SPATIAL RELATIONSHIP OF PHOTOSYSTEM I, PHOTOSYSTEM II, AND THE LIGHT-HARVESTING COMPLEX IN CHLOROPLAST MEMBRANES

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ABSTRACT

We have previously demonstrated (Armond, P. A., C. J. Arntzen, J.-M. Brian-tais, and C. Vernotte. 1976. Arch. Biochem. Biophys. 175:54–63; and Davis, D. J., P. A. Armond, E. L. Gross, and C. J. Arntzen. 1976. Arch. Biochem. Biophys. 175:64–70) that pea seedlings which were exposed to intermittent illumination contained incompletely developed chloroplasts. These plastids were photosynthetically competent, but did not contain grana. We now demonstrate that the incompletely developed plastids have a smaller photosynthetic unit size; this is primarily due to the absence of a major light-harvesting pigment-protein complex which is present in the mature membranes. Upon exposure of intermittent-light seedlings to continuous white light for periods up to 48 h, a light-harvesting chlorophyll-protein complex was inserted into the chloroplast membrane with a concomitant appearance of grana stacks and an increase in photosynthetic unit size. Plastid membranes from plants grown under intermediate light were examined by freeze-fracture electron microscopy. The membrane particles on both the outer (PF) and inner (EF) leaflets of the thylakoid membrane were found to be randomly distributed. The particle density of the PF fracture face was approx. four times that of the EF fracture face. While only small changes in particle density were observed during the greening process under continuous light, major changes in particle size were noted, particularly in the EF particles of stacked regions (EF$_S$) of the chloroplast membrane. Both the changes in particle size and an observed aggregation of the EF particles into the newly stacked regions of the membrane were correlated with the insertion of light-harvesting pigment-protein into the membrane. Evidence is presented for identification of the EF particles as the morphological equivalent of a “complete” photosystem II complex, consisting of a photochemically active “core” complex surrounded by discrete aggregates of the light-harvesting pigment protein. A model demonstrating the spatial relationships of photosystem I, photosystem II, and the light-harvesting complex in the chloroplast membrane is presented.
Chloroplast membranes provide a useful system for correlating membrane structure and function. In structural studies utilizing the technique of freeze-fracture electron microscopy, the membranes have been shown to have two distinctly different types of membrane subunits on the fracture faces (5, 12, 17, 25, 32). The inner leaflet of a normal, wild type chloroplast thylakoid membrane is characterized by a population of relatively large, widely spaced particles. This fracture face, by current terminology convention, is termed the EF face. The outer leaflet of the membrane which is exposed during freeze fracture is characterized by the presence of tightly packed, smaller particles. This fracture face has now been termed the PF face (14).

Functional studies of chloroplast lamellae have led to the widely held view that two light reactions act in series to catalyze noncyclic electron transport. Detergent fractionation of chloroplast membranes has established that each of these photosystems is a structurally distinct entity. The photosystem I complex contains P700, the reaction center chlorophyll of photosystem I, and light-harvesting chlorophyll composed of almost entirely chlorophyll a. In contrast, the complete photosystem II complex contains P680, the reaction center chlorophyll of photosystem II, and light-harvesting (antenna) chlorophyll which are composed of both chlorophylls a and b (6, 10, 34). Recent fractionation studies have shown that most of the light-harvesting chlorophyll protein which is normally bound to photosystem II can be dissociated by detergents in cation-free media (7). The light-harvesting complex recovered in these procedures is a structurally distinct protein aggregate which will reassociate with photosystem II in the presence of cations.

Freeze-fracture analysis of the detergent-derived photosystem I and II complexes has shown that the functional differences are related to a structural dimorphism. In digitonin-derived photosystem I preparations, only the smaller freeze-fracture particles (equivalent to the size of those of the PF face of normal plastids) were observed (5). The digitonin-derived submembrane preparations which contained photosystem II and which were enriched in chlorophyll b were found to be enriched in the large size-class particles (analogous to the EF fracture face subunits of normal thylakoids). These observations led Arntzen et al. (5) to propose that the two different freeze-fracture particles may represent structural counterparts to the two different functional units found in the chloroplast membrane.

This viewpoint was challenged after studies of an algal mutant lacking stacked membrane regions which was found to have complete photochemical activity, but which had very few large freeze-fracture particles (17). Investigation of wildtype chloroplast membranes of algal and higher plant chloroplasts has subsequently shown that most large freeze-fracture particles are localized in the appressed membranes of grana stacks. It has therefore been suggested that the large freeze-fracture subunit is in some way involved in the membrane stacking process (17, 28), and need not necessarily be related to the photosystem II functional complex.

It is now generally accepted that the insertion of functional components into chloroplast lamellae occurs in a step-wise manner during the normal greening process in continuous light (see reference 4). We have recently shown that exposure of dark-grown pea plants to intermittent illumination (cycles of 2-min light, 118-min dark) results in incomplete differentiation of the chloroplast membrane (3, 16). Chloroplasts developed under the intermittent-light regime exhibit high rates of photosystem I and II activity, but are deficient in the light-harvesting assembly of photosystem II. If plants which were previously exposed to intermittent light were subsequently subjected to continuous illumination, the plastids accumulated chlorophyll b which was specifically associated with a light-harvesting chlorophyll a/b protein (16). In this paper, we will examine submembrane structural changes of the chloroplast lamellae during the time in which there is an increase in the light-harvesting pigment content of the membrane. We will demonstrate that the increase in content of light-harvesting pigment-proteins within the membrane results in a preferential increase in size of the subunits of the EF fracture face.

MATERIALS AND METHODS

Preparation of Chloroplast Samples

Leaf material used for the isolation of control chloroplasts was collected from pea (Pisum sativum L.,

1 Mention of a trademark or commercial company does not imply endorsement by the U.S. Department of Agriculture.
Morse's Progress no. 9) seedlings grown under a 16-h photoperiod. Developing chloroplasts were isolated from seedlings that had been germinated in the dark for 7 days, exposed to intermittent illumination (2-min light, 118-min dark cycles) for 2 days, and then subjected to continuous illumination for periods of 0-48 h. Further details of growth conditions have been previously described (3).

Chloroplasts were isolated by grinding the leaves in a cold solution of 0.4 M sorbitol and 0.1 M sodium tricine, pH 7.8. The brei was filtered through 4, then 12 layers of cheesecloth and centrifuged at 1,000 g for 10 min to yield to chloroplast pellet. Samples which were to be used for freeze-fracture studies were resuspended in 0.4 M sorbitol, 0.1 M tricine (pH 7.8) and 5 mM MgCl₂. Glycerol (final concentrations of 35%) was added with stirring over a 30-min period. Chloroplast pellets to be used for particle density measurements were resuspended in 0.05 M tricine (pH 7.8) and incubated at 4°C for 30 min, followed by 10 min at 20°C to allow for unstacking of the membranes and intermixing of intra-membranous particles from stacked and unstacked membrane regions (32). Glycerol was added in the manner described above. Replicas were prepared on a Balzers BA 360 freeze-etching apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) and examined on a Philips EM 300 electron microscope.

Chloroplasts prepared for thin-sectioning analysis were treated as described earlier (3). Measurements of linear membrane distances on micrographs of thin-sectioned material were made with a map distance measuring device.

**Measurements and Calculations of Structural Membrane Parameters in Freeze-Fractured Samples**

Particle sizes were determined from micrographs taken at ×40,000, and enlarged to ×200,000 and viewed through a 10× objective lens equipped with a micrometer grating. The width of the shadow of a given particle was measured over the shadowed half of the particle. Where the edge of the shadow appeared fuzzy or irregular, a minimum width was always taken. Between 300 and 500 particles were measured in flat continuous areas on 5-8 micrographs for each histogram of particle size distribution (Figs. 7-10). Particle distribution counts were made on 8-10 micrographs of large flat membrane regions enlarged to ×100,000 by imposing a transparent sheet of graph paper and counting all particles within quadrants on the paper. Membrane areas equivalent to a total area of 1 μm² were used for particle distribution counts.

The procedures used for particle size and distribution determinations used herein are identical to those recently discussed by Staehelin (32). He has demonstrated that these techniques allow detection of particle size-class changes of as little as 4 Å with high statistical significance.

**Disc Gel Electrophoresis**

Membrane samples to be used for electrophoretic analysis were washed by resuspending and thoroughly dispersing the initial chloroplast pellets (described above) in 0.75 mM Na-EDTA, pH 7.2, and sedimenting the membranes by centrifugation at 5,000 g for 15 min. This wash was repeated once. The resultant membranes were extracted twice in 90% acetone and then subjected to solubilization in sodium dodecyl sulfate (SDS) and electrophoresis as described by Hoober (19). The current used for electrophoresis was 1.75 mA/tube, using 6-mm diameter gel tubes.

**Measurement of Photochemically Active Membrane Components**

Photosynthetic unit size determinations were conducted by titrimetrically monitoring ferricyanide reduction in flashing light. The number of electrons transported per flash is calculated from:

\[
\frac{1}{2} \text{H}_2\text{O} + \text{FeCN}^{3+} \rightarrow \frac{1}{2}\text{O} + \text{FeCN}^{2+} + \text{H}^+.
\]

Reaction mixtures contained 5 mM MgCl₂, 50 mM KCl, 0.5 mM potassium ferricyanide, 10⁻⁸ M gramicidin and 0.1 M sorbitol. The pH of the suspension was adjusted to 7.4 with NaOH or HCl. Saturating flashes (10 μs flashes, 720 flashes/min) were obtained with a General Radio type 1539-A Strobosclave flash lamp (General Radio Co., Concord, Mass.).

Fluorescence transients of isolated plastids were detected with an Aminco 10-280 microphotometer (American Instrument Co., Inc., Silver Spring, Md.), stored on a Nicolet model 535 digital signal averager (Nicolet Instrument Corp., Madison, Wis.), and plotted on an x, y recorder. The reaction medium for fluorescence measurements was 1 mM Na-tricine, pH 7.8, 10 mM NaCl, 5 mM MgCl₂ and 5 μg chlorophyll/ml. The areas above the induction curves were analyzed by cutting out the appropriate areas of the recorder record and weighing the paper. Weights were used to give relative sizes of the A pool and the Q pool [measured in the presence of 1 × 10⁻⁵ M DCMU (3-3,4-dichlorophenyl)-1,1-dimethylethra].

Cytochromes f, b₅₅₀, and b₅ were quantitatively determined according to the procedures of Bendall et al. (8) with an Aminco DW-2 spectrophotometer. All cytochrome b₅₅₀ was converted to the low potential form by Triton X-100 treatment before assays.

**RESULTS**

**Photochemical Activity of Developing Plastids**

We have earlier demonstrated (3) that plastids greened only under intermittent light have full...
photochemical activities (photosystem I, photosystem II, ion pumping, phosphorylation). Further analysis of these plastids and of chloroplasts developed by greening under continuous light is described in Table I. Photosynthetic unit size calculations were based upon analysis of the number of chlorophylls participating in the transfer of one electron from water to ferricyanide in flashing light. These determinations indicated that the incompletely differentiated intermittent-light plastids contained only 40% of the light-harvesting chlorophyll of normal membranes.

We have also attempted to determine whether the various membrane components involved in photochemistry undergo changes in relative amounts with respect to one another during membrane differentiation. Malkin and Kok (21) have previously demonstrated that the record of fluorescence inductions in isolated chloroplasts can provide an accurate analysis of the concentrations (per chlorophyll) of the electron carriers involved in the light reactions of photosynthesis. In dark-adapted chloroplasts treated with DCMU, fluorescence typically rises very rapidly to a maximal level after illumination; the area over this induction curve provides a relative measure of the concentration of "Q," the primary electron acceptor of photosystem II (22). In samples of chloroplasts which do not contain DCMU but which are otherwise identical, the fluorescence rise to a maximum is much slower; the area above this induction curve defines the relative amount of a secondary electron acceptor pool called the "A" pool. A ratio of the relative amounts of the A:Q pools calculated from the fluorescence induction allows an estimation of the concentration of the number of electron carriers in the photosynthetic electron-transport chain with respect to the number of photosystem II centers (assuming one Q per center). As is shown in Table I, the ratio of A:Q pools varied only slightly between samples greened in intermittent as compared to continuous light. These data indicate that the relative content of electron carriers in the photosynthetic electron-transport chain did not change significantly during the increase in photosynthetic unit size which occurred during chloroplast greening in continuous light.

We have also determined the cytochrome content of plastids isolated from plants grown either in intermittent light or in intermittent and then continuous light. All cytochromes (b6, b559, and f) were found to be enriched in the undifferentiated plastids when data are presented on a chlorophyll basis (Table I). It should be noted, however, that the content of each cytochrome on a photosynthetic unit basis remained quite constant during greening. These data again suggest that the content of electron carriers per electron-transport chain remained constant during the final stages of membrane greening.

The polypeptide composition of EDTA-extracted lamellae of intermittent-light plastids and fully greened chloroplasts was examined by disc-gel electrophoresis. A gel scan of representative samples is shown in Fig. 1. The position of each visually detectable band is indicated on the top of the figure by an arrow. Detailed analysis of each of the stained bands observed on the gel is described in Table II. Data in the table are organized such that corresponding bands on the two samples which had relative mobilities (Rm) which differed by 0.01 or less are considered identical molecular weight species. Three polypeptide species were detected in intermittent light but not in the controls: bands 3, 4, and 10 (band 10 may be presented in the fully greened sample but is masked by the broad, intensely stained band 11). Two new polypeptide species were observed in the sample greened under continuous light: bands 11 and 15.

### Table I

**Characterization of Membrane Constituents of Chloroplast Membranes from Pea Seedlings Greened for 2 Days under Intermittent Light plus either 0 or 48 h of Greening in Continuous Light**

| Sample (time of greening in continuous light) | Photosynthetic unit size (no. of chlorophylls participating in a one electron transfer from H2O to Fe(III)) | A:Q (Ratio of pool sizes) | Cytrome f content Cytochlorophyll | Cytrome 559 content | Cytrome 559 content |
|---------------------------------------------|-------------------------------------------------|---------------------------|-----------------------------------|---------------------|---------------------|
| 0 h                                         | 261                                             | 8:1                       | 1/215                             | 1/172               | 1/194               |
| 48 h                                        | 650                                             | 10:1                      | 1/557                             | 1/680               | 1/523               |

**Armond, Staehelin, and Arntzen**

*Spatial Relationships in Chloroplast Membranes* 403
FIGURE 1  SDS-polyacrylamide gel electrophoresis of membrane polypeptides from (A) chloroplasts from pea seedlings greened for 48 h under intermittent illumination and (B) chloroplasts from pea seedlings greened for 48 h under continuous light. Gels were stained with Coomassie blue and scanned at 580 nm. Arrows at the top of the figure represent positions of visibly distinguishable bands. Numbers at arrows correlate with band identification in Table II.

The amount of protein in each of the bands of the gels can be approximated by the amount of bound Coomassie blue stain. This has been done for the fully greened sample by calculating the area under each of the peaks of the gel scan and presenting these values as a percent of the entire area under the gel scan (Table II). Two bands (nos. 11 and 12; $R_M = 0.55$ and 0.61) contain more than 50% of the total Coomassie blue stain on the gel. These bands are almost totally absent in the intermittent-light plastids (Fig. 1). There was also a very significant increase in the amount of band 15 ($R_M = 0.84$) in the fully greened membranes.

It should be noted that the polypeptides migrating with $R_M = 0.55$ and 0.61 in our mature membrane protein preparation appear to be identical to polypeptides IIb and IIc described by Anderson and Levine (2). Components IIb and IIc in their studies were identified as proteins of complex II (the light-harvesting pigment protein) of chloroplast membranes. Band 15 in our gels ($R_M = 0.84$) can now also be suggested to be related to the light-harvesting pigment-protein complex, since it was not present in the functionally active intermittent-light plastids.

Membrane Structure

When plants grown for 48 h in continuous light were transferred to continuous illumination, there was an increase in the total amount of membranes per plastid. Measurement of total length of membranes in randomly selected thin-sectioned samples of intermittent-light plastids averaged $19.2 \pm 3.9 \, \mu m$ (linear membrane distance) per plastid. This value increased to $23.7 \pm 5.7$ and $33.3 \pm 10.8$ after 4 and 8 h of continuous-light greening, respectively.
Table II

Summary of Characteristics of Polypeptide Species Shown in Gel Scans of Figure 1.

| Identifying no. on gel scan | Band width R_w (Center of trailing edge of band) | Maximum absorbance of band (Relative absorbance) | Total protein (continuous light sample only) |
|----------------------------|-----------------------------------------------|-----------------------------------------------|---------------------------------------------|
| 1                          | 0.13                                          | 0.11/0.15                                       | 0.05                                        | 1                                           |
| 2                          | 0.20                                          | 0.19/0.23                                       | 0.08                                        | 2                                           |
| 3                          | 0.25                                          | 0.24/0.25                                       | 0.11                                        |                                             |
| 4                          | 0.27                                          | 0.26/0.28                                       | 0.12                                        |                                             |
| 5                          | 0.30                                          | 0.28/0.32                                       | 0.29                                        | 5                                           |
| 6                          | 0.33                                          | 0.32/0.34                                       | 0.03                                        | 1                                           |
| 7                          | 0.38                                          | 0.36/0.40                                       | 0.11                                        |                                             |
| 8                          | 0.42                                          | 0.41/0.43                                       | 0.07                                        | 3                                           |
| 9                          | 0.45                                          | 0.43/0.47                                       | 0.11                                        | 4                                           |
| 10                         | 0.53                                          | 0.51/0.54                                       | 0.17                                        |                                             |
| 11                         | 0.62                                          | 0.60/0.63                                       | 0.03                                        | 37                                          |
| 12                         | 0.74                                          | 0.69/0.76                                       | 0.25                                        |                                             |
| 13                         | 0.80                                          | 0.78/0.84                                       | 0.27                                        | 15                                          |
| 14                         | 0.84                                          | 0.81/0.87                                       | 0.39                                        |                                             |

R_w of polypeptide bands is based on a mobility equal to 1.0 for the tracking dye (Bromphenol blue). Identifying no. of polypeptide bands on gel scans are indicated at the top of Fig. 1. The amount of protein in each band was calculated for the continuous-light sample by measuring the area under each peak of the gel scan and presenting that area as a percent of the total area under the gel scan.

Our previous studies demonstrated that plastids in leaf material grown under intermittent light had occasional regions of membrane overlap and apparent membrane appression (3). In samples of isolated plastids of similar material used for the present study, it was found that these regions of membrane contact were lost and that the lamellae formed separate vesicles. Addition of cations (10 mM MgCl_2 or 150 mM KCl) did not restore any stacking. Since these plastids had full photochemical activity in electron-transport, ion pumping, and photophosphorylation (3), it is not likely that the membranes were artifically damaged during isolation. We conclude that the random regions of membrane appression observed in vivo are not comparable to the normal grana stacking observed in control samples since cation-mediated membrane fusion could not be detected in vitro; the membrane contact seen in vivo may be related to one stage of membrane development in the partially differentiated plastids. In membrane samples isolated from plants exposed to 4 or more hours of continuous light, fused membranes were observed in all isolated membranes (in the presence of cations).

In chloroplasts from plants which had been exposed to intermittent illumination followed by 4 h of continuous light, membrane appression (formation of grana stacks) was observed (Fig. 3). Aggregation of particles into regions of membrane fusion occurred on the EF face. After 36 h of continuous light greening, the thylakoids (Fig. 4) were
found to possess fracture face characteristics nearly indistinguishable from those of plastids isolated from control (16 h photoperiod) pea plants (Fig. 5). Four different types of fracture faces were observed: \(PF_s\) (stroma half membrane leaflet from the stacked, grana membrane region), \(PF_u\) (stroma leaflet of unpaired stroma lamellae), \(EF_s\) (lumenal half membrane leaflet of stacked, grana membrane region), and \(EF_u\) (lumenal leaflet of unstacked stroma lamellae). Both the \(PF_s\) and \(PF_u\) faces contained rather tightly packed particles but were distinguishable by the size of the particles and by the clarity with which the particles stood out from their background matrix. The \(EF_s\) fracture face clearly differs from the \(EF_u\) face in that the particles are aggregated in the regions of membrane appression.

**Particle Density Measurements**

To determine whether there was a change in the number of particles within the chloroplast membrane at various stages of greening, we made particle density measurements of the \(EF\) and \(PF\) faces of intermittent-light plastids and of samples from leaves exposed to various periods of continuous illumination. One difficulty in this type of measurement arises because the intramembranous particles are not uniformly distributed between the stacked and unstacked regions of the mature thylakoids (Figs. 4 and 5). In studies of control chloroplasts, we have found 1,466 ± 110 particles per square micrometer on the \(EF_s\) face as opposed to 416 ± 128 per square micrometer particles on the \(EF_u\) face. No significant difference was observed on the \(PF\) face of control chloroplasts (4,520 ± 331 particles per square micrometer on the \(PF_s\) face vs. 4,440 ± 385 particles on the \(PF_u\) face).

In order to obtain particle density values which would average out the differences in particle distribution between stacked and unstacked membrane regions, we have experimentally unstacked the different lamellar samples by washing the membranes in solutions containing low concentrations of cations. It has previously been demonstrated with algal chloroplasts (26) and higher plant chloroplasts (32) that "low-salt" washing leads to the unstacking of thylakoids and to the randomization of particles throughout the membranes. Fig. 6 illustrates thylakoid membranes of an experimentally unstacked control chloroplast whose particles have become evenly distributed as a result of the low salt treatment. Particle samples from plants exposed to various times of continuous-light greening were prepared under low-salt conditions, and particle density measurements were made. Table III shows the results of these measurements. The data reveal that little change in particle densities occurs during plastid greening in continuous light.

**Analysis of Particle Sizes in Stacked Membrane Regions**

Particle size changes have also been investigated during the greening of chloroplast membranes. For these experiments, the plastids were isolated in the presence of 5 mM MgCl₂ to maintain any existing grana stacks. The upper left portion of Fig. 7 shows the particle size histogram for the \(EF\) fracture face of plastids isolated from plants exposed only to intermittent illumination (0 h of...
FIGURE 5 Replica image of freeze-fractured thylakoid membranes isolated from a control pea plant and exhibiting four distinct fracture faces: PFu, PFv, EFu, and EFv. × 76,000.

FIGURE 6 Normal thylakoid membranes (as in Fig. 5) subjected to low cationic strength media to induce experimental unstacking before freezing. Freed from the constraints of the stacked membrane regions, the particles have become evenly distributed on both EF and PF fracture faces. × 70,000.
Table III

Particle Density on Fracture Faces of Experimentally Unstacked Chloroplast Membranes

| Continuous white light | EF fracture face | PF fracture face |
|------------------------|-----------------|-----------------|
| h                      | Particles/μm² ± SE | Particles/μm² ± SE |
| 0                      | 1,213 ± 210 | 5,412 ± 357 |
| 4                      | 1,216 ± 174 | 4,720 ± 481 |
| 8                      | 1,133 ± 119 | 4,735 ± 487 |
| 12                     | 1,128 ± 132 | 4,688 ± 373 |
| 48                     | 1,072 ± 210 | 4,416 ± 401 |

Chloroplast samples were isolated from dark-grown pea plants which had received intermittent light followed by 0, 4, 8, 12, or 48 h of continuous white light.

continuous light. It should be noted that at 0 h of continuous-light greening there was no stacking and hence no EF,F,EF,F differentiation. The particle size histogram of this fracture face shows a single maximum at approx. 80 Å.

In plastid samples which had received 4 h of continuous light after the intermittent-light regime (Fig. 7) it was observed that a proliferation of EF,F particle size-classes had occurred. In addition to the 80-Å histogram maximum present in the 0 h sample, other maxima appeared at 105 Å, 132 Å, and 164 Å (these values represent average maxima obtained from the various greening times indicated in Fig. 7). As time of continuous-light greening was extended (shown in the histograms for samples greened for 8, 12, and 48 h in continuous light; Fig. 7), the peak heights of the histogram changed in relation to one another. In the most highly differentiated sample (48 h of continuous light greening) only two peaks in the particle size classes of the EF,F face were predominant: one at 105 Å, the other at 164 Å (these values are nearly identical to measurements previously reported for the EF,F face of normally grown chloroplast membranes [31, 32]). These data suggest that discrete structural components are being added to a basic 80-Å EF,F particle during greening, resulting in defined size increases of the particle.

The discrete increasing particle size steps from 80 Å to 164 Å allow us to estimate the volume of material being added to an EF “core” particle. For maximum simplicity in these calculations, we have assumed that the particles are regular geometric shapes. That is, we have assumed that the initial 80-Å particle is a sphere in the membrane. We have made the additional assumption that components added to this initial sphere do not significantly change the thickness of the membrane but are added in such a way as to make the resulting particles oblate ellipsoids. (Although we do not mean to imply that the particles must actually be spheres and ellipsoids in the membrane, such simple geometric shapes best approximate the membrane particles. These assumptions are also consistent with a previous analysis of these membranes at various stages of greening in which no change in membrane thickness was detected in thin-sectioning studies (3, and unpublished observations).) Calculations of volumes for an 80-Å sphere and ellipsoids with a minor axis diameter of 80 Å and major axis diameters of 105 Å, 132 Å, or 164 Å are shown in Table IV. It is evident that the change in volume, ΔV, occurs in increments of approx. 215,000 Å³ for the 80 Å → 105 Å and 105 Å → 132 Å increases, and approx. twice that value for the 132 Å → 164 Å diameter increase.

From these calculations we postulate that the basic 80-Å unit has added to it 1, 2, or 4 subunits. (A three subunit addition was not observed.)

Histograms of particle size distributions on the PF,F fracture face (the complementary fracture face of the EF,F face) are shown in Fig. 8. The sample which was exposed only to intermittent illumination (0-h continuous-light sample) displays a single maximum in the histogram with an average particle size of 70 Å (Fig. 8). Examination of the PF,F fracture face for samples taken during continuous-light greening showed only small changes in the particle size histograms for the PF,F face with respect to the 0-h sample; a histogram of the sample exposed to 36 h of continuous light is shown. The only detectable changes in this sample were a slight increase in average size of the particles (77 Å) and a minor shift in the mode of the histogram.

Analysis of Particle Size in Unstacked Membrane Regions

Examination of the EF,F fracture face of plastids exposed to continuous light during greening (Fig. 4) revealed that in addition to a maximal size category at 80 Å, which had been seen in the 0-h sample, a second size class with a maximum at 105 Å developed under continuous light greening (Fig. 9). The 105-Å size class was observed in samples at all stages of continuous-light greening; the 36-h sample is shown here since it was characteristic of nearly fully differentiated lamellae. On the basis of its size, the 105-Å particle of the EF,F face
FIGURE 7 Histograms of particle size classes on the EF-fracture face of membranes isolated from plants grown under intermittent light and the EF$_{fracture}$ face of chloroplast membranes isolated from plants which received the intermittent-light treatment followed by 4, 8, 12, or 48 h continuous white light.

appears to be analogous to the 105-Å particle of the EF$_{fracture}$ face (Fig. 7 and Table IV). It should also be noted that a high percentage of the 80-Å particles were still present in the EF$_{fracture}$ face, even at 36 or 48 h of continuous-light greening.

An analysis of size-classes of particles on the complementary PF$_{fracture}$ fracture face (Fig. 10) revealed that a substantial change occurred during the continuous-light greening. From an initial single size class with a maximum of about 70 Å, an additional maximum at approx. 105 Å appeared after 12 h of exposure to continuous light. After 48 h of continuous-light greening, the PF$_{fracture}$ fracture face demonstrated an increased number of particles in this second size class, but no additional maxima in the particle size classes were noted. We have analyzed the changing size-classes of the PF$_{fracture}$ particle histograms in a manner similar to that used for the EF$_{fracture}$ fracture face; that is, a 70-Å diameter sphere was assumed for the basic unit, and an ellipsoid for the 105-Å particle. The change in volume for the 70 Å $\rightarrow$ 105 Å size
increase (Table V) is similar to that observed for the corresponding size change of \( \text{EF}_s \) particles, i.e., for the addition of one subunit.

**DISCUSSION**

Pea seedlings that are exposed to intermittent illumination develop plastids that have full photochemical competence but that do not have grana stacks and do not contain chlorophyll \( \text{b} \) (3). When these plants are transferred to continuous illumination, the photosynthetic unit size increases dramatically while the relative amounts of several biochemical constituents of the membrane remain constant on a photosynthetic unit basis (Table I). These data suggest that the final stages of membrane differentiation, which occur during seedling growth under continuous illumination, result in the addition of light-harvesting chlorophylls to the otherwise totally functional photosynthetic complexes in the membrane.

A major portion of the light-harvesting chlorophyll in normal chloroplast membranes is thought to be associated with the light-harvesting chlorophyll \( \text{a/b} \) protein (33). We have previously shown that intermittent-light chloroplasts do not contain this pigmented light-harvesting protein (16). In the present study it has been found that the intermittent-light chloroplasts contain almost no polypeptides corresponding to the light-harvesting chlorophyll complex (polypeptides of \( R_M = 0.55 \) and 0.61 in Fig. 1; previously identified as part of the light-harvesting complex in the studies of Anderson and Levine [2]). During greening in continuous light, pea plastids accumulated large amounts of the polypeptides with \( R_M \) of 0.55 and 0.61. In the samples analyzed in Table II, these two polypeptides accounted for approx. 50% of the total protein content of the membrane extract (quantitation based on Coomassie blue staining).

In this paper, we have analyzed the changes in structural organization of the plastid lamellae that occur during the final stages of membrane differentiation in continuous light. Since the increase in

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**Table IV**

*Calculated EF Particle Volumes and Changes in Particle Volumes Which Occurred during Continuous-Light Greening*

| Particle diameter (Å) | Theoretical geometric shape | Calculated vol | \( \Delta V \) | No. of subunit additions |
|-----------------------|-----------------------------|----------------|----------------|-------------------------|
| 80                    | Sphere                      | 268,083        | 0              | 0                       |
| 105                   | Ellipsoid                   | 461,814        | 193,731        | 1                       |
| 132                   | Ellipsoid                   | 729,856        | 461,773        | 2                       |
| 164                   | Ellipsoid                   | 1,126,619      | 858,536        | 4                       |

* The initial 80 Å particle vol was calculated by assuming the particle to be a sphere; other particle vol were calculated by assuming the particles to be oblate ellipsoids with a minor axis diameter of 80 Å, and a major axis diameter of 105 Å, 132 Å, or 164 Å.

† An estimated number of subunit additions is calculated on the basis of a size increase of 80 Å-105 Å corresponds to the addition of one subunit aggregate of light-harvesting pigment protein.

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**Figure 8** Histograms of particle size classes on the PF fracture face of intermittent-light chloroplast membranes (0 h, Fig. 2) and the \( \text{PF}_s \)-fracture face of chloroplast membranes isolated from plants which received 36-h continuous white light after the intermittent-light regime (36 h, Fig. 4).
Particle Diameter (~ Å)

FIGURE 9  A comparison of the particle size histograms of the EF particles of chloroplast membranes from plants grown under intermittent light with the EF\textsubscript{u} particle size histogram of chloroplast membranes isolated from plants which had received 36-h continuous white light after the intermittent-light treatment (36 h).

Particle Diameter (~ Å)

FIGURE 10  Particle size histograms for the PF fracture face in unstacked membrane areas (PF\textsubscript{u}) for chloroplast membranes isolated from plants which had received 0, 12, or 48 h of continuous white light after the intermittent-light treatment.

TABLE V

| Particle diameter (Å) | Theoretical geometric shape | Calculated volume | ΔV |
|-----------------------|-----------------------------|-------------------|----|
| 70  Sphere            | 179,594                     |                   |    |
| 105 Ellipsoid         | 404,087                     | 224,493           |    |

* Geometric shapes of the particles were assumed as described in Table II.

Calculated PF Particle Volumes and Changes in Particle Volumes Which Occurred during Continuous-Light Greening

Changes in Number of Membrane Subunits during Membrane Differentiation

Photonsynthetic unit size which occurred during this period primarily consisted of the addition of a new set of dominant polypeptide species forming the light-harvesting complex, we had expected that substantial changes in structural organization within the membrane must occur concomitant with the insertion of the pigment-protein. These changes were anticipated to be either (a) an increase in the number of intramembranous particles within the membrane (appearance of distinctly new structural components corresponding to the pigment-proteins) or (b) an increase in size of existing structural subunits (corresponding to the addition of the newly synthesized protein to an existing complex). These possibilities are considered below.

Changes in Number of Membrane Subunits during Membrane Differentiation

Particulate subunits observed within the membrane by freeze-fracture analysis are thought to represent protein complexes (12, 13, 29). Analy-
sis of the number of freeze-fracture particles per unit area of the EF face showed a slight but statistically insignificant decrease during membrane differentiation (Table III). The density of PF particles also decreased by a small but statistically significant value. This latter decrease may reflect a change in packing of the particles within the membrane; during the continuous-light greening the increase in size of the EF particles may cause a wider separation of the PF fracture face subunits. Since the total number of particles remained constant, the data indicate that insertion of the new protein during membrane differentiation does not result in the appearance of new, separate morphological entities within preexisting regions of the membrane.

We must also consider another possibility, however. The synthesis of the light-harvesting pigment proteins must be accompanied by further membrane synthesis since membrane surface per plastid increased in continuous-light greening. It would be conceivable that the preexisting membrane particles were simply diluted during new membrane synthesis in continuous light, and that some undetermined factor maintained the number of particles per unit area nearly constant even though new, larger structural units had been formed. If this idea were correct, we should be able to determine the extent of new membrane synthesis on the basis of the magnitude of dilution of preexisting subunits. This is most easily accomplished by examining the histogram data for the EF fracture face (Fig. 7). During the first 4 h of continuous-light greening, the 80-Å subunit size class was diluted from 100% to about 30% of the total EF face particle population; this would correspond to an increase of more than 300% for the total membrane area during these 4 h of greening if new membrane synthesis and the associated particle dilution were solely responsible for the membrane subunit changes. On the basis of measurements of thin-sectioned plastids (described in Results), we can state that this extensive new membrane synthesis did not occur. We have determined that there was no more than a 50% increase in total membrane surface during the first 4 h of greening in continuous light.

Our evaluation of particle numbers per unit area of membrane and the conclusions reached above are valid only if components of the membrane did not aggregate during differentiation. Since the particles of the EF face increased in size, it might be suggested that the new, larger EF, membrane subunits of fully greened membranes are aggregates of preexisting, separate protein subunits. This suggestion can be ruled out on the basis of our protein and photochemical activity analysis, however. Aggregation of one particle type but not the other, i.e., EF subunit aggregation without PF subunit change, would have resulted in dramatic changes in the relative proportions of those proteins associated with the different particle types; the components making up the EF particles should have increased in relative content by 300-400% during the greening process on a unit membrane area basis when compared to the components making up the PF face. Our analysis of membrane constituents (Table I) does not show any major changes in relative content of functional membrane components that would support such an idea. Our protein gel analysis also did not show a three- to fourfold increase in any polypeptide species that would be compatible with this idea.

The data discussed above are consistent with our proposal that the incorporation of the light-harvesting chlorophyll proteins into chloroplast lamellae during continuous-light greening does not lead to a corresponding increase in density of structural subunits within the chloroplast membrane; that is, the pigment protein does not seem to be organized into separate morphological units in a normal, mature membrane. We are therefore left with the possibility that when the new polypeptide is incorporated into the membrane, it associates with certain preexisting intramembranous particles.

Analysis of Changes in Particle Size during Thylakoid Differentiation

The histograms describing particle sizes of the freeze-fracture faces at various stages of continuous-light greening (Figs. 7-10) reveal two main features. First, a major increase in size of nearly all EF fracture face particles, and a minor increase in size of a small proportion of PFu particles. (Although there was a slight increase in the size of the PFu particles, the unstacked lamellar regions only account for about one-third of the total membrane. The total volume change of particles on the PF face is therefore a small fraction of that of the EF face.) Secondly, the change in particle size appears to be gradual and to occur in discrete steps. The EF face of intermittent-light plastids
These data imply a stepwise addition of one, two, or four subunits to the basic 80-Å unit (see Table IV). It should be noted that there were very few particles in the 80-Å size class in the EF\textsubscript{a} fracture face of plastids greened for 48 h under continuous light (Fig. 7), whereas more than one-half of the particles on the EF\textsubscript{a} face at 48 h were in the 80-Å size class (Fig. 9). Arntzen and Briantais (4) have recently summarized evidence suggesting that new membrane components are synthesized and incorporated into the membrane in the unstacked stroma lamellae. We can suggest that the 80-Å subunits of the EF\textsubscript{a} face are incompletely differentiated structural components. This idea can be extended to hypothesize that the fully developed, larger EF subunits (105-164-Å size classes) tend to migrate into and concentrate within the appressed lamellar regions that form the point of membrane contact in grana stacks (thus forming the EF\textsubscript{a} face). It is not yet clear what factors (other than the presence or absence of cations; 4, 26, 32) control the tendency of EF face particles to aggregate or “patch” within certain areas of the membrane. Since this study has shown that the particle aggregation only occurs in membranes containing the light-harvesting pigment protein complex, we conclude that this membrane component is in some way involved in regulating particle distribution and particularly the aggregation of certain categories of particles into stacked membrane regions.

Localization of the Light-Harvesting Complex within the Chloroplast Membrane

In the previous section we have related the incorporation of the light-harvesting pigment protein complex to changes in particle size on the freeze-fracture faces of the differentiating lamellae. To more quantitatively define the localization of the added pigment protein within the membrane, we have summarized the details of our structural analysis in Table VI. For the intermittent-light plastids (0-h sample), and for the plastid sample which had received 48 h of continuous illumination subsequent to an intermittent-light regime, we have listed the average size class maxima for the particles observed, and particle densities for each of the fracture faces. Assuming that the 70, 77, and 80 Å particles were spheres and that 105 or 164 Å particles were ellipsoids, we have used the data of Table VI to calculate the total particle volumes (cubic angstroms) per square micrometers of membrane surface for each fracture face. Since the PF and EF fracture faces are complementary surfaces, we have compared the particle volume values to estimate the percent of total membrane particle volume which is associated with each fracture face. In the case of the plastids greened in continuous light for 48 h, it was necessary to introduce a value for the amount of paired vs. unpaired membranes for calculations of average particle volume over the entire plastid; measurements of thin-sectioned plastids showed 66% of the membrane surface in appressed lamellae (grana) regions in the 48-h sample. The overall conclusion from Table VI is that the total particle volume of subunits on the EF fracture face increased dramatically during the period of light-harvesting pigment protein synthesis. This is true either when the data are considered in an absolute quantitative sense, i.e., a particle volume change from 3.27 to 8.0 x 10^8 Å^3/μm^2 or in a relative sense with respect to the PF face, i.e., a change from 25% of fractional particle volume to 43% (Table VI).

A summary of the hypothesized organization of the light-harvesting complex within the chloroplast membrane is diagrammatically depicted in Fig. 11. Proteinaceous subunits are thought to be embedded within a lipid matrix; the 70-Å particles are more externally localized and cleave with the PF fracture face during freeze-fracture, whereas the more internally localized 80-Å particles cleave with the EF fracture face. In the intermittent-light plastids (0 h), these two main size classes are the only intramembranous particles observed. In the seedlings exposed to 48 h of continuous light, the PF faces still exhibit a sizable number of small (70 Å) particles besides 105-Å particles, whereas the EF faces possess essentially only particles that fall into two larger size classes (105 and 164 Å) and no 80-Å particles. We propose that the 105- and 164-Å EF particles both contain identical 80-Å core particles and that their different sizes are caused by varying amounts of attached light-harvesting pigment-protein complexes (Fig. 11, shaded material). (The membrane model of Fig. 11 is meant to diagrammatically indicate the organization of intramembranous particles in a fused membrane.
### TABLE VI
*Calculations of the Relative Volume Change Which Occurred during the Continuous-Light Greening Process*

| Sample | Fracture face (diameter) | Particle density | Particle vol of fracture face | Σ of vol of given fracture face/μm² | Fractional particle vol |
|--------|--------------------------|------------------|-----------------------------|-----------------------------------|-------------------------|
| 0 h PF | 70                       | 5,412            | 9.72 × 10⁸                  | 9.72 × 10⁸                        | 75                      |
| 0 h EF | 80                       | 1,213            | 3.27 × 10⁸                  | 3.27 × 10⁸                        | 25                      |
| EFₜₙ | 70                       | 2,930            | 5.26 × 10⁸                  | *3.79 × 10⁸*                      | 19                      |
|       | 105                      | 1,510            | 6.10 × 10⁸                  | *7.20 × 10⁸*                      | 38                      |
|       | 105                      | 1,510            | 6.10 × 10⁸                  | *7.20 × 10⁸*                      | 38                      |
| 48 h EFₜₙ | 80               | 241              | 0.65 × 10⁸                  | *0.49 × 10⁸*                      | 31                      |
|       | 105                      | 175              | 0.81 × 10⁸                  | *7.51 × 10⁸*                      | 40                      |
| 164 EFₜₙ | 762                   | 3.52 × 10⁸      | 7.74 × 10⁸                  | 7.74 × 10⁸                        | 43                      |

Samples taken at 0 and 48 h of continuous-light greening are compared with regard to partitioning of total particle vol on the membrane and the present changes which occurred. The major change in percentages is due to an increase in size of the intramembranous particles of the EF face (3.27 × 10⁸ Å²/μm² at 0 h vs. 11 × 10⁸ Å²/μm at 48 h).

* Based on 2/3 fused lamellae, 1/3 unpaired lamellae as determined by measurements of thin-sectioned plastid samples.

The data discussed above demonstrate that an 80-Å core subunit can be found on the EF face of the basic core component of the EF face with little or no attached light-harvesting pigment protein.

A chlorophyll b-less barley mutant has been described which does not contain the light-harvesting pigment protein complex (33). Several studies of the membrane protein composition of this mutant and normal barley chloroplast membranes have been conducted; these studies have recently been reviewed by Boardman et al. (11). It was concluded that the light-harvesting complex of normal barley contains two polypeptides. In the mutant, one of these polypeptides is absent and one is present. It might be suggested, therefore, that the EF particles of the mutant chloroplast membranes would be reduced in size. This has, in fact, been observed in a recent study by Miller et al. (23). They showed that particle size histograms of the EF particles in mutant membranes were very significantly reduced in size as compared to the normal plants.

**Core Subunit of the EF Fracture Face**

The idea that the >80-Å EF particles contain, in part, the light-harvesting chlorophyll a/b protein (as shown in Fig. 11) is consistent with several earlier observations in the literature. The EF fracture face of a chlorophyll b-deficient Chlamydomonas reinhardtii mutant contains fewer large size class particles (17). A soybean mutant which is partially chlorophyll b-deficient was found to contain EF particles which were somewhat reduced in size (20). Euglena chloroplasts, which normally have low levels of the chlorophyll a/b protein (15), have also been found to have reduced size particles on the EF fracture face (24). It should be noted here that Ophir and Ben-Shaul (27) have observed changes in the size of intramembranous particles of the EF face (B face in the earlier terminology) during greening of Euglena chloroplasts. We can now suggest that those authors also may have been observing additions of light-harvesting chlorophyll a/b protein to the differentiating lamellar membranes, and can interpret these observations as showing the presence of the grana stack region. For that reason, no 105-Å particles are indicated for the particles associated with the PF face.

The data presented show that an 80-Å core subunit can be found on the EF face of the basic core component of the EF face with little or no attached light-harvesting pigment protein.
Particles associated with the
PF face after freeze-fracture

Particles associated with the
EF face after freeze-fracture

Core of EF particle (photosystem II)

Light harvesting complex

Figure 11 A proposed model for the particulate subunits of the chloroplast lamellae. In plastids from plants grown under intermittent light (0-h sample), 75-Å and 80-Å particles are embedded in a lipid phase of the membrane; the more externally localized 75-Å particles cleave with the PF face during freeze fracture, whereas 80-Å particles cleave with the EF face. During greening in continuous light (48-h sample), the light-harvesting pigment protein (shaded material) is inserted into the membrane and primarily associates with the 80-Å "core" unit of the EF face. The total aggregate (80-Å core unit plus various amounts of light-harvesting pigment protein) results in EF particles of discretely variable size classes. Particle spacing in this diagram conforms to average separation distances measured on freeze-fracture micrographs.

Undifferentiated pea thylakoids, and that the light-harvesting pigment protein is added to this core subunit during greening. While we have no direct biochemical evidence for the identity of the core unit, the following lines of evidence suggest that it could be the photosystem II reaction center complex. It is thought that the light-harvesting chlorophyll a/b protein binds all of the chlorophyll b in the membrane (33). It is generally agreed that chlorophyll b primarily sensitizes photosystem II (18). Detergent fractionation studies have indicated that the chlorophyll b-containing protein is physically associated with the photosystem II complex; both digitonin and Triton X-100 solubilization of chloroplast membranes release “heavy” submembrane fragments which are enriched in photosystem II activity and in chlorophyll b (1, 9, 10, 34, 35). Arntzen et al. (5) demonstrated that these detergent-derived submembrane fragments are also enriched in particles that appear structurally identical to those of the EF fracture face. They concluded that the EF particles were “markers” of photosystem II activity. We now add further support for this hypothesis by suggesting that the EF fracture face particles consist of an 80-Å photosystem II core complex onto which may be added 0, 1, 2, or 4 aggregates of light-harvesting chlorophyll a/b protein. It should be noted that the aggregation of the >80-Å EF particles into regions of stacked membranes (EF, faces) follows
insertion of the pigment protein complex into the membrane (Figs. 3 and 4). This is consistent with the observations that isolated grana (stacked lamellae) are enriched in chlorophyll $a$ and photosystem II activity, whereas isolated stroma lamellae have few EF particles, low photosystem II activity and high chlorophyll $a/b$ ratios (4, 6, 30).

It was previously suggested that the small, densely packed particles of the PF face may correspond to the morphological equivalent of the photosystem I complex (4). This concept is compatible with the present study, since little chlorophyll $b$ or light-harvesting chlorophyll $a/b$ protein was previously found to be structurally associated with photosystem I in detergent fractionation studies (1, 9, 34, 35), and we have found only a small increase in the size of the average particle of the PF face during plastid greening in continuous light, with the increase being confined to a certain proportion of particles that are found only in $P_F^a$ regions. This increase in size of some $P_F^a$ particles may mean that some light-harvesting chlorophyll $a/b$ protein associates with the photosystem I complex in these unpaired lamellae, perhaps during membrane assembly. Alternatively, the 105-Å subunits on the $P_F^a$ face could conceivably represent a new but minor form of the total photosystem I components which reside only in unstacked lamellar regions.

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Armond, Staehelin, and Arntzen Spatial Relationships in Chloroplast Membranes 417
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