

SYNTHESIS OF THYLAKOID MEMBRANE PROTEINS BY CHLOROPLASTS ISOLATED FROM SPINACH

Cytochrome *b559* and P700-Chlorophyll *a*-Protein

RAYMOND E. ZIELINSKI and C. A. PRICE

From the Waksman Institute of Microbiology, Rutgers University, Piscataway, New Jersey 08854.
Dr. Zielinski's present address is The Rockefeller University, New York 10021.

ABSTRACT

Intact chloroplasts, purified from spinach leaves by sedimentation in density gradients of colloidal silica, incorporate labeled amino acids into at least 16 different polypeptides of the thylakoid membranes, using light as the only source of energy. The thylakoid products of chloroplast translation were visualized by subjecting membranes purified from chloroplasts labeled with [³⁵S]methionine to electrophoresis in high-resolution, SDS-containing acrylamide gradient slab gels and autoradiography. The apparent mol wt of the labeled products ranged from <10,000 to >70,000. One of the labeled products is the apoprotein of the P700-chlorophyll *a*-protein (CPI). The CPI apoprotein is assembled into a pigment-protein complex which is electrophoretically indistinguishable from the native CPI complex. Isolated spinach chloroplasts also incorporate [³H]leucine and [³⁵S]methionine into cytochrome *b559*. The radioactive label remains with the cytochrome through all stages of purification: extraction of the thylakoid membranes with Triton X-100 and urea, adsorption of impurities on DEAE cellulose, two cycles of electrophoresis in Triton-containing polyacrylamide gels and electrophoresis in SDS-containing gradient gels. Cytochrome *b559* becomes labeled with both [³H]leucine and [³⁵S]methionine and accounts for somewhat <1% of the total isotopic incorporation into thylakoid protein. The lipoprotein appears to be fully assembled during the time-course of our labeling experiments.

Chloroplasts contain transcriptional and translational machinery which closely resembles that of bacterial cells and is distinct from that of the nuclear-cytoplasmic systems of higher plants and algae. Considerable effort has been directed for the past 15 years toward ascertaining the subcellular origins of chloroplast macromolecules and the contributions which the chloroplast makes to its own development and maintenance within the cell. One of the most fruitful approaches to the identification of chloroplast translation products

has been to feed radioactive amino acids to isolated, intact chloroplasts followed by separation of the newly synthesized products by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)¹ (reviewed in references 14 and 15). By

¹ *Abbreviations used in this paper:* ALA, 5-aminolevulinic acid; CF1, chloroplast coupling factor 1; CPI, the P700-chlorophyll *a*-protein; DTT, dithiothreitol; LA, levulinic acid; PMSF, phenylmethyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

use of autoradiography of two-dimensional slab gels, Ellis et al. (16) counted no fewer than 90 newly synthesized polypeptides in the stroma alone.

Several of the polypeptides synthesized by isolated chloroplasts have been identified as specific proteins or as subunits of specific proteins. In the stromal fraction these include the large subunit of ribulose-1,5-bisphosphate carboxylase (3, 6, 33, 43) and the elongation factors EF-G and EF-T (42); in the thylakoid fraction, these include the α , β , and ϵ subunits of chloroplast coupling factor 1 (CF1) (23) and cytochrome *f* (12). A 30,000–32,000 mol wt polypeptide of the thylakoids, also known as “peak D” (see, e.g., reference 13), is widely recognized as the most rapidly labeled polypeptide in the thylakoid fraction, but it has not been identified beyond its mobility on SDS gels. In fact, most of the thylakoid polypeptides that are typically visualized by SDS-PAGE remain unidentified.

In this study, we employed specific extractions and high-resolution gel electrophoresis techniques to characterize better and to determine the identities of some of the thylakoid polypeptides translated on ribosomes in isolated spinach chloroplasts. Our results support the general conclusions of other investigators who have either studied the synthetic activities of isolated plastids (6, 13, 20, 21, 33, 43) or examined the synthesis of plastid proteins under rigorously controlled conditions of site-specific inhibition of protein synthesis *in vivo* (9). More importantly, our results extend these earlier studies by identifying two specific, integral thylakoid polypeptides, the apoproteins of cytochrome *b559* and the P700-chlorophyll *a*-protein (CPI), as translation products of the plastid. In addition, we present evidence that suggests that these two apoproteins become associated with their respective porphyrin prosthetic moieties during the time-course of our experiments. Preliminary accounts of parts of this work have been presented elsewhere (47, 48).

MATERIALS AND METHODS

Materials

Spinach seedlings (*Spinacia oleracea* L. var. Bloomsdale Long-standing) were harvested 2–3 wk after the appearance of the first primary leaves. Leaves of seedlings raised in the greenhouse were used immediately for the preparation of chloroplasts; plants grown in the field were washed thoroughly with tap water and stored at 4°C for 24–48 h before plastid isolation. This incubation period was obligatory with field-grown leaves in order to separate

plastids from stripped thylakoids by density gradient sedimentation.

Methods

CHLOROPLAST PREPARATION, LABELING, AND FRACTIONATION: Intact spinach chloroplasts were purified by centrifugation in gradients of silica according to Morgenthaler et al. (32, 34). The isopycnicly banded plastids were collected, washed, and resuspended in sorbitol/Tricine medium (330 mM sorbitol, 50 mM Tricine/KOH, pH 8.4) at a chlorophyll concentration of ~ 1 mg/ml⁻¹. Protein synthesis was performed as described previously (33). Additions, when made to the basic labeling mixture, were made from concentrated reagent stocks dissolved in sorbitol/Tricine. To minimize bacterial contamination, all media and glassware used in chloroplast preparation and incubation were sterilized by autoclaving.

After incorporation of radioactivity, the chloroplasts were washed once by differential centrifugation in fresh sorbitol/Tricine to remove any unincorporated amino acids. The plastids were then lysed by resuspending them in freshly prepared 50 mM HEPES, pH 6.8, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and the thylakoid membranes were purified by sedimentation in discontinuous gradients of sucrose as described by Mendiola-Morgenthaler and Morgenthaler (30) except that the middle (23% wt/vol) sucrose step was omitted and 1 mM PMSF was added to all gradient solutions just before centrifugation. After sedimentation, the thylakoid pellets were homogenized in a sterile solution of 50 mM Tris-HCl, pH 6.8, and divided into suitable aliquots in 0.5-ml Eppendorf microfuge tubes. The washed membranes were then pelleted, the wash fluid was discarded, and the membrane pellets were stored at -80°C until needed for electrophoretic analyses.

SDS-PAGE: Thylakoid membranes dissociated in SDS were subjected to electrophoresis in $20 \times 32 \times 0.1$ -cm slab gels containing various concentrations of acrylamide (indicated in the text and in legends to figures). The gels were topped with $1 \times 32 \times 0.1$ -cm stacking gels which contained 5% (wt/vol) acrylamide. In all cases, the discontinuous buffer system of Laemmli (28) was employed. Membranes were suspended in 50 mM Tris-HCl, pH 6.8, 1% (wt/vol) SDS, 0.1 M dithiothreitol (DTT), and 10% (vol/vol) glycerol at a chlorophyll concentration of 1 mg·ml⁻¹. The solubilized membrane proteins were held on ice for ~ 10 min before electrophoresis. When dissociation of chlorophyll-protein complexes was desired, the membrane suspension was heated in a boiling water bath for 30 s and then chilled on ice for 5–10 min before loading the membrane samples onto gels. Gels were stained for 1–2 h in 0.07% Coomassie Brilliant Blue R-250 made up in 50% (vol/vol) methanol:10% (vol/vol) acetic acid, and destained in two changes of 40% (vol/vol) methanol:6% (vol/vol) acetic acid. The gels were dried on Whatman 3 MM paper, photographed, and exposed for various intervals to Cronex 2DC medical x-ray film (E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.) at -80°C .

EXTRACTION AND PURIFICATION OF CYTOCHROME *b559*:

Cytochrome *b559* was purified according to the method of Garewal and Wasserman (18) with minor modifications (46). Cytochrome-containing extracts, which were made in a medium of 50 mM Tris-HCl, pH 8, 4 M urea, and 2% (vol/vol) Triton X-100, were concentrated by ultrafiltration using Diaflo XM100A membranes (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). Final purification of the protein was achieved by polyacrylamide gel electrophoresis in the presence of Triton X-100 (Triton-PAGE). In some cases, the cytochrome was purified by

two successive rounds of Triton-PAGE as follows: the greenish eluate obtained after binding most of the noncytochrome impurities to DEAE-cellulose was concentrated and subjected to Triton-PAGE in 9% (wt/vol) acrylamide gels; bands of cytochrome from these gels were hand-sliced and forced into siliconized gel tubes containing fresh Triton gels and subjected to a second electrophoretic separation. The second gels contained longer than usual (3-cm) stacking gels. The entire electrophoretic operation took ~48 h and all steps were carried out at 4°C.

For electrophoretic analyses in SDS-containing gels, samples of cytochrome *b559* purified by Triton-PAGE were lipid-extracted according to the following procedure. Cytochrome-containing gel bands obtained from the second Triton-PAGE separation were cut from the gels with a clean razor blade and finely minced. The cytochrome was extracted from these slices into a solution containing 0.5% (wt/vol) SDS by incubating the gel pieces in solutions at 100°C for 5 min and repeating the extraction twice with fresh SDS solutions. The SDS extracts were pooled and cytochrome *b559* was precipitated by the addition of 9 vol of ice-cold acetone. The precipitated protein was collected by centrifugation, washed three to five times with 10 vol of ethyl ether, and dried under a stream of N₂. The extracted residue was then resuspended in 50 mM NaPO₄ buffer, pH 7.0, 3% (wt/vol) SDS, and 5% 2-mercaptoethanol, and boiled for 2 min. Aliquots of this suspension were used for SDS-PAGE.

RESULTS

Isolated chloroplasts purified from spinach in density gradients of colloidal silica were incubated with radiolabeled amino acids in the light for 1 h and the thylakoid membranes were collected and purified. Plastids incubated in the dark or in the light in the presence of D-threo-chloramphenicol show very low (typically <5%) levels of incorporation compared to control plastids incubated in the light. Protein synthesis by our plastid preparations, moreover, is largely unaffected by ribonuclease or cycloheximide. We concur with the earlier conclusions (3, 6, 33) that protein synthesis by chloroplasts prepared in this way can be attributed almost entirely to the activity of plastid ribosomes.

When labeled chloroplasts are lysed by resuspension in hypotonic buffer and the different chloroplast subfractions are separated by density gradient sedimentation, we find the thylakoids to account for between 40 and 65% of the total radioactivity. Incorporation into the soluble stromal fraction varies between ~60 and 35% of the total, whereas the envelope membranes typically account for <0.5% of the counts.

Another important consideration when examining the synthesis of a membrane-bound protein by isolated organelles is that the labeling observed in the membrane preparations be attributable solely to protein synthesis and not to conversion of labeled amino acid into lipid. We examined this

possibility by extracting aliquots of [³H]leucine-labeled thylakoid membranes with chloroform-methanol according to Bligh and Dyer (4). Radiolabel recovery was measured in the lipid extract and in the extracted membrane residue. Table I shows that only 1–2% of the radioactivity is extracted into the chloroform phase. Chloroform-methanol mixtures can extract up to 50% of the total membrane protein of thylakoids (10, 25), including the chloroplast coupling factor (45). Thus, it is likely that the labeling we observe is attributable to protein synthesis only.

Products of Protein Synthesis in Isolated Chloroplasts

We examined the distribution of apparent molecular weights of the thylakoid products of chloroplast translation by subjecting membranes purified from plastids labeled for one h with [³⁵S]-methionine to SDS-PAGE and autoradiography.

In Fig. 1 we compare the polypeptide and autoradiographic profiles of labeled spinach thylakoids separated by SDS-PAGE either with or without heating the solubilized membrane proteins. In typical preparations, we observe more than 30 polypeptides in the thylakoid fraction, the exact number depending upon the conditions of electrophoretic separation (cf. Figs. 1 and 2); the polypeptides range in mol wt from <10,000 to >70,000. Autoradiography of the labeled, separated thylakoid proteins reveals a number of ra-

TABLE I
Distribution of Radioactivity in Chloroform-Methanol Extracts of Thylakoid Membranes Previously Labeled with [³H]Leucine

Fraction	Radioactivity ³ H dpm × 10 ⁻⁶	Percent- age of Total	Pro- tein μg	Percent- age of Total
Whole thylakoids	1.50	100	373	100
Methanol-water phase	0.05	3.3		
Chloroform phase	0.02	1.4		
Protein precipitate	1.42	94.7	307	82.3
Recovery		99.4		82.3

Protein precipitated by mixing thylakoid membranes with chloroform-methanol (4) was collected by centrifugation after removing the chloroform layer by aspiration. The protein residue was dissolved in 0.1 N NaOH and aliquots were analyzed for radioactivity and total protein.

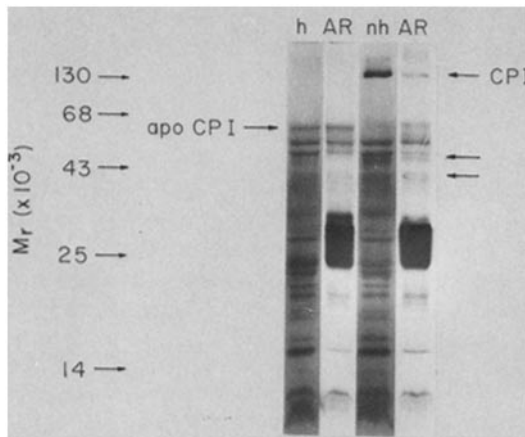


FIGURE 1 Synthesis and assembly of CPI by isolated spinach chloroplasts detected by SDS-PAGE and autoradiography (AR) of nonheated (nh) and heated (h) thylakoid membranes. The gel lanes of a 9–15% SDS-containing gradient gel were loaded with identical [³⁵S]methionine-labeled thylakoid membranes corresponding to 30 μg of chlorophyll (~1.2 × 10⁹ cpm) either with or without prior heating in SDS sample buffer. The dried gel was exposed to x-ray film for 10 d at -80°C. The positions of two other labeled polypeptides that show small changes in mobility upon heating and probably correspond to the CPIII and CPIV apoproteins (7, 10) are also indicated in the figure.

radioactive polypeptides as shown in Fig. 1. The apparent mol wt of the labeled products range from <10,000 to ~68,000. None of these labeled polypeptides appear to arise from contamination of the membranes by stromal proteins (data not shown). All of the labeled bands coincide with authentic stainable thylakoid proteins with the exception of the diffuse zone of highly labeled material which migrates in the mol wt range of 25 to 30 × 10³. By underexposing our autoradiographs, we find that discrete labeled bands appear in this diffuse zone with mobilities corresponding to those of each of the major stainable polypeptides. The diffuse labeled zones that do not correspond to discrete polypeptides may arise from partial degradation of one or more of the labeled products, but it is puzzling that such diffuse zones should occur only in this molecular weight range.

The principal differences between the electropherograms of heated and nonheated membranes are indicated by arrows in Fig. 1. In nonheated samples, a pigmented zone of low mobility appears in the gel that is absent in the heated sample.

Spectral analyses showed that this material possesses a visible absorption spectrum identical to that of CPI described by many others (cf. references 10 and 41). Heating in the presence of SDS causes the CPI complex to dissociate, resulting in the appearance of a polypeptide possessing an apparent mol wt of ~68,000, which is not observed in nonheated thylakoid samples.

By comparing the autoradiographic patterns of heated and nonheated membranes, we find a change in the pattern of radioactivity which exactly coincides with the major change in Coomassie Blue staining. Our results thus confirm the findings of Cederblad and Vasconcelos (7) and of B. R. Green (personal communication) that isolated chloroplasts are able to synthesize the apoprotein of CPI. In Fig. 1, we find some radioactivity associated with the intact CPI complex and some associated with the 68,000 apoprotein in the nonheated thylakoid sample. The free apoprotein present in this gel arose by dissociation of the pigments normally associated with the intact complex. Under more favorable electrophoretic conditions, as in low temperature SDS-PAGE, in which no CPI apoprotein can be seen by staining, we find that nearly all of the CPI-associated radioactivity detectable by autoradiography co-migrates with the intact CPI complex. We conclude that most, if not all, of the newly formed CPI apoprotein is assembled into the intact pigment-protein complex by our chloroplast preparations.

Fig. 1 also shows that we observe radioactivity coincident with two polypeptides whose electrophoretic mobilities are very similar to those of the apoproteins of two pigment-protein complexes that were recently described by Chua and Blomberg (8) and Delepelaire and Chua (11), and whose apoproteins are synthesized on plastid ribosomes in *Chlamydomonas* (8). These polypeptides are indicated in the figure and have mol wt of ~48,000 and 42,000.

The gels in Fig. 1 reveal the presence of radioactivity which migrates nearly with the detergent-complexed free pigment and is not well resolved in our 9–15% gradient gels. To determine whether these labeled zones correspond to authentic thylakoid polypeptides, we separated [³⁵S]methionine-labeled thylakoid membranes in a gel containing a 12–18% linear gradient of acrylamide and 8 M urea (R. P. Piccioni, P. Bennoun, and N.-H. Chua, manuscript in preparation), stained and dried the gel, and subjected the dried gel to autoradiography. Fig. 2 shows the results of this

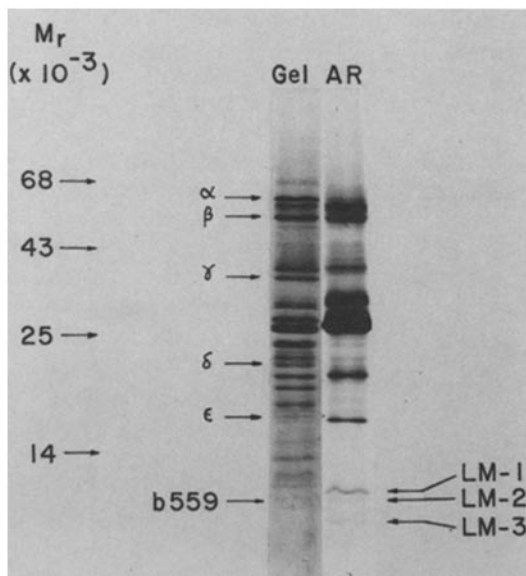


FIGURE 2 SDS electropherogram showing low molecular weight products of chloroplast translation. Thylakoid membranes purified from [^{35}S]methionine-labeled spinach chloroplasts were subjected to SDS-PAGE in a 12–18% (wt/vol) polyacrylamide gel containing 8 M urea. The gel was stained, dried, and exposed to x-ray film for 10 d at -80°C (AR). The gel contained membranes corresponding to $30\ \mu\text{g}$ of chlorophyll ($\sim 1.2 \times 10^5$ cpm) that had been heated for 30 s at 100°C and then chilled for 5 min on ice just before electrophoresis. The positions of the subunits of the chloroplast coupling factor and of cytochrome *b559* that were run in an identical gel along side unlabeled thylakoids on a different occasion are indicated in the figure.

experiment. We find at least three labeled polypeptides of $<10,000$ mol wt in our thylakoid preparations that we have designated LM-1, LM-2, and LM-3 in order of decreasing size. We estimate the mol wt of these polypeptides to be 6,400; 5,800; and 3,300, respectively. On another occasion, as additional standards in this experiment, we included samples of highly purified CF1 and cytochrome *b559* in parallel slots of the gel. The mobilities of these proteins are indicated in the figure. The electrophoretic mobility of cytochrome *b559* is identical to that of LM-2. The ϵ subunit of CF1, which was shown by Mendiola-Morgenthaler et al. (31) to be a translation product of isolated spinach chloroplasts, migrates with the same mobility as a somewhat larger polypeptide of $\sim 16,000$ in this gel system. The α and β subunits of CF1 also co-migrate with labeled thylakoid polypeptides; nearly all of the radioactivity associated with

polypeptides which co-migrate with authentic CF1 subunits are extracted from the membranes by washing with 1mM EDTA, pH 8 (26) (data not shown).

Extraction of Cytochrome *b559* from Labeled Spinach Chloroplasts

In mitochondria the principal products of translation are associated with the enzyme complexes of the inner mitochondrial membrane (reviewed in reference 38). These include three of the seven subunits of cytochrome oxidase (29) and the polypeptide(s) of cytochrome *b* (44). Previous studies on the biosynthesis of plastid cytochromes using inhibition of protein synthesis *in vivo* suggested that the same might be true for both *b*- and *c*-type chloroplast cytochromes (reviewed in reference 14). Consequently, we decided to reinvestigate the possibility that cytochrome *b559* is synthesized on plastid ribosomes by applying Garewal and Wasserman's procedures (18) for the extraction and purification of this protein to chloroplasts labeled by our standard methods.

Disruption of labeled thylakoid membranes by sonication in Triton-urea solubilized 20–40% of

TABLE II
Recovery of Radioactivity in the Extraction of
Cytochromes from ^3H -labeled thylakoid membranes

Fraction	Protein mg	Percent- age of Total	Radioac- tivity $^3\text{H dpm} \times 10^{-6}$	Percent- age of Total
Purified thyla- koids*	104.30	100	17.69	100
Ethanol extracts‡			6.32	35.7
Tris washes‡			1.32	7.4
Extracted parti- cles	71.00	68.1	10.06	56.9
Crude extract	11.48	11.0	2.11	11.9
DEAE eluate	0.63	0.6	0.41	2.3

* Purified thylakoids were obtained from ^3H -labeled intact chloroplasts by osmotically shocking plastids immediately after 60 min of labeling by resuspending in 5 vol of 50 mM HEPES/NaOH, pH 6.8. Thylakoids were separated from the stroma and envelope membranes by centrifugation in a discontinuous gradient of sucrose in the same buffer as described previously (30).

‡ The purified membranes were extracted twice with 95% ethanol and washed twice in 50 mM Tris-HCl, pH 8 before solubilizing the membrane proteins in 50 mM Tris-HCl, pH 8, 4 M urea, and 2% vol/vol Triton X-100. All steps were carried out at 4°C .

the radioactivity and 30–60% of the total membrane protein (Table II), including all three species of chloroplast cytochromes. Removal of most of the noncytochrome impurities (and cytochrome *b6*) by binding to DEAE-cellulose yields a fraction which contains 1–2% of the total thylakoid protein and ~1% of the radiolabel incorporated into thylakoid proteins. Further purification of this extract by gel electrophoresis in the presence of Triton X-100 yields a visibly pink zone which, upon elution from the gel, possesses a visible absorption spectrum characteristic of low potential cytochrome *b559*, as shown in Fig. 3.

Cytochrome *b559* from chloroplasts that had been labeled for 1 h with a mixture of [³H]leucine and [³⁵S]methionine was first purified by electrophoresis on 9% (wt/vol) acrylamide gels, and the visible cytochrome zone was cut from the gels and subjected to a second round of electrophoresis on 9 or 10.5% gels. The results (Fig. 4) show coincident zones of cytochrome absorbance, stainable protein, and both ³H and ³⁵S radioactivity in these gels. The ratio of leucine to methionine, moreover, is constant in the cytochrome-containing zones. In another set of experiments, we subjected [³H]leu-

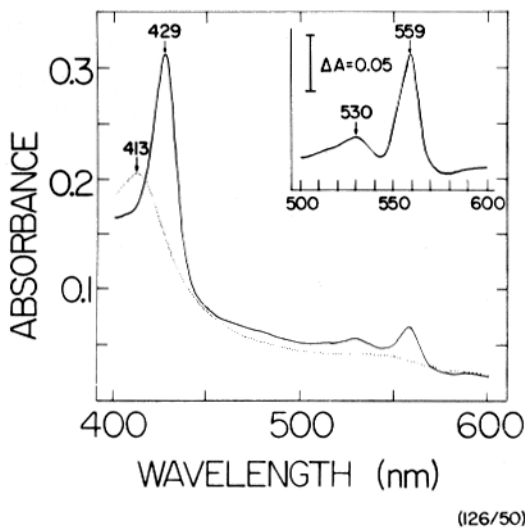


FIGURE 3 Visible absorption spectra at room temperature of reduced (—) and oxidized (···) cytochrome *b559* prepared by electrophoresis in Triton-containing polyacrylamide gels. The cytochrome was eluted from the gel into a medium of 50 mM Tris-HCl, pH 8, 15% (vol/vol) glycerol, and 5 mM DTT. The protein was oxidized immediately before recording its spectrum by adding ammonium persulfate to a final concentration of 0.01% (wt/vol). The inset shows a reduced-minus-oxidized spectrum of the protein in greater detail.

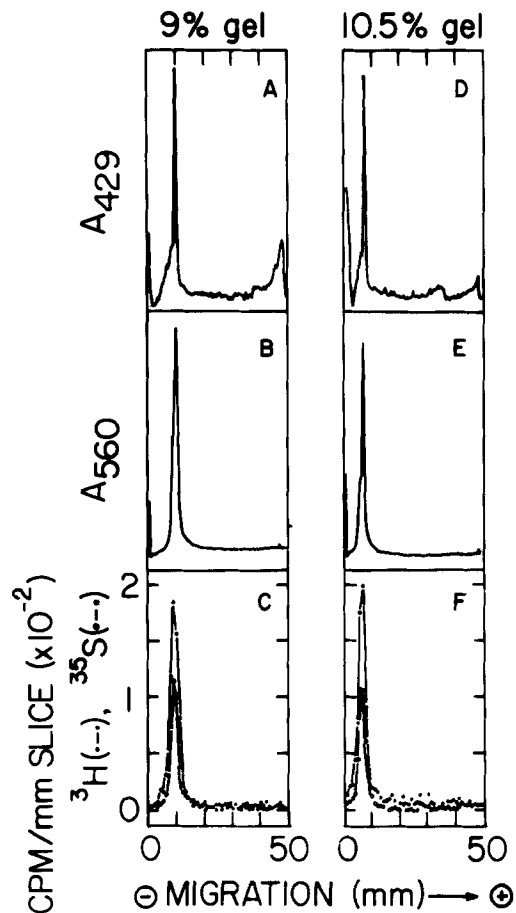


FIGURE 4 Electrophoresis of cytochrome *b559* extracted from chloroplasts labeled with [³H]leucine and [³⁵S]methionine. The protein was first separated in a 9% (wt/vol) acrylamide gel, cut from the gel, and subjected to a second electrophoretic separation in either a 9% (A-C) or a 10.5% (D-F) acrylamide gel (see Materials and Methods for details). The gels were scanned (A_{429}) before staining to locate the cytochrome, then stained with Coomassie Blue (A_{560}), and sliced sequentially into 1-mm segments to detect radioactivity.

cine-labeled cytochrome *b559* to electrophoresis in Triton-containing gels ranging from 6 to 12% (wt/vol) in acrylamide concentration. Fig. 5 shows that when the mobility of the cytochrome and its associated radioactivity are analyzed according to the method of Hedrick and Smith (24), they yield molecular weight estimates differing by <5%.

We also purified the cytochrome-containing Triton-urea extract by gel filtration on a column of Bio-Gel A 1.5 m (Bio-Rad Laboratories, Richmond, Calif.) followed by gel electrophoresis in

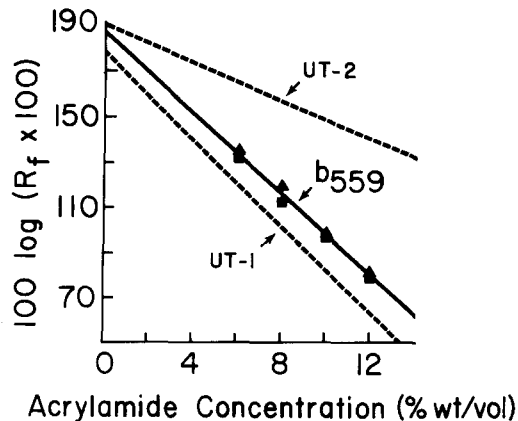


FIGURE 5 Analysis of the mobility of cytochrome *b559* (■) and its associated radioactivity (▲) in nondenaturing Triton-containing acrylamide gels. Cytochrome *b559* was extracted from [³H]leucine-labeled thylakoid membranes and purified by a single electrophoretic step in gels containing different concentrations of acrylamide which were run simultaneously. After staining for total protein, the gels were sliced and radioactivity determined in each gel segment. The mobilities of the cytochrome protein zone, determined by densitometry, and the zone of highest radioactivity were calculated with respect to the bromphenol blue tracking dye according to Hedrick and Smith (24). The mobilities of two unidentified, nonradioactive thylakoid proteins (UT-1 and UT-2) also present in these gels are indicated (---).

Triton-containing gels. Once again, we observed coincident zones of cytochrome absorbance, stainable protein, and radioactivity (data not shown). These results strongly argue that the cytochrome zones in these gels contain but a single protein species. Therefore, if the labeling we detected in the cytochrome-containing regions of the gels was attributable to a contaminant, such a protein would have to possess physical properties and an amino acid composition virtually identical to that of cytochrome *b559*. Considering the fact that we are performing our extraction on membranes which contain only ~30 major polypeptide species, we feel that this possibility is unlikely.

We extracted cytochrome *b559* from these nondenaturing acrylamide gels, exhaustively extracted the residual Triton X-100 and lipids, and subjected the protein residue to SDS-PAGE.

Fig. 6 shows that nearly all of the material that stains with Coomassie Blue runs as a polypeptide with a mol wt of ~6,000, which is characteristic of the polypeptide subunit of cytochrome *b559* (19; and R. Zielinski, unpublished data). A fluoro-

graph of the gel shows a small amount of radioactivity coincident with the authentic cytochrome polypeptide, but most of the label is slightly retarded, corresponding to that of a polypeptide with a mol wt of ~7,000.

The inexact coincidence of radioactivity and Coomassie Blue-stainable material is also shown in an experiment in which a sample of the lipid-depleted cytochrome was resuspended in buffer containing Triton X-100 and 4 M urea and subjected to re-electrophoresis in Triton-containing acrylamide gels (no urea). Fig. 7 shows that the cytochrome *b559* sample extracted from labeled membranes yields a protein zone whose mobility corresponds to that of the cytochrome extracted from unlabeled membranes. The labeled cytochrome sample, however, yielded a second, slower-moving protein zone in these gels that was not present in the unlabeled cytochrome preparation (indicated by an arrow in Fig. 7). Fluorography of

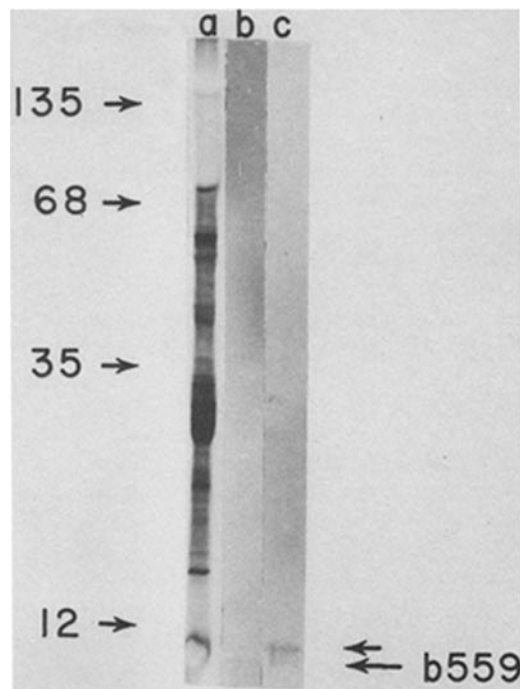


FIGURE 6 Electrophoresis and fluorography of dissociated cytochrome *b559* apoprotein in an SDS-containing gel. Cytochrome *b559* labeled with [³H]leucine and [³⁵S]methionine as in Fig. 2 was dissociated in SDS and separated on an SDS slab gel containing a gradient of 10–18% wt/vol acrylamide. The contents of the channels are: (a) fluorograph of unfractionated thylakoids, (b) electropherogram of stained cytochrome *b559*, (c) fluorograph of b.

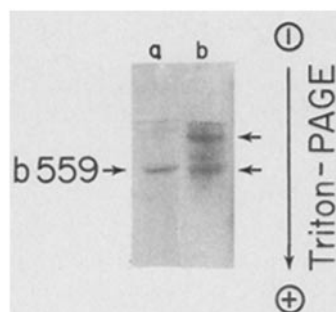


FIGURE 7 Electrophoresis of lipid-depleted cytochrome *b559* in a Triton-containing 9% (wt/vol) polyacrylamide gel. (a) Coomassie-Blue-stained gel of cytochrome *b559* purified from chloroplasts labels with [³⁵S]methionine. (b) Fluorograph of labeled cytochrome, performed for 2 mo at -70°C .

the gel revealed that part of the radioactivity coincided with the extract of authentic cytochrome and part with the slower-moving zone.

DISCUSSION

Our results clearly show that isolated spinach chloroplasts synthesize at least 15 of the 30 or more polypeptides comprising the thylakoid membranes. We have identified, moreover, two specific thylakoid products of plastid protein synthesis as the apoproteins of cytochrome *b559* and CPI. Both of these polypeptides are assembled into molecules which are indistinguishable electrophoretically from the native biologically active cytochrome and CPI complex, respectively, during the time-course of our labeling experiments.

When cytochrome *b559* purified from labeled thylakoids is depleted of lipids and subjected to electrophoresis in SDS-containing gradient slab gels, a small amount of radiolabel coincides with the 6,000 mol wt polypeptide characteristic of cytochrome *b559* (19), but most of it moves slightly behind the authentic subunit (Fig. 6). Resolution of the newly synthesized, labeled material from the apoprotein of cytochrome *b559*, which had been prepared from unlabeled cytochrome purified by identical procedures, is also shown in Triton-containing gels (Fig. 7). This result could be interpreted to mean that our cytochrome *b559* preparations are contaminated with small amounts of a more rapidly synthesized polypeptide whose apparent mol wt is somewhat larger than apocytochrome *b559*. However, because our cytochrome preparations used in the gels depicted in Figs. 6 and 7 were subjected first to purification condi-

tions designed to optimize recovery of the native 110,000 mol wt cytochrome, we think it more likely that cytochrome *b559* may be synthesized in a precursor form, a "precytochrome *b559*", which is slightly less charged than the native polypeptide. The lower mobility of the additional species in Triton-containing gels indicates that the charge-to-mass-ratio of the protein in this band is lower than that of the native cytochrome. If the extra length of the "precytochrome *b559*" polypeptides were caused by hydrophobic amino acids, the charge-to-mass ratio would indeed be lower than that of the native cytochrome. We are tempted to speculate that the additional length of such a precursor might be caused by a "signal" sequence, as had been detected for proteins synthesized on microsomal membranes (5).

More experiments will be required to establish the relation of our putative precursor to cytochrome *b559*, but we think it significant that the newly synthesized higher molecular weight polypeptide appears to assemble into a membrane-bound lipoprotein that is inseparable from native cytochrome *b559* under nearly all conditions. This suggests to us the possibility that precytochrome *b559* may be processed into the mature protein after it is assembled in the thylakoid membrane.

Under ideal conditions, as is the case for soluble proteins, a very sensitive indication of the purity of a specific protein can be obtained by a two-dimensional electrophoretic analysis. In our hands, however, none of these electrophoretic methods were able to separate purified cytochrome *b559* satisfactorily. Similar behavior has been noted for the hydrophobic polypeptides of cytochrome oxidase (35).

Previous attempts to localize the sites of synthesis of chloroplast cytochromes have generally relied on the selective inhibition of protein synthesis in vivo (1, 17, 22, 39). Specifically, the formation of *b*- and *c*-type cytochromes in the plastids of higher plants and algae is inhibited by chloramphenicol and streptomycin and, to a lesser extent, by cycloheximide. Although indirect and inherently ambiguous (cf. criticisms in references 14 and 38), such findings have been interpreted to mean that the activities of both plastid and cytoplasmic ribosomes are required for the formation of these molecules. Our data provide the first direct evidence that chloroplasts are capable of synthesizing the polypeptides of cytochrome *b559* and assembling them into the functional cytochrome molecule. Similarly, Doherty and Gray

(12) have shown that isolated intact pea chloroplasts synthesize cytochrome *f*. A great advantage in this experimental approach over the more traditional method of detecting synthesis of proteins in plastids during inhibition *in vivo* by "site-specific" antibiotics is the relatively straightforward way in which data can be interpreted. Our results, however, are in excellent agreement with those of Chua and Gillham (9) who employed short-term, site-specific inhibition of protein synthesis in exponentially growing cultures of *Chlamydomonas* and high-resolution SDS-PAGE and autoradiographic analysis of thylakoid polypeptides.

Two factors greatly enhanced our resolution of radioactively labeled thylakoid polypeptides. Brief heating (30 s maximum) followed by immediate cooling of heated membrane suspensions improved recovery of CPI apoprotein specifically and, with the exception of the zone of 25,000–30,000 mol wt, also decreased the general heterodisperse background found in samples heated for extended periods of time. We also found that gradient gels of different concentration limits aided the identification of labeled thylakoid polypeptides, particularly in the low molecular weight range (cf. Figs. 1 and 2). Our gradient gel analyses show that all of the labeled products associated with the thylakoids, including the 32,000 mol wt polypeptide, co-migrate with authentic, stainable thylakoid polypeptides. Using similar electrophoretic techniques and fluorography, Grebanier et al. (20, 21) found that in isolated maize chloroplasts, the 32,000 mol wt polypeptide did not correspond to a stainable thylakoid polypeptide. They attributed this finding to an inability of the isolated organelles to process the newly synthesized polypeptide. The discrepancy between their results and ours may be caused by species differences or to a selective enrichment of fully competent chloroplasts by the purification technique (32, 34) we employed: chloroplast preparations of lesser integrity may be unable to process and complete the assembly of membrane polypeptides into functional complexes as a result of loss of necessary enzymes or co-factors or because of subtle changes in thylakoid conformation.

Table III lists the labeled polypeptides that we can identify with varying degrees of confidence. It is clear that isolated chloroplasts are capable of synthesizing a wide variety of membrane polypeptides, including both peripheral proteins (e.g., subunits of CF1) and integral proteins (e.g., cytochrome *b559* and CPI).

TABLE III
*Identification of Membrane Polypeptides
Synthesized by Chloroplasts Isolated from Spinach**

Apparent mol wt	Identity
$\times 10^{-3}$	
130‡	CPI native complex
68	CPI apoprotein
58	CF1 α subunit
53	CF1 β subunit
50	CPIII§
42	CPIV§
32	"Peak D"
13	CF1 ϵ subunit
5.8	Cytochrome <i>b559</i>

* Chloroplasts were labeled, as described in Materials and Methods, with [32 S]methionine for 60 min in the light. Thylakoid membranes were purified and fractionated by SDS-PAGE as described in the legend to Fig. 1.

‡ The native CPI complex has been shown to migrate anomalously in SDS-PAGE (10), and the value presented here for its apparent mass should only be regarded as a way of identifying the complex in non-heated membrane electropherograms.

§ These complexes were recently described in references 8 and 11. Our identification of these polypeptides is tentative, based only on similar electrophoretic behavior in SDS gradient gels.

The results we presented in Fig. 2 show that isolated spinach chloroplasts synthesize at least three small polypeptides. Could these polypeptides arise from proteolysis not normally associated with plastid membranes? We do not believe so, for the following reasons: Our starting material was intact chloroplasts purified in density gradients, so that nonspecific degradation by subcellular contaminants seems unlikely. We performed organelle lysis and purification of thylakoids in the presence of a potent protease inhibitor. We stored the membranes for only a short period of time at -80°C before electrophoretic analysis and found no evidence of changes in the electrophoretic or autoradiographic pattern over the period of 3 d to 2 wk after labeling. And we found the mobilities of the three labeled products to correspond to the mobilities of three authentic thylakoid polypeptides detectable by staining. One of the low molecular weight polypeptides, LM-2, is likely to be the apoprotein of cytochrome *b559*. In addition, Chua and Gillham (9) have detected the production of low molecular weight polypeptides associated with thylakoids by chloroplast ribosomes by inhibition of protein synthesis *in vivo* in *Chlamydomonas*.

The polypeptides we have shown to be translation products of the plastid would require a minimum of ~15 kilobase pairs of DNA for their structural genes, or ~12% of the informational content of the chloroplast DNA. Such an accounting of chloroplast genetic information assumes, of course, that all mRNAs of the chloroplast are transcribed from chloroplast DNA. There have been proposals that some mRNAs of nuclear origin may be transported into the chloroplast for translation (cf. references 23 and 27), but the evidence for such a process remains indirect.

Because CPI and cytochrome *b559* appear to be synthesized and fully assembled by isolated chloroplasts during the rather brief duration of our experiments, we were hopeful that the addition of 5-aminolevulinic acid (ALA) might stimulate the formation of plastid proteins. We note, however, that in preliminary experiments neither ALA nor levulinic acid (LA) stimulated or inhibited incorporation of [³H]leucine or [³⁵S]methionine into thylakoid proteins generally or into CPI specifically. This result may mean that coupling of protein synthesis and porphyrin synthesis does not occur in plastids, as it does in mitochondria (37) and in photosynthetic bacteria (40); but as there are Mendelian mutants of *Chlamydomonas* that lack CPI (10), there is also a strong possibility that factors produced in the cytoplasm may control the synthesis of CPI apoprotein more stringently than does the availability of porphyrins. Ellis (15) has suggested mechanisms by which the synthetic activity of plastid ribosomes may be regulated according to the needs of the cell. There exists, however, no clear-cut instance of regulation of plastid translation (cf. reference 36). Thus, the question of what the factors are that control chloroplast protein synthesis remains largely unanswered.

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