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Comparative Studies of the Thylakoid Proteins of Mesophyll and Bundle Sheath Plastids of *Zea mays*[1]

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ABSTRACT

The proteins from both grana and stroma lamellae of maize (*Zea mays*) mesophyll plastids and from maize bundle sheath plastid membranes have been compared by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels using a discontinuous buffer system. Peptide differences between grana and stroma lamellae were essentially quantitative and not qualitative. Bundle sheath plastid membrane peptides more closely resembled those of the ultrastructurally similar stroma lamellae. However, bundle sheath membranes contained several peptides not apparent in the stroma lamellae.

The unappressed membranes (stroma lamellae and bundle sheath plastid membranes) were enriched in heavy (60-40 kilodaltons) peptides and depleted in light (31-20 kilodaltons) peptides as compared to stacked grana membranes. The heavier peptides were tentatively identified as subunits of chloroplast coupling factor. These peptides in unappressed membranes were much more resistant to removal by washing with ethylenediaminetetraacetate (under conditions of low ionic strength) than they were in granal membranes.

Ribulose-1,5-diphosphate carboxylase was identified on the gels and was localized exclusively in the bundle sheath cells. It is suggested that sodium dodecyl sulfate electrophoresis is a simple method to test for the localization of carboxylase in various C_4 plastid fractions.

A recent ultrastructural model (25) holds that the differentiation of chloroplast membranes of higher plants into stroma lamellae and grana lamellae is correlated with biochemical differentiation into membranes which contain PSI activity and PSI and PSII activity, respectively. The implication of this model has been that grana formation is in someway related to PSII activity. Algal exceptions to this pattern were found by Goodenough *et al.* (10) who studied a *Chlamydomonas* mutant with normal PSII activity but lacking grana. A similar exception for higher plants was reported by Andersen *et al.* (1) who demonstrated appreciable PSII activity in the essentially grana-free bundle sheath plastids of *Zea mays*.

Our goal in this study has been to elucidate the relations between membrane composition and membrane structure and function. The choice of a C_4 plant such as *Zea mays* with its dimorphic chloroplasts seemed ideal for such a study. Anderson and Levine (3) have recently used SDS-acrylamide electrophoresis to compare the mesophyll and bundle sheath chloroplasts of *Zea*. From their study they concluded that certain membrane peptides may be responsible for membrane appression into grana stacks. Since both whole mesophyll plastids and bundle sheath plastids of *Zea* contain PSII activity it was not possible for them to separate structural effects from functional effects. We have extended the study by comparing appressed grana (PSI and PSII) and stroma lamellae (PSI) from maize mesophyll plastids with nonappressed lamellae from maize bundle sheath plastids (PSI and PSII). In addition we have attempted to consider the effects of C_4 metabolism in these plastid types.

MATERIALS AND METHODS

Plant Material. Seedlings of *Zea mays* L. (Burpee No. 6205) were grown in soil in a greenhouse under natural illumination supplemented with artificial lighting to maintain 16-hr daylengths. Mature leaves were harvested from plants of between 4 and 6 weeks of age. Mesophyll chloroplasts and bundle sheath chloroplast fragments were isolated by a modification of the methods of Woo *et al.* (30) in which 20 g batches of midrib-free 1-cm leaf strips were ground in a Waring Blendor in 250 ml of 0.5 M sucrose and 0.01 M KCl in 0.05 M K-phosphate (pH 7.2). Fraction purity was monitored by light and electron microscopy.

Membrane Fractions. Both mesophyll plastids and bundle sheath plastid fragments were resuspended in 0.15 M KCl in 0.05 M K-phosphate (pH 7.2) and sedimented by centrifugation at 1,000*g* for 18 min. This washing was repeated three times to remove adhering cytoplasmic proteins; the final plastids consisted primarily of internal lamellae and lacked both outer plastid envelopes and stromal contents. Mesophyll plastids were resuspended in the washing buffer at a Chl concentration of 0.3 mg/ml and were passed through a French pressure cell at 6,000 p.s.i. Differential centrifugation yielded a number of fractions (27): 10,000*g* yielded the 10k or grana fraction; 160,000*g* yielded the 160k or stroma lamellae fraction. Table I shows typical Chl *a/b* ratios for the fractions extracted in 80% (v/v) acetone (5) and measured with a Cary 14 R spectrophotometer and scattered transmission accessory.

Electrophoretic Samples. For some experiments membranes were further purified before electrophoresis. Some membranes were subjected to an extensive salt washing (0.5 M NaCl in 0.1 M tris-HCl [pH 8]) to remove adhering stromal proteins such as RuDP[3] carboxylase; a portion of each fraction was further purified by washing for 30 min at 0 C in 1 mM EDTA (pH 8) to remove peripheral membrane proteins such as chloroplast coupling factor (12). The solubilized proteins were recovered for analysis by precipitation with 15% (w/v) trichloroacetic acid. Lipids were extracted by washing the membranes seven times in chloroform-methanol (1:2, v/v), three times in anhydrous methanol, followed by drying *in vacuo*. Dried proteins were dissolved by grinding (in a glass tissue grinder) in sample buffer containing 62.5 mM tris-HCl (pH 6.8), 1% (w/v) SDS, 5% (v/v) 2-mercap-

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[3] Abbreviations: RuDP: ribulose 1,5-diphosphate; TMD: tetramethylethylenediamine; AP: ammonium persulfate; kD: kilodaltons.

Table I. *Chlorophyll a/b Ratios of Plastids and Plastid Fractions*

Fraction	Ratio
Mesophyll plastids	3.2
10k grana membranes	2.8
160k stroma lamellae	5.0
Bundle sheath plastids	5.5

toethanol, and 40 mM dithiothreitol followed immediately by a 10-min immersion in a boiling water bath. Insoluble material was removed by centrifugation and pellets were tested for residual nitrogen by the micro-Kjeldahl method (19). About 96% of the nitrogen in the original samples was dissolved by the above procedure. Protein content of samples was monitored by a modification of the Folin procedure (18) using BSA as a standard.

Electrophoresis. Samples were analyzed by discontinuous buffer SDS-acrylamide electrophoresis. All gels were prepared from recrystallized acrylamide and N,N'-methylenebisacrylamide (17) and were made by diluting a stock solution containing 30% (w/v) acrylamide and 0.8% (w/v) N,N'-methylenebisacrylamide. The electrode buffer (0.192 M glycine-0.025 M tris [pH 8.3] with 0.1% SDS, w/v) of Laemmli (13) was used while the gel buffers were modified from those of Nelville (23). The 1-cm stacking gel (5%, w/v, acrylamide) contained 0.1 M tris-HCl (pH 6.1), 0.16% (v/v) TMD, 0.025% (w/v) AP, and 0.1% (w/v) SDS; the 8-cm resolving gel [10% (w/v) acrylamide] contained 1.3147 M tris-HCl (pH 9.8), 0.04% (v/v) TMD, 0.0125% (w/v) AP, and 0.1% (w/v) SDS. After a run, gels were removed from their tubes, fixed for 3 hr in 35% (w/v) trichloroacetic acid followed by 12 hr in several changes of 25% isopropyl alcohol-10% acetic acid, v/v. The gels were stained in Coomassie blue (29) and diffusion destained in alcohol-acetic acid mixtures (24). The gels were scanned at 560 nm with a Beckman DU spectrophotometer equipped with a Gilford phototube and linear transport.

Molecular Weights. One gel in each run received a sample containing the following purified proteins: bovine albumin, catalase, aldolase, fumarase, carbonic anhydrase, chymotrypsinogen, trypsin, myoglobin, and lysozyme. The spectrophotometric scans of the gels containing standard proteins were used to prepare a standard curve from which the mol wt of the peptides were calculated (29). The weights given below in kD represent the average of at least 10 runs. Actual values for a particular run were normally within ±500 daltons of the reported value.

RuDP Carboxylase. Carboxylase was prepared from maize leaves by ammonium sulfate precipitation (26) or by density gradient centrifugation (9). The enzyme assays were performed as described by Paulsen and Lane (26) with modifications of the assay mixture. Fifty μl of enzyme solution were added to 100 μl of assay solution which contained in μmol: Tricine-OH^- (pH 8): 15; RuDP: 0.2; $NaH^{14}CO_3$ (5 μCi): 10; $MgCl_2$: 5; and 2-mercaptoethanol: 8. The mixture was incubated at 25 C for 3 min and the reaction was stopped by adding 200 μl of 25% acetic acid, v/v.

RESULTS

Electrophoresis of Non-EDTA-washed Membranes. Figure 1a shows the peptide composition of mesophyll grana membranes. These membranes show the same general peptide profile as has been previously reported for many green plants (3, 15). There is a prominent doublet at 60 kD and 57 kD. Minor peptides are consistently apparent at 68 kD and 40.5 kD. In the lower mol wt regions prominent peaks are found at 30.5 kD and 25.5 kD with smaller peaks at 23.5 kD and 22 kD. A prominent doublet is present at 18 kD and 17 kD while the extreme end of the gel displays a peak, frequently with several shoulders, which falls below the range of calibration for these gels.

The stroma lamellae fraction (Fig. 1b) is considerably different from the granal fraction. The 60 kD, 57 kD, and 40.5 kD peptides are more prominent than in the granal membranes while the 30.5 kD and 25.5 kD peptides are depleted as compared to the grana. The depletion of the 25.5 kD peak apparently allows the 23.5 kD and 22 kD peptides to be more clearly resolved on the gels.

If a sample of bundle sheath plastid membranes is prepared without additional salt washing, the resulting gels show a very

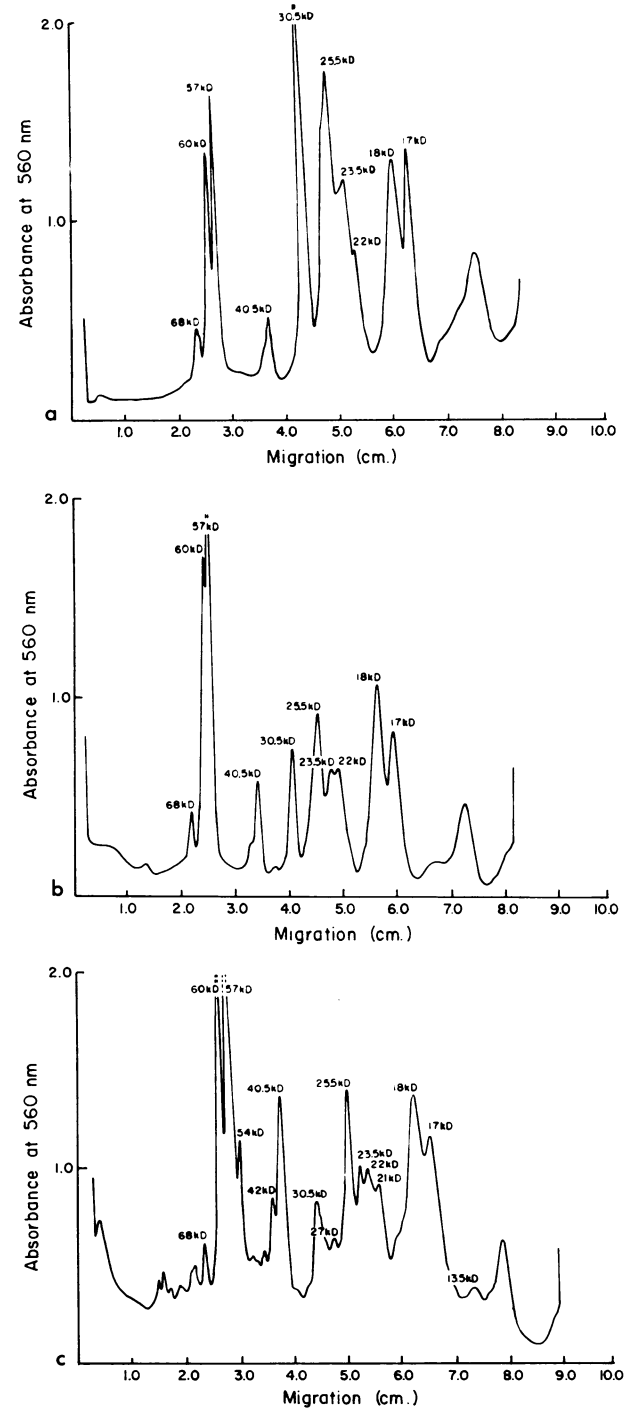

FIG. 1. Scans of Coomassie-stained SDS gels of maize chloroplast membrane fractions. Full scale = 2 *A*. a: 10k grana fraction of mesophyll plastids; b: 160k stroma lamellae fraction of mesophyll plastids; c: salt-washed bundle sheath plastid membranes.

large peak in the heavy peptide region [see Fig. 6 of Anderson and Levine (3)]. Reference to the calibration curves shows that this peptide has a weight of 54 kD and hence is completely missing from either of the mesophyll fractions. Extensive washings of the membranes in 0.5 M NaCl in 0.1 M tris-HCl (pH 8) almost completely removes this peptide yielding the result seen in Figure 1c. As compared to granal membranes the 60 kD, 57 kD, and 40.5 kD are more abundant thus more closely resembling stroma lamellae. Note the presence of the 54 kD peptide and 42 kD peptide neither of which are visible in mesophyll fractions. Compared to the granal membranes the 30.5 kD and 25.5 kD peptides are depleted. Again this resembles the stroma lamellae; however, bundle sheath plastids consistently show more 25.5 kD and less 30.5 kD peptides than do the stroma lamellae. Note the 27 kD peptide; this peptide is also present in granal fractions but is too near the massive 25.5 kD peak to be resolved by the gel scanner. It has not been observed in stroma lamellae fractions. The bundle sheath plastids also contain a 21 kD peptide and 13.5 kD peptide neither of which appears to be present in the mesophyll fractions. Overlooking the above differences, bundle sheath membranes most closely resemble mesophyll stroma lamellae. However, unlike mesophyll fractions bundle sheath fractions show several minor peptides above 68 kD and a visible stain of peptides that failed to enter the top of the gels. There appears to be some problems of peptide aggregation in bundle sheath samples.

Electrophoresis of EDTA-washed Membranes. EDTA washing has frequently been used to remove peripheral membrane proteins, and washing of chloroplast membranes in 1 mM EDTA (pH 8) has been shown partially to remove chloroplast coupling factor (12). Figure 2a shows the result of washing grana lamellae with EDTA. The most apparent change is the loss of the 60 kD, 57 kD, and to a lesser extent the 40.5 kD peptides. In addition the ratio of the 30.5 kD to the 25.5 kD peptide is altered. Apparently this is due primarily to a loss of 30.5 kD peptide. The behavior of the stroma lamellae (Fig. 2b) is different in that the 60 kD, 57 kD, and 40.5 kD components are not greatly reduced by the EDTA treatment. In addition the 30.5 kD peptide shows no change in ratio with the 25.5 kD peptide. Bundle sheath plastid membranes that have been EDTA-washed (Fig. 2c) reveal a pattern similar to that of washed stroma lamellae in that little peptide loss appears to have taken place. It might be argued (particularly with the 160,000*g* stroma fraction) that the solubilized proteins are being co-sedimented with the membranes. However, whole plastids broken within the French press and sedimented at 160,000*g* show considerable loss of the above mentioned peptides. In addition, bundle sheath membranes are sedimented at 10,000*g* after EDTA washing—hardly sufficient acceleration to sediment proteins. It appears that unstacked membranes bind certain proteins more strongly than do stacked membranes.

Supernatant Proteins from EDTA-washed Membranes. The above assertion of differential binding of proteins can be reenforced by looking at the supernatant proteins released by EDTA treatment. The gels represent supernatant proteins from membranes containing equal quantities of Chl. Figure 3a shows that granal membranes release a large amount of peptide at 58 kD; this most likely represents the 60 kD and 57 kD peptides which are not separately resolved after the extraction procedure. The 40.5 kD peptide is also present and as was expected from the analysis of the washed membranes the 30.5 kD peptide is present in the supernatant. The stroma lamellae (Fig. 3b) and bundle sheath membrane (Fig. 3c) supernatants show a much lower amount of the 58 kD peptide even though the membranes were much richer in the high mol wt peptides. Very little 30.5 kD peptide is released from these membranes; however, they do release 18 kD and 17 kD peptides which are not released from grana membranes. The total protein on a Chl basis released from

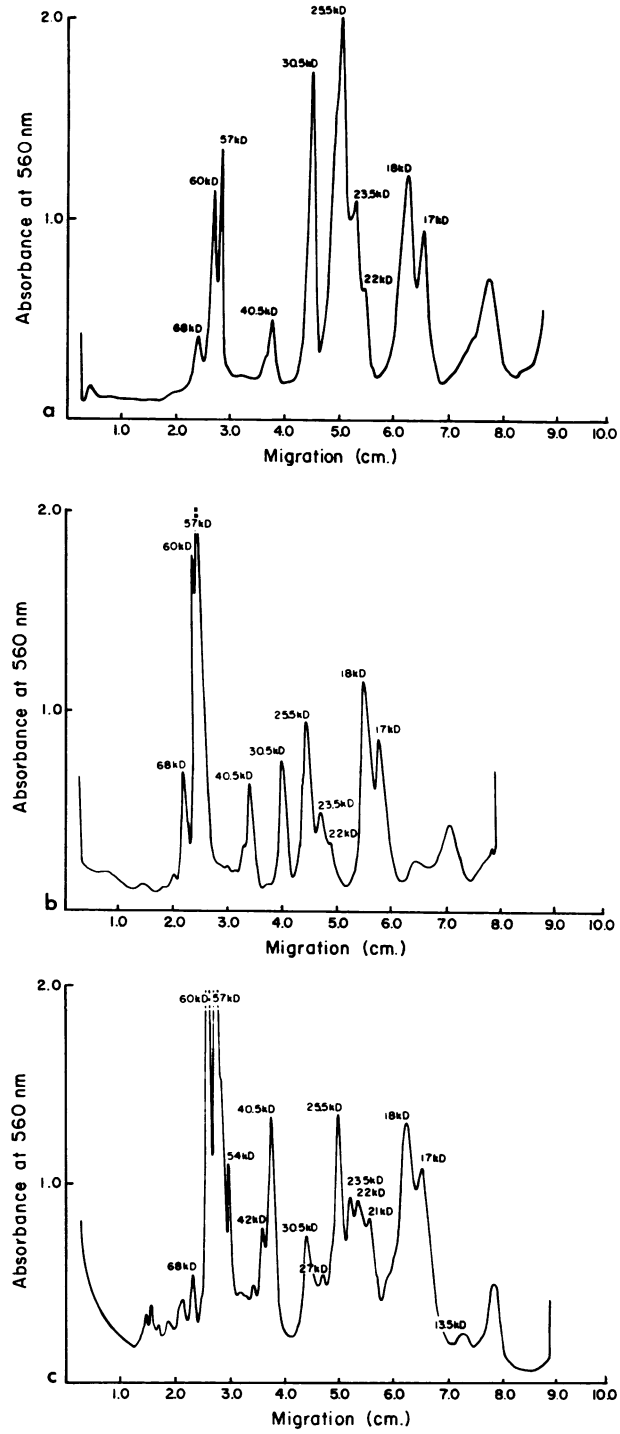

FIG. 2. Scans of Coomassie-stained SDS gels of EDTA-washed maize chloroplast membrane fractions. Full scale = 2 *A*. a: 10k grana fraction of mesophyll plastids; b: 160k stroma lamellae fraction of mesophyll plastids; c: salt-washed bundle sheath plastid membranes.

the unstacked membranes is less than that released from the stacked membranes even though stroma lamellae and bundle sheath membranes initially have more protein on a Chl basis than do grana membranes.

RuDP Carboxylase. The rather tightly bound peripheral peptide at 54 kD in the bundle sheath plastids was intriguing since this is close to the mol wt reported for the large subunit of RuDP carboxylase (20). The trace of a gel made from the ammonium sulfate II fraction (26) which showed a RuDP carboxylase activ-

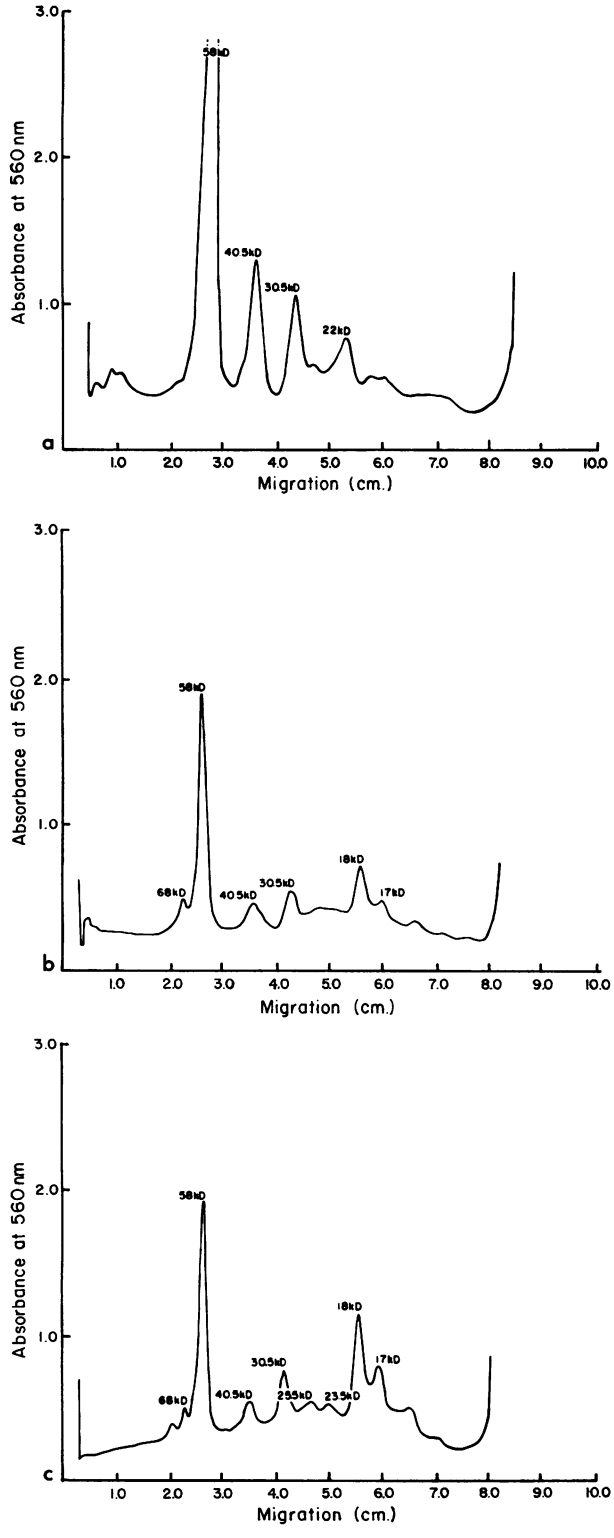

FIG. 3. Scans of Coomassie-stained SDS gels of EDTA supernatants from maize chloroplast membrane fractions representing protein released from membrane samples with equal amounts of chlorophyll. Full scale = 3 *A*. a: supernatant from 10k grana fraction of mesophyll plastids; b: supernatant from 160k stroma lamellae fraction of mesophyll plastids; c: supernatant from salt-washed bundle sheath plastid membranes.

ity of 0.5 unit/mg of protein is illustrated in Figure 4. The two major peptide peaks have calculated weights of 54 kD and 13.5 kD. When the carboxylase was purified by density gradient centrifugation (9), the fractions showing maximal activity contained the 54 kD and 13.5 kD peptides. The entire supernatant fraction (1 liter) from the first grinding (isolation of mesophyll plastids) was precipitated with trichloroacetic acid and analyzed on gels. Virtually no 54 kD or 13.5 kD peptides were found. On the other hand, the supernatant from the final grinding which broke open bundle sheath cells contained an abundance of 54 kD and 13.5 kD peptides.

DISCUSSION

We hoped that comparison of unstacked membrane containing PSI activity (stroma lamellae) with unstacked membranes containing PSI and PSII activity (bundle sheath membranes) might reveal peptide differences attributable to PSII. At the same time comparison of stacked membrane fractions with unstacked membrane fractions might reveal peptide differences responsible for the structural differences. Attempts to discover PSII peptides were disappointing. We did not confirm the unique peptides observed earlier in spinach grana (24); the 27 kD peptide that is present in bundle sheath plastids and in grana membranes (although not resolved on the presented gel traces) but not in stroma membranes may be of some significance. The number of various electron transport enzymes can be calculated on a Chl basis. Their concentration in most cases seems to be too low to be resolved on these gels especially in the presence of massive amounts of "structural proteins" (by this we mean membrane proteins whose functions is yet to be discovered).

Previous investigators (3, 15) have described chloroplast membrane peptides as belonging to two main groups: a group of higher mol wt peptides between approximately 60 kD and 40 kD (Group I peptides [15]); and a group of lower mol wt peptides between approximately 30 kD and 20 kD (Group II peptides [15]). It has also been shown (3, 15) that the Group I peptides tend to be enriched in PSI fractions while Group II peptides are enriched in PSII regions. This study confirms the above observations.

Anderson and Levine (4) identify the Group I peptides with the protein portion of the 110 kD p700-Chl *a*-protein. It is difficult to compare the present study with that of Anderson and Levine since they do not give mol wt calibrations for their gels. Because EDTA removes chloroplast coupling factor (12) and since Nelson *et al.* (22) have shown that chloroplast coupling factor has 5 subunits of 59 kD, 56 kD, 37 kD, 17.5 kD, and 13 kD, we conclude that the majority of the Group I peptides (60 kD, 57 kD, and 40.5 kD in this study) probably represent subunits of chloroplast coupling factor. If other observed pep-

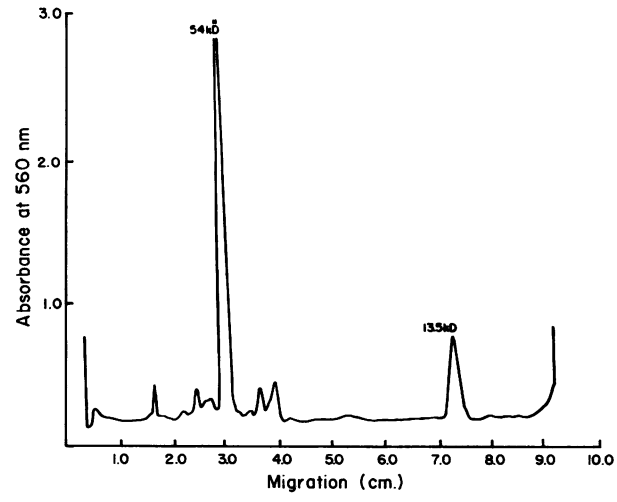

FIG. 4. Scan of Coomassie-stained SDS gel of ammonium sulfate fraction II representing partially purified RuDP carboxylase. Full scale = 3 *A*.

tides (*i.e.* the 17 kD peptide) are also subunits of the coupling factor, they do not show the same response to EDTA washing as do the aforementioned subunits. Silicotungstic acid has been shown to be far more efficient than EDTA in the removal of chloroplast coupling factor (16). Preliminary experiments in our laboratory show that washing whole mesophyll plastids in silicotungstic acid removes the majority of the Group I peptides without removing any Chl. This is not the expected result if the group I peptides are primarily derived from the P700-Chl *a*-protein. Certainly regions rich in PSI activity (particularly cyclic photophosphorylation) would be expected to be rich in coupling factor.

Studies with mutant plants which lack Chl *b* (3, 11, 28) have shown a lack of Group II peptides, particularly those around 25 kD. Bundle sheath chloroplasts of *Zea* have been shown to be deficient in the light-harvesting *a*/*b*-protein (8). Anderson and Levine (4) have concluded that two of the Group II peptides are derived from the light-harvesting Chl *a*/*b*-protein. It would appear that their IIb and IIc peptides are the same as our 25.5 kD and 23.5 kD peptides, respectively. Our data are consistent with the origin of these peptides from the light-harvesting Chl *a*/*b*-protein. Anderson and Levine's peptide IIa appears to be similar to our 30.5 kD peptide; it is probably not part of a pigment complex since it is partly removed by washing under low ionic conditions without loss of Chl.

Investigations of both green algae and higher plant chloroplast peptides (3, 15) have indicated the possible involvement of Group II peptides in membrane appression. Recently, Anderson (2) has hypothesized that cation-induced aggregation of the light-harvesting Chl *a*/*b*-protein is primarily responsible for the formation of grana. This idea is supported by the discovery of cation binding sites (7) on the pigment protein and by the overall correlation of the presence of the light-harvesting pigment protein with the presence of grana (see ref. 2). The presence of Chl *b* and the Group II peptides in the grana are explained by such a model, and the, as yet unisolated, reaction center for PSII might also become aggregated into the grana stacks along with the light-harvesting complex. Anderson (2) also attempts to equate the light-harvesting pigment protein complex with the large particle revealed by the freeze fracture of granal membranes since she finds that the Chl *b*-less mutant of barley which lacks the light-harvesting complex (28) also lacks large free fracture particles. Yet the Chl *b*-less mutant has a fair number of grana stacks, and this laboratory (11) has found the Group II peptides to be lacking from the mutant, but the large freeze fracture particles to be still present.

Anderson (2) also believes that the chloroplast coupling factor is important in the formation of grana stacks. With this we concur, since our data suggest a difference in the binding of the coupling factor to different membrane fractions. Murakami and Packer (21) have suggested that some nonionic interaction must be responsible for the stability of grana stacks. Perhaps this interaction could be provided by a more loosely attached coupling factor in the grana.

The presence of RuDP carboxylase exclusively in the bundle sheath plastids have been reported by many workers [see Black (6)]. There has been much controversy surrounding these reports. Laetsch (14) has presented clearly the position of those scientists who believe that the carboxylase is present in both mesophyll and bundle sheath plastids. They attribute the failure of many workers to find RuDP carboxylase in mesophyll plastids to denaturation of the enzyme by phenols or other inhibitors released during plastid isolation. Such denaturation makes localizations based on enzyme activity or immunological techniques questionable. The identification of carboxylase by SDS-acrylamide electrophoresis represents a novel approach since SDS completely denatures all proteins and previous denaturation has no effect on the results of SDS electrophoresis. Thus denaturation by phenols will not alter the localization detected by this method. Significantly, SDS electrophoresis localizes the carboxylase exclusively in the bundle sheath cells.

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