Characterization of Envelope Membrane Polypeptides from Spinach Chloroplasts*

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Chloroplast envelope polypeptides from spinach were separated into several fractions on the basis of their solubility in a mixture of chloroform/methanol (2:1, v/v) or in 0.1 N NaOH. Using a simple two dimension procedure for gel electrophoresis, we resolved up to 70 polypeptides. The envelope is characterized by the preponderance of high molecular weight polypeptides (Mr > 54,000), many of which are not extracted by NaOH, indicating that they are integral membrane proteins. The polypeptide pattern of envelope membranes is very different from that of the stroma or of the thylakoids: very few polypeptides from the three fractions have identical mobility in the different electrophoretic systems used. Most of the major envelope polypeptides are peripheral proteins. Two of these, E14 and E54, were identified by crossed immunoelectrophoresis as the small and the large subunit of ribulose bisphosphate carboxylase, respectively. However, polypeptide E30 (involved in phosphate transport across the envelope) was the only major polypeptide to be extracted by chloroform/methanol. This polypeptide is also an integral membrane polypeptide since it is not extracted by NaOH. Using double immunodiffusion of envelope, stroma, and thylakoids against antibodies to two integral polypeptides, we demonstrated that these four polypeptides are not found in the thylakoid membranes or in the stroma. Furthermore, we also have shown that there is no cross-reaction between the envelope membranes and antibodies prepared against the major thylakoid membrane polypeptides (α, β, γ, and δ subunits of coupling factor CF1 and polypeptides 5, 6, and 11). These results do not support a direct biogenetic relation between the two chloroplast membrane systems (envelope and thylakoids). If indeed thylakoid membranes are derived from the inner envelope membranes, considerable changes must occur in the composition of the vesicles following the initial steps of membrane invagination.

Higher plant chloroplasts are made up of three morphologically and functionally distinct compartments: envelope, stroma, and thylakoids. The envelope consists of two membranes which together provide a flexible boundary between the chloroplast and the surrounding cytosol (1). This double membrane system plays an important role in chloroplast biogenesis (1, 2). The plastid envelope is involved in the synthesis of chloroplast components such as galactolipids (3), carotenoids (4, 5), and prenylquinones (5, 7). Furthermore, the envelope membranes form a selective barrier for the passage of molecules into the organelle. For instance, the inner envelope membrane regulates the flow of metabolites between the cytosol and chloroplast (8), and the outer envelope membrane probably mediates the post-translational uptake of cytoplasmically synthesized polypeptides (2, 9). Furthermore, there is circumstantial evidence, based on electron microscopic studies, that the inner envelope membrane gives rise to the thylakoid membrane by invagination during chloroplast development (10).

A better understanding of the enzymatic and transport functions of the chloroplast envelope requires a detailed knowledge of its constituent polypeptides. Early studies of the polypeptide composition of the chloroplast envelope by sodium dodecyl sulfate gel electrophoresis resolved approximately 20 bands and a group of high molecular weight polypeptides (Mr > 70,000) (11-18), including two dominant components at 30,000 and 52,000 daltons. Since these studies, resolution of polypeptides by SDS-polyacrylamide gel electrophoresis has improved considerably (19, 20). Therefore, we have reexamined the polypeptide composition of the envelope using both one- and two-dimensional gel electrophoresis. Moreover, to probe the possible biogenetic relationship between the envelope and the thylakoids, we have investigated immunochemical cross-reactivity of their constituent polypeptides. The envelope fraction contains at least 70 polypeptide bands with 7 major components (E125, E54, E41, E37, E30, E24, and E14) which together account for more than 70% of the total protein mass. At least 40 envelope polypeptides are integral components which are not dislodged from the membranes by 0.1 N NaOH and half of these are hydrophobic by virtue of their solubility in a 2:1 (v/v) mixture of chloroform/methanol. Based on electrophoretic and immunochemical analyses, the major polypeptides of the envelope and the thylakoids are exclusively localized in only one of the two chloroplast membrane systems. However, several stromal enzymes, including ribulose bisphosphate carboxylase are detected.

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tected in the envelope fraction. Some of these stromal proteins could have become trapped in envelope vesicles during chloroplast lysis, and stringent washing of the envelope fraction prior to assay removes them. Rubi-P\textsubscript{2} carboxylase, on the other hand, is not removed by such treatments and thus probably is more tightly bound to the envelope fraction.

**MATERIALS AND METHODS**

**Isolation of Intact Chloroplasts and Preparation of Envelope Membranes**—Chloroplasts were isolated from 1-2 kg of spinach leaves obtained from Long Island farms. Deveined leaves were homogenized for 2 s in a 4:1 Waring Blendor and a crude chloroplast pellet was obtained from the leaf homogenate according to Douce and Joyard (21). To avoid contamination by “micromeres” and swollen thylakoid membranes, the chloroplast preparation was purified further by isopycnic centrifugation in silicone sol (Percoll, Pharmacia) gradients (21). Envelope, thylakoids, and stroma were prepared from purified, intact chloroplasts after swelling in a hypotonic medium followed by centrifugation through a step sucrose gradient (22). The swelling medium as well as the different sucrose layers contained the following protease inhibitors: phenylmethylsulfonylfluoride, 1 mM; \(-\)aminocaproic acid, 5 mM; and benzamidine-HCl, 1 mM.

The yield of envelope membranes was 2–3 mg of protein/kg of spinach leaves. The essential absence of NADH:cytochrome \(c\) oxidoreductase activity and phosphatidylethanolamine precludes any significant contamination of the envelope fraction by extrachloroplast membranes (7, 22).

**Fractionation of Stromal Proteins**—The stromal fraction (8-10 mg of protein) obtained as described above was layered on top of a 10–30% sucrose density gradient and centrifuged at 140,000 \(\times\) g, for 16 h (19). Two fractions were resolved: an 18 S peak containing the Rubi-P\textsubscript{2} carboxylase holoenzyme and a top fraction containing all the other stromal enzymes (9).

**Electrophoretic Analysis of Chloroplast Proteins**—Polypeptides of the chloroplast envelope, thylakoid membranes, and stroma (total, 18 S, and top fraction) were analyzed by SDS- or LDS-polyacrylamide gel electrophoresis. Samples were first solubilized at room temperature in 50 mM Na\( \text{CO}_3\), 50 mM diethiothreitol, 12% sucrose, 2% SDS or LDS. Electrophoresis was performed either at 4 \(^\circ\)C in LDS slab gels containing a 7.5–15% linear acrylamide gradient or at room temperature in SDS slab gels containing 8 M urea and a 12–18% linear acrylamide gradient (19, 20). Several types of samples were analyzed: unextracted, chloroform/methanol (2:1, v/v) extracts and residues, and 0.1 M NaOH extracts and residues (20). The experimental conditions for gel preparation, sample extraction and solubilization, electrophoresis, and gel staining have been detailed by Chua (19) and Piccioni et al. (20).

**Preparation of Monospecific Antibodies to Chloroplast Envelope Polypeptides**—Chloroplast envelope polypeptides were fractionated first into two groups by extraction with a 2:1 (v/v) mixture of chloroform/methanol. Polypeptides in the two fractions were then separated in SDS-urea gels containing 8 M urea and a 12–18% linear acrylamide gradient (19, 20). Bands corresponding to envelope polypeptides E23 and E24 in the C/M residues and E30 and E37 in the C/M extracts (cf. Fig. 1) were excised and pooled, and the polypeptide was eluted by electrodialysis (23). The eluted SDS-poly- peptide complex was checked for purity before injection into rabbits for raising antibodies (23).

**Preparation of Monospecific Antibodies to Stromal and Thylakoid Polypeptides**—Coupling factor CF\textsubscript{s} was prepared from spinach (24) and the subunits were purified by preparative SDS gel electrophoresis (19, 20). Ferredoxin-NAD\textsuperscript{+} oxidoreductase was purchased from Sigma and purified further by SDS gel electrophoresis (19, 20). Purified tobacco ribulose-\(5\)-phosphate kinase was a gift of Dr. Tak Kagawa, University of Missouri. Antibodies to all these proteins were raised in rabbits by injection of the SDS-protein complexes (23). Monospecific antibodies against spinach fructose-1,6-bisphosphatase were kindly provided by Dr. Bob B. Buchanan, University of California, Berkeley. Preparation of monospecific antibodies against the large and small subunit of Rubi-P\textsubscript{2} carboxylase (9) and against Chlamydona\(s\) thylakoid membrane polypeptides (25) have been published.

**Immunochromatographic Studies of Envelope Membranes**—IgG fractions were purified from antisera according to Harboe and Ingild (26). Double immunodiffusion assays according to Ouchterlony and Nilsson (27) were performed at 37 \(^\circ\)C overnight in agarose gels containing 1% agarose, 1% gelatin, 1% SDS, and 10% glycerol.
Spinach Chloroplast Envelope Membrane Polypeptides

In each gel corresponds to 100 μg of total envelope protein. Gel A, total polypeptides from envelope membranes; gel B, chloroform/methanol (2:1, v/v) insoluble fraction of envelope polypeptides; gel C, chloroform/methanol-soluble fraction of envelope polypeptides.

Electrophoretic Analysis of Envelope Membranes—Since efficient techniques of the separation of the two envelope membranes still are not available, the results presented here pertain to a mixture of outer and inner membranes (1). We first analyzed the envelope fraction by LDS-polyacrylamide gel electrophoresis at 4 °C. The total envelope fraction contains at least 60 stained gel bands, with 7 major components (E125, E54, E41, E37, E30, E24, and E14) ranging in Mr from 14,000 to 125,000 (Fig. 1, lane 1). The envelope polypeptides fall roughly into three groups on the basis of their mobility. There are approximately 25 polypeptides with Mr > 54,000, 25 polypeptides of between 54,000 and 24,000 daltons, and a group of polypeptides with Mr < 24,000.

Due to the high lipid content of the chloroplast envelope (1), the migration of the low Mr polypeptides often is distorted by lipid-SDS micelles (data not shown). To overcome this problem and also to increase polypeptide resolution, we fractionated envelope polypeptides into two groups on the basis of their solubility in C/M. Polar lipids were removed from the C/M-soluble fraction by extraction with diethyl ether. The C/M-soluble and -insoluble fractions were then analyzed separately by electrophoresis in LDS gradient gels. Only 10 envelope polypeptides including the major components E30 and E41 are extracted by C/M (Fig. 1, lane 3). Some polypeptide bands, e.g. E14, are only partly extracted by C/M, suggesting that such bands may contain more than one polypeptide (Fig. 1, lanes 2 and 3). Most of the high molecular weight (Mr > 54,000) polypeptides are insoluble in the organic solvent.

The 7.5-15% gradient gel system (Fig. 1) poorly resolves polypeptides with Mr < 14,000. Better resolution of these small polypeptides was achieved in a 12-18% gradient gel containing 8 M urea (19, 20) (Fig. 2).

The two stained bands in the region of E14 in the 7.5-15% gradient gel can be resolved into 5 polypeptides in the SDS-

urea gel (cf. Fig. 1, lane 1, and Fig. 2, lane 1). The doublet at E14 is found exclusively in the C/M-soluble fraction (Fig. 2, lane 2) whereas E11 and E10 are recovered in the C/M-insoluble fraction (Fig. 2, lane 3). E12 appears to be composed of at least two polypeptides, only one of which is extracted by the organic solvent (Fig. 2, lanes 2 and 3). The complexity of the envelope polypeptide pattern prompted us to analyze the C/M-soluble and -insoluble frac-

3 In most experiments, E10 is completely extracted by a 21 (v/v) mixture of chloroform/methanol. However, in the experiment shown in Figs. 1 and 2, the extraction of this polypeptide is incomplete.

RESULTS

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**Spinach Chloroplast Envelope Membrane Polypeptides**

**Fig. 5. Immunodiffusion of envelope (E), stroma (S), and thylakoid (T) polypeptides with various antibodies raised against spinach stroma polypeptides.** The antigen loads in the wells were 30 μl of envelope membranes in 2% Triton X-100, 50 mM Tris-HCl (pH 8.6) (10 mg of protein/ml); 20 μl of stroma in 2% Triton X-100, 50 mM Tris-HCl (pH 8.6) (10 mg of protein/ml); and 10 μl of thylakoid membranes in 2% Triton X-100, 50 mM Tris-HCl (pH 8.6) (1 mg of chlorophyll/ml). Antisera loads were: A, 20 μl of anti-large Rbu-P2 carboxylase subunit IgG (anti-L; 8.42 mg/ml) and 30 μl of anti-small Rbu-P2 carboxylase subunit IgG (anti-S; 25.4 mg/ml); B, C, and D, 30 μl of anti-fructose-1,6-bisphosphate phosphatase IgG (30 mg/ml) and 30 μl of anti-ribulose-5-phosphate kinase IgG (16 mg/ml).

Envelope membranes were used before (A, B, and C) and after (D) sonication in the presence of 0.4 M NaCl. Es, envelope membranes prepared as follows. In this case, envelope membranes equivalent to 2 mg of protein were suspended in 5 ml of 10 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine-NaOH (pH 7.8) and 0.4 M NaCl. After sonication with a Branson sonifier for 3 × 20 s (at 0 °C), the vesicles were centrifuged at 40,000 rpm (Beckman Ti-50 rotor) for 1 h. The pellet obtained was then suspended in 2% Triton X-100, 50 mM Tris-HCl (pH 8.6) to a final concentration of 10 mg of protein/ml and used for immunodiffusion.

**Fig. 6. Immunodiffusion of envelope (E), stroma (S), and thylakoid (T) polypeptides with various antibodies raised against spinach envelope polypeptides.** The antigen loads in the upper wells were the same as in Fig. 5. Antisera loads were: A, antiserum against E37 polypeptide, 50 μl of anti-E37 IgG (6 mg/ml); B, antisera against E30 polypeptide, 50 μl of anti-E30 IgG (6 mg/ml); C, antisera against E24 polypeptide, 50 μl of anti-E24 IgG (6.6 mg/ml); D, antisera against E10 polypeptide, 50 μl of anti-E10 IgG (10 mg/ml). The presence of Rbu-P2 carboxylase raised the possibility that the envelope fraction contains Rbu-P2 carboxylase. Similar conclusions have been reported by Pineau and Douce (11) and Pineau et al. (32).

The presence of Rbu-P2 carboxylase subunits in each gel system, strongly suggesting that the envelope fraction contains Rbu-P2 carboxylase. Similar conclusions have been reported by Pineau and Douce (11) and Pineau et al. (32).

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Fig. 7. Immunodiffusion of envelope (E), stroma (S), and thylakoid (T) polypeptides with various antisera raised against spinach thylakoid polypeptides. The antigen loads in the upper wells were the same as in Fig. 5. Antisera loads (in the lower wells) were A, antisera against coupling factor CFI polypeptides, 30 μl of anti-α IgG (56 mg/ml); 30 μl of anti-β IgG (32 mg/ml); 30 μl of anti-γ IgG (33 mg/ml); 30 μl of anti-δ IgG (43 mg/ml); B, antisera against polypeptides 5, 6, and 11 of thylakoids (see Ref. 4 for a nomenclature), 30 μl of anti-5 IgG (43.6 mg/ml); 30 μl of anti-6 IgG (40 mg/ml); and 30 μl of anti-11 IgG (45 mg/ml); C, antisera against ferredoxin-NADP⁺-oxidoreductase (a thylakoid protein released in the soluble phase during the course of chloroplast fractionation), 30 μl of anti-oxidoreductase IgG (48.7 mg/ml).

S fraction; cf. Ref. 9) reveals that a few minor envelope polypeptides have the same electrophoretic mobility as stromal polypeptides in addition to E54 and E14 (Fig. 4, lanes 3-5).

Immunochemical Analysis of Polypeptides of the Chloroplast Envelope and Stroma—To investigate further the possibility of cross-contamination of the envelope fraction by stromal proteins, we carried out double immunodiffusion assays using antisera raised against several stromal polypeptides. The envelope fraction gives a single strong precipitin line with antibodies to the large or small subunits of Rbu-P₂ carboxylase (Fig. 5A) and a weak precipitin reaction with antibodies to fructose-1-6-bisphosphatase (Fig. 5B) but not with those to ribulose-5-phosphate kinase (Fig. 5C). By crossed immunoelectrophoresis, we have identified envelope polypeptides E54 and E14 as the large and small subunit, respectively, of Rbu-P₂ carboxylase (data not shown).

To determine whether the subunits of Rbu-P₂ carboxylase in the envelope fraction exist as free pools or in the form of assembled holoenzyme, envelope membranes were solubilized in 2% Triton X-100 and the extract was sedimented in a 10-30% sucrose gradient (9). The E54 and E14 components in the detergent extract sedimented together as a discrete peak at 18 S. Electrophoretic and immunochemical analyses confirmed that this peak is the Rbu-P₂ carboxylase holoenzyme (data not shown).

A trivial explanation for the presence of some stromal proteins in the envelope fraction is that they are contaminants which were trapped in envelope membrane vesicles during chloroplast lysis. Indeed, sonication in the presence of high salt (0.4 M NaCl) completely removed fructose-1,6-bisphosphatase from the envelope preparation (Fig. 5D). In contrast, a portion of the Rbu-P₂ carboxylase is associated persistently with the envelope membrane vesicles even after repeated sonication in a high ionic strength buffer (data not shown), suggesting that a subpopulation of Rbu-P₂ carboxylase might interact specifically with the envelope membranes.

Immunoechemical Analysis of Envelope and Thylakoid Polypeptides—Previous electron microscopic studies have suggested that thylakoid membranes arise via invaginations of the inner envelope membrane during chloroplast development (10). One prediction of this hypothesis is that the envelope and the thylakoid may share common polypeptides, especially integral membrane polypeptides. Although results of our electrophoretic analysis indicate the absence of any major common polypeptides in the two chloroplast membrane systems a major polypeptide of one membrane may have become modified in the other membrane and therefore assumed a different electrophoretic mobility. Double immunodiffusion assays showed that antibodies to E10, E24, and E37 are monospecific whereas those against E30 are bispecific (Fig. 6). None of these antibodies reacts with either the thylakoid membranes or the stromal fraction (Fig. 6). These results were confirmed by crossed immunoelectrophoresis (data not shown). The chloroplast ATPase is the most abundant peripheral protein of the thylakoid membranes (33) whereas the apoproteins of chlorophyll-protein complex II (34), III, and IV (35) are predominant integral polypeptides. Antibodies raised against each of these thylakoid polypeptides do not cross-react with either the envelope or the stromal fraction (Fig. 7). Together, these results show that the major integral and peripheral polypeptides of the envelopes and the thylakoids are localized exclusively in their respective subchloroplast compartment.
inhibitors in the isolation medium does not alter visibly the polypeptide profile. We conclude that the greater number of polypeptides obtained in our studies is real and is due to the higher resolving power of the gel systems employed.

Comparison of the polypeptide patterns of the envelope and stroma as well as immunological analysis of these two fractions reveal that the envelope fraction contains some stromal proteins. Chloroplast envelope membranes are negatively charged (36) and, in the presence of Mg\(^{2+}\), some stromal proteins may be bound nonspecifically. Stromal proteins also may be trapped in envelope vesicles during chloroplast lysis. In either case, the stromal proteins should be removed by sonication of the envelope vesicles in high salt buffer. Indeed, such treatment removed all of the fructose-1,6-bisphosphatase from the envelope preparation, but a portion of the Rub-P\(_2\) carboxylase remained persistently bound even after repeated sonication, suggesting that association of the carboxylase with the envelope may be of physiological significance. Pineau and Douce (11) and Pineau et al. (32) have reported previously the presence of Rub-P\(_2\) carboxylase subunits in the envelope fraction. Our results confirm their observations and show further that the Rub-P\(_2\) carboxylase subunits are in fact the 18 S holoenzyme.

The chloroplast envelope is involved in the biosynthesis of galactolipids (3), carotenoids (5), geranyleranoid derivatives (4), tocopherol (6, 7), flavonoids (3, 4), and plastiquinone (7). The inner envelope membrane contains specific transporters which work to anion permeability to a limited number of anions (8), e.g., phosphate, sulfate, nucleotides, etc. (cf. Ref. 1). Indirect evidence suggests that the outer envelope membrane contains specific receptors for the post-translational transport of proteins into the chloroplasts (3, 37). Although these enzymatic activities and transport functions have been studied extensively, little is known about the polypeptides that mediate them. So far, only one envelope polypeptide has been identified structurally as well as functionally. Flugge and Heldt (17, 38, 39) identified a 30,000-dalton polypeptide involved in the specific transport of phospholipids across the envelope inner membrane. Thence translocator may be the E30 component, a hydrophobic, integral membrane polypeptide. Translocators which mediate protein transport and transport of other metabolites have not yet been identified but they are likely to be integral membrane polypeptides and therefore should be present in the 0.1 N NaOH-insoluble fraction. The presence of E30, an inner envelope membrane component, in our prepartation does not support the suggestion that envelope membranes prepared by lysis consist mainly of outer membranes (40).

An important point that emerges from our comparative electrophoretic and immunological studies is that each chloroplastic compartment contains a unique set of constituent polypeptides. With the few exceptions discussed above and within the limits of our analytical techniques, the three chloroplast compartments share no common polypeptides. These results imply that mechanisms must exist which ensure the specific localization of chloroplast proteins in the correct compartment after their synthesis in the cytosol or within the organelle (9). Many cytoplasmically synthesized chloroplast proteins are made as larger precursors. The additional sequence (transinit sequence) presumably is involved in the post-translational transport of the precursor into the chloroplast (9). Whether the transit sequence is also involved in targeting thylakoid polypeptides such as the CP II apoprotein to the appropriate membrane is not known.

Results from electron microscopic studies suggested that the thylakoid membranes may be derived from the inner envelope membrane by invagination during chloroplast development (10). If this were the case, we might expect the two membranes to share common polypeptides. However, our results demonstrate clearly no detectable immunonchemical relationship between the major polypeptides of the two membrane. Furthermore, the chemical composition (lipids, pigments, prenylquinones) of the plastid envelope is qualitatively and quantitatively different from that of the thylakoid membranes (1. 41-43). Finally, analysis of images of freeze-fractured membranes of mature chloroplasts of Fortulaco olaracea and barley has shown that the thylakoids are different from the inner envelope membranes in terms of the particles size and distribution (40). Taken together, these results do not support the proposed direct biogenetic relationship between the two chloroplast membranes. If indeed thylakoid membranes are from the inner envelope membranes, considerable changes must occur in the polypeptide, lipid, and pigment composition of the vesicles following the initial steps of membrane invagination.

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