount of tissue analyzed (0.8 mg per gel lane), it can be estimated that dark grown leaves contain between 0 and 20 μg of apoprotein per gram leaf fresh weight. The upper limit of this range represents sufficient protein to bind 1–2 μg chlorophyll *a* per gram fresh weight (2, 15).

The P700 chlorophyll *a*-protein binds ~40 molecules of chlorophyll *a* per reaction center, or about 10% of the total thylakoid membrane chlorophyll (2, 15, 26). In our system then, 60 μg of chlorophyll *a* is associated with the P700 chlorophyll *a*-protein after 24 h illumination. Therefore, the amount of apoprotein present in the dark could represent at most <4% of the total accumulated after 24 h illumination.

DISCUSSION

Studies of P700 chlorophyll *a*-protein synthesis have previ-

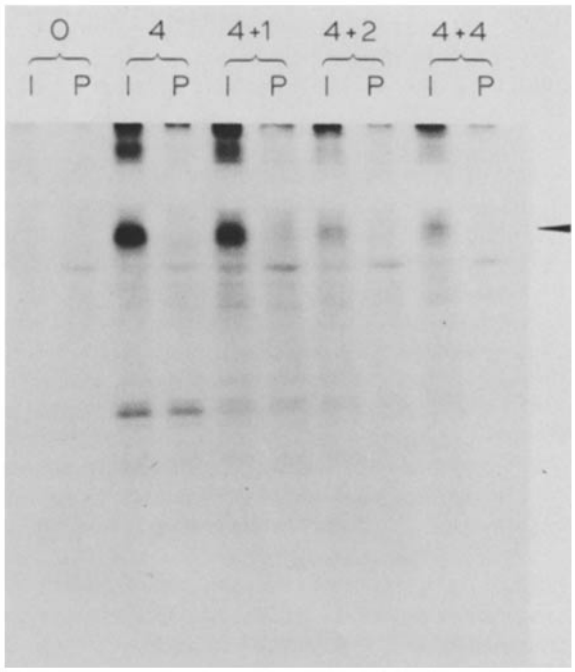

FIGURE 7 Decrease in P700 chlorophyll *a*-apoprotein synthesis in the dark. Fluorogram of immune (*I*) and preimmune (*P*) precipitates prepared from total membrane proteins. Plants were labeled in the dark (*0*), during the fourth hour of illumination (*4*), during the first hour in the dark after 4 h illumination (*4 + 1*), the second hour in the dark (*4 + 2*), or the fourth hour in the dark (*4 + 4*). Amount of protein assayed (cpms) for each time point was as follows: *0*, 78,000 cpm; *4*, 65,000 cpm; *4 + 1*, 82,000 kcpm; *4 + 2*, 81,000 cpm; *4 + 4*, 70,000 cpm. Arrow indicates the position of the P700 chlorophyll *a*-apoprotein.

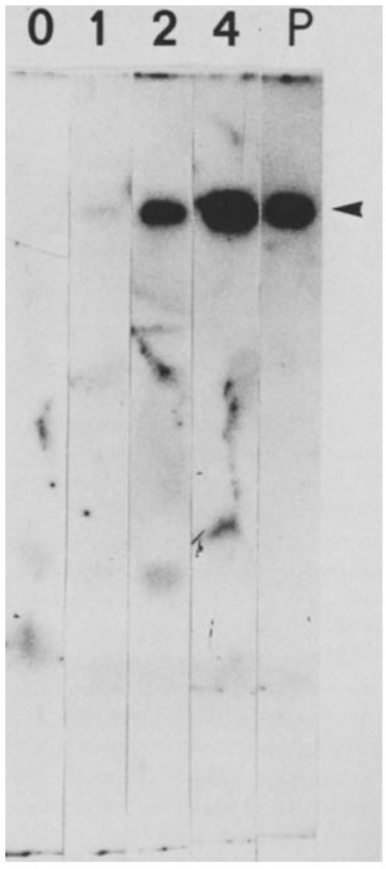

FIGURE 8 Autoradiogram of a protein blot showing P700 chlorophyll a-apoprotein content of membranes isolated after 0, 1, 2, or 4 h of illumination as indicated. Membranes from ~0.8 mg leaf fresh wt were applied per gel lane. Lane *P* is 50 ng of isolated P700 chlorophyll *a*-protein.

ously been limited to the description of the accumulation of the apoprotein or chlorophyll-binding protein as analyzed by conventional gel electrophoretic techniques (18–23, 34–36). While these studies have shown that the amount of P700 chlorophyll *a*-protein increases during chloroplast development, the factors regulating this increase could not be examined using these techniques.

We have performed several experiments that demonstrate that synthesis of the P700 chlorophyll *a*-apoprotein changes rapidly and dramatically in response to illumination and that this response is an example of differential chloroplast gene expression. *De novo* synthesis of the apoprotein was observed during the first 15 min of illumination, and during the first hour of illumination the net rate of synthesis increased from undetectable levels (<0.1%) to as much as 4% of total membrane protein synthesis. In contrast, no dramatic change in synthesis of several other polypeptides produced by the chloroplast results from the light-dark transition. These data indicate that the observed increase in P700 chlorophyll *a*-apoprotein synthesis does not simply result from an overall light-regulated increase in chloroplast protein synthesis relative to total cellular protein synthesis. The high percentage value emphasizes the extent to which chloroplast protein synthesis is committed to production of this chlorophyll-protein during the early hours of chloroplast development.

The general pattern of P700 chlorophyll *a*-apoprotein synthesis appears to parallel the change in chlorophyll synthesis during development. That is, the rate of chlorophyll accumulation is increasing during the time that P700 chlorophyll *a*-apoprotein synthesis is increasing and chlorophyll synthesis reaches a maximal linear rate at the time (4–5 h) that P700 chlorophyll *a*-protein synthesis plateaus. Little or no apoprotein has been detected in the soluble protein fraction, therefore it is assumed that examination of the membrane fraction represents total P700 chlorophyll *a*-apoprotein synthesis. In other experiments we have observed that incorporation into the P700 chlorophyll *a*-apoprotein is declining between 13 and 16 h of illumination. The conclusion that this pattern of synthesis correlates with the observed pattern of chlorophyll synthesis is confounded by the fact that total protein synthesis may also be changing during this time period. However, Apel and Kloppstech (3) observed that total mRNA levels increased during the first 4–6 h and remained stable during the subsequent 6–24 h of chloroplast development in barley. Assuming that mRNA levels are a general measure of protein synthesis rates, we conclude that overall changes in protein synthesis affect only the magnitude of the increase in P700 chlorophyll *a*-apoprotein synthesis during the first 4 h of development, but do not otherwise change the pattern of synthesis. Therefore, the observed rates of P700 chlorophyll *a*-apoprotein synthesis can still be correlated with the observed rates of chlorophyll accumulation. Furthermore, the increase in P700 activity concomitant with the increase in chlorophyll synthesis suggests that there is no significant lag between apoprotein synthesis and assembly into a functional chlorophyll-protein complex.

As noted above, synthesis of the P700 chlorophyll *a*-apoprotein is not observed in seedlings germinated and grown in the dark. However, after plants have been illuminated for ≥4 h, leaves continue to synthesize and accumulate the apoprotein when plants are returned to darkness. This is consistent with previous observations of other workers (34–36). It has been shown that when etiolated plants are illuminated for 2-min intervals separated by 98 min of darkness, P700 chlorophyll *a*-protein accumulated to 50% of the level present in light grown leaves by 48 h (34–36). The continued synthesis and stable accumulation of the P700 chlorophyll *a*-apoprotein in the dark demonstrates that synthesis is not dependent on concurrent chlorophyll production. It is possible that continued synthesis of the apoprotein in the dark is dependent on an available pool of chlorophyll produced either during the previous illumination period or released by turnover of existing chlorophyll proteins.

Our studies demonstrate that the bulk of the P700 chlorophyll *a*-apoprotein is synthesized and accumulated after the onset of illumination. Owing to the limits of sensitivity of our assay, we cannot conclude that the apoprotein is completely absent in dark grown seedlings. However, because synthesis and net accumulation of the P700 chlorophyll *a*-apoprotein are observed during the first hour of illumination, we conclude that the rapid appearance of PSI activity during development is dependent on the light-mediated synthesis of this protein.

It is not possible from our experiments to determine at what level, transcriptional and/or posttranscriptional, light acts to initiate P700 chlorophyll *a*-apoprotein synthesis. Illumination has been demonstrated to increase or decrease the pool sizes of specific chloroplast mRNAs in developing chloroplasts (37, 38). In contrast to the rapid induction we have demonstrated, these apparently transcriptionally regulated changes have been measured after a period of ≥3 h of illumination, and may therefore only secondarily result from illumination. Stability of the altered mRNA pools upon return of plants to darkness was not examined.

We have been unable to detect the P700 chlorophyll *a*-apoprotein among the in vitro translation products of total RNA from dark-grown or 10-h–illuminated barley seedlings using either wheat germ or rabbit reticulocyte cell free translation systems. This result may be caused by inefficient translation of this message on 80S eukaryotic ribosomes. Further experiments of this type or analyses using a specific chloroplast DNA probe containing the P700 chlorophyll *a*-protein gene will be required to resolve at what level light control is exerted.

We hypothesize that synthesis is initiated by light at the transcriptional level and that continued, but declining, synthesis in the dark reflects the half-life of the mRNA and/or a light-activated "factor" necessary for transcription. Furthermore, we suggest that coordination of the rate of P700 chlorophyll *a*-apoprotein synthesis with the production of chlorophyll is controlled posttranslationally. As has been proposed regarding chlorophyll *a*/*b*-protein synthesis (4, 16, 10), association of chlorophyll with the protein may be required for stable insertion of the protein into the developing thylakoid membranes.

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Note added in proof: A report describing the identification of the P700 chlorophyll *a*-apoprotein gene on the spinach chloroplast genome appeared after submission of this manuscript. (Westhoff, P., J. Alt, N. Nelson, W. Bottomley, H. Bünemann, and R. G. Herrmann.

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