Biosynthetic Pathways of Two Polypeptide Subunits of the Light-harvesting Chlorophyll *a/b* Protein Complex

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ABSTRACT We have used an in vitro reconstitution system, consisting of cell-free translation products and intact chloroplasts, to investigate the pathway from synthesis to assembly of two polypeptide subunits of the light-harvesting chlorophyll-protein complex. These polypeptides, designated 15 and 16, are integral components of the thylakoid membranes, but they are products of cytoplasmic protein synthesis. Double immunodiffusion experiments reveal that the two polypeptides share common antigenic determinants and therefore are structurally related. Nevertheless, they are synthesized in vitro from distinct mRNAs to yield separate precursors, p15 and p16, each of which is 4,000 to 5,000 daltons larger than its mature form. In contrast to the hydrophobic mature polypeptides, the precursors are soluble in aqueous solutions. Along with other cytoplasmically synthesized precursors, p15 and p16 are imported into purified intact chloroplasts by a post-translational mechanism. The imported precursors are processed to the mature membrane polypeptides which are recovered exclusively in the thylakoids. The newly imported polypeptides are assembled correctly in the thylakoid lipid bilayer and they bind chlorophylls. Thus, these soluble membrane polypeptide precursors must move from the cytoplasm through the two chloroplast envelope membranes, the stroma, and finally insert into the thylakoid membranes, where they assemble with chlorophyll to form the light-harvesting chlorophyll protein complex.

Many cellular membranes contain specific mechanisms by which newly synthesized proteins are transported into their destined subcellular compartments. The two modes of protein transport across membranes that have been recognized, co-translational and post-translational, reflect the intracellular sites of protein synthesis by membrane-bound and free ribosomes, respectively.

Details of the co-translational transport mechanism have been resolved largely by in vitro studies of the segregation of secretory proteins into microsomal vesicles during mRNA translation on membrane-bound ribosomes (cf. reference 19). In contrast, many, if not all, cytoplasmically synthesized proteins that are destined for mitochondria or chloroplasts are products of free ribosomes (cf. references 15, 19, 50). Post-translational transport of organelle proteins was first demonstrated using the small subunit (S) of ribulose-1,5-bisphosphate carboxylase (RuBPCase), which is synthesized by free cytoplasmic ribosomes as a higher molecular weight precursor (10, 14, 21, 26, 40, 49). In *Chlamydomonas*, the small subunit precursor (pS) possesses an extension of 44, mostly apolar, amino acids at its amino terminus (42). The peptide extension has been termed the "transit sequence" because of its presumptive role in the post-translational transport of pS through the chloroplast envelope (15, 42). In vitro reconstitution studies have shown that pS can be taken up by purified intact chloroplasts after it is completely synthesized (14, 26, 44). During or immediately after transport across the envelope membranes, the transit sequence is removed by a specific protease yielding the mature small subunit that assembles with the chloroplast-synthesized large subunit (14, 44). Cytoplasmically synthesized soluble and membrane proteins of mitochondria also follow a post-translational mode of transport from the cytoplasm into the organelles (17, 29, 33, 37, 38, 51).

The demonstration that pS enters chloroplasts after protein synthesis raises the question whether a similar post-translational mechanism also applies to the import of other chloroplast proteins that are made on cytosolic ribosomes. The thylakoid membrane polypeptides are particularly interesting because, in

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contrast to S, they are localized in a different subchloroplastic compartment and many of them are hydrophobic. After synthesis in the cytosol and passage through the two envelope membranes the polypeptides must be integrated into yet another membrane system, the thylakoids.

The most predominant component of the thylakoids is the light-harvesting chlorophyll protein complex (LHC). This complex enhances the distribution of light energy to photosystem II upon binding cations (cf. references 4, 6, 47) and it also mediates thylakoid pairing and the formation of grana stacks (34, 45). In this study we have reconstructed in vitro the synthesis, transport, processing, and assembly of thylakoid membrane polypeptides. Specifically, we have examined in detail the biosynthetic pathway of two polypeptide subunits (polypeptides 15 and 16) of the LHC. Although these two integral membrane polypeptides are extremely hydrophobic, they are synthesized in vitro as soluble precursors, p15 and p16, which are 4,000–5,000 daltons larger than the mature forms. After synthesis, the precursors are imported into intact chloroplasts, processed to their mature sizes, and inserted correctly into the thylakoids to form the light-harvesting complex. A preliminary account of this work has been presented in a meeting (43).

MATERIALS AND METHODS

[^{35}S]sulfate and [^{35}S]methionine were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). *Staphylococcus aureus*, strain V-8 protease, from Miles Laboratories, Inc. (Elkhart, Ind.), proteinase K from MCB Manufacturing Chemists, Inc. (Cincinnati, Ohio), Trasylol from FBA Pharmaceuticals, Inc. (New York, N. Y.), *Micrococcal* nuclease and pTp from P-L Biochemicals, Inc. (Milwaukee, Wis.), urea and sucrose from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.), lithium dodecyl sulfate (LDS) from Gallard-Schlesinger Chemical Mfg. Corp. (Carle Place, N. Y.), and poly(U)-Sepharose, Percoll, and Ficoll from Pharmacia (Uppsala, Sweden). Other proteases, HEPES, and other reagents for in vitro protein synthesis were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RNA Extraction and Purification of Poly(A) RNA

Poly(A) RNA was purified from 7- to 10-d-old pea (*Pisum sativum* Progress No. 9) seedlings grown in a greenhouse. The seedling leaves were removed and frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. The material was thawed in an extraction buffer of 50 mM Tris-HCl (pH 8.4), 10 mM EDTA, 2% SDS, 100 mM NaCl, and then proteinase K was added to 0.5 mg/ml. After incubating with continuous mixing for 30 min at 37°C, residual proteins were removed with 2 vol of chloroform and phenol (1:1). The aqueous phase obtained upon centrifugation was re-extracted with 2 vol of chloroform to remove phenol and some of the plant phenolics. The final aqueous extract was then adjusted to pH 5.0 by dropwise addition of acetic acid and to 0.2 M NaCl. RNA was precipitated with 0.6 vol of isopropanol at −20°C overnight. This procedure facilitates minimal contamination of the RNA with phenolics and avoids coprecipitation of the masses of polysaccharides that are endemic to plant extracts. Some polysaccharides occasionally precipitate in isopropanol, but these tend to float in a mass that can be easily removed with a sterile spatula. The precipitated nucleic acid was redissolved in sterile distilled H_2O, and the high molecular weight RNA precipitated overnight at 4°C in 1 mM $MgCl_2$ and 2 M LiCl. After dissolving the LiCl pellet in sterile H_2O, the solution was adjusted to 0.2 M NaCl and the RNA precipitated with 2 vol of ethanol. Finally, the RNA was subjected to chromatography on poly(U)-Sepharose to obtain poly(A) RNA (14).

Cell-free Protein Synthesis and Immunoprecipitation of Translation Products

The conditions for in vitro protein synthesis in the wheat germ cell-free system were described recently (24, 25). Reticulocyte lysates were prepared according to Pelham and Jackson (35), except that 0.4 mM pTp (2′-deoxythymidine 3′,5′-diphosphate) was employed to arrest micrococcal nuclease (18) after digestion of endogenous mRNAs. Reticulocyte translation mixtures contained 10 mM HEPES-KOH (pH 7.5), 0.65 mM Mg acetate, 90 mM K acetate, 10 mM phosphocreatine, 24 μM of 19 amino acids minus methionine, 0.5 mM ATP, 12 μM GTP, 0.3 mM neutralized spermine, 1 mM dithiothreitol (DTT), 300 μCi/ml [^{35}S]methionine (>600 Ci/mmol), 40 μg/ml creatine phosphokinase, 50 μg/ml poly(A) RNA, and 0.4 vol of reticulocyte lysate. Wheat germ tRNAs were purified by the method of Rogg et al. (39) and used at a concentration 100 μg/ml. Translation of mRNA in this system was at 37°C for 1 h.

Immunoprecipitation of the in vitro translation products was performed with purified IgG from rabbits immunized with *Chlamydomonas* thylakoid polypeptide 11 which is a constituent of the chlorophyll-protein complex II (CP II). This antibody has been characterized previously and shown to cross-react with the homologous polypeptides of higher plants (12). Immunoprecipitation of translation products directed by pea poly(A) in the wheat germ system was performed following TCA precipitation of postribosomal supernates (42). The preponderance of globin in the reticulocyte lysates required modification of this technique. Postribosomal supernates of the reticulocyte-lysate translation mixtures were adjusted to 0.1 M Tris-HCl (pH 8.6) and 0.5% SDS. After incubation at 37°C for 15 min to denature the translation products, the mixture was diluted to 0.2% SDS by addition of Triton X-100 to 1% and NaCl to 0.15 M while Tris-HCl (pH 8.6) was maintained at 0.1 M. Trasylol to 100 U/ml and anti-11 IgG to 1 mg/ml were then added for immunoabsorption at 26°C overnight. Adsorption of the IgG-antigen complexes to formalin-fixed *S. aureus* (Cowan's strain), washing the bacteria, and elution of the IgG-antigen conjugates were performed as described before (42).

Post-translational Transport of Cell-free Translational Products into Purified Intact Chloroplasts

Chloroplasts from pea seedlings or market Romaine lettuce for in vitro transport were purified by centrifugation through Percoll (silica sol) gradients (32). Post-translational uptake experiments were performed basically as described in reference 24. Briefly, postribosomal supernates of translation mixtures were incubated for 1 h at room temperature under an illumination of ~4,000 lux. Manipulation during transport experiments in darkness was performed under a dim green safelight. The incubation mixtures contained 0.5 vol of translation mixture, 750 μg chlorophyll/ml of pea chloroplasts or 375 μg chlorophyll/ml Romaine lettuce plastids in a dilution buffer of 50 mM HEPES-KOH (pH 7.5)–0.33 M sorbitol. However, the experiment in Fig. 11 employed HEPES-KOH (pH 8.0) to enhance transport of proteins during darkness. From hemocytometer counts we determined that pea and Romaine lettuce preparations contained roughly 7×10^{-7} and 2×10^{-7} μg chlorophyll/chloroplast, respectively. The chlorophyll *a* to *b* ratio of acetone extracts (3) of pea chloroplasts was ~2.8 while that of Romaine lettuce plastids fluctuated at ~4. After transport, the chloroplasts were harvested by brief centrifugation at 4,000 *g* and resuspended in dilution buffer containing 40 μg/ml each of trypsin and α-chymotrypsin. The chloroplasts were incubated on ice for 30 min to digest adsorbed, but not transported, ^{35}S-labeled translation products. PMSF was added to 2 mM and the chloroplasts were layered over 10–80% Percoll gradients, and the surviving intact chloroplasts were reisolated (32).

After uptake, the intact chloroplasts were lysed in a hypotonic buffer of 25 mM HEPES-NaOH (pH 7.5), 1 mM PMSF, 5 mM ε-amino-N-caproic acid and 1 mM benzamidine. The chloroplast lysate was centrifuged at 15,000 *g* for 15 min. Stromal proteins in the supernate were concentrated by precipitation with 10% TCA and thylakoid membranes were purified from the 15,000 *g* pellet by flotation through discontinuous sucrose gradients (11). Both fractions were processed for PAGE as described in a later section.

Synthesis of Thylakoid Membrane Polypeptides in the Presence and Absence of Inhibitors

The sites of synthesis of thylakoid membrane polypeptides were analyzed with 7-d-old pea seedling that were excised at the epicotyl. These were inserted in vials of sterile water, and protein synthesis inhibitors were given for a 30-min preincubation period with cycloheximide or chloramphenicol at 20 μg/ml and 200 μg/ml, respectively (9). Carrier-free $^{35}SO_4^{2-}$ was then added to 10 μCi/seedling and the seedlings were incubated under a bank of fluorescent lamps for 3 h. Intact chloroplasts were prepared from each sample by centrifugation through Percoll gradients (32). Chloroplast subfractionation was carried out as described in the previous section.

Subfractionation of Thylakoid Membrane Polypeptides

Thylakoid membranes were subfractionated by extraction with a 2:1 (vol/vol) mixture of $CHCl_3/CH_3OH$ or dilute alkali. $CHCl_3/CH_3OH$ extractions of thy-

lakoids were carried out as described (13). In this procedure, most of the hydrophobic membrane polypeptides are solubilized by the organic solvent mixture. To separate peripheral from integral membrane polypeptides, we suspended a thylakoid membrane pellet in 0.1 N NaOH (46) to a final chlorophyll concentration of 0.5 mg/ml. After incubation on ice for 30 min the membrane residue was pelleted by centrifugation 140,000 *g* for 1 h. The pellet which contained the integral polypeptides was solubilized at a chlorophyll concentration of 1 mg/ml in 60 mM Tris base, 12% sucrose, 2% SDS, and 60 mM DTT, with heating at 100°C for 1 min before PAGE. The 140,000 *g* supernate which contained the peripheral polypeptides was neutralized with 1 vol of 0.1 N HCl and then precipitated with 10% TCA before solubilization in the same gel sample buffer.

Protease Digestion of Thylakoid Membranes

Thylakoid membrane vesicles (300 μg chlorophyll/ml) were treated with protease (50 μg/ml) in 50 mM HEPES-KOH (pH 7.5) and 100 mM NaCl at 26°C for 30 min. After incubation, PMSF was added to 1 mM and the membranes were pelleted at 140,000 *g* for 1 h. The membrane pellet was immediately solubilized in gel sample buffer, with heating at 100°C for 1 min.

Peptide Mapping

Thylakoid membrane polypeptides labeled in vivo with $^{35}SO_4^{2-}$ and [^{35}S]-methionine-labeled translation products of pea mRNA were resolved by SDS PAGE in 1-mm slab gels containing 9% acrylamide, 4 M urea in the first dimension. Adjacent lanes of unlabeled thylakoid membrane polypeptides were also electrophoresed, excised, and stained to locate the CP II polypeptides. These regions were then cut out of each appropriate lane and transferred at right angles to the top of 1.2-mm second-dimension gels. The 5% acrylamide stacking gels, 50 mm in length, contained, for the second dimension, *S. aureus*, strain V-8 protease (16), which was introduced by brief pre-electrophoresis to a concentration of 0.8 μg/cm width. This step was devised to minimize inactivation of the proteases in SDS and urea which might occur if the first-dimension samples were overlayed with solutions of the proteases as described by Bordier and Crettol-Jarvinen (7). The second-dimension resolving gel was 180 mm in length and was composed of a 12/0.08% to 18/0.12% gradient of acrylamide/bisacrylamide with 8 M urea throughout to achieve maximal resolution of low molecular weight peptides. Electrophoresis was performed at a constant current of 17.5 mA, during which partial proteolysis of the first-dimension samples occurred in the stacking phase. The resulting peptides were visualized by fluorography (28).

PAGE

All polyacrylamide gels in this paper contained the discontinuous buffer system of Laemmli (27), with 0.1% LDS or SDS in the upper reservoir. Gels supplemented with urea contained this reagent only in the resolving gel. Samples were applied in 60 mM Na_2CO_3, 2% LDS or SDS 60 mM DTT, and 12% sucrose, except where otherwise noted. Stained gels were processed for fluorography as described by Laskey and Mills (28).

Other Analytical Techniques

Purification, electrophoretic elution, and immunological characterization of the CP II polypeptides were performed as described by Chua and Blomberg (12). Chlorophyll concentrations were measured in 80% acetone extracts (3).

RESULTS

Characterization of the Constituent Polypeptides of Purified CP II from Peas

PAGE of thylakoid membranes solubilized in SDS give two chlorophyll protein (CP) complexes, designated CP I and CP II (cf. references 4, 6). CP II, which contains both chlorophylls *a* and *b*, is devoid of any photochemical activity and is believed to function in the capture and transfer of light energy (4). A chlorophyll *a*/*b*–protein complex, designated the light-harvesting chlorophyll-protein complex (LHC), has been purified as a subfraction of chloroplast membranes solubilized with Triton X-100 (8, 45). Because CP II and LHC are prepared by different methods, their exact relationship is still unclear. Nevertheless, it is likely that the two complexes are equivalent or at least that CP II accounts for the bulk of LHC. For this article, we regard CP II and LHC as synonymous.

There is, as yet, no consensus as to the number of polypeptides comprising CP II, the identity of the polypeptides which actually bind chlorophyll, or whether distinct CP II polypeptides bind exclusively chlorophyll *a* and *b* (8, 22, 31, 41). As a first step in the analysis of the synthesis and assembly of the CP II polypeptides, we undertook to determine the minimum number of polypeptides which comprise CP II in peas. This was accomplished by solubilizing purified thylakoid membranes with LDS followed by PAGE of the sample at 4°C. As reported previously (20), CP complexes related to PS II reaction center, which dissociate if exposed to room temperatures, remain stable during low temperature electrophoresis. Additionally, there is a marked increase in the amount of chlorophylls *a* and *b* associated with CP II. When solubilized thylakoids are heated at 100°C for 45 s before electrophoresis, all CP complexes are dissociated. As seen in Fig. 1 (first two lanes from the left), CP II comigrates with a major and a minor band in the heated sample. Because of this comigration, it is difficult to determine the true constituent polypeptides of CP II purified by PAGE in SDS.

To determine unambiguously the constituent polypeptides of CP II, we exploited the observation of Aro and Valanne (5) that inclusion of magnesium in SDS gels and anodal buffer results in an altered mobility of CP II. When this was adopted to the LDS gel system (Fig. 1), we found that the electrophoretic mobility of CP II is progressively retarded relative to other thylakoid membrane polypeptides as the concentration of Mg^{2+} is increased. Thus, we were able to select a Mg^{2+} concentration which will cause CP II to migrate to a region of the gel where

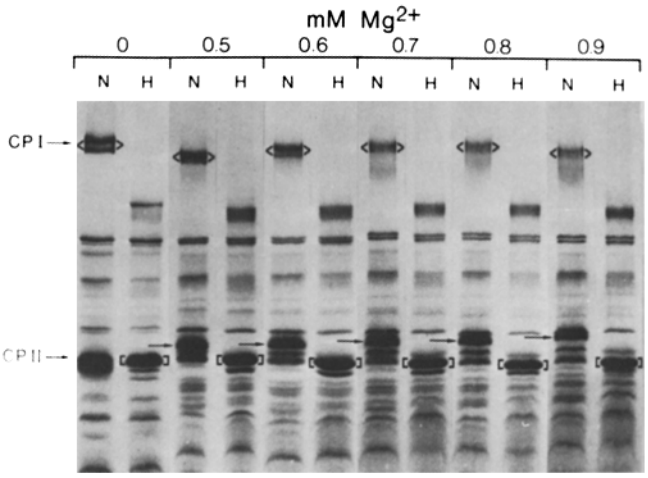

FIGURE 1 Effect of Mg^{2+} on the electrophoretic mobility of CP II from pea thylakoids. A composite is presented of six LDS gels composed of 9% polyacrylamide. The gels differ in the concentration of $MgCl_2$, that was included in the stacking gel, resolving gel, and anodal reservoir buffers. Pea thylakoid membranes were solubilized at 4°C with LDS in the sample buffer described in Materials and Methods. An aliquot of the membranes (*H*) was heated at 100°C for 45 s to dissociate chlorophyll from the CP II polypeptides (in brackets) before loading in wells adjacent to nonheated (*N*) membranes. Electrophoresis was carried out at 4°C. The positions of CP I (<>), the chlorophyll *a*-P700-protein complex, and CP II (→) are indicated. CP I appears to deteriorate and migrate faster as a function of increasing Mg^{2+} concentrations, whereas CP II is stabilized and exhibits decreased electrophoretic mobility relative to proteins that do not bind chlorophylls in both nonheated and heated samples.

few polypeptides in samples that are heated before electrophoresis are located. Consequently, CP II can be obtained in a state of high purity simply by subjecting LDS-solubilized thylakoid membranes to gel electrophoresis in which 0.7 mM Mg^{2+} is included in the lower reservoir and resolving gel buffers.

CP II was excised from preparative gels containing 0.7 mM Mg^{2+}, heat-dissociated, and re-electrophoresed on polyacrylamide gels containing no Mg^{2+}. Only one stained gel band could be resolved on a variety of gels that differed in polyacrylamide concentration or buffer composition (data not shown). However, Süss et al. (48) reported that two polypeptides which comigrate with CP II could be resolved in polyacrylamide gels containing urea. The electrophoretic mobility of many thylakoid membrane polypeptides is profoundly altered by urea, which probably affects the amount of detergent that binds to certain polypeptides. This effect is illustrated in Fig. 2*a*, in which a 10% polyacrylamide gel was cast so that a 0–8 M gradient of urea was formed in the transverse direction. A sample of SDS-solubilized, heat-treated thylakoid membranes was then layered across the top of the gel. The electrophoretic mobility of many thylakoid membrane polypeptides from pea seedlings is dramatically retarded as the concentration of urea is increased. The order of migration of several polypeptides, especially those of low molecular weight, is altered. Moreover, the major thylakoid membrane polypeptides that normally comigrate with CP II are resolved into at least two major species. Subsequently, the CP II region from a preparative polyacrylamide gel containing 0.7 mM Mg^{2+} was excised, subjected to heat-dissociation, and then analyzed on a 10% gel containing a transverse 0–8 M urea gradient. As seen at the far left of the gel (Fig. 2*b*), only one stained gel band of highly purified CP II is obtained in the absence of urea. However, when the urea concentration of the resolving gel is increased to 4 M, this band is resolved into two polypeptide species. We designate these as polypeptides 15 and 16 on the basis of their electrophoretic migration with respect to the other major components of pea thylakoid membranes. As yet, we do not know whether both polypeptides 15 and 16 bind both chlorophyll *a* and *b*. However, polypeptides 15 and 16 are, certainly, the minimal components required for formation of CP II.[1]

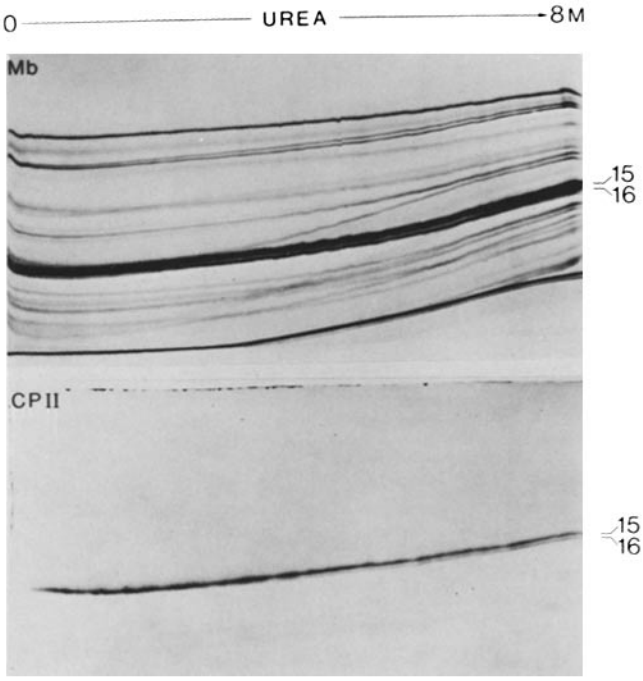

FIGURE 2 Effect of urea on the electrophoretic mobility of thylakoid membrane proteins and resolution of the CP II polypeptides. A gradient of 0–8 M urea was established in the transverse direction of 10% polyacrylamide gels by casting at right angles to the direction of electrophoresis. After polymerization, a 5% polyacrylamide stacking gel without sample slots was formed in the normal position. In the upper gel, heated thylakoid membranes were applied in a continuous layer across the top of the gel. In the lower gel, the CP II region of a preparative 9% polyacrylamide, 0.7 mM $MgCl_2$, LDS gel of nonheated thylakoids electrophoresed at 4°C was excised. After heating the gel strip in 50 mM DTT, 2% SDS, it was transferred to the top of the second-dimension urea gradient gel. The positions of CP II polypeptides 15 and 16 are indicated.

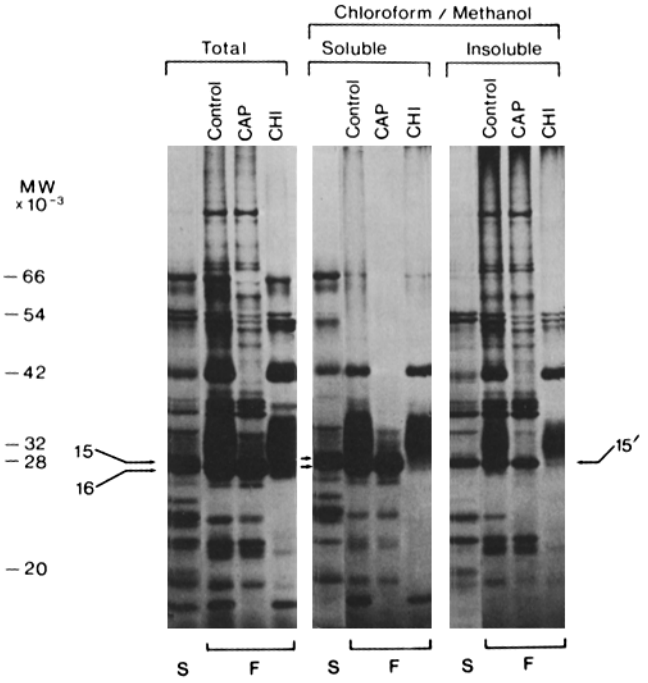

FIGURE 3 In vivo sites of synthesis and solubility properties of pea thylakoid membrane proteins. Thylakoid membranes were purified from intact chloroplasts of pea seedlings that were pulse-labeled for 3 h with $^{35}SO_4^{2-}$ in the absence of protein synthesis inhibitors (Control) or presence of 200 μg/ml chloramphenicol (*CAP*) or 20 μg/ml cycloheximide (*CHI*). Aliquots of the membranes were extracted with 2:1 (vol/vol) mixtures of $CHCl_3$ and CH_3OH to obtain soluble and insoluble subfractions. Unextracted membranes (Total) and the organic solvent subfractions were solubilized with SDS, heated at 100°C for 45 s, and then electrophoresed in a 9% polyacrylamide, 4 M urea gel. The gel was stained and photographed (*S*) before processing for fluorography (*F*) to visualize the labeled polypeptides. The positions of CP II polypeptides 15, 16, and 15′ are indicated.

The polypeptides associated with CP II are the major $CHCl_3/CH_3OH$-soluble components of photosynthetic membranes, and a substantial portion of polypeptide 15 and all of polypeptide 16 are soluble in the organic solvent (Fig. 3). A band which comigrates with polypeptide 15 is recovered in the

[1] We have found recently that when CP II is obtained from gels without $MgCl_2$ it contains two to three additional minor polypeptides in the 23,000- to 25,000-dalton range (P. Delepelaire and N.-H. Chua, unpublished data). Thus, $MgCl_2$ may cause release of chlorophylls from these minor constituents of CP II.

$CHCl_3/CH_3OH$-insoluble phase. As will be shown later, this polypeptide, designated 15′, is structurally and immunologically identical to polypeptide 15 and is the result of an incomplete extraction of the latter by the organic solvent.

Intracellular Site of CP II Polypeptide Synthesis

The thylakoid membrane component which comigrates with CP II was shown by Machold and Aurich (30) and Cashmore (9) to be a product of cytoplasmic protein synthesis. Since two polypeptides in this region of polyacrylamide gels could be resolved, we re-examined the sites of synthesis of pea thylakoid synthesized on cytoplasmic ribosomes.

More than 30 thylakoid membrane polypeptides were intensely labeled with $^{35}SO_4^{2-}$ in vivo (Fig. 3, lane *2*). These include all but two of the major stained constituents of the membranes. In addition, some radioactive bands are poorly visualized in the stained gel profiles, particularly in the high molecular weight (>66,000) region and in the region of 32,000 daltons above polypeptides 15 and 16. Cycloheximide blocks synthesis of most of the low molecular weight polypeptides (Fig. 3, lane *4*). However, labeling of eight polypeptides persists, indicating that these are products of chloroplast protein synthesis. The major chloroplast synthesized thylakoid polypeptide is a diffuse band (32,000 mol wt), corresponding to the D protein which is a product of protein synthesis in isolated chloroplasts (23). The diffuse nature of the 32,000-dalton protein partially obscures polypeptides 15 and 16 in the control sample. However, the results with the inhibitors show clearly that both polypeptides 15 and 16 are products of cytoplasmic protein synthesis. They are labeled in the presence of chloramphenicol at concentrations that completely inhibit synthesis of the 32,000-dalton polypeptides but are not synthesized in the presence of low concentrations of cycloheximide.

Immunological Characterization of CP II Polypeptides

In *Chlamydomonas reinhardtii*, three thylakoid membrane polypeptides, 11, 16, and 17, have been shown to be subunits of the CP II complex (12). Antibodies raised against polypeptide 11 were found to react with polypeptides 16 and 17, forming partial immunological identity (12). These results indicate that the three constituent polypeptides of the CP II share common antigenic determinants and are probably structurally related. Crossed immunoelectrophoresis revealed that anti-*Chlamydomonas* polypeptide 11 IgG reacts with two homologous thylakoid polypeptides in spinach that migrate near one another in the CP II region during SDS PAGE (12). Similar analysis with pea thylakoid polypeptides resulted in the formation of a broad immunoprecipitin arc that spanned polypeptides 15 and 16 (data not shown). We interpret these results to mean that pea polypeptides 15 and 16, which are constituents of CP II, both cross react with the antibodies showing complete immunological identity with respect to the antigenic domains recognized by the heterologous antibodies. Because the two polypeptides migrate closely together in SDS gels, only a broad precipitin arc resulted. To obtain additional evidence on this point, we purified polypeptides 15 and 16 and polypeptide 15′ by preparative electrophoresis (12) of the $CHCl_3/CH_3OH$ extracts and residues. These polypeptides were analyzed by immunodiffusion using antibodies raised against *Chlamydomonas* polypeptide 11. Fig. 4 shows that a single precipitin line is formed against each of these polypeptides as well as the total complement of SDS-solubilized thylakoid membranes or the $CHCl_3/CH_3OH$ subfractions. Since the precipitin lines fuse completely with each membrane component, we conclude that the antigenic determinants recognized by the heterologous antibodies are identical in the three polypeptides 15, 15′, and 16. We wish to emphasize that immunological differences between polypeptides 15 and 16 may be revealed if antibodies raised against one of these polypeptides were used.

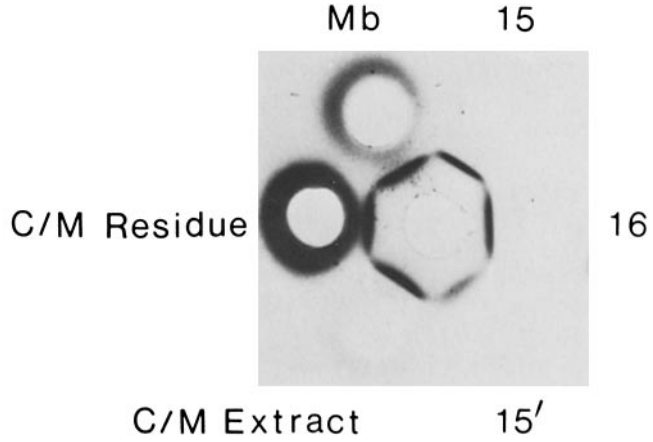

FIGURE 4 Immunological resemblance of the CP II polypeptides. Polypeptides 15 and 16 were purified by preparative electrophoresis in 9% acrylamide, 4 M urea gels of $CHCl_3/CH_3OH$ extracts of pea thylakoid membranes, whereas polypeptide 15′ was similarly obtained from gels of the $CHCl_3/CH_3OH$-insoluble fraction. 5 μg of each polypeptide, 25 μg of the total (*Mb*) thylakoid membranes, and the equivalent portions of the $CHCl_3/CH_3OH$-derived subfractions were applied to the peripheral wells of 1% agarose gels containing 50 mM Tris-HCl (pH 8.6), 0.1 M NaCl, and 1% Triton X-100. The antigens were solubilized in 1% SDS and then were diluted with a tenfold excess weight of Triton X-100 to sequester SDS before they were applied to the agarose wells (12). The center well contained 0.75 mg of IgG purified from antiserum against the major CP II polypeptide (polypeptide 11) of *Chlamydomonas reinhardtii*.

Immunological cross-reacting of polypeptides 15, 15′, and 16 could be interpreted as evidence for a common biosynthetic pathway. Thus, polypeptides 15 could be a relatively stable precursor to polypeptide 16. Alternatively, polypeptide 16 might originate by spurious proteolysis of polypeptide 15 during purification and analysis of the membranes. However, this possibility appears unlikely since the inclusion of several protease inhibitors during all steps did not alter the ratio of the two polypeptides. A third possibility, which is supported below, is that polypeptides 15 and 16 are distinct protein synthesis products. Their immunological relatedness could result from an evolutionary duplication and divergence of a structural gene or alternative pathways by which a single primary transcript is subsequently processed to form somewhat different mRNAs encoding the separate polypeptides.

Identification of Higher Molecular Weight Precursors to the Major CP II Polypeptides

The primary translation product of mRNA encoding the CP II polypeptides was identified by immunoprecipitation of translation mixtures primed with pea leaf poly(A) RNA (Fig. 5). Two polypeptides were reproducibly immunoprecipitated with antibodies against *Chlamydomonas* polypeptide 11 from the

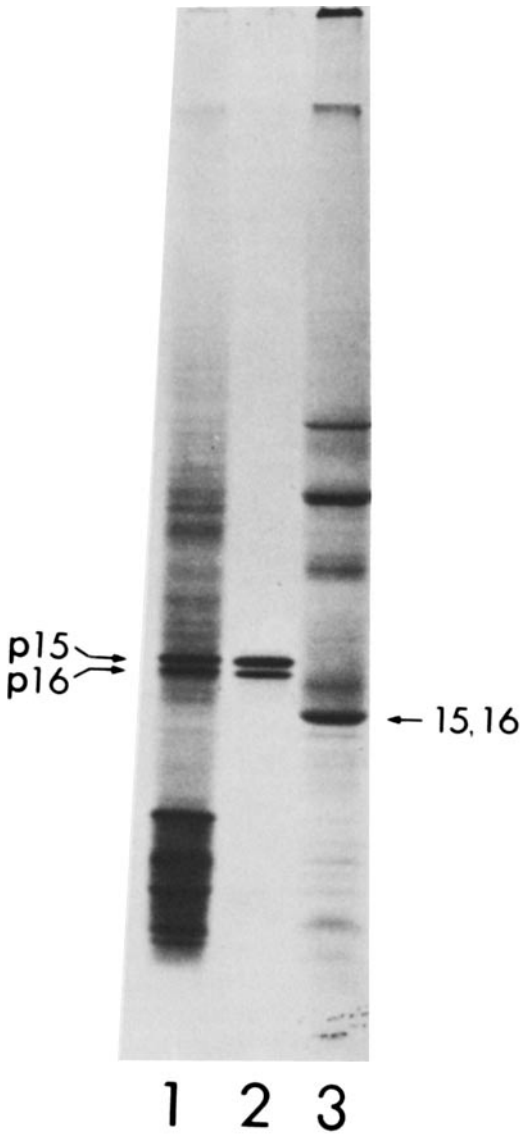

FIGURE 5 Immunological identification of higher molecular weight precursors of the CP II polypeptides. Poly(A) RNA from pea seedlings was translated in the wheat germ system (25). A portion of the postribosomal supernate obtained from the translation mixture was precipitated with 10% TCA (lane *1*), and the remainder was subjected to immunoprecipitated with antibodies to *Chlamydomonas* polypeptide 11 (lane *2*). The fluorograph of a 7.5–15% gradient polyacrylamide gel is presented with $^{35}SO_4^{2-}$-labeled pea thylakoid membrane polypeptides for reference in lane *3*. In this gel system, polypeptides 15 and 16 are not resolved from one another.

products formed in the wheat germ system (lane *2*).[2] These have molecular weights of ~33,000 and 32,000 and are designated p15 and p16, respectively, on the basis of the peptide-mapping studies presented below. A single higher molecular weight precursor to a CP II polypeptide was identified previously among the translation products of barley poly(A) RNA (1, 2). However, our results clearly show that pea thylakoids contain at least two CP II polypeptides, so the presence of two precursors in the cell-free translation mixture is not unexpected. We consider it unlikely that p15 is converted to p16 by proteases or other modifying enzymes present in the wheat germ system since both precursors are also synthesized in the rabbit reticulocyte lysate system (reference 43, data not shown). Moreover, p16 probably does not result from premature chain termination during translation of p15 mRNA, since supplementing the reticulocyte lysate with wheat germ tRNAs has no effect on the relative abundance of these two products (reference 43, data not shown).

Further evidence that polypeptides 15 and 16 are synthesized independently as higher molecular weight precursors is shown by peptide mapping (Fig. 6). The peptide profiles generated by V8 protease from p15 and p16 are different, although some fragments do comigrate. Further, many of the fragments observed in the digestion products of p15 and p16 comigrate with some products of polypeptides 15 and 16, respectively. There are prominent peptides produced from the in vivo–labeled polypeptides which are not apparent in the precursors; these differences could arise from the use of $^{35}SO_4^{2-}$ for in vivo labeling and [^{35}S]methionine for in vitro protein synthesis. Several fragments of polypeptides 15 and 16 comigrate, sug-

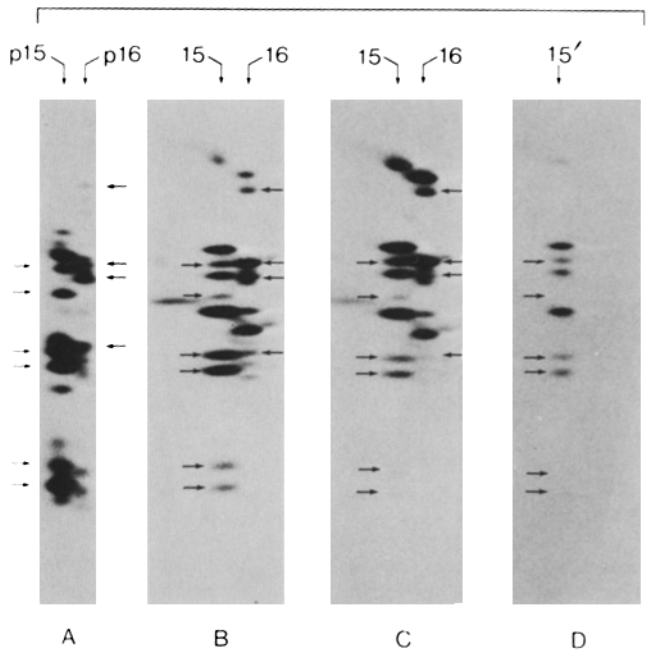

FIGURE 6 Confirmation of higher molecular weight precursors to the CP II polypeptides by peptide mapping. [^{35}S]methionine-labeled translation products of pea mRNA (*A*) and thylakoid membrane proteins that were pulse-labeled with $^{35}SO_4^{2-}$ (in the presence of 200 μg/ml chloramphenicol) (*B*) were electrophoresed in first-dimension gels of 9% polyacrylamide, 4 M urea. Thylakoid membranes subfractionated into $CHCl_3/CH_3OH$-soluble (*C*) and -insoluble (*D*) constituents were also electrophoresed in parallel lanes of the first-dimension gel. Regions of the respective lanes containing the CP II polypeptides or presumptive precursors were excised and subjected to proteolysis with *S. aureus* V-8 protease during stacking in the second-dimension 8 M urea, 12–18% polyacrylamide gradient gel. The first-dimension positions of p15 and p16 and mature $CHCl_3/CH_3OH$-soluble CP II polypeptides 15 and 16 and $CHCl_3/CH_3OH$-insoluble polypeptide 15′ are indicated at the top of the fluorographs. Peptides which are common to p15 and polypeptides 15 and 15′ (→) and peptides common to p16 and polypeptide 16 (←) are indicated.

[2] In addition to p15 and p16, a faint band at 31,000 daltons is seen in the immunoprecipitate upon over exposure of the fluorographs. With some batches of wheat germ extracts, polypeptide bands that comigrate with polypeptides 15 and 16 are also recovered. It is likely that some extracts contain processing enzymes, probably derived from proplastids in the wheat embryo, which convert a limited amount of the precursors to the mature forms.

gesting that these polypeptides are structurally related. Finally, Fig. 6 also shows that digestion products of polypeptide 15′ are indistinguishable from those of polypeptide 15, indicating that the two polypeptides are identical.

We have recently isolated a cDNA clone which is complementary to the mRNA encoding only the 33,000-dalton polypeptide, p15 (R. Broglie et al., unpublished data). Translation of this mRNA in a wheat germ system followed by uptake of the translation product shows that p15 is processed to polypeptide 15 by intact pea chloroplasts. These results reinforce our conclusion that the 33,000-dalton polypeptide is the precursor to polypeptide 15.

Precursors to the CP II Polypeptides Are Soluble

The occurrence of p15 and p16 in the postribosomal supernates of in vitro translation mixtures does not eliminate the possibility that these polypeptides are sequestered in lipid or low-density membrane vesicles that could be present in wheat germ extracts or reticolocyte lysates. In either case, the precursors should be completely or partially protected against proteolysis by virtue of the hydrophobic domains of such structures. However, treating postribosomal supernates of the pea poly(A) RNA translation products with trypsin results in complete digestion of p15 and p16 as well as pS (data not shown). These results contrast starkly with the effects of proteases on the mature forms of the CP II polypeptides that are embedded in thylakoid membranes (Fig. 10). Therefore, the data indicate that p15 and p16 do not reside in membrane or membranelike structures in translation mixtures, even though their mature forms display marked hydrophobicity. The physiological significance of this finding was established by showing that transport of water-soluble p15 and p16 into isolated intact chloroplasts occurs in a post-translational manner.

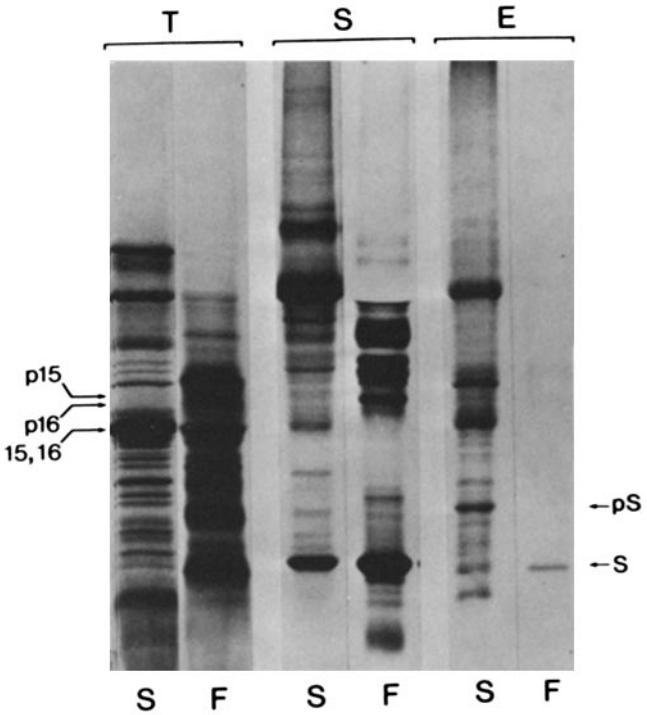

FIGURE 7 Distribution of post-translationally imported polypeptides in chloroplast subfractions. Post-translational transport of in vitro translation products of pea mRNA into isolated intact pea chloroplasts was performed as described in Materials and Methods. Adsorbed, but not transported translation products were removed by proteolysis, and intact chloroplasts were repurified. A composite is presented of the stained 7.5–15% polyacrylamide gradient gel profiles (*S*) and their respective fluorographs (*F*) to visualize imported polypeptides recovered with the total thylakoid (*T*), soluble stroma (*S*) and envelope membrane (*E*) fractions that were subsequently purified as described in Materials and Methods. The positions of pS, p15, and p16 were determined by coelectrophoresis of an aliquot of the postribosomal supernate of the translation mixture used for transport.

Post-translational Transport of CP II Polypeptides into Isolated Intact Chloroplasts

To assess the physiological role of p15 and p16, we investigated whether they can be imported into intact chloroplasts in vitro. Thus, the postribosomal supernate of a translation mix, which contained labeled pea polypeptides including p15 and p16, was incubated with intact pea chloroplasts under conditions shown to be optimal for post-translational uptake of chloroplast polypeptides (25). After incubation, the chloroplasts were fractionated into envelope, stroma and thylakoid membranes by sucrose density gradient centrifugation (36). Fig. 7 shows that the major translation product that is imported into chloroplasts is S, which is found most abundantly in the stroma fraction and to a minor extent in association with the envelope fraction. Several other in vitro synthesized polypeptides are also prominent in the stromal fraction. The CP II polypeptide precursors, p15 and p16, are not found in any of the chloroplast fractions as determined by mobilities in gels (Fig. 7) or immunological methods (data not shown). In contrast, thylakoid membranes contain labeled polypeptides 15 and 16 and as well as several other as yet unidentified membrane polypeptides. From these results we conclude that p15 and p16 and precursors of the other membrane polypeptides are transported into chloroplasts by a post-translational mechanism. During or immediately after passage through the envelope membranes or upon association with the thylakoids, the precursors must be converted rapidly to their mature forms. Significantly, neither the stromal nor the envelope fraction contains labeled polypeptides 15 and 16 (Fig. 7), demonstrating that in our in vitro reconstitution system the post-translational import of these polypeptides culminates in their specific localization in the thylakoid membranes. The absence of newly imported CP II polypeptides and their precursors in the stromal and envelope fractions suggests that the transit time through these chloroplast compartments must be very rapid.

Transport, Processing, and Assembly of CP II Polypeptides into Chloroplasts from Homologous and Heterologous Sources

In many experiments we employed heterologous systems consisting of chloroplasts from the inner leaves of Romaine lettuce and pea poly(A) RNA translation products to maximize uptake and assembly of p15 and p16. The small plastids of Romaine lettuce are relatively undifferentiated and their thylakoids contain proportionally lower amounts of native CP II polypeptides. Also, the Romaine lettuce CP II polypeptides exhibit electrophoretic mobilities different from those of pea. This has facilitated analysis of heterologous processing of p15 and p16 to their mature forms and their subsequent capacity to integrate into the lipid bilayer of photosynthetic membranes.

The pea mRNA translation products that are imported into Romaine lettuce chloroplasts and are recovered specifically in

thylakoids show electrophoretic mobilities different from those of Romaine lettuce but identical to those observed in pea thylakoids (Fig. 8). Hence, heterologous transport of these proteins is accompanied by correct processing of precursors with respect to the source of the mRNA and not the origin of the chloroplasts. Therefore, the processing enzymes and the sites on precursors that they attack must be evolutionarily conserved.

To determine that the imported CP II polypeptides are inserted into the thylakoid lipid bilayer, we suspended the membranes in 0.1 N NaOH, a treatment which extracts peripheral membrane components (46). Polypeptides 15 and 16 that were transported in vitro, like those synthesized in vivo, are not removed by this treatment (Fig. 8). Thus, our in vitro transport system faithfully mimics the in vivo events which result in the complete integration of CP II polypeptides into thylakoids as integral membrane components. Several other peripheral and integral membrane polypeptides also undergo post-translational transport into both pea and Romaine lettuce chloroplasts. In each instance, processing and assembly is correct with regard to the mature, native pea membrane components and not those of Romaine lettuce.

Further evidence that polypeptides 15 and 16 are processed and assembled correctly following in vitro transport into either pea or Romaine lettuce chloroplasts was obtianed by $CHCl_3$/CH_3OH extraction of purified thylakoid membranes. Solvent

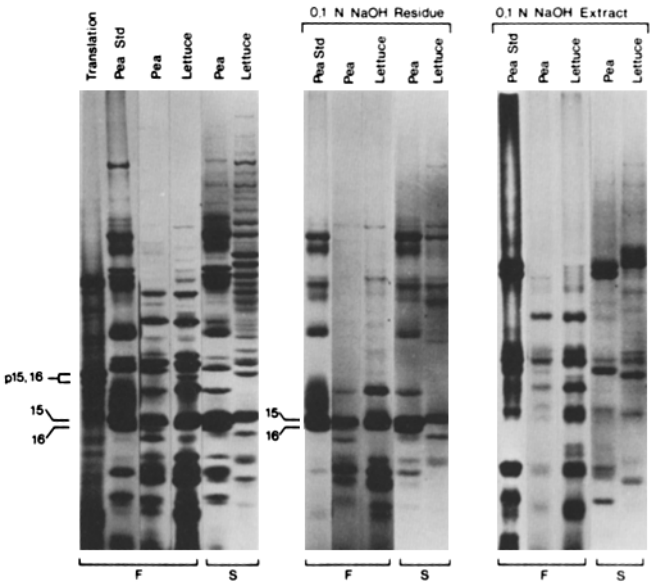

FIGURE 8 Post-translational transport, processing, and thylakoid membrane assembly of pea thylakoid polypeptides with chloroplasts from homologous and heterologous sources. Postribosomal supernates of translation products of pea mRNA were incubated with intact chloroplasts from pea and Romaine lettuce as described in Materials and Methods. Subsequently, thylakoid membranes were isolated from protease-treated and repurified plastids. Portions of the thylakoids were solubilized directly with SDS and heated at 100°C for 45 s before loading on the 9% polyacrylamide, 4 M urea gel. Another aliquot of each of the thylakoid preparations was extracted with 0.1 N NaOH to separate extrinsic proteins from the integral proteins, recovered in the NaOH residue. Composites of fluorographs (*F*) to visualize imported proteins are displayed with the corresponding stained gel profiles (*S*) of the thylakoid membranes and NaOH-derived subfractions from pea and Romaine lettuce chloroplasts. The positions of pea CP II polypeptides 15 and 16 are indicated as are those of their precursors.

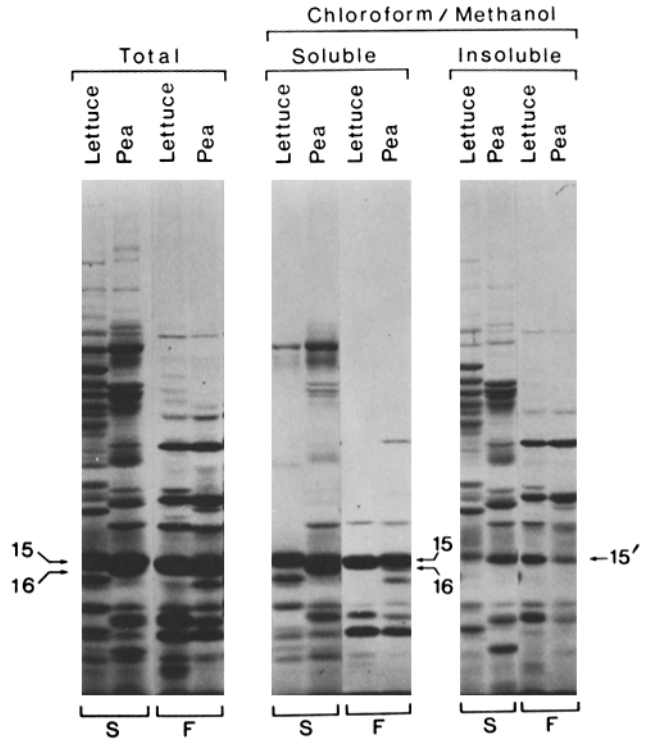

FIGURE 9 Imported thylakoid polypeptides acquire solubility properties identical to those native to pea upon post-translational transport and processing with homologous and heterologous chloroplasts. Thylakoid membranes were purified from reisolated intact chloroplast employed for in vitro import of pea mRNA translation products. A portion of the thylakoids from pea and Romaine lettuce were subjected to extraction with a 2:1 (vol/vol) mixture of $CHCl_3$/CH_3OH as described in the legend of Fig. 3. The stained profiles (*S*) and respective fluorographs (*F*) to visualize imported proteins in the total and organic solvent subfractions of thylakoids resolved in a 9% polyacrylamide, 4 M urea gel are presented in composite form.

partitioning of the CP II polypeptides imported in vitro is identical to that from native pea thylakoids (Fig. 9). Despite the solubility of p15 and p16 in aqueous solutions, after they are transported through both membranes of the chloroplast envelope, processed, and inserted into the thylakoids, they are recovered as mature forms that are hydrophic, integral membrane components.

The extractability of newly imported polypeptides 15 and 16 with alkali and organic solvents indicates that they are firmly lodged in the thylakoid membranes. However, these experiments do not define the degree to which the respective polypeptide chains are embedded in the lipid bilayer. Transmembrane disposition was investigated by determining the extent to which imported polypeptides are protected against proteolysis by the lipid bilayer of the thylakoid membrane. Several high molecular weight membrane polypeptides are extensively digested by either trypsin or acetylated trypsin, but there is only a small effect on endogenous or imported polypeptides 15 and 16 (Fig. 10). In both cases, the altered electrophoretic mobilities of imported 15 and 16 are indistinguishable from those of the partially digested native pea CP II constituents. The CP II polypeptides imported into Romaine lettuce chloroplasts appear to attain dispositions in the lipid bilayer identical to those that are endogenous to pea thylakoids. These results are the strongest evidence that in vitro synthesis and transport of the CP II polypeptide precursors results in correct processing and insertion into the thylakoid membranes.

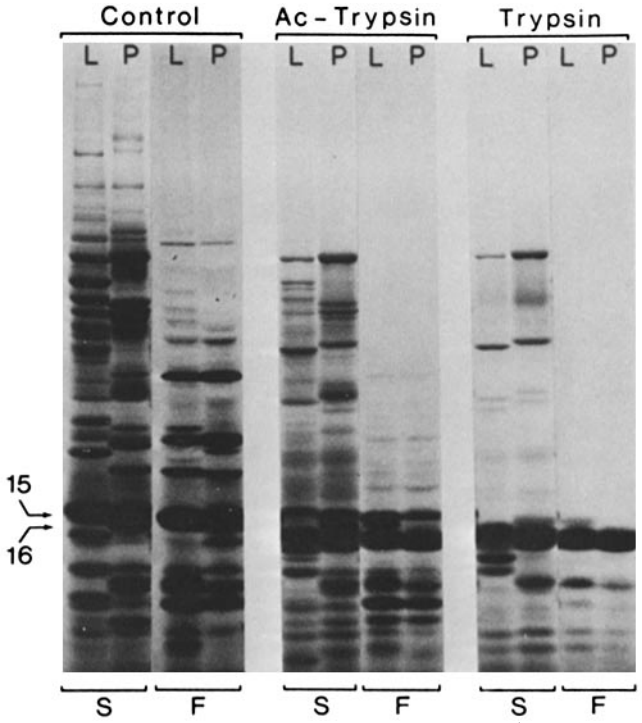

FIGURE 10 Imported polypeptides acquire conformations in the thylakoids membrane lipid bilayer indistinguishable from those native to pea upon post-translational transport and processing with homologous and heterologous chloroplasts. Thylakoid membranes were purified from chloroplasts of pea (*P*) and Romaine lettuce (*L*) that were employed for import of pea mRNA translation products. The membranes were incubated with the indicated proteases for 30 min at room temperature as described in Materials and Methods. The membranes were solubilized with SDS and heated at 100°C in gel sample buffer before those portions of the polypeptides that were protected from degradation by the lipid bilayer were resolved in a 9% polyacrylamide, 4 M urea gel. Stained gel profiles (*S*) are presented for comparison with those of imported polypeptides visualized by fluorography (*F*).

In Vitro Assembly of the CP II Polypeptides Results in Chlorophyll Binding

The ultimate test of our attempts to reconstruct with fidelity the in vitro synthesis, transport, and assembly of the CP II polypeptides is to demonstrate that the imported proteins attain functional roles in the photosynthetic membranes. Although we cannot show conclusively that the newly imported CP II polypeptides harvest light energy for photosynthetic electron transport, we show that they bind chlorophylls to form CP II. Imported polypeptides 15 and 16 are recovered in CP II after electrophoresis in LDS containing Mg^{2+} (Fig. 11). Like endogenous polypeptides 15 and 16, the imported polypeptides are dissociated from the complex upon heating of thylakoids before electrophoresis. The polypeptide that migrates most slowly in the CP II samples is a thylakoid polypeptide not associated with CP II, since its mobility in Mg^{2+}-LDS gels is not altered by heating the thylakoids before electrophoresis. These results provide evidence that assembly of the in vitro–synthesized proteins into thylakoid membranes is accompanied by chlorophyll binding. However, we cannot rule out the possibility that the altered mobility of the imported polypeptides 15 and 16 in Mg^{2+}-LDS gel may be due to their association with lipids rather than with chlorophyll.

Results of numerous in vivo studies have indicated that either synthesis or membrane assembly of the CP II polypeptides is tightly coupled to light-dependent chlorophyll synthesis in higher plants (cf. reference 6). We addressed this aspect of thylakoid membrane biogenesis by examining whether the CP II polypeptides can integrate into thylakoid membranes without concomitant chlorophyll formation during uptake experiments. Transport and assembly of polypeptides 15 and 16 occur in darkness, although to a lesser extent than in the light (Fig. 11). We have shown elsewhere (24) that the reduced level of transport and assembly of CP II polypeptides in the dark can be restored to the light level by the addition of ATP. Thus, partial dark inhibition is due to a requirement by the envelope protein transport mechanism for ATP (24), the most accessible source of which is photosynthetic phosphorylation. Despite

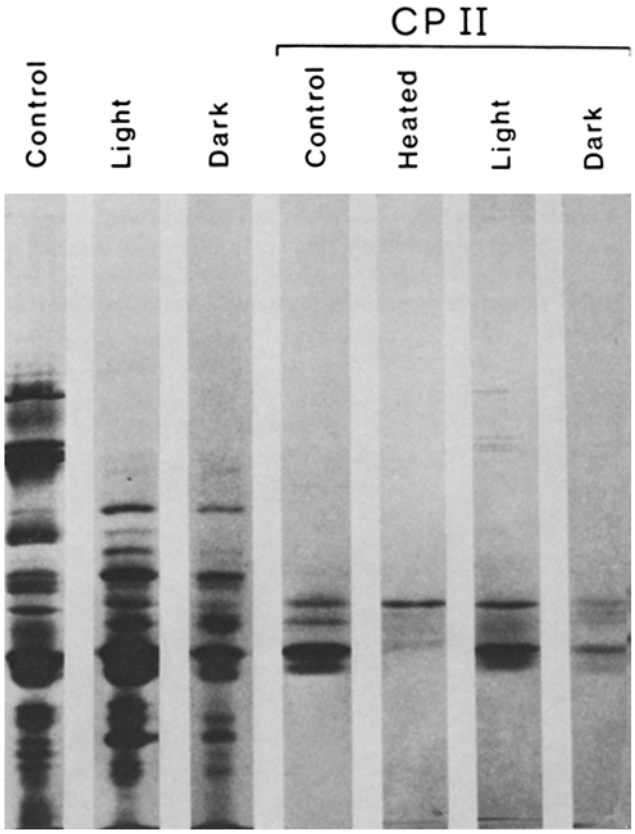

FIGURE 11 Assembly of imported CP II polypeptides culminates in chlorophyll binding during post-translational transport in light and darkness. Thylakoid membranes were purified from pea chloroplasts that were incubated with postribosomal supernates of in vitro translation mixtures in light or darkness. In the latter, exposure to light did not occur until membrane pellets were obtained from lysed chloroplasts. Aliquots of the membranes were solubilized with LDS at 4°C and subjected to LDS PAGE in a 9% polyacrylamide, 0.7 mM $MgCl_2$ gel as described in the legend to Fig. 2. A CP II control sample was prepared from pea thylakoids labeled in vivo with $^{35}SO_4^{2-}$ in the presence of chloramphenicol. A sample of thylakoids from chloroplasts incubated with translation products in the light was heated before Mg^{2+} LDS gel electrophoresis to identify contaminants of the CP II preparation. The CP II regions were excised and the gel slices were heated at 100°C for 1 min to dissociate the chlorophyll-protein complexes before they were transferred to a second-dimension 9% polyacrylamide, 4 M urea gel. Samples of the total thylakoid membrane polypeptides of the chloroplasts used for import were resolved in adjacent lanes in the second-dimension gel. Only ^{35}S-labeled polypeptides visualized by fluorography are shown.

reduced transport of p15 and p16 in darkness, detectable amounts of the in vitro synthesized proteins are recovered in the CP II from chloroplasts that were incubated with translation products in darkness. These results suggest that a pool of chlorophyll is present in thylakoid membranes and available for binding to newly imported CP II polypeptides or that exchange of chlorophyll can occur between the newly imported and extant CP II proteins. Thus, membrane assembly of polypeptides 15 and 16 can be uncoupled at least to some extent from chlorophyll biosynthesis.

DISCUSSION

In this study, we have reconstructed in vitro the major events in the biosynthesis of the two constituent polypeptides of the LHC. The salient features of our findings are: (*a*) the primary in vitro translation products of mRNAs encoding the two CP II polypeptides (polypeptides 15 and 16) are soluble, higher molecular weight precursors (p15 and p16); (*b*) the precursors are imported into intact chloroplasts post-translationally; (*c*) during or shortly after passage through the envelope membranes, the precursors are processed correctly to their mature sizes by proteases that are present in the chloroplasts; (*d*) the newly imported precursors are recovered exclusively in thylakoid membranes where they, like their native counterparts, reside as hydrophobic integral components; (*e*) the dispositions of the newly integrated polypeptides in the thylakoid membranes are indistinguishable from those of the endogenous forms, as probed by limited proteolysis of isolated thylakoid vesicles; (*f*) the CP II polypeptides synthesized in vitro most likely bind chlorophyll and therefore are presumed to have attained a functional role in the photosynthetic membranes; and (*g*) transport and assembly of the CP II polypeptides can be uncoupled from chlorophyll biosynthesis; however, we cannot rule out an absolute requirement for chlorophyll since a small pool of the pigments may exist in our chloroplasts incubated in the dark.

Transport of the CP II polypeptide precursors, p15 and p16, occurs by a post-translational mechanism identical to that demonstrated previously for the soluble small subunit of RuBPCase (15). The soluble protein is synthesized initially as a precursor with a 4,000- to 5,000-dalton chain extension at the amino terminus. The chain extension in the membrane polypeptide precursors is of a similar size. We assume that this chain extension functions as a transit sequence (15, 42) in that it plays a role in the post-translational uptake of the precursors into chloroplasts. Furthermore, since polypeptides 15 and 16 are hydrophobic membrane proteins, the precursor chain extension must also contain special properties that render the precursors soluble.

Our in vitro reconstitution system for uptake and processing of p15 and p16 provides the opportunity to clarify one aspect of the assembly pathway of the LHC. The complex may be assembled by two possible routes: (*a*) the newly imported polypeptides 15 and 16 combine first with newly synthesized chlorophyll *a* and *b* as a CP complex before insertion into the thylakoids. Thus, the formation of the complex is an obligatory intermediate step before membrane insertion. (*b*) Thylakoid insertion of polypeptides 15 and 16 occurs independent of new chlorophyll synthesis. The inserted polypeptides may then recruit pre-existing chlorophyll in the thylakoid membranes to form the LHC. Since uptake and processing of the precursors and assembly of the imported polypeptides 15 and 16 can occur in the dark, a condition which prevents chlorophyll synthesis in higher plants, our results demonstrate that assembly of CP II occurs in the thylakoid membrane after insertion of the apoprotein.

An important point that emerges from our work is that the newly transported polypeptides 15 and 16 are localized exclusively in the thylakoid membranes. Thus, mechanisms must exist to ensure their specific thylakoid insertion after passage through the chloroplast envelope. One could argue that since these polypeptides are hydrophobic they associate spontaneously with the thylakoid membranes. However, this property alone is insufficient to explain why polypeptides 15 and 16 are not detected in the chloroplast envelope membranes. Among the many differences between the thylakoids and the envelope is the exclusive localization of chlorophylls *a* and *b* in the former membrane system (36). Since polypeptides 15 and 16 are chlorophyll-binding proteins, we consider it reasonable to propose that both chlorophyll *a* and *b* serve as receptors for these membrane polypeptides. However, recent results showed that the chlorophyll *b*-less mutant of barley is completely competent in the uptake, processing, and thylakoid assembly of polypeptides 15 and 16 (G. Bellemare, S. G. Bartlett, and N.-H. Chua, unpublished observations). This observation rules out unequivocally chlorophyll *b* as a receptor but still leaves open the possibility of chlorophyll *a* for this role.

In addition to polypeptides 15 and 16, many labeled proteins are recovered in the repurified chloroplasts employed for uptake of in vitro translation products. Nearly every one of these corresponds in mobility to a chloroplast polypeptide which is a product of cytoplasmic protein synthesis in vivo. Significantly, the imported polypeptides of thylakoid membranes become correctly localized in the extrinsic or intrinsic aspects of the membranes with respect to their putative native counterparts. Moreover, the mobilities of the newly integrated polypeptides match those of pea thylakoid membranes whether uptake is into pea or Romaine lettuce chloroplasts. Since few of these membrane polypeptides have been identified, we do not know whether they are all synthesized as larger precursors. Nevertheless, our results demonstrate unequivocally that the post-translational mode of polypeptide transport applies to most, if not all, thylakoid membrane polypeptides. As shown elsewhere, this mode of transport requires ATP generated by cyclic photosynthetic phosphorylation (24).

Although the post-translational transport of p15 and p16 is firmly established, nothing is known regarding the molecular events that occur during precursor-receptor interaction at the envelope or the fate of the CP II polypeptides within the chloroplast before their complete assembly in membranes. Precursor processing presumably precedes thylakoid insertion; however, the possibility that the precursors are processed only after their assembly with chlorophylls in the membrane cannot be ruled out. Furthermore, the precursor chain extension of these integral membrane polypeptides may contain information not only for transport across the chloroplast envelope but also specific localization in the thylakoids. If this were the case, p15 and p16 may be processed in two steps. Future experiments are needed to clarify these aspects of the biosynthetic pathway of the LHC.

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REFERENCES

1. Apel, K. 1979. Phytochrome-induced appearance of mRNA activity for the apoprotein of the light-harvesting chlorophyll *a/b* protein of barley (*Hordeum vulgare*). *Eur. J. Biochem.* 97:183–188.
2. Apel, K., and K. Kloppstech. 1978. The plastid membranes of barley (*Hordeum vulgare*). Light-induced appearance of mRNA coding for the apoprotein of the light-harvesting chlorophyll *a/b* protein. *Eur. J. Biochem.* 85:581–588.
3. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. *Plant Physiol. (Bethesda).* 24:1–15.
4. Arntzen, C. J. 1978. Dynamic structural features of chloroplast lamellae. *Curr. Top. Bioenerg.* 8:111–160.
5. Aro, E.-M., and N. Valanne. 1978. Effect of magnesium on chlorophyll protein complexes. *Physiol. Plant.* 43:261–265.
6. Boardman, N. K., J. M. Anderson, and D. J. Goodchild. 1978. Chlorophyll-protein complexes and structure of mature and developing chloroplasts. *Curr. Top. Bioenerg.* 8: 35–109.
7. Bordier, C., and A. Crettol-Jarvinen. 1979. Peptide mapping of heterogeneous protein samples. *J. Biol. Chem.* 254:2565–2567.
8. Burke, J. J., C. L. Ditto, and C. J. Arntzen. 1978. Involvement of the light-harvesting complex in cation regulation of excitation energy distribution in chloroplasts. *Arch. Biochem. Biophys.* 187:252–263.
9. Cashmore, A. R. 1976. Protein synthesis in plant leaf tissue. The sites of synthesis of the major proteins. *J. Biol. Chem.* 251:2848–2853.
10. Cashmore, A. R., M. K. Broadhurst, and R. E. Gray. 1978. Cell-free synthesis of leaf protein: identification of an apparent precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. U. S. A.* 75: 655–659.
11. Chua, N.-H., and P. Bennoun. 1975. Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii*: wild-type and mutant strains deficient in photosystem II reaction center. *Proc. Natl. Acad. Sci. U. S. A.* 72:2175–2179.
12. Chua, N.-H. and F. Blomber. 1979. Immunochemical studies of thylakoid membrane polypeptides from spinach and *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 254:215–223.
13. Chua, H.-H., K. Matlin, and P. Bennoun. 1975. A chlorophyll-protein complex lacking in photosystem I mutants of *Chlamydomonas reinhardtii*. *J. Cell Biol.* 67:361–377.
14. Chua, N.-H., and G. W. Schmidt. 1978. Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase. *Proc. Natl. Acad. Sci. U. S. A.* 75:6110–6114.
15. Chua, N.-H., and G. W. Schmidt. 1979. Transport of proteins into mitochondria and chloroplasts. *J. Cell Biol.* 81:461–483.
16. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.
17. Conboy, J. G., F. Kalousek, and L. E. Rosenberg. 1979. In vitro synthesis of a putative precursor of mitochondrial ornithine transcarbamoylase. *Proc. Natl. Acad. Sci. U. S. A.* 76:5724–5727.
18. Cuatrecasas, P., S. Fuchs, and C. B. Anfinsen. 1967. Catalytic properties and specificity of the extracellular nuclease of *Staphylococcus aureus*. *J. Biol. Chem.* 242:1541–1547.
19. Davis, B. D., and P.-C. Tai. 1980. The mechanism of protein secretion across membranes. *Nature (Lond.).* 283:433–438.
20. Delepelaire, P., and N.-H. Chua. 1979. Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of thylakoid membranes at 4°C: characterization of two additional chlorophyll a-protein complexes. *Proc. Natl. Acad. Sci. U. S. A.* 76:111–115.
21. Dobberstein, B., G. Blobel, and N.-H. Chua. 1977. In vitro synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* 74:1082–1085.
22. Dunkley, P. R., and J. M. Anderson. 1979. The light-harvesting chlorophyll a/b-protein complex from barley thylakoid membranes. *Biochim. Biophys. Acta.* 545:174–187.
23. Ellis, R. J. 1977. Protein synthesis by isolated chloroplasts. *Biochim. Biophys. Acta.* 463: 185–215.
24. Grossman, A., S. Bartlett, and N.-H. Chua. 1980. Energy-dependent uptake of cytoplasmically-synthesized polypeptides by chloroplasts. *Nature (Lond.).* 285:625–628.
25. Grossman, A. R., S. Bartlett, G. W. Schmidt, and N.-H. Chua. 1979. Post-translational uptake of cytoplasmically-synthesized proteins by intact chloroplasts in vitro. *In* Precursor Processing and Biosynthesis of Proteins. D. Steiner and M. Zimmerman, editors. *Ann. N. Y. Acad. Sci.* 343:266–274.
26. Highfield, P. E., and R. J. Ellis. 1978. Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase. *Nature (Lond.).* 271:420–424.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
28. Laskey, R. A., and A. D. Mills. 1976. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
29. Maccecchini, M.-L., Y. Rudin, G. Blobel, and G. Schatz. 1979. Import of proteins into mitochondria: precursor forms of the extramitochondrially made F_1-ATPase subunits in yeast. *Proc. Natl. Acad. Sci. U. S. A.* 76:343–347.
30. Machold, O., and O. Aurich. 1972. Sites of synthesis of chloroplast lamellar proteins in *Vicia faba*. *Biochim. Biophys. Acta.* 281:103–112.
31. Machold, O., and A. Meister. 1979. Resolution of the light-harvesting chlorophyll a/b-protein of *Vicia faba* into two different chlorophyll-protein complexes. *Biochim. Biophys. Acta.* 546:472–480.
32. Morgenthaler, J.-J., M. P. F. Marsden, and C. A. Price. 1975. Factors affecting the separation of photosynthetically competent chloroplasts in gradients of silica sols. *Arch. Biochem. Biophys.* 168:289–301.
33. Mori, M., S. Miura, M. Tatibana, and P. P. Cohen. 1979. Cell-free synthesis and processing of a putative precursor for mitochondrial carbamyl phosphate synthetase I of rat liver. *Proc. Natl. Acad. Sci. U. S. A.* 76:5071–5075.
34. Mullet, J. E., and C. J. Arntzen. 1980. Stimulation of grana stacking in a model membrane system. *Biochem. Biophys. Acta.* 589:100–117.
35. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–256.
36. Poincelot, R. P., and P. R. Day. 1974. An improved method for the isolation of spinach chloroplast envelope membranes. *Plant Physiol (Bethesda).* 54:780–783.
37. Raymond, Y., and G. C. Shore. 1979. The precursor for carbamyl phosphate synthetase is transported to mitochondria via a cytosolic route. *J. Biol. Chem.* 254:9335–9338.
38. Ries, G., E. Hundt, and B. Kadenbach. 1978. Immunoprecipitation of a cytoplasmic precursor of rat-liver cytochrome oxidase. *Eur. J. Biochem.* 91:179–191.
39. Rogg, H., W. Wehrli, and M. Staehelin. 1969. Isolation of mammalian transfer RNA. *Biochim. Biophys. Acta.* 195:13–15.
40. Roy, H., B. Terenna, and L. C. Cheong. 1977. Synthesis of the small subunit of ribulose-1,5-bisphosphate carboxylase by soluble fraction polyribosomes of pea leaves. *Plant Physiol. (Bethesda).* 60:532–537.
41. Satoh, K. 1979. Polypeptide composition of the purified photosystem I pigment-protein complex from spinach. *Biochim. Biophys. Acta.* 546:84–92.
42. Schmidt, G. W., A. Devillers-Thiery, H. Desruisseaux, G. Blobel, and N.-H. Chua. 1979. NH_2-terminal amino acid sequences of precursor and mature forms of the ribulose-1,5-bisphosphate carboxylase small subunit from *Chlamydomonas reinhardtii*. *J. Cell Biol.* 83:615–622.
43. Schmidt, G. W., S. Bartlett, A. R. Grossman, A. R. Cashmore, and N.-H. Chua. 1980. In vitro synthesis, transport, and assembly of the constituent polypeptides of the light-harvesting chlorophyll a/b protein complex. *In* Genome Organization and Expression in Plants. C. L. Leaver, editor. Plenum Publishing Corp., N. Y. 337–351.
44. Smith, S. M., and R. J. Ellis. 1979. Processing of small subunit precursor of ribulose bisphosphate carboxylase and its assembly into whole enzyme are stromal events. *Nature (Lond.).* 278:662–664.
45. Staehelin, L. A., D. P. Carter, and A. McDonnel. 1980. Adhesion between chloroplast membranes: experimental manipulation and incorporation of the adhesion factor into artificial membranes. *In* Membrane-Membrane Interactions. N. B. Gilula, editor. Raven Press, N. Y. 179–193.
46. Steck, T. L., and J. Yu. 1973. Selective solubilization of proteins from red blood cell membranes by protein perturbants. *J. Supramol. Struct.* 1:220–232.
47. Steinback, K. E., J. J. Burke, and C. J. Arntzen. 1979. Evidence for the role of surface-exposed segments of the light-harvesting complex in cation-mediated control of chloroplast structure and function. *Arch. Biochem. Biophys.* 195:546–557.
48. Süss, K.-H., O. Schmidt, and O. Machold. 1976. The action of proteolytic enzymes on chloroplast thylakoid membranes. *Biochim. Biophys. Acta.* 448:103–113.
49. Tobin, E. M. 1978. Light regulation of specific mRNA species in *Lemma gibba* L. G-3. *Proc. Natl. Acad. Sci. U. S. A.* 75:4749–4753.
50. Wickner, W. 1980. Assembly of proteins into membranes. *Science (Wash. D. C.).* 210:861–868.
51. Zimmerman, R., U. Paluch, M. Sprinzl, and W. Neupert. 1979. Cell-free synthesis of the mitochondrial ADP/ATP carrier protein of *Neurospora crassa*. *Eur. J. Biochem.* 99:247–252.