

# MEMBRANE PROTEINS SYNTHESIZED BUT NOT PROCESSED BY ISOLATED MAIZE CHLOROPLASTS

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## ABSTRACT

One-dimensional maps of proteolytic fragments generated by digestion with *Staphylococcus aureus* protease in sodium dodecyl sulfate (SDS) were used to identify three polypeptides synthesized by isolated *Zea mays* chloroplasts. This technique does not depend upon proper incorporation of the newly synthesized polypeptides into a more complex structure for their identification. The only preliminary purification required is electrophoretic separation on SDS-polyacrylamide gels.

The pattern of radioactive fragments from labeled proteins which co-migrate with the  $\alpha$  and  $\beta$  subunits of chloroplast coupling factor ( $CF_1$ ) corresponds precisely to the pattern of stainable fragments derived from subunits of the purified enzyme.

A 34,500-dalton protein is the major membrane-associated product of protein synthesis by isolated maize chloroplasts. From the similarity in the fragments formed by digestion with *S. aureus* protease, it appears that this radioactive protein is probably a precursor of a 32,000-dalton protein which is a component of the thylakoid.

The  $\alpha$  and  $\beta$  subunits of  $CF_1$  newly synthesized by isolated chloroplasts are not fully extractable by procedures which normally solubilize the enzyme from membranes. The 34,500-dalton protein is not processed to the 32,000-dalton form in any great amount by isolated chloroplasts. A 19,000-dalton fragment of the 32,000-dalton protein is protected from digestion when thylakoids are treated with proteases, while the newly synthesized 34,500-dalton protein is fully susceptible. The isolated chloroplast does not appear to be able to fully integrate these newly made proteins into the membrane structure.

**KEY WORDS** chloroplast coupling factor ·  
limited proteolysis · etioplasts · thylakoid

energy source (1, 3, 5, 8, 14, and footnote 1).  
Both soluble and membrane-associated products  
are formed.

Isolated chloroplasts from a number of different  
plants can synthesize proteins, using light as an

<sup>1</sup> A. E. Grebanier, K. E. Steinback, and L. Bogorad.  
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The principal soluble protein synthesized by isolated chloroplasts is the large subunit of ribulose 1,5-bisphosphate carboxylase (1). It was identified by comparison of autoradiographs of two-dimensional separations of tryptic peptides of the soluble protein synthesized *in vitro* with those derived from carboxylase purified from radioactively labeled plants.

The most successful approach thus far for identification of membrane-bound products has been determination of incorporation of labeled amino acids into proteins which can be specifically solubilized and purified. In this way, it has been shown that the  $\alpha$ ,  $\beta$ , and  $\epsilon$  subunits of chloroplast coupling factor 1 (CF<sub>1</sub>) (footnote 1 and reference 3) and cytochrome *b*<sub>559</sub> (17) are made by isolated chloroplasts. However, this approach requires the polypeptides synthesized by isolated chloroplasts to be incorporated into the membrane exactly as components synthesized *in vivo*. Otherwise, specific extraction and purification procedures may not work with the newly synthesized polypeptides.

We have employed an approach which should unambiguously identify membrane-associated products, regardless of whether they are correctly integrated into the membrane structure or not. The techniques developed by Cleveland et al. (4) for partial proteolysis of proteins during electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels give distinctive one-dimensional maps for fragments derived from different proteins. We have used their procedures, together with fluorography (2, 10), to identify radioactive proteins, separated solely by gel electrophoresis of dissociated membranes, with proteins which can be purified from these membranes. To test the applicability of this approach for identification of polypeptides synthesized by isolated chloroplasts, we first used these techniques to extend the identification of subunits of CF<sub>1</sub> as products of protein synthesis by isolated chloroplasts. Next, we used a combination of the one-dimensional mapping of proteolytic peptides in SDS-polyacrylamide gels and two-dimensional mapping of tryptic peptides on thin-layer plates to evaluate possible relationships between the 34,500-dalton protein made by isolated maize chloroplasts (6) and slightly smaller proteins which are part of the thylakoid membrane. Additionally, by using the one-dimensional peptide mapping method in conjunction with specific extraction and topographical analysis procedures, we were able to get a qualitative estimate of the capability of isolated chloroplasts to integrate these proteins into the thylakoid membrane.

## MATERIALS AND METHODS

### *Plant Material*

Seeds of *Zea mays* (FR9CMS × FR37, Illinois Foundation Seeds) were soaked for 20 h, then grown in vermiculite in a greenhouse. When etiolated plants were required, trays were kept in a darkroom instead of the greenhouse.

### *Radioactive Labeling of Proteins*

For *in vitro* protein synthesis, chloroplast isolation and illumination were performed essentially by the methods of Blair and Ellis (1). 15 g of 7- to 9-day old, greenhouse-grown, seedling leaves were homogenized in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) for 5 s in 100 ml of 0.35 M sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate-NaOH (pH 7.6), 2 mM Na ascorbate, 2 mM EDTA. The homogenate was passed through two layers of Miracloth (Chicopee Mills Inc., Milltown, N. J.), and the filtrate was centrifuged for 1 min at 2,000 *g*. The resulting pellet was resuspended with 0.2 M KCl, 66 mM tricine-KOH (pH 8.2), 6.6 mM MgCl<sub>2</sub>, so that the chlorophyll concentration was 700–800  $\mu$ g/ml. 50–100  $\mu$ Ci of <sup>35</sup>S-methionine (400 to 900 Ci/mmol, Amersham/Searle Corp., Arlington Heights, Ill.) were added to 0.5 ml of this crude chloroplast suspension, which was then illuminated with white light at 20°C for 1 h. After illumination, chloroplasts were diluted with 10 ml of 5 mM Tris-HCl (pH 8.0) and repelleted. This wash step was repeated. The pellet consisted primarily of thylakoid membranes. These were either stored at –20°C or else immediately solubilized for SDS-polyacrylamide gel electrophoresis (9).

For *in vivo* labeling in leaves, Tween (Atlas Chemical Industries, Inc., Wilmington, Del.), at a final concentration of 1%, was added to a solution of <sup>35</sup>S-methionine, and 5–10  $\mu$ l (25  $\mu$ Ci) were spread on a leaf of an intact seedling. After 4 h, the leaf was cut from the plant and ground in a mortar with a pestle with 2 ml of the homogenization buffer described above. The homogenate was filtered through Miracloth (Chicopee Mills Inc.). The filtrate was centrifuged at 2,000 *g* for 5 min. The pellet was washed twice with 5 mM Tris-HCl (pH 8.0).

### *CF<sub>1</sub> Solubilization and Purification*

CF<sub>1</sub> was removed from freshly prepared thylakoid membranes by three methods. In the first, extraction was made with 0.75 mM EDTA (11). In the second, membranes which had first been washed four times with 10 mM Na-pyrophosphate (pH 7.4) (16) were extracted with 0.2 M sucrose, 2 mM tris-(hydroxymethyl)-methylglycine (Tricine)-NaOH (pH 7.8). In the third method, extraction was done with 0.8% silicotungstic acid (STA) after the membranes had been swollen by washing with 20 mM Tricine-NaOH (pH 7.8) (12).

CF<sub>1</sub> was purified from EDTA extracts of chloroplasts

by diethylaminoethyl-Sephadex chromatography and sucrose density gradient centrifugation (11).

### *Proteolytic Digestion during SDS-Polyacrylamide Gel Electrophoresis*

Labeled membrane proteins or subunits of purified CF<sub>1</sub> were first separated on 10 or 12% polyacrylamide gels with a 5% stacker, using Laemmli's discontinuous buffer system (9). Polypeptides of interest were located, excised, and treated as described by Cleveland et al. (4). Gel slices were stored at -20°C until immediately before use for a proteolytic gel. The latter gels consisted of a 15-20% acrylamide gradient running gel (120 × 140 × 1.5 mm) and a 5% stacking gel (60 × 140 × 1.5 mm). In all gels, the ratio of acrylamide to bis-acrylamide was 37.5:1. The wells in the stacking gel were 24 mm deep. For CF<sub>1</sub> subunits, one or two slices containing radioactive polypeptides, excised from the first gel, were placed at the bottom of each well. Sometimes, slices of gel containing the same polypeptide, unlabeled, were added to increase the amount of protein in the slot. For most of the experiments on the 34,500-dalton protein, the stained region containing proteins of 32,000 and 34,000 daltons was cut out of an SDS-polyacrylamide gel of labeled thylakoid proteins, but the stained band containing only a 32,000-dalton polypeptide was excised from similar gels of unlabeled thylakoid proteins. Slices of radioactive and nonradioactive material were combined for digestion. In this way, the bulk of the stainable fragments was derived from the 32,000-dalton protein; these could be compared with radioactive digestion products.

2 μl of *S. aureus* protease was added to each well in the manner described by Cleveland et al. (4). Electrophoresis was carried out at 25 mA for 6-8 h. Gels were stained for 1 h with 0.2% Coomassie Brilliant Blue, 50% methanol, 7% acetic acid, and destained overnight in 20% methanol, 7% acetic acid. They were prepared for fluorography as described previously (2).

### *Tryptic Peptide Analysis*

<sup>35</sup>S-methionine-labeled polypeptides were located in SDS-polyacrylamide gels by autoradiography of the wet gel. The exposure time was 16 h. The gel slices were excised, chopped into small pieces, swollen with SDS gel electrophoresis buffer and eluted into dialysis bags by electrophoresis overnight at 100 V. The eluted proteins were dialyzed extensively against distilled water. 130 μg of unlabeled maize leaf proteins was added as carrier per milliliter of sample, and the proteins were precipitated by making the sample 10% (wt/vol) in TCA. The precipitate was washed once with 90% (wt/vol) ethanol, 0.1 M HCl, once with absolute ethanol, and dried under vacuum. The precipitates were resuspended in 0.2 ml of 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, containing 5 μg of trypsin, and digested for 4 h at 37°C. Then, 5 μg of additional trypsin was added and digestion was continued overnight, during

which time the precipitate dissolved. The sample was lyophilized, redissolved in water, and re-lyophilized. Finally, the digests were dissolved in 0.05 M pyridine-acetate (pH 3.5) and spotted on cellulose-coated, thin-layer plates, 1/4 μl at a time, with intervening drying. The plates were subjected to electrophoresis in 0.05 M pyridine-acetate buffer (pH 3.5) for 45 min, at 950 volts, followed by ascending chromatography in sec-butanol:*n*-propanol:isoamyl alcohol:pyridine:water (1:1:1:3:3). The plates were subsequently chromatographed in 7% 2,5-diphenyloxazole (wt/vol) in ether, and fluorographed.

### *Proteolytic Digestion of Chloroplast Membranes*

Washed thylakoids were resuspended with 50 mM Tris-HCl (pH 8.0) so that the chlorophyll concentration was 100 μg/ml. The incubations were carried out at room temperature. After 1 h with 100 μg/ml trypsin, or 20 min with 100 μg/ml pronase, the chloroplasts were washed twice with 5 mM Tris-HCl (pH 8.0), 100 μg/ml phenyl-methyl sulfonyl fluoride.

## RESULTS AND DISCUSSION

### *α and β Subunits of CF<sub>1</sub>*

PROTEOLYTIC ANALYSES OF SUBUNITS SEPARATED ELECTROPHORETICALLY FROM SOLUBILIZED CF<sub>1</sub> AND FROM THYLAKOID MEMBRANES: To verify the identification by Mendiola-Morgenthaler et al. (13) of the α and β subunits of CF<sub>1</sub> as products of in vitro protein synthesis, CF<sub>1</sub> was specifically extracted from labeled chloroplasts by the method of Strotmann et al. (16), then mixed with unlabeled, purified CF<sub>1</sub>. The subunits were separated electrophoretically on an SDS-polyacrylamide gel, and the bands containing α and β subunits were excised and digested with *S. aureus* protease on a second gel. A band of radioactive material is seen corresponding in position to each band of stainable material for digests of both α and β subunits of CF<sub>1</sub> (Fig. 1). It should be noted that, although the α and β subunits are close in molecular weight (62,000 and 58,000 daltons), the patterns are distinctive showing that samples are not cross-contaminated. The identification of these radioactive products as subunits of CF<sub>1</sub> is therefore unambiguous.

We wanted to see if the same technique could be used to identify radioactive proteins which had not been separated from the thylakoids by extraction. The bands co-migrating with the α and β subunits during electrophoresis were excised from SDS-polyacrylamide gels of labeled, solubilized

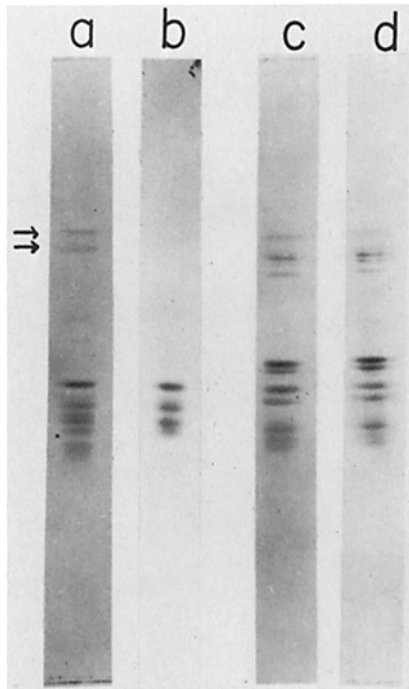


FIGURE 1 Digestion pattern of  $\alpha$  and  $\beta$  subunits from solubilized  $CF_1$ . The protein in a sucrose-tricine extract of labeled thylakoids was precipitated with 5% trichloroacetic acid, washed once with ether, then dissolved together with 30  $\mu$ g of purified  $CF_1$ . The subunits were separated on a 10% acrylamide gel containing SDS. The  $\alpha$  and  $\beta$  subunits were excised and digested with *S. aureus* protease as described in Materials and Methods. (a) Photograph of stained fragments of  $\alpha$ . Arrows indicate bands contributed by *S. aureus* protease. (b) Fluorograph of Fig. 1a. (c) Photograph of stained fragments of  $\beta$ . (d) Fluorograph of Fig. 1c.

thylakoid proteins, and similarly digested (Fig. 2). The pattern of fragments from each polypeptide visualized by staining is identical to that of those in digests of proteins in bands excised from polyacrylamide gels on which these two subunits of purified  $CF_1$  had been separated electrophoretically. This result further substantiates the identification of these stained bands among the thylakoid proteins as subunits of  $CF_1$ . In this case, there are radioactive fragments which do not correspond to  $CF_1$  subunit fragments. However, for both the  $\alpha$  and  $\beta$  subunit preparations, it is clear that a substantial proportion of the radioactive material is identical to the  $CF_1$  subunit.

ANALYSIS OF  $CF_1$  SUBUNITS AFTER STA EXTRACTION OF LABELED THYLAKOIDS: Although precise, quantitative data were not avail-

able, it seemed that more radioactive material with the properties of subunits of  $CF_1$  could be recovered from gels of disassociated membranes than from gels of subunits of extracted  $CF_1$ . The low ionic strength extraction does not remove all of the  $CF_1$  from the thylakoids (16), and this may account for the lower recovery of label in the extract. However, it is also possible that a significant proportion of the newly synthesized polypeptide, though associated with the membrane, is not incorporated into functioning enzyme molecules, and may not be extracted under the same conditions as the enzyme.

To test the hypothesis that the newly synthesized proteins are not entirely properly integrated into the membrane, another extraction technique was used. The only procedure known to remove almost completely  $CF_1$  is treatment of thylakoids with STA (12). This extraction is not so specific as low ionic strength (16), but it is more complete. Gels of membranes extracted with STA before solubilization with SDS show that very little re-

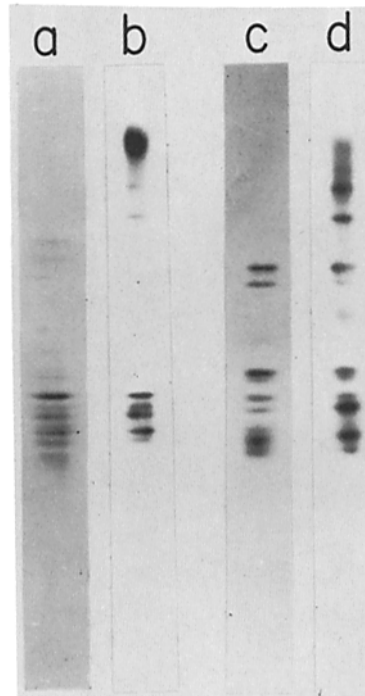


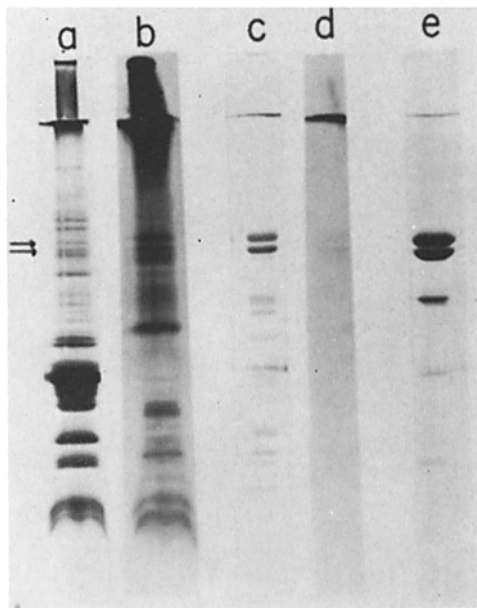
FIGURE 2 Digestion pattern of  $\alpha$  and  $\beta$  subunits from gels of thylakoid proteins. Proteins were separated on a 12% acrylamide gel containing SDS. The  $\alpha$  and  $\beta$  subunits were excised and digested with *S. aureus* protease as described in Materials and Methods. (a-d) Exactly as in Fig. 1.

mains of the stained bands corresponding to the  $\alpha$  and  $\beta$  subunits of  $CF_1$  (Fig. 3a). Most of the  $CF_1$  is found in the extract (Fig. 3c). In contrast, the fluorographs of these gels show that most of the radioactivity in polypeptides of that size remains with the membrane, with relatively less in the extract (Figure 3b and d). Although exact calculations cannot be made, it is evident from the greater amounts of  $CF_1$   $\alpha$  and  $\beta$  proteins in the extract and the greater amounts of radioactivity in the extracted membranes that the specific radioactivity of the  $\alpha$  and  $\beta$  subunit proteins is higher in the residual extracted membrane than in the extract.

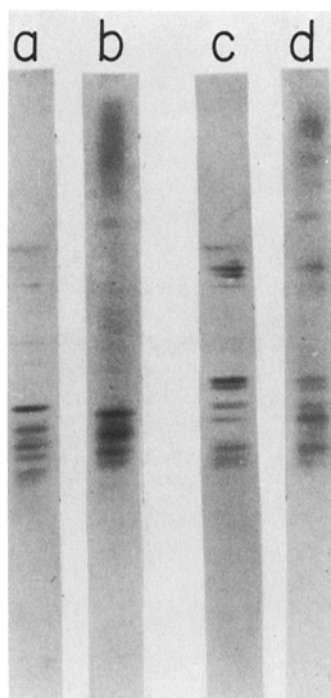
Confirmation that the radioactive proteins of 62,000 and 58,000 daltons remaining with the membranes after STA extraction are  $CF_1$  subunits comes from examination of the *S. aureus* protease digestion products. Extracted membranes were

mixed with unlabeled purified  $CF_1$ , dissolved in SDS solution, and analyzed on a 10% acrylamide gel containing SDS. The  $\alpha$  and  $\beta$  subunits were excised and digested on a second gel. The radioactive products again correspond to the digestion products of these  $CF_1$  subunits (Fig. 4).

**EVALUATION OF THE TECHNIQUE FOR IDENTIFICATION OF MEMBRANE PROTEINS:** The results presented above show that a specific polypeptide separated electrophoretically in an SDS-polyacrylamide gel of total membrane proteins can be unequivocally identified with a specific purified component, using the method of Cleveland et al. (4) for limited proteolysis. This technique provides much stronger evidence than simple coincidence in molecular weight of the intact proteins. Because the proteolytic enzymes cleave only certain peptide bonds, the pattern of products formed is dependent on the sequence of amino acids in the protein. With a polypeptide the



**FIGURE 3** Silicotungstic acid-extracted thylakoids:  $^{35}S$ -methionine in vitro-labeled thylakoids were treated as described in Materials and Methods. The protein in the silicotungstic acid extract was precipitated with trichloroacetic acid. The extracted proteins and the remaining thylakoid proteins were separated on a 10-15% acrylamide gradient gel containing SDS. (a) Extracted membranes. Arrows indicate positions of  $\alpha$  and  $\beta$  subunits of  $CF_1$ . (b) Fluorograph of Fig. 3a. (c) Proteins in extract. (d) Fluorograph of Fig. 3c. (e) Purified  $CF_1$ . Fig. 3a and c contain proteins from equivalent amounts of starting material.



**FIGURE 4** Digestion pattern of  $\alpha$  or  $\beta$  subunits from gels of  $^{35}S$  methionine-labeled membranes, extracted with silicotungstic acid and then mixed with unlabeled purified  $CF_1$ . Membrane proteins together with  $CF_1$  subunits were separated on a 10% acrylamide gel containing SDS. The  $\alpha$  and  $\beta$  subunits were excised and digested with *S. aureus* protease as described in Materials and Methods. (a-d) Exactly as in Fig. 1.

size of the  $\alpha$  or  $\beta$  subunit of  $CF_1$ , the pattern is sufficiently complex to allow unambiguous identification. Through comparison of the patterns of the fragments of the  $\alpha$  and  $\beta$  subunits of  $CF_1$  visualized by staining with the patterns of radioactive fragments visualized by fluorography, these subunits of  $CF_1$  were clearly identified as products of protein synthesis by isolated chloroplasts. With smaller polypeptides, this approach may not be appropriate because of the smaller number of fragments which would result from proteolytic digestion, and the consequent greater possibility of ambiguity in the interpretation of results.

A particular advantage of the method of comparing proteolytic products is that an incompletely processed polypeptide may be identified. The data on the effects of extraction with STA indicate that much of the material synthesized in isolated chloroplasts is not extractable in the same way that the bulk of functional  $CF_1$  is. For this reason, previous estimates of the proportion of products of protein synthesis by isolated plastids which are  $CF_1$  subunits, based on the number of specifically extractable counts (6, 13), are probably too low.

#### The 34,500-Dalton Protein

**RADIOACTIVE LABELING IN VITRO AND IN VIVO:** Isolated maize chloroplasts incubated with  $^{35}S$ -methionine produce relatively large amounts of a labeled, 34,500-dalton, membrane-associated polypeptide which does not co-migrate with any of the thylakoid proteins seen on a stained, SDS polyacrylamide gel after electrophoresis (peak D in reference 6). A band of material appearing at about the 33,000-dalton position on SDS-polyacrylamide gel electrophoretographs is a major synthetic product of intact green maize seedlings supplied  $^{35}S$ -methionine, but is not produced by etiolated maize seedlings (Fig. 5). Re-electrophoresis (see below) showed this latter band of radioactivity to be a poorly resolved doublet of proteins with mol wt of 34,500 and 32,000.

We thought that the in vitro 34,500-dalton product might be an incompletely processed form of a 32,000-dalton protein seen by staining of SDS-polyacrylamide gels of electrophoretically separated thylakoid protein. The 32,000-dalton protein appears during greening of etiolated seedlings.<sup>1</sup> To test this hypothesis, we used two mapping procedures. The two-dimensional maps of radioactive fragments generated by trypsin diges-

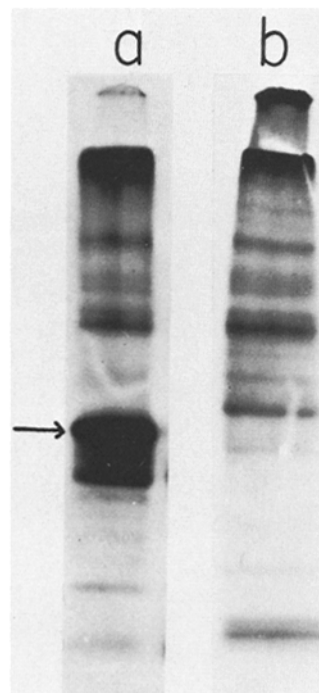


FIGURE 5 Proteins labeled in vivo in green and etiolated leaves. Thylakoid and etioplast membranes from labeled leaves were electrophoresed on 10–15% acrylamide gradient gels containing SDS. (a) Fluorograph of material labeled in green leaves. Arrow indicates the 33,000-dalton protein. (b) Fluorograph of material labeled in etiolated leaves.

tion of proteins labeled in vivo and in vitro were compared. We also mapped by one-dimensional polyacrylamide gel electrophoresis the stainable and radioactive fragments of the 32,000- and 34,500-dalton proteins generated by digestion with *S. aureus* protease. Through these comparisons, we have obtained evidence that the 34,500-dalton polypeptide produced in vitro by isolated maize chloroplasts is probably a precursor of a 32,000-dalton, stainable polypeptide produced during greening of etiolated maize.

**PROTEOLYTIC ANALYSES OF PREEXISTING AND NEWLY SYNTHESIZED PROTEINS:** *S. aureus* protease digestion products of the radioactive 32,000- and 34,500-dalton polypeptides and of the stainable 32,000-dalton polypeptides were compared by SDS-polyacrylamide gel electrophoresis by the method of Cleveland et al. (4). A combination of labeled and unlabeled proteins was prepared and treated as described in Materials and Methods. For simple re-electrophoresis to test

the purity of the excised proteins, the gel slices were treated as described for digestion with *S. aureus* protease, except that the proteolytic enzyme was omitted from the wells.

Upon re-electrophoresis of nonradioactive 32,000-dalton protein combined with material excised from an SDS-polyacrylamide gel of thylakoid proteins labeled in vitro with  $^{35}\text{S}$ -methionine, essentially a single, highly labeled band, 34,500 in mol wt, was detected (fig. 6*b*). In some experiments, a faint band of radioactivity co-migrating with the 32,000-dalton protein was observed. Treatment of protein in comparable excised gel pieces with *S. aureus* protease during electrophoresis resulted in the appearance of three radioactive products of 18,000, 11,500, and 10,800 daltons (Fig. 6*d*). The relative proportions of these varied from experiment to experiment, although

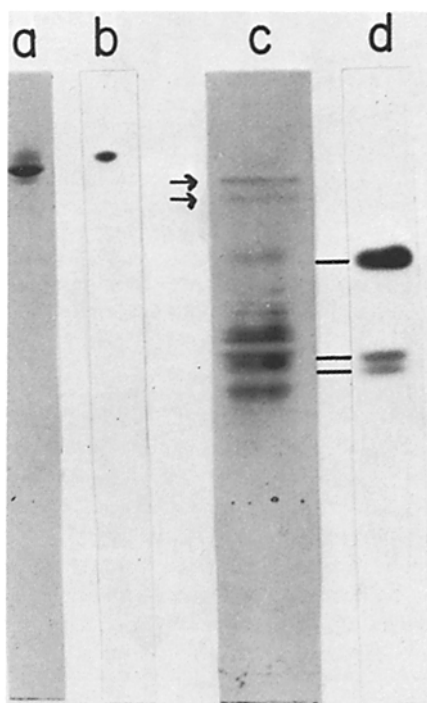


FIGURE 6 Digestion pattern of 32,000-dalton protein after in vitro protein synthesis. Material was prepared from labeled thylakoids and digested with *S. aureus* protease as described in Materials and Methods. (a) Photograph of stained gel of 32,000-dalton protein run onto second gel without protease. (b) Fluorograph of Fig. 6*a*. (c) Photograph of stained gel of 32,000-dalton protein digested with *S. aureus* protease. Arrows indicate bands contributed by the protease. (d) Fluorograph of Fig. 6*c*.

all were present each time. The two smaller fragments were not always clearly resolved on those gels in which there was less of the largest fragment and more of at least one of the two smaller ones. Since the sum of the apparent weights of the three fragments is greater than the weight of the protein from which they are derived, the 18,000-dalton fragment is thought to be a partial digestion product which includes one of the smaller pieces. The largest digestion product co-migrates with a faint, diffuse band which can be detected by staining (Fig. 6*c*). The second largest digestion product co-migrates with a sharper, more heavily stainable band. The smallest does not correspond to any stainable band. Occasionally, a very faintly radioactive band is seen which co-migrates with a 9,900-dalton, stainable fragment.

SDS-polyacrylamide gels of thylakoid polypeptides from whole maize seedlings supplied  $^{35}\text{S}$ -methionine display a radioactive band at about the "33,000-dalton" position. Re-electrophoresis of material in this excised gel segment shows the presence of two radioactive bands, 32,000 and 34,500 in mol wt (Fig. 7*b*). When this doublet is digested with *S. aureus* protease, the pattern of radioactive fragments formed is similar to that produced by the digestion of material labeled in isolated chloroplasts (compare Figs. 7*d* and 6*d*). Generally, the digest of the material labeled in vivo contains less radioactivity in the largest fragment, and the doublet of 11,500- and 10,800-dalton fragments is usually blurred. The smallest radioactive fragment, 9,900 daltons, is produced more consistently and in greater amounts than in the digests of material labeled in vitro. The principal radioactive fragments of the in vivo labeled proteins correspond in migration to certain of the stainable fragments of the 32,00-dalton protein (Fig. 7*c* and *d*).

Although the principal stainable proteins which were digested with *S. aureus* protease in experiments shown in Figs. 6 and 7 were of 32,000 daltons, some stainable 34,000-dalton material was present as well (Figs. 6*a* and 7*a*). To eliminate the possibility that the stainable fragments which co-migrate with radioactive ones are derived from the larger protein, gel slices containing principally the 34,000-dalton protein were treated with *S. aureus* protease during electrophoresis. The major stainable polypeptide fragments formed do not correspond in size to any radioactive fragments of material labeled in vitro (Fig. 8).

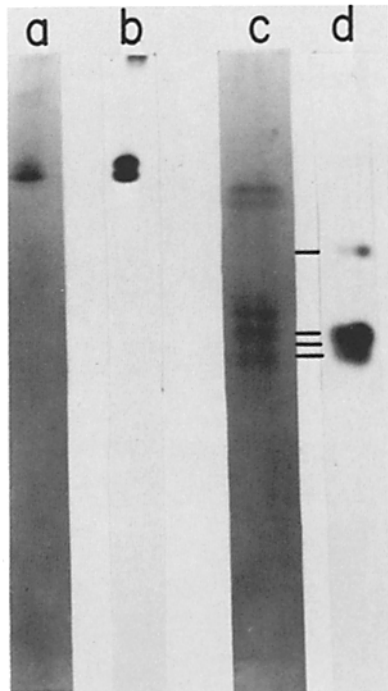


FIGURE 7 Digestion pattern of 32,000-dalton protein after in vivo labeling of proteins. Treatment of labeled thylakoids was exactly the same as in Fig. 6. (a-d) Exactly the same as in Fig. 6.

The information in Figs. 6, 7, and 8 is compiled and summarized in Fig. 9. From these data, it appears that the 32,000-dalton protein in thylakoid membranes is derived from a 34,500-dalton protein which is produced by isolated plastids and is present in small amounts in vivo. There is too little of the 34,000-dalton polypeptide to detect it in vivo but it is detectable by its radioactivity after  $^{35}\text{S}$ -methionine is administered to leaves. The 32,000- and 34,500-dalton  $^{35}\text{S}$ -methionine-labeled polypeptides are not completely separated from one another, when a dissolved thylakoid preparation is subjected to gel electrophoresis; both are present in a 33,000-dalton band. Independent of these polypeptides is an unrelated 34,000-dalton membrane protein which is not made by isolated maize plastids nor is it made in large amounts to become strongly radioactive when  $^{35}\text{S}$ -methionine is administered to intact leaves.

To clarify further the possible relationships between the different-sized proteins, methionine-containing tryptic peptides of the 33,000-dalton in vivo labeled protein were compared with those of

the 34,500-dalton in vitro labeled protein. Autoradiographs of the two-dimensional maps show that the radioactive material recovered from labeled, intact leaves is comprised of tryptic peptides identical to those present in the 34,500-dalton protein synthesized by isolated chloroplasts in vitro (Fig. 10). Much of the radioactivity in both is in three major spots with the same electrophoretic mobility toward the cathode. Both maps also contain two conspicuous, acidic peptides migrating toward the anode. The very highly acidic character of the latter suggests that they are modified.

The identity of the two halves of Fig. 10 is curious, since the 33,000-dalton in vivo labeled protein is a doublet, while there is only one major radioactive protein in the in vitro labeled material. The coincidence of the maps would be explained if the amino acid sequence of the 32,000-dalton, radioactive protein is contained within the 34,500-

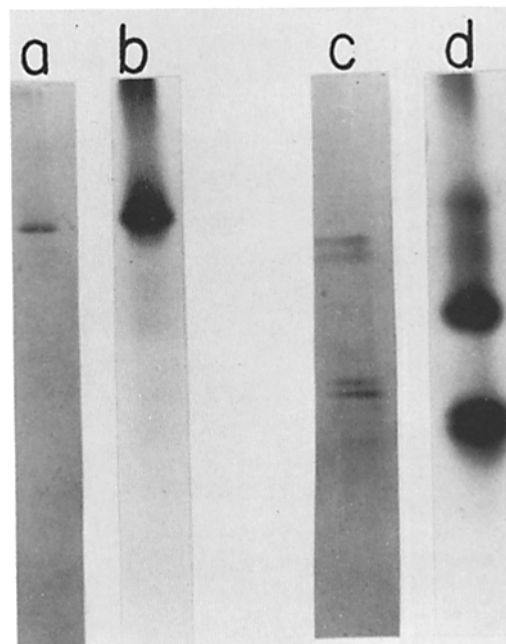
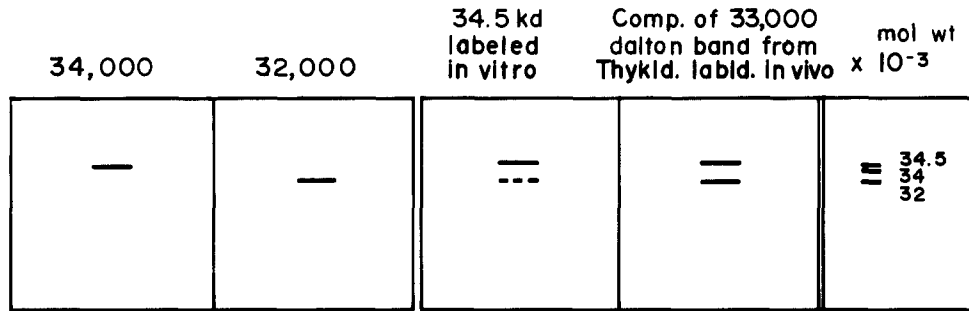


FIGURE 8 Digestion pattern of 34,000-dalton protein synthesized by isolated maize chloroplasts in vitro. The 34,000-dalton protein band was excised from SDS-polyacrylamide gels of labeled thylakoids and digested with *S. aureus* protease as described in Materials and Methods. (a) Photograph of stained gel of 34,000-dalton protein run onto second gel without protease. (b) Fluorograph of Fig. 8a. (c) Photograph of stained gel of 34,000-dalton protein digested with *S. aureus* protease. (d) Fluorograph of Fig. 8c.



Isolated from maize leaves.  
Stained after gel electro-  
phoresis in SDS.

Synthesized in presence of  $^{35}\text{S}$  - methionine in  
vivo or by isolated chloroplasts in vitro.  
Fluorographed after gel electrophoresis in SDS



SDS Gel electrophoresis after treatment with *S. aureus* protease of ex-  
cised gel slices.

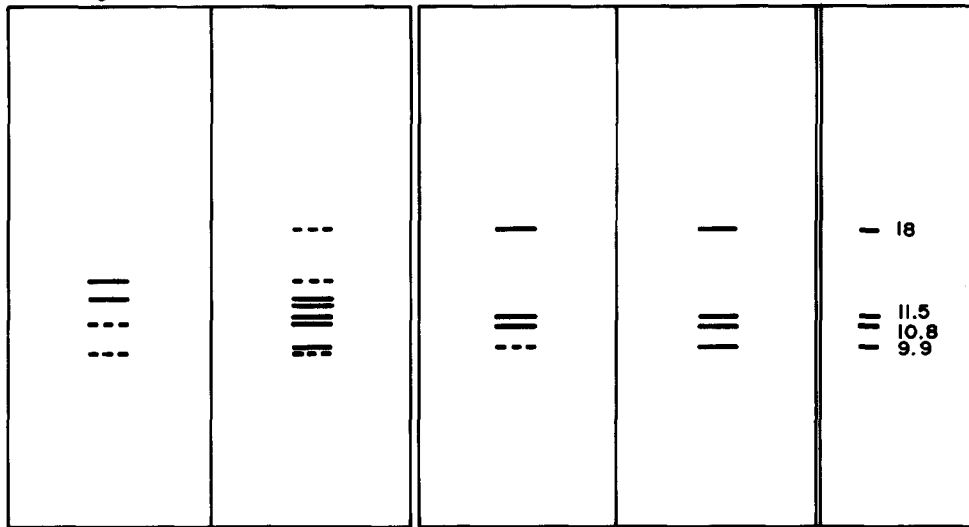


FIGURE 9 Summary of relations among 32,000-, 34,000-, and 34,500-dalton thylakoid proteins. Diagram of bands seen on stained gels and fluorographs after reelectrophoresis of slices excised from SDS-polyacrylamide gels of thylakoid proteins.

dalton one, and if there are no methionine residues in those segments present in the 34,500-dalton protein but absent from the 32,000-dalton one. It is also possible that a tryptic oligopeptide derived from the larger protein might co-migrate with, or be unresolvable from, a fragment present in both proteins. The oligopeptides produced by tryptic digestion are small; thus, differences in molecular weights of the original proteins would not necessarily be reflected in the map of the methionine-containing fragments.

Differences would be discerned more easily in the larger digestion products from treatment with

*S. aureus* protease in the presence of SDS. Most of the radioactive fragments in the digest of the 32,000- to 34,500-dalton doublet, labeled in vivo, correspond in size to stained fragments derived from the 32,000-dalton protein alone, but the digest of material labeled in vivo or in vitro includes a 10,800-dalton radioactive fragment which does not co-migrate with any stained polypeptide. This 10,800-dalton fragment may include the sequence of the 9,900-dalton fragment, the former being derived from the 34,500-dalton protein and the latter from the 32,000-dalton protein. The 9,900-dalton fragment is more heavily la-

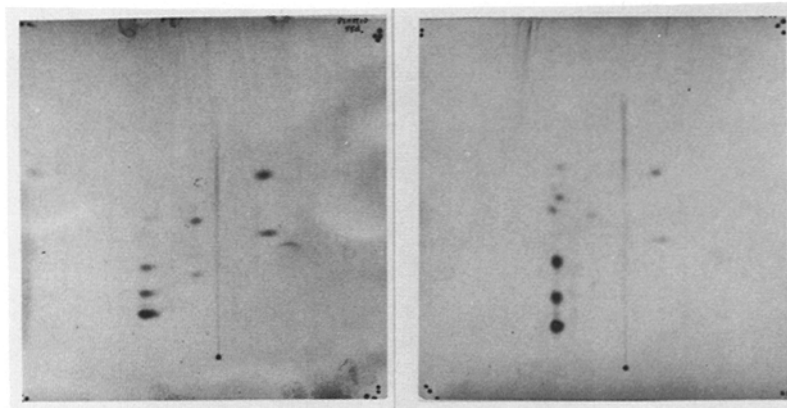


FIGURE 10 Two-dimensional tryptic fingerprints of: (left) 33,000-dalton band polypeptides labeled with  $^{35}\text{S}$ -methionine in isolated maize chloroplasts and (right) 33,000-dalton polypeptide labeled with  $^{35}\text{S}$ -methionine in intact leaves. (left) Autoradiographed for 55 days with prefogged film (8). (right) Autoradiographed for 55 days with XR-5 film. Cathode is to the right, anode to the left.

beled in digests of material labeled in vivo, in which there is heavier labeling of the 32,000-dalton protein.

Alternatively, the differences in mobility of the 32,000- and 34,500-dalton, radioactive proteins during electrophoresis could be due to differences in conformation, even in the presence of SDS, rather than in molecular weight. (We have designated molecular weights solely on the basis of mobility during SDS-polyacrylamide gel electrophoresis.) After digestion with proteases, the varied property might be detected more readily by SDS-polyacrylamide gel electrophoresis than by cellulose thin-layer electrophoresis and chromatography.

From the characteristics of the partial and complete digestion products, it seems likely that the 34,500-dalton protein labeled in vitro is a precursor of a 32,000-dalton protein seen on stained SDS-polyacrylamide gels of thylakoid proteins. No relationship of the 34,500-dalton protein to the 34,000-dalton protein is indicated.

The absence of radioactivity from many stainable fragments in the *S. aureus* protease digest of protein in the 32,000-dalton gel band can be explained by the fact that there are at least two different proteins of this size which cannot be distinguished in this electrophoretic system. Evidence that there is more than one protein in the single-stained band is presented in the next section. The presence of two membrane proteins in a single-stained band points up the difficulty of relying solely on coincidence in molecular weights

for identification of proteins.

**THE LOCATION OF THE 32,000-DALTON PROTEIN IN MAIZE THYLAKOIDS:** Two experimental approaches have shown that a 32,000-dalton protein is exposed at the outer surface of the thylakoid membrane. When thylakoids are radioiodinated, using lactoperoxidase as the catalyst (7), under conditions which should allow labeling only of proteins which are exposed at the outer surface, a 32,000-dalton protein is heavily labeled (15). When thylakoids are treated with proteolytic enzymes, only those proteins at the outer surface should be susceptible to digestion. Under the conditions used, pronase removes a portion of the 32,000-dalton protein, while trypsin removes all of it (Fig. 11).

If the 32,000-dalton protein remaining after pronase treatment of thylakoids is digested with *S. aureus* protease, fewer fragments are found than in the digest of material from untreated thylakoids (Fig. 12). Thus, we conclude that there is more than one protein in the band at the 32,000-dalton position in preparations from untreated thylakoids. Among the fragments missing from the *S. aureus* protease digest of the protein in the 32,000-dalton band from pronase-treated thylakoids are those of the same size as radioactive digestion products of in vivo or in vitro material. Thus, the 32,000-dalton protein, which is derived from the 34,500-dalton protein, is susceptible to both pronase and trypsin digestion of thylakoids. (Interestingly, the *S. aureus* protease digest of 32,000-dalton protein from pronase-treated thy-

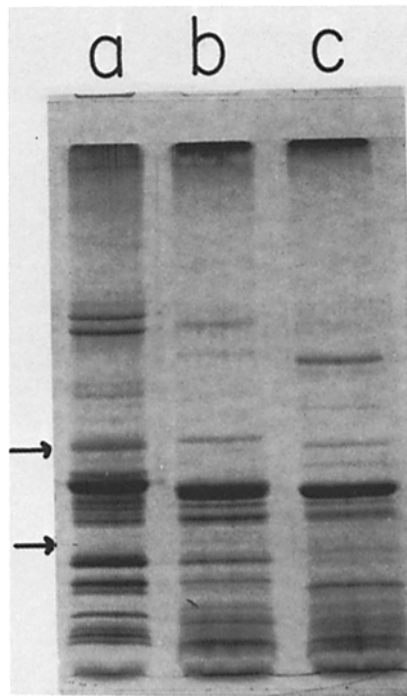


FIGURE 11 Trypsin and pronase digestion of thylakoid membranes. Thylakoid membranes were treated as described in Materials and Methods. The polypeptides remaining after digestion were separated on a 10-15% acrylamide gradient gel containing SDS. Arrows indicate positions of 32,000- and 20,500-dalton proteins. (a) Untreated thylakoids. (b) Thylakoids digested with trypsin. (c) Thylakoids digested with pronase.

lakoids is remarkably similar to the digest of the unlabeled 34,000-dalton protein, shown in Fig. 8.)

All of the 33,000-dalton radioactive material disappears after thylakoids isolated from  $^{35}\text{S}$ -methionine-labeled leaves are treated with either pronase or trypsin. The most heavily labeled products of trypsin digestion have mol wt of 20,500 and 19,000 (Fig. 13b). The most heavily labeled product of pronase digestion has a mol wt of 19,000 (Fig. 13c). Considering the relative intensities of labeling (compare Fig. 13a, b, and c), it seems likely that all of these radioactive products are derived from the 33,000-dalton material. The presence of a major protein of 19,000 daltons in the thylakoid makes it impossible to detect additional proteolytic fragments of this size by staining gels. However, a small amount of a 20,500-dalton polypeptide is generated upon trypsin treatment of the thylakoids. Since the band is

very faint, it does not show up well in a photograph of the stained gel (Fig. 11).

The results with digestion of in vivo labeled thylakoids indicate that the 32,000-dalton protein which is derived from the 34,500-dalton protein is partially exposed at the surface of the thylakoid and partially protected by the membrane, so that a 19,000-dalton segment is not susceptible to proteolytic digestion. In contrast, when chloroplasts labeled in vitro with  $^{35}\text{S}$ -methionine are treated with either trypsin or pronase, all of the 34,500-dalton radioactive protein disappears but all radioactive fragments migrate at the ion front during SDS-polyacrylamide gel electrophoresis (Fig. 14). It appears that isolated chloroplasts are unable to process the 34,500-dalton protein and

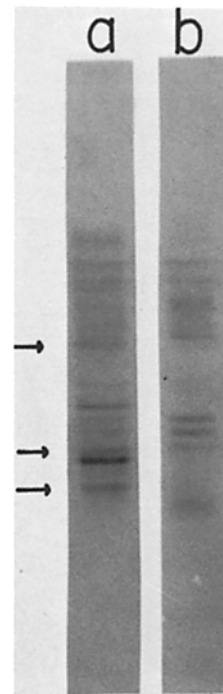


FIGURE 12 Digestion pattern of 32,000-dalton protein from thylakoids which had been treated with pronase. Thylakoids were treated with pronase as described in Materials and Methods. The 32,000-dalton protein was isolated and digested with *S. aureus* protease as described in Methods. (a) Digestion pattern of 32,000-dalton material from untreated chloroplasts. The arrows indicate bands which co-migrate with radioactive fragments derived from materials labeled in vivo. (b) Digestion pattern of 32,000-dalton material from pronase-treated membranes.

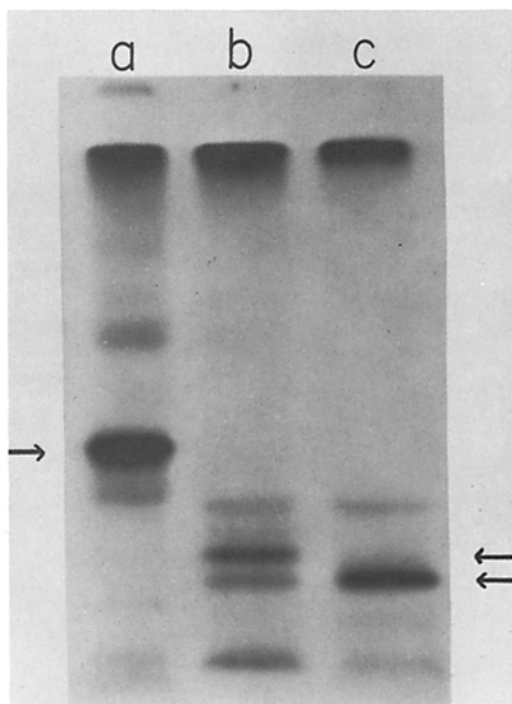


FIGURE 13 Trypsin and pronase digestion of thylakoid membranes after *in vivo* labeling of proteins (14-day-old seedling). Conditions for digestion and gel electrophoresis were exactly the same as for Fig. 11. Left-hand arrow indicates position of 33,000-dalton protein. Right hand arrows indicate position of 20,500- and 19,000-dalton proteins. (a) Fluorograph of untreated membranes. (b) Fluorograph of trypsin-digested thylakoids. (c) Fluorograph of pronase-digested thylakoids.

to integrate the product into the membrane in the same way as occurs *in vivo*.

#### CONCLUSIONS

The one-dimensional proteolytic mapping technique of Cleveland et al. (4) is particularly suitable for the identification of newly synthesized membrane proteins. The technique requires only electrophoretic separation of components of the membrane before mapping, and permits direct comparison of fragments of radioactively labeled proteins with purified proteins available in stainable quantities.

We have used this procedure to identify three polypeptides synthesized by isolated maize chloroplasts. The 62,000- and 58,000-dalton proteins are identical to the  $\alpha$  and  $\beta$  subunits of  $CF_1$ . The 34,500-dalton protein seems to be a precursor of a 32,000-dalton protein made in only

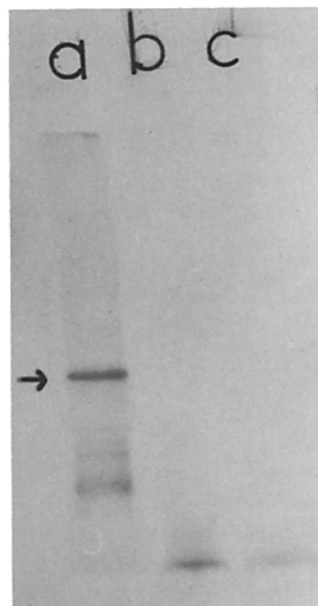


FIGURE 14 Trypsin and pronase digestion of thylakoid membranes after *in vitro* labeling of proteins (chloroplasts from 14-day-old seedlings). Conditions for digestion and gel electrophoresis exactly as for Fig. 11. Arrow indicates 34,500-dalton protein. (a-c) Exactly the same as in Fig. 13.

minor amounts by isolated chloroplasts. The 32,000-dalton protein can be detected in stained SDS-polyacrylamide gels of electrophoretically separated thylakoid proteins.

The bulk of the newly synthesized  $\alpha$  and  $\beta$  subunits of  $CF_1$  and the 34,500-dalton protein do not appear to have the same position in the membrane as proteins which are synthesized *in vivo*, so that the nature of their association with the membrane is unclear. The chloroplast may be dependent on a continuing supply of some unidentified material from elsewhere in the cell to fully process and/or integrate the proteins it makes; or isolated chloroplasts may lose essential factors into the suspending medium. Whatever the cause, under the conditions used, isolated chloroplasts can synthesize proteins for a period of time, but, at least in these cases, cannot fully integrate them into the membrane to any great extent.

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