THE SITES OF SYNTHESIS OF THE PRINCIPAL THYLAKOID MEMBRANE POLYPEPTIDES IN *CHLAMYDOMONAS REINHARDTII*

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ABSTRACT

The sites of synthesis of the major thylakoid membrane polypeptides have been studied in the green alga *Chlamydomonas reinhardtii* by pulse labeling of cells with [^{14}C]acetate in the presence of inhibitors specific for chloroplast and cytoplasmic protein synthesis. The labeled membrane polypeptides were separated by an improved method of sodium dodecyl sulfate (SDS) gradient gel electrophoresis, and autoradiographs were made of the dried gels. The results demonstrate that of the 33 polypeptides resolved in the gels, at least nine are made on chloroplast ribosomes. Two of these (polypeptides 2 and 6) are associated with the reaction centers of photosystems I and II. Another polypeptide (polypeptide 5) appears from genetic data to be coded by chloroplast DNA. Experiments with a mutant whose chloroplast ribosomes are resistant to spectinomycin (*spr-u-1-6-2*) show that polypeptides whose synthesis takes place on chloroplast ribosomes are made in the presence of spectinomycin in the mutant although their synthesis is blocked by this antibiotic in wild type cells.

It has been known for some time that the chloroplast contains its own distinctive protein-synthesizing system, and one of the important questions in the biogenesis of this organelle is why this should be so. What are the functions of the chloroplast protein-synthesizing system, and are there reasons why these functions cannot be preempted by the protein-synthesizing machinery of the cytosol? Before these questions can be answered, it is first necessary to have a precise knowledge of the proteins that are made inside the chloroplast.

So far, two approaches have been employed in attempts to identify the products of chloroplast protein synthesis in both higher plants and algae (see review by Gillham et al., reference 18). The first approach makes use of the differential sensitivity of chloroplast and cytoplasmic ribosomes to inhibitors of protein synthesis in vivo. Most often, such experiments have been done during greening of etiolated seedlings (16) or algal cells (17, 21) when the labeling patterns of newly synthesized proteins can easily be compared in the presence or absence of given inhibitors. However, since relatively long periods of treatment have been used in most of these experiments, indirect effects of the inhibitors on membrane assembly have not been ruled out rigorously. The second approach is to isolate intact chloroplasts capable of light-dependent protein synthesis and examine the products of chloroplast protein synthesis in vitro. Generally, chloroplasts are allowed to incorporate radioactive precursors, and the labeled proteins are identified by sodium dodecyl sulfate (SDS) gel electrophoresis (6, 14, 16, 30, 32). Although this approach is

more direct, it appears to be effective only for those proteins which either occur in great abundance in the chloroplast, have high rates of turnover, or do not require the presence of cytoplasmically made partner proteins for their assembly (e.g., into membranes). The results obtained with these approaches have shown that the large subunit of ribulose-1,5-bisphosphate carboxylase (5, 13, 20, 23); three of the subunits of the chloroplast coupling factor, CF1 (29); and one or two chloroplast envelope proteins of unknown function (24, 30) are made inside the chloroplast. It is also generally agreed that several of the thylakoid membrane polypeptides are made inside the chloroplast (3, 7, 14, 16, 17, 21, 27, 30, 32), but the precise number has not yet been determined, partly because of the experimental limitations mentioned above and also, because the experimental design as well as the methods used for gel electrophoresis and for extracting thylakoid membranes vary widely.

Consequently, we decided to reexamine the sites of synthesis of the thylakoid membrane polypeptides in the green alga *Chlamydomonas reinhardtii*. In our experiments, membrane polypeptides were pulse-labeled for 30 min with [^{14}C]acetate in the presence of inhibitors. Anisomycin (ANISO) was used to block protein synthesis on cytoplasmic ribosomes (26) whereas chloramphenicol (CAP) or spectinomycin (SPEC) was used to inhibit protein synthesis on chloroplast ribosomes (cf. references 10, 12, 22). To provide a more rigorous demonstration that polypeptides whose synthesis was inhibited by CAP or SPEC were really made on chloroplast ribosomes, the pulse-labeling pattern of thylakoid membrane polypeptides of a spectinomycin-resistant mutant (*spr-u-1-6-2*) was compared to that of wild type. Since the mutant has chloroplast ribosomes resistant to SPEC both in vivo and in vitro (12), it was expected that the synthesis of thylakoid membrane polypeptides on chloroplast ribosomes in this mutant would be blocked only by CAP whereas in wild type both CAP and SPEC were expected to interfere with the synthesis of these polypeptides. After labeling, the membrane polypeptides were extracted and separated by an improved method of SDS gel electrophoresis (9). Autoradiographs were then made of the stained gel to establish the inhibition patterns.

The results obtained both with wild type and *spr-u-1-6-2* showed that of the 33 or more polypeptides fractionated by the SDS gels, at least nine are made inside the chloroplast. The products of chloroplast protein synthesis include polypeptides 2 and 6, previously shown to be associated with the reaction center activity of photosystems I and II, respectively (11, 4, 9); polypeptide 5, whose function has not yet been identified but which, on the basis of genetic criteria (see review by Adams et al., reference 1), appears to be coded for by chloroplast DNA (8); and several other polypeptides whose functions are unknown.

MATERIALS AND METHODS

Pulse-Labeling of Cells with [^{14}C]Acetate

Cells of wild type and the *spr-u-1-6-2* mutant were grown in the Tris-acetate-phosphate medium of Gorman and Levine (19) under cool white fluorescent lights at an intensity of 15,000 lx at 25°C, and log phase cultures at a density of approx. 1×10^6 cells/ml were used in all experiments. Cells were harvested by low-speed centrifugation, washed once in minimal (Tris-HCl-phosphate) medium (33), and resuspended in the same medium to a final density of 6×10^6 cells/ml. Aliquots (100 ml) of the cell suspension were transferred to 500-ml Erlenmyer flasks and agitated on a rotary shaker at 25°C under a light intensity of about 10,000 lx. After 15 min of adaptation, appropriate amounts of inhibitors were added to the samples from stock solutions. The stock solution of SPEC sulfate (15 mg of SPEC base per ml) was made up in distilled H_2O, whereas those of ANISO (0.125 M) and CAP (100 mg/ml) were prepared as ethanolic solutions. All samples received ethanol to a final concentration of 0.3%. The final concentrations of the inhibitors were: ANISO, 2.5×10^{-4} M; CAP, 100 μg per ml; and SPEC, 300 μg/ml. The concentration of ANISO was found to be saturating as determined by dosage-inhibition experiments (data not shown). Optimal concentrations of CAP and SPEC were established previously by Chua et al. (10) and Conde et al. (12), respectively. 5 min after the addition of the inhibitors, cells were pulse-labeled for 30 min with 2 μCi/ml of sodium [1-^{14}C]acetate. At the end of the pulse-labeling period, 100-μl aliquots of each sample were removed in duplicate for radioactivity measurement. 50 ml of the Tris-acetate-phosphate medium was then added to each flask, giving an ~200-fold dilution of the [^{14}C]acetate specific activity, and the flasks were chilled immediately in an ice bath. Cells were harvested by centrifugation at 2,000 *g* for 10 min.

Analysis of Pulse-Labeled Thylakoid Membrane Polypeptides

Thylakoid membranes were purified by the flotation procedure described previously (9). Membrane polypeptides were fractionated by SDS gel electrophoresis using a modification of Neville's procedure (31). The stacking

gel contained 6% acrylamide whereas the resolving gel was made up of a 7.5–15% linear acrylamide concentration gradient (9). Membranes were dissolved at room temperature in a mixture containing 50 mM Na_2CO_3, 50 mM dithiothreitol (DTT), 2% SDS, 12% sucrose, and 0.04% bromophenol blue. Each sample was divided into two equal portions: one portion was incubated at 100°C for 1 min to dissociate chlorophyll-protein complexes (heated) whereas the other portion was not treated further (nonheated). Thylakoid membrane polypeptides were fractionated by extraction with a 2:1 (vol/vol) mixture of chloroform:methanol (C/M), and the extract was taken to dryness under a stream of nitrogen (11). Polypeptides in the C/M residue and extract were analyzed by SDS gel electrophoresis. Gels were stained with 0.025% Coomassie Brillant Blue F 250, 7% acetic acid, and 50% methanol for 3 h, and excess dye was eluted by shaking the gels in a mixture of 7% acetic acid and 40% methanol. Gels were dried by suction onto Whatman 3 MM chromatographic papers, and the dried gels were placed in contact with Cronex 2 DC medical X-ray films (E. I. du Pont de Nemours & Co., Wilmington, Del.) for 4–7 days. Autoradiographs were scanned with a Joyce-Loebl microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, Eng.). The relative amount of radioactivity under each peak was then quantified with the aid of a Hewlett-Packard digitizer (Hewlett-Packard Corp., McMinnville, Oreg.).

Measurement of Radioactivity, Chlorophyll, and Cell Number

Radioactivity was measured by the filter paper disk method of Mans and Novelli (28) using a Beckman liquid scintillation spectrometer (model LS-350, Beckman Instruments, Inc., Fullerton, Calif.). The counting efficiency for ^{14}C is ~80%. Chlorophyll was determined according to Arnon (2), and cell number was measured with Cytograf (model 6302; Bio/Physics Systems, Inc., Mahopac, N. Y.).

Chemicals and Solutions

SDS (sequanal grade) was purchased from Pierce Chemical Co., Rockford, Ill.; Coomassie Brillant Blue R 250, bromophenol blue, DTT, and CAP from Sigma Chemical Co. (St. Louis, Mo.); SPEC sulfate (64% SPEC base) was a gift from Dr. G. B. Whitfield, Jr., Upjohn Co. (Kalamazoo, Mich.); ANISO was obtained from E. I. du Pont de Nemours & Co.; and sodium [1-^{14}C]-acetate (sp act 58 mCi/mmol) was purchased from New England Nuclear (Boston, Mass.).

RESULTS

Effects of Inhibition on Pulse-Labeling of Whole Cells and Total Thylakoid Membrane Polypeptides with [^{14}C]Acetate

Under our conditions of pulse-labeling, the specific radioactivity incorporated into whole cell proteins varies from 1.0 to 2.0 × 10^5 cpm per 10^6 cells. Approx. 30–50% of the pulse-radioactivity is incorporated into the thylakoid membrane proteins.

ANISO inhibits [^{14}C]acetate incorporation into whole cell proteins dramatically whereas CAP causes only a small decrease in incorporation and SPEC actually stimulates incorporation slightly in both wild type and *spr-u-1-6-2* (Table I). Different results are obtained, however, when incorporation into the thylakoid membrane polypeptides is examined. In wild type, ANISO lowers [^{14}C]acetate incorporation to 33% of the control whereas CAP and SPEC only reduce the incorporations to 70–80% of the control (Table I). It should be noted that the ANISO-resistant incorporation (15% of control) in whole cells can be accounted for largely by the incorporation of radioactivity into the thylakoid membrane polypeptides. When CAP and ANISO or SPEC and ANISO are added together, incorporation of [^{14}C]acetate into thylakoid membrane polypeptides is reduced to around 12 and 17%, respectively. In the mutant *spr-u-1-6-2*, SPEC does not inhibit the incorporation, but the pattern of inhibition for the other drugs is the same as for wild type cells. Furthermore, thylakoid membranes of this mutant become labeled to the same extent in ANISO or ANISO plus SPEC.

Pattern of Pulse-Incorporation of [^{14}C]Acetate into Thylakoid Membrane Polypeptides of Wild Type in the Presence of Inhibitors

Fig. 1*a* compares the Coomassie Blue-staining pattern of thylakoid membrane polypeptides (labeled Gel") and the corresponding autoradi-

TABLE I

Effects of Inhibitors on the Pulse-Incorporation of [^{14}C]Acetate into Whole Cell and Thylakoid Membrane Proteins in C. reinhardtii

	Wild type		*spr-u-1-6-2*	
Samples	Whole cells	Thylakoids	Whole cells	Thylakoids
	%			
Control	100	100	100	100
ANISO	15	33	18	37
CAP	89	66	91	70
ANISO + CAP	8.5	11.5	12	13
SPEC	104	80.6	108	112
ANISO + SPEC	11.3	17	19	37

Values were averages of four separate experiments for each genotype.

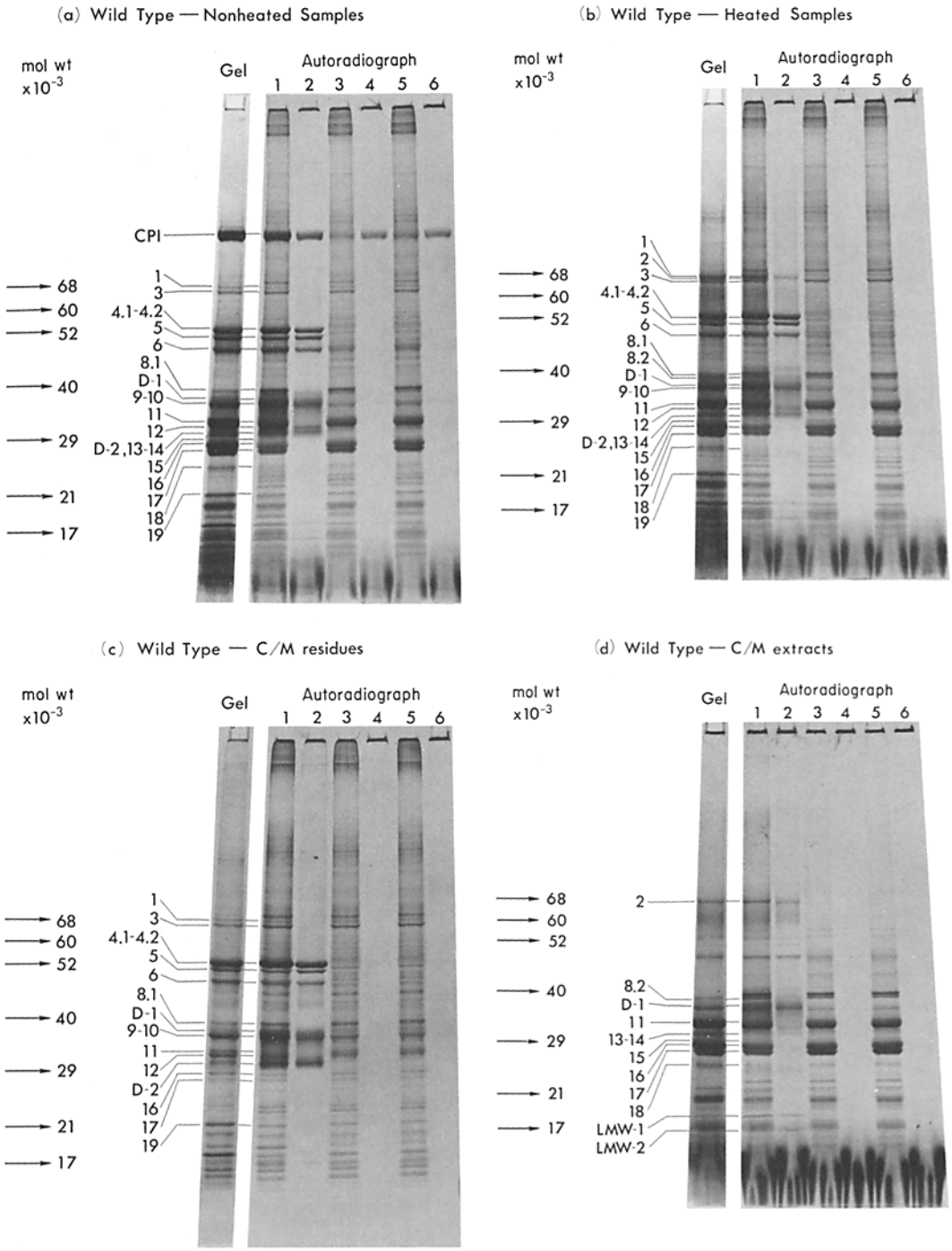

FIGURE 1 Pulse-incorporation of radioactive precursors into thylakoid membrane polypeptides of wild type in the presence of different inhibitors. (*a*) nonheated samples, 20 μg chlorophyll per slot; (*b*) heated samples, 20 μg chlorophyll per slot; (*c*) C/M membrane residues, equivalent to 30 μg chlorophyll per slot; and (*d*) C/M extracts, equivalent to 30 μg chlorophyll per slot. The slots on the left (labeled "Gel") show the Coomassie Blue-staining pattern of membrane polypeptides whereas slots *1–6* show the corresponding autoradiographs. (*1*) control; (*2*) ANISO, 2×10^{-4} M; (*3*) CAP, 100 μg/ml; (*4*) ANISO (2×10^{-4} M) plus CAP (100 μg/ml); (*5*) SPEC, 300 μg/ml; and (*6*) ANISO (2×10^{-4} M) plus SPEC (300 μg/ml). Membrane polypeptides are identified by consecutive numbers starting from the high molecular weight region, and the arrows on the far left side indicate the position of molecular weight markers as described in reference 9. For other details, see Materials and Methods.

ograph (slot 1) obtained with a 30-min pulse-labeling with [^{14}C]acetate. Under our conditions, all the stained gel bands are labeled and, in addition, several radioactive bands which do not coincide with any stained gel bands or correspond only to minor components are also detected. One nonstaining band migrates in the high molecular weight region (ca. 70,000) but nonstaining bands also appear elsewhere in the gel. The nature of these bands is not clear at present: they could be contaminating polypeptides not derived from the thylakoids, membrane polypeptides which stain poorly, minor thylakoid membrane polypeptides with high turnover rates, or precursors of membrane polypeptides.

The effects of various inhibitors on the pulse-incorporation pattern of thylakoid membrane polypeptides are illustrated in Fig. 1 *a–d*. In the nonheated samples (Fig. 1 *a*), chlorophyll-protein complex I (CP I) as well as polypeptides 4.1 plus 4.2, 5, and 6 are labeled in the presence of ANISO (Fig. 1 *a*, slot 2). Incorporation into these polypeptides but not into CP I is greatly reduced in samples treated with CAP (Fig. 1 *a*, slot 3) or SPEC (Fig. 1 *a*, slot 5). With the exception of CP I, little incorporation into membrane polypeptides is observed in the presence of ANISO plus CAP (Fig. 1 *a*, slot 4) or ANISO plus SPEC (Fig. 1 *a*, slot 6). It should be noted that, under the conditions of gel electrophoresis used, CP II dissociates during electrophoresis and the chlorophyll released migrates with the zone of SDS-pigment complexes (11).

The residual radioactivity of CP I in samples treated with CAP (Fig. 1 *a*, slot 3) or SPEC (Fig. 1 *a*, slot 5) could be due to either incomplete inhibition of polypeptide synthesis by the antibiotics or incorporation of newly synthesized chlorophylls into the complex, since acetate is a good precursor of chlorophyll biosynthesis. To distinguish between these two possibilities, we made use of the previous observation that CP I is heat-labile and can be dissociated into free chlorophyll and its constituent polypeptide (polypeptide 2) by incubation at 100°C for 1 min (11). Fig. 1 *b* shows that, in the heated samples, polypeptide 2 is labeled in the control autoradiograph (slot 1) and in the presence of ANISO (slot 2), but labeling of this polypeptide is not apparent in the presence of CAP (slot 3) or SPEC (slot 5) or in the presence of each of these inhibitors plus ANISO (slots 4 and 6). These results show that labeling of CP I in the presence of inhibitors that block both chloroplast and cytoplasmic protein synthesis occurs because chlorophyll synthesis takes place in the presence of the inhibitors on preexisting enzymes. The radioactive chlorophyll must then be assembled into CP I using a pool of unlabeled polypeptide 2 molecules made before the pulse.

To determine whether both polypeptides 4.1 and 4.2 were made on chloroplast ribosomes, thylakoid membrane polypeptides were separated on 5–10% gradient gels (Fig. 2). Under these conditions, the two polypeptides are clearly resolved and it is seen that the synthesis of both is sensitive to CAP or SPEC but not to ANISO. In addition to the aforementioned polypeptides, two broad, fuzzy bands of radioactivity are seen between polypeptides 8.1 and 9–10 (diffuse band -1, D-1) and in the region of polypeptides 13–14 (diffuse band -2, D-2) in the control (Fig. 1 *a*, slot 1) and ANISO-treated sample (Fig. 1 *a*, slot 2). Incorporation into these bands is inhibited by the 70S ribosome-specific antibiotics (Fig. 1 *a*, slots 3 and 5). The labeling pattern of these two diffuse bands becomes clearer after C/M extraction as discussed below. Finally, several polypeptides with a molecular weight of <20,000 also appear to be labeled in the presence of ANISO (Fig. 1 *b*, slot 2), but because of inadequate resolution in the low molecular weight region, it is not known whether they are inhibited by CAP or SPEC.

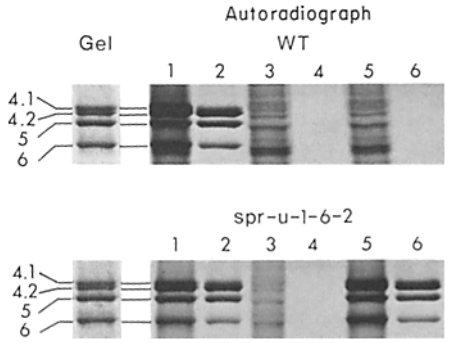

FIGURE 2 Resolution of polypeptides 4.1 and 4.2 by SDS gels containing a 5–10% acrylamide concentration gradient. Only the gel region containing polypeptides 4.1 to 6 are shown. The slots on the left show the Coomassie Blue-staining pattern of the gel. All samples were nonheated and the load was 20 μg chlorophyll per slot. (*1*) control; (*2*) ANISO, 2 × 10^{-4} M; (*3*) CAP, 100 μg/ml; (*4*) ANISO (2 × 10^{-4} M) plus CAP (100 μg/ml); (*5*) SPEC, 300 μg/ml; and (*6*) ANISO (2 × 10^{-4} M) plus SPEC (300 μg/ml). *WT*, wild type. For other details, see Materials and Methods.

Because numerous radioactive bands are evident in the autoradiographs shown in Fig. 1 *a* and *b*, it seemed important to reduce the complexity of the labeling pattern by differential extraction of the polypeptide mixture. It has been reported previously that the thylakoid membrane polypeptides can be fractionated into two groups on the basis of their differential solubility in a 2:1 (vol/vol) mixture of C/M (11). Thus, polypeptides of similar apparent molecular weights may be resolved from one another by this technique. We have made use of this differential extraction procedure to separate polypeptide 2 from polypeptides 1 and 3 and to increase further the resolution of other membrane polypeptides, especially those in the region of polypeptides 8–17. Fig. 1 *c* and *d* confirm previous observations that polypeptide 2 is quantitatively extracted by C/M whereas polypeptides 1 and 3 remain in the C/M residue (11). The accompanying autoradiographs show that polypeptide 2 is clearly labeled in the presence of ANISO (Fig. 1 *d*, slot 2) but not in the presence of CAP (Fig. 1 *d*, slot 3) or SPEC (Fig. 1 *d*, slot 5). These results confirm the suggestion made earlier that the residual radioactivity of CP I in samples treated with CAP or SPEC (Fig. 1 *a*) can be attributed largely to the incorporation of [^{14}C]acetate into chlorophylls associated with the complex. The radioactivity in band D-1 appears to be distributed equally between the C/M residue and extract (Fig. 1 *c* and *d*), but it should be emphasized that we do not know whether this band contains more than one polypeptide. Nevertheless, incorporation into this band in both fractions (Fig. 1 *c* and *d*) is clearly resistant to ANISO (slot 2) but sensitive to CAP (slot 3) and SPEC (slot 5). The labeling patterns of the other diffuse band, D-2, and of two low molecular weight polypeptides, LMW-1 and LMW-2, become distinct after C/M extraction. Fig. 1 *c* and *d* show that there are at least three polypeptides which migrate in between polypeptide 12 and polypeptide 15. One of them (D-2) is insoluble in C/M (Fig. 1 *c*) whereas the other two (polypeptides 13 and 14) are extracted (Fig. 1 *d*). Autoradiographs of the C/M residues (Fig. 1 *c*) reveal that D-2 is labeled in the presence of ANISO (slot 2) but not CAP (slot 3) or SPEC (slot 5). The amount of radioactivity incorporated into polypeptides 13 and 14 is usually low in comparison to the other membrane polypeptides (Fig. 1 *d*, slot 1). However, upon longer exposure of the autoradiographs, we found that these polypeptides are labeled in the presence of CAP and SPEC but not ANISO (data not shown, but see Fig. 3 *b*). These results demonstrate that in the ANISO-treated samples not extracted with C/M (Fig. 1 *a* and *b*) the radioactivity in this gel region can be attributed solely to the labeling of D-2. LMW-1 and LMW-2 are extracted by C/M (Fig. 1 *d*), and their labeling patterns in the presence of various inhibitors resemble that of D-2, indicating that these polypeptides are also made inside the chloroplast.

The patterns of incorporation of radioactivity into two closely migrating polypeptides, 11 and 12, also become clearer after C/M extraction. In the nonheated samples (Fig. 1 *a*), polypeptide 12 is not resolved from polypeptide 11, whereas in the heated samples (Fig. 1 *b*) the former migrates only slightly faster than the latter. In confirmation of previous results (11), we found that polypeptide 11 is largely soluble in C/M (Fig. 1 *d*) but, in contrast, polypeptide 12 is not extracted (Fig. 1 *c*). Autoradiographs of the C/M residues (Fig. 1 *c*) show that incorporation into polypeptide 12 is inhibited by ANISO (slot 2) but not by CAP (slot 3) or SPEC (slot 5). There is a faint radioactive band which migrates slightly ahead of polypeptide 12 in the ANISO-treated sample (Fig. 1 *c*, slot 2) but this band is not consistently observed in all experiments. In similar experiments carried out with the mutant *spr-u-1-6-2*, this band is not detected in the C/M residues (Fig. 3 *a*), and here it can be seen clearly that the synthesis of polypeptide 12 is sensitive to ANISO (slot 2) but insensitive to CAP (slot 3).

Pulse-labeling experiments with wild type cells were also carried out with [^{14}C]arginine or $^{35}SO_4^{2-}$ as radioactive precursor instead of [^{14}C]-acetate, and essentially similar patterns of incorporation were obtained in the absence or presence of various inhibitors (results not shown). These results rule out the possibility that any of the radioactivity bands are due to polypeptide-associated lipids into which [^{14}C]acetate was incorporated.

Pattern of Incorporation of [^{14}C]Acetate into Thylakoid Membrane Polypeptides of spr-u-1-6-2 in the Presence of Inhibitors

So far, our results show that, on a qualitative basis, at least nine membrane polypeptides (nos. 2, 4.1, 4.2, 5, 6, D-1, D-2, LMW-1, and LMW-2) are labeled in the presence of ANISO but not in CAP or SPEC, whereas the converse situation holds true for the remaining polypeptides.

The remarkable complementary pattern of in-

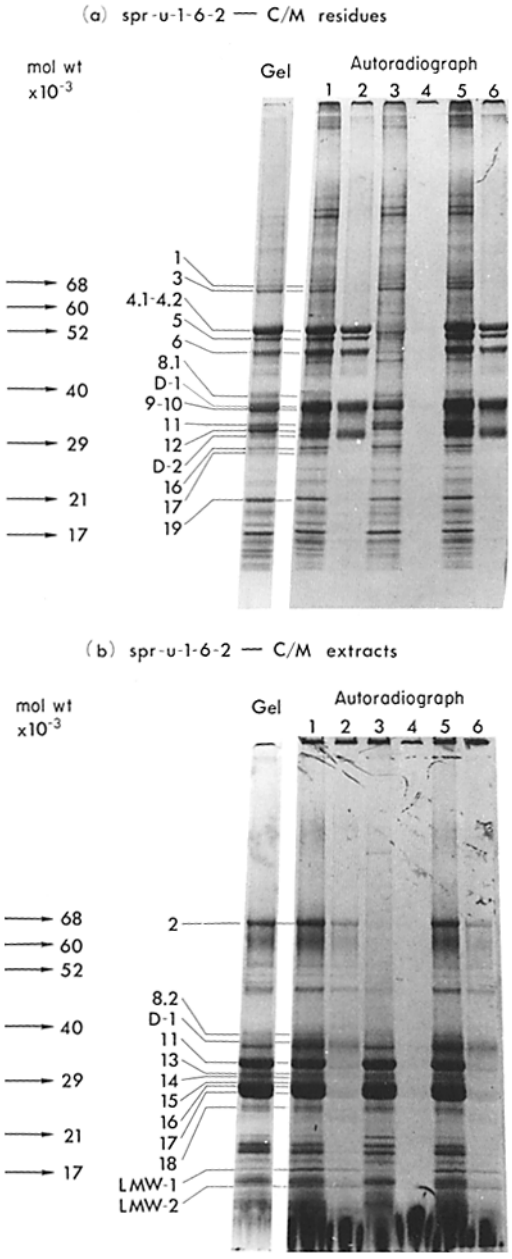

FIGURE 3 Pulse-incorporation of radioactive precursors into thylakoid membrane polypeptides of *spr-u-1-6-2* in the presence of different inhibitors. (*a*) C/M membrane residues, equivalent to 30 μg chlorophyll per slot; and (*b*) C/M extracts, equivalent to 30 μg chlorophyll per slot. The slots of the left (labeled "Gel") show the Coomassie Blue-staining pattern of the gels whereas slots *1-6* show the corresponding autoradiographs. (*1*) control; (*2*) ANISO, 2×10^{-4} M; (*3*) CAP, 100 μg/ml; (*4*) ANISO (2×10^{-4} M) plus CAP (100 μg/ml); (*5*) SPEC, 300 μg/ml; and (*6*) ANISO (2×10^{-4} M) plus SPEC (300 μg/ml). For other details, see legend to Fig. 1.

corporation of radioactivity into the membrane polypeptides obtained with ANISO and each of the two 70S ribosome-specific antibiotics in wild type strongly reduces the possibility of nonspecific effects of the inhibitors. To prove that polypeptides whose synthesis is inhibited by CAP or SPEC really were made on chloroplast ribosomes, we repeated the above experiments using the *spr-u-1-6-2* mutant instead of wild type. This mutant has chloroplast ribosomes resistant to SPEC both in vivo and in vitro (12). We predicted that incorporation of radioactivity into polypeptides made on chloroplast ribosomes in the mutant would show the same pattern of inhibition seen for wild type in the presence of CAP, but that synthesis of these polypeptides would be resistant to SPEC. The patterns of pulse-label incorporation into thylakoid membrane polypeptides of *spr-u-1-6-2* in the presence of the inhibitors are illustrated in Figs. 2 and 3 *a* and *b*. Comparisons of the results obtained with the mutant (Figs. 2 and 3) and with wild type (Figs. 1 and 2) show that only in the presence of SPEC is a difference seen between the labeling patterns. Incorporation of radioactivity into polypeptides synthesized on chloroplast ribosomes is sensitive to CAP in both wild type and the mutant, but resistant to SPEC in the mutant. This point is best illustrated through a comparison of samples treated with ANISO and ANISO plus CAP or ANISO plus SPEC. Treatment with ANISO plus CAP (Fig. 3, slot 4) virtually eliminates all incorporation into membrane polypeptides although chlorophyll at the bottom of the gel and carotenoids which are retained on top of the stacking gel are still labeled by [^{14}C]acetate. In the presence of ANISO plus SPEC (Fig. 3, slot 5), the incorporation of radioactivity into polypeptides thought to be made on chloroplast ribosomes continues to the same extent as it does in the presence of ANISO alone (Fig. 3, slot 2). These results provide strong evidence that our interpretation of the inhibitor data is correct and that we are not observing nonspecific effects of the inhibitors.

Quantitative Effects of Inhibitors on [^{14}C]Acetate Incorporation into Thylakoid Membrane Polypeptides

To estimate the quantitative effects of the inhibitors on incorporation, the autoradiographs were scanned with a microdensitometer, and peak areas were measured as described in Materials and Methods. The microdensitometric tracings show good resolution in much of the high molecular

weight region, but in the low molecular weight region there are numerous poorly resolved peaks (data not shown). For this reason we did not attempt quantification of polypeptide peaks with a molecular weight smaller than that of polypeptide 17. Control experiments in which different amounts of radioactive thylakoid membrane polypeptides were loaded on the gels and exposed to X-ray film established that under our conditions film darkening as estimated by microdensitometry was related linearly to the amount of radioactivity loaded (data not shown).

The quantitative results support the qualtitative observations (Table II). The polypeptides synthesized on chloroplast ribosomes are cleanly partitioned between the C/M extract and residue, expect for band D-1. It is clear that synthesis of polypeptides 4.1 + 4.2, 5, and 6 is largely, if not completely resistant to ANISO, but incorporation of radioactivity into these polypeptides is blocked by CAP or SPEC in wild type. In the *spr-u-1-6-2* mutant, incorporation into these polypeptides takes place at close to control rates in the presence of SPEC but is inhibited by CAP. Incorporation of radioactivity into polypeptide 2 is blocked by CAP and SPEC in wild type and by CAP alone in *spr-u-1-6-2*, indicating that it is synthesized on chloroplast ribosomes. However, inhibition of incorporation is also seen with ANISO, although the extent of this inhibition is highly variable. In four separate experiments with wild type, the incorporation in the presence of ANISO varied from 0 to 42% of the control values.

We felt that the variable inhibition of incorporation into polypeptide 2 might be explained if the integration of this polypeptide into thylakoid membranes required other components, e.g. a "partner protein", lipids, and chlorophylls, whose synthesis requires active cytoplasmic protein synthesis. During ANISO inhibition, synthesis of the partner protein would be blocked but synthesis of polypeptide 2 molecules would continue on chloroplast ribosomes. The pool size of the putative component before ANISO inhibition would, therefore, determine the number of newly synthesized radioactive polypeptide 2 molecules which could be incorporated into the thylakoid membranes during pulse labeling in the presence of ANISO. To test this hypothesis, we preincubated wild type cells for 30 min with CAP to permit the pool size of the putative cytoplasmically synthesized component to build up relative to polypeptide 2. The cells were then pulsed with [^{14}C]acetate for 30 min in the presence or absence of ANISO, and autoradiographs were made of the gels obtained from the C/M extract and residue of the thylakoid membranes as before. Under these conditions, the level of label incorporation into polypeptide 2 was high in the presence of ANISO. Quantification of the autoradiographs indicated that the mean level of incorporation in the presence of ANISO after the CAP preincubation in four separate experiments was 80% of the control (data not shown), as compared to 22% of the control without CAP preincubation (Table II).

Quantification of D-1, which partitions between the C/M extract and residue, could only be done on the extract, and it is evident that synthesis of this portion of D-1 is partly inhibited by ANISO but completely blocked by CAP and SPEC in wild type. Whether cytoplasmically synthesized components are necessary for the appearance of la-

TABLE II

Incorporation of Radioactivity into Thylakoid Membrane Polypeptides

		Mean % incorporation of radioactivity as a percent of control									
Inhibitor	Genotype	1	2	3	4.1 + 4.2	5	6	9-10	11	15-17	D-1
ANISO	Wild type	n.d.	22	n.d.	80	103	100	n.d.	n.d.	n.d.	58
	spr-u-1-6-2	n.d.	42	n.d.	78	135	74	n.d.	n.d.	n.d.	51
CAP	Wild type	103	n.d.	108	n.d.	n.d.	n.d.	85	92	103	n.d.
	spr-u-1-6-2	169	n.d.	118	n.d.	n.d.	n.d.	91	81	62	n.d.
SPEC	Wild type	165	n.d.	113	n.d.	n.d.	n.d.	76	90	111	n.d.
	spr-u-1-6-2	168	74	92	133	105	131	109	125	105	104
ANISO + SPEC	*spr-u-1-6-2*	n.d.	34	n.d.	83	217	86	n.d.	n.d.	n.d.	42

Radiographs of the chloroform:methanol extracts and precipitates were scanned with a microdensitometer, and the area under each polypeptide peak was quantified as described in Materials and Methods. Control experiments in which different amounts of radioactive polypeptides were exposed to X-ray film established that there is a linear relation between film darkening and the amount of radioactivity loaded under the experimental conditions used. Mean percent incorporation is shown for three to four experiments for each genotype. Satisfactory quantification could not be obtained for peak D-2 due to the presence of interfering polypeptides.

n.d. = radioactivity not detectably above background.

beled D-1 in the membrane, we do not know. The microdensitometric resolution in the region of polypeptides 8–17 of the C/M residue was poor so that quantification of that part of the autoradiograph, which included D-2, was not attempted. Quantitative data were also obtained for polypeptides 1, 3, 9–10, 11, and 15–17 and support the qualitative observations that these polypeptides are products of cytoplasmic protein synthesis.

Effects of Acetone Extraction on Thylakoid Membrane Polypeptides

In the course of their investigations on thylakoid membrane polypeptide synthesis, several workers (6, 21) have extracted their membrane preparations with 90% acetone in order to remove photosynthetic pigments which they thought might interfere with SDS gel electrophoresis. However, it has been shown previously (11) that acetone treatment of membranes leads to a selective disappearance of polypeptides from the electrophoretogram. We have confirmed this observation (Fig. 4). Gels of thylakoid membrane polypeptides extracted with 90% acetone lack polypeptide 2 and are deficient in polypeptides 5 and 6. As demonstrated previously (11), the missing or deficient polypeptides are not solubilized since they are not present in the 90% acetone extract. Rather, these polypeptides become aggregated specifically after acetone extraction and as a result they are retained on top of the stacking gel (Fig. 4). These results suggest that polypeptides 2, 5, and 6, which are made on chloroplast ribosomes, might not have been seen by other workers who have studied the sites of synthesis of thylakoid membrane polypeptides and who have employed acetone extraction.

Effects of CAP on the Synthesis and Assembly of Thylakoid Membrane Polypeptides in Long-Term Experiments

Although our results with wild type and the *sprU-1-6-2* mutant show that protein synthesis inhibitors are specific when used in 30-min pulse experiments, it was not known whether the same inhibitors would produce nonspecific and indirect effects in long-term experiments. To investigate this possibility, thylakoid membranes of wild type cells grown in the presence of CAP (100 μg/ml) for 6–7 generations were analyzed for alterations in

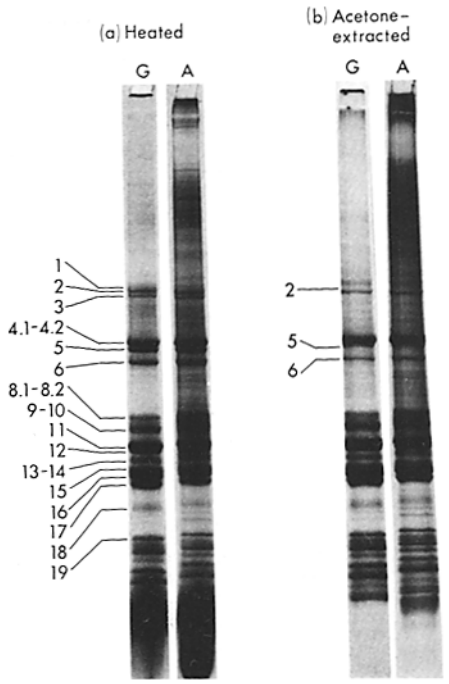

FIGURE 4 Effects of acetone extraction on thylakoid membrane polypeptide profiles. *G*, gel; *A*, autoradiograph. (*a*) heat sample, 20 μg chlorophyll; (*b*) sample extracted with 90% acetone, 30 μg chlorophyll.

polypeptide profiles. Fig. 5 shows that membranes of wild type cells treated with CAP are deficient in CP I, polypeptides 2, 4.1, 4.2, 5, 6, 8.2, 9, 10, 12, 19, D-1, D-2, LMW-1, and several other low molecular weight polypeptides. Since the pulse experiments indicate that polypeptides 8.2, 9, 10, 12, and 19 are made on cytoplasmic ribosomes, the deficiencies of these polypeptides must be due to indirect effects of CAP. One obvious possibility is that such cytoplasmically synthesized polypeptides may require a product of chloroplast protein synthesis either for transport across the chloroplast envelope or for their final assembly into the thylakoids. In addition to these membrane alterations, two new polypeptides (marked by arrows in Fig. 5) that migrate in between polypeptides 6 and 7, are detected in membranes of CAP-treated cells. The nature of these polypeptides is unknown at present.

DISCUSSION

The results presented in this paper show that among the 33 or more thylakoid membrane polypeptides found in *C. reinhardtii,* at least nine are

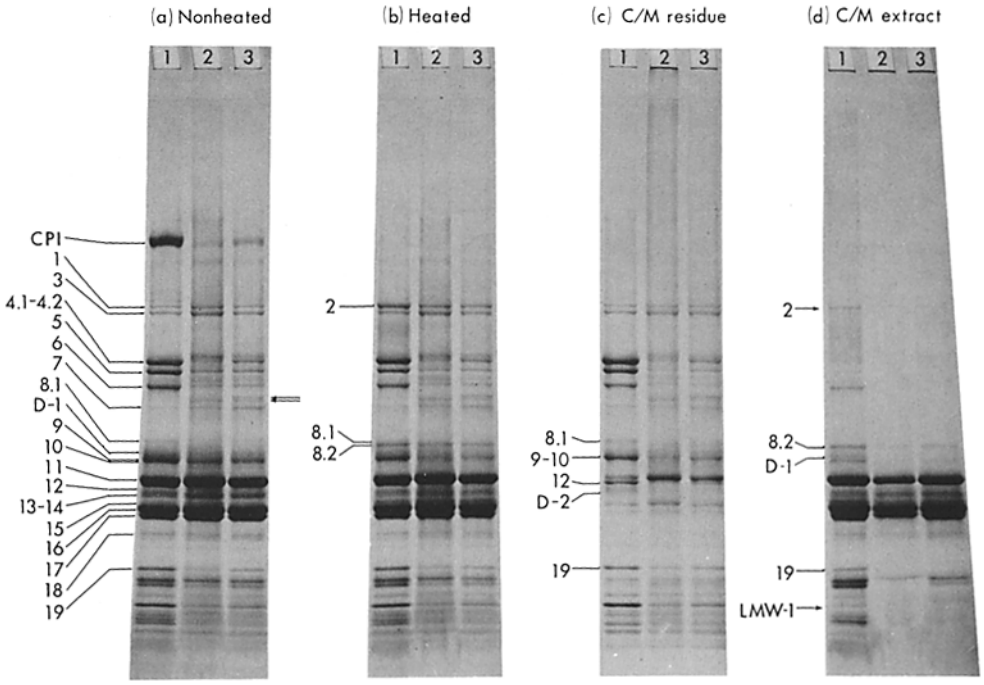

FIGURE 5 Long-term effects of CAP on thylakoid membrane polypeptides of wild type. (*1*) control; (*2*) and (*3*) cells grown in CAP (100 μg/ml) for 6–7 generations under mixotrophic conditions. For heated and nonheated samples, the load was 20 μg chlorophyll per slot, and for the C/M residues and extracts, the load was equivalent to 30 μg chlorophyll per slot. For other details, see legend to Fig. 1.

pulse-labeled in the presence of a saturating concentration of ANISO, an inhibitor of cytoplasmic protein synthesis. Pulse-label incorporation into these nine polypeptides is specifically inhibited by either CAP or SPEC in wild type, and little incorporation into membrane polypeptides occurs in the presence of CAP plus ANISO or SPEC plus ANISO. Similar results were obtained with the mutant *spr-u-1-6-2* which possesses chloroplast ribosomes resistant to spectinomycin, except that SPEC does not inhibit incorporation. Furthermore, the pulse-incorporation pattern with SPEC plus ANISO is identical to that with ANISO alone. *In toto,* these results seem to exclude any nonspecific effects on the patterns of incorporation seen in the presence of these protein synthesis inhibitors. The chloroplast protein synthesis products include polypeptides 2, 4.1, 4.2, 5, 6, two low molecular weight polypeptides, and two diffuse regions of labeling, bands D-1 and D-2. It is interesting to note that polypeptides 2 and 6 have been reported previously to be associated with PS I and PS II reaction centers, respectively (11, 9). Also, the integration of polypeptide 2 into thylakoid membranes apparently requires the presence of a component(s) made in the cytosol. The function of polypeptide 5 has not yet been ascertained but a molecular weight variant of this polypeptide is synthesized by a mutant, *thm-u-1,* whose defect is inherited uniparentally (8) and probably is localized in chloroplast DNA (see Adams et al., reference 1, for a review). The nature of the two diffuse polypeptide bands (D-1 and D-2) is not clear; they are either minor polypeptide components which turn over rapidly, or polypeptides that are not stained well by Coomassie Blue dye. Since CAP inhibits ^{14}C-incorporation into thylakoid membrane polypeptides by about 30–35%, we estimate that membrane polypeptides which are made in the cytosol account for at least 65% in mass of the total (Table I). However, this number may be higher if D-1 and D-2 indeed have high turnover rates. Prominent among the cytoplasmic products is polypeptide 11 which accounts for ~20% in mass of the total membrane proteins. This polypeptide is a constituent of the chlorophyll-protein complex II (11) which has been suggested to be the light-harvesting pigment complex for PS II (25, 34).

Long-term growth of cells in the presence of

CAP leads to the loss of specific thylakoid membrane polypeptides which are synthesized in the cytoplasm in addition to the polypeptides which are made on chloroplast ribosomes. These include polypeptides 8.2, 9, 10, 12, 19, and several low molecular weight proteins. A possible explanation of this finding is that these cytoplasmically synthesized proteins cannot be integrated into the thylakoid membranes in the absence of specific components made on chloroplast ribosomes. Even if this explanation is incorrect, our results emphasize the danger of drawing conclusions from experiments in which cells have been treated with protein synthesis inhibitors over an extended period.

The sites of synthesis of the thylakoid membrane polypeptides also have been investigated in regreening cells of the *y-1* mutant of *Chlamydomonas,* using specific inhibitors of chloroplast and cytoplasmic protein synthesis, by Hoober (21), Bar-Nun and Ohad (3), and Eytan and Ohad (17). Cashmore (7), Machold and Aurich (27), and Ellis (15, 16) have used specific inhibitors to study the sites of synthesis of thylakoid membrane polypeptides in vivo in higher plants. Finally, a number of workers have examined thylakoid membrane polypeptide synthesis in higher-plant chloroplasts in vitro (6, 14, 16, 30, 32). Extraction procedures and gel methodologies vary widely among different experimenters. We have attempted to bring all of these data together, in light of our own findings, in a recent review (18). That seems to us a better forum for a general discussion, as it allows a more leisurely dissection of the literature than does a research paper.

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