ANALYSIS OF THE THYLAKOID OUTER SURFACE

Coupling Factor is Limited to Unstacked Membrane Regions

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ABSTRACT

The structure of the spinach thylakoid outer surface has been examined by deep-etching, a technique which exposes the true surfaces of biological membranes by sublimination of frozen dilute buffer. The membrane surface is covered with large (150 Å average diameter) and small (90 Å average diameter) particles. Approximately 30% of the large particles can be removed under conditions reported to selectively remove carboxydismutase from the membrane surface. The remaining large particles can be removed only under conditions which cause a loss of coupling factor activity. When purified coupling factor is readded to membranes from which all coupling factor activity has been removed, large particles reappear, indicating that they represent coupling factor molecules. Since the number of particles and the amount of ATPase activity in the reconstituted and control membranes were the same, coupling factor molecules may be attached to specific binding sites. Analysis of antibody labeling experiments, enzyme assays, and experiments involving the unstacking and restacking of thylakoid membranes indicate that coupling factor is excluded from regions of membrane stacking (grana) and is present only in unstacked membrane regions. The exclusion of coupling factor from grana, which are known to be centers of intense photosynthetic activity, strongly suggests that the mechanism coupling electron transport to photophosphorylation is indirect. In addition to the large and small particles, in some cases regularly spaced ridges are visible on the outer surface after unstacking. Coupling factor binding sites seem to be excluded from regions where these structures occur.

The elements of the energy-transducing light reaction of photosynthesis are known to be bound to the thylakoid membranes found within chloroplasts of green plants and algae. The structure of these membranes has been the subject of a number of recent reviews (3, 18, 31, 39), and a consistent feature of the thylakoid has proven to be the presence of particulate subunits both within the membrane and at its surface. Although some subunits can be visualized in negatively stained (10, 12, 30, 32, 33) and thin-sectioned material (35), the greatest wealth of structural information about the thylakoid has been derived from the use of freeze-fracturing and freeze-etching techniques. It is now well established that when frozen biological membranes are fractured, and a replica quickly made of
the frozen surface, two roughly complementary fracture faces are formed by the splitting of individual membranes along an internal plane (4, 5, 6). The particles on such fracture faces are believed to represent structural equivalents of the integral membrane proteins of the fluid mosaic membrane model (40, 41). In contrast, when membranes frozen in distilled water or a suitably dilute buffer are “etched” by allowing the sublimation of ice from the frozen surface for several minutes or seconds after freeze fracturing, the true surfaces of the membranes can be exposed and examined in the replica (6, 37, 38) as well as the fracture faces.

Although a complete analysis of all the available information has not been completed, combining the results of freeze-fracturing and etching permits the thylakoid membrane to be examined from four aspects: two complementary internal fracture faces and the two true membrane surfaces. Studies examining the four images of the membrane which can be obtained in this way show that distinct classes of particles exist on each surface and fracture face (10, 26, 38).

Several recent reports have indicated that the presence of a large particle on the thylakoid outer surface can be correlated with chloroplast coupling factor 1 (CF1) activity (2, 10, 26, 27, 35). The CF1 is an enzyme thought to couple electron transport in the thylakoid membrane to the synthesis of ATP (14, 17, 24). However, in none of these studies was an attempt made to distinguish between CF1 and carboxydismutase molecules, which are also known to be associated with the external surface of the membrane (16). This report examines the structure of the thylakoid outer surface in greater detail, provides for a distinction between CF1 and carboxydismutase and coupling factor molecules, and demonstrates that the CF1 particle is confined to unstacked regions of the thylakoid outer surface.

MATERIALS AND METHODS
Isolation of Chloroplasts

Spinach leaves were obtained fresh from a local market, devenined, and macerated in isolation buffer (either 300 mM sucrose, 5 mM Tris/HCl pH 7.6; or 300 mM sucrose, 10 mM sodium pyrophosphate, pH 7.5) in a Waring blender for 15-20 s. The cell homogenate was passed through several layers of gauze, then centrifuged for 2 min at 200 g. The pellet from this centrifugation was discarded. The supernate was then centrifuged at 3,000 g for 10 min, the supernate discarded, and the pellet resuspended. This suspension was centrifuged at 3,000 g for 10 min. The resulting pellet contained nearly pure chloroplasts, and was shown by electron microscopy to be free from contamination by mitochondria and other cell organelles. All these operations were carried out at 4°C.

Unstacking and Restacking of Thylakoids

The techniques used for unstacking and restacking isolated chloroplasts are modifications of methods first reported by Izawa and Good (13). For the unstacking of isolated thylakoids, the membranes were washed several times in 50 mM Tricine-NaOH pH 7.6 over the course of an hour at room temperature. Membranes unstacked by the procedure could be restacked by suspension in “TM” buffer (2 mM MgCl2, 5 mM Tris pH 7.5). The restacking process was allowed to proceed over the course of an hour at room temperature.

Freeze-Etching

Membrane samples for etching experiments were prepared by suspending thylakoids in either TM buffer (stacked membranes) or 5 mM Tris pH 7.5 (unstacked membranes) before freezing. Fixation of membranes by 3% glutaraldehyde for 30 min before freezing made no difference in the appearance of the outer surfaces of stacked membranes. However, unstacked membranes (in which the attachment of the CF1 to the outer surface could not be stabilized by the addition of divalent cations) were routinely fixed for 30 min in 3% glutaraldehyde before resuspension in dilute (5 mM) Tricine-NaOH pH 7.6 to ensure that CF1 molecules would not be removed from the surface by action of the dilute buffer. Control experiments showed that this procedure prevented loss of membrane surface particles during resuspension in the dilute buffer. After the pretreatment steps, individual droplets of the suspensions were placed on copper specimen support disks, frozen in liquid freon 12, and transferred to liquid nitrogen for storage.

Replicas were prepared at −100°C according to the method of Moor and Mühlethaler (29) on a Balzers freeze-etching apparatus. For etching, the cold microtome knife was placed over the specimen when fracturing was completed to increase the rate of sublimation and to prevent contamination of the exposed surface. After 2-5 min of etching in this manner, the temperature of the specimen stage was reduced to −104°C, the knife removed to retard further sublimation, and the frozen surface replicated with platinum and carbon. Successful replicas were cleaned in bleach and 40% chromic acid, and examined in a Philips 300 electron microscope.

Thin Sectioning

For thin sectioning, isolated chloroplasts were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer for 30 min,
washed twice in the buffer, and postfixed in 2% aqueous osmium tetroxide for 90 min, or processed according to the method of Oleszko and Moudrianakis (35). After repeated rinses in distilled water, the material was dehydrated through an acetone series and embedded in either Epon-Araldite or Spurr's plastic, and polymerized under vacuum at 60°C. Sections were cut on a Porter-Blum MT-2 microtome, and examined in a Philips 300 electron microscope.

**Preparation of Antisera**

CF₁ was purified from dilute EDTA washes of isolated chloroplast membranes as described by Lien and Racker (20). A specific antiserum to the CF₁ was prepared according to methods developed by Kannangara, van Wyk, and Menke (16). Immunoglobulin was prepared from this antiserum according to standard techniques. (9) Double-diffusion tests of the antiserum, immunoglobulin, and of crude and purified samples of the CF₁ were performed according to the method of Ouchterlony (36).

**Washing and Reconstitution Experiments**

For some experiments, isolated chloroplasts were prepared according to methods described by Strotmann, Hesse, and Edelmann (44). These chloroplasts were washed five to seven times in 10 mM sodium pyrophosphate pH 7.6. This procedure results in total removal of carboxydismutase (ribulose-1,5-diphosphate carboxylase) from the membrane surface, according to these authors. Thylakoids so treated were then washed three times in 300 mM sucrose, 2 mM tris pH 7.5. This procedure was found to cause a near complete loss of CF₁ activity from the membrane sample.

Membranes from which the CF₁ had been removed were washed several times in TMC buffer (5 mM Tris, 2 mM MgCl₂, 5 mM CaCl₂ pH 7.5). To a sample of these membranes containing 1 mg of chlorophyll was added 5 mg of purified CF₁ which had been dialyzed overnight against TMC buffer. This mixture was allowed to mix gently for 1 h at room temperature. Membranes reconstituted in this manner were washed five times in large volumes of the buffer in order to remove all soluble or loosely bound protein, and were then analyzed microscopically and biochemically.

**Ferritin Labeling**

Suspensions of isolated thylakoids containing 500 μg of chlorophyll were mixed with 40 μg of immunoglobulin prepared against the CF₁. Control samples were prepared with an identical amount of nonspecific immunoglobulin, and all samples were allowed to mix gently at room temperature. Excess antibody was removed by four repeated washes in the same buffer, and the membranes were prepared for thin sectioning or etching as described.

**Measurements**

For measurements of particle density, 10 micrographs of each sample were selected on which particles were easily recognized, and particles within a relatively flat area 1 μm square were counted. The micrographs used for this purpose were enlarged to 100,000 ×. Measurements of particle diameter were made on similar micrographs enlarged to 150,000 ×. Particle diameters were determined through a magnifying ocular with a measurement grating calibrated in 0.1-mm steps. The percentages given for surface areas in stacked and unstacked regions were determined from 10 micrographs of each sample type where lengths of stacked and unstacked membrane profiles were measured with a map reader.

**Analytical Methods**

Protein was estimated according to the method of Lowry et al. (22). Chlorophyll was estimated according to the method of MacKinney (23). The activity of the CF₁ was expressed as a specific activity for the membrane sample in question. A unit of enzyme activity was defined as the amount of the enzyme required to release 1 μmol of inorganic phosphate per minute at 37°C. For membrane samples, the specific activity was defined as units of enzyme activity per milligram of chlorophyll. Activation and ATPase assays of the CF₁ were carried out as described (20).

**RESULTS**

**Sequential Removal of Carboxydismutase and CF₁**

The appearance of the thylakoid outer surface is illustrated in Fig. 1. It is covered with numerous larger particles (130–180 Å), with smaller particles (70–110 Å) frequently visible in the spaces in between. These smaller particles, because they are often obscured by the larger ones, are difficult to count and measure in preparations such as this, and therefore we have not attempted such analyses. The measured sizes and distributions of the larger particles have been determined, however (see Fig 5 a and Table 1). The surface which these measurements and Fig. 1 illustrate is similar in most respects to the image of the thylakoid outer surface reported by other workers (2, 10, 37, 38). Only stromal, or unstacked, membrane outer surfaces can be observed in thylakoid preparations, since the close appression of another thyla-
FIGURE 1  The outer surface of the spinach thylakoid membrane, washed in the dilute "FM" buffer, and defined as control. The surface is covered with large particles averaging 150 Å in diameter, at a concentration of 1,051 ± 143 particles per µm². Smaller particles averaging 90 Å in diameter are also visible (compare with particle size histogram, Fig. 5 a). × 100,000.

FIGURE 2  Thylakoid outer surface after repeated washes in 10 mM sodium pyrophosphate pH 7.6 as described in text, a treatment reported to selectively remove the enzyme carboxydismutase from the membrane surface. The same size classes of particles as in Fig. 1 are visible, but the concentration of large particles has now been reduced to 710 ± 67 per µm², indicating a possible loss of enzyme particles from the thylakoid surface (compare with particle size histogram, Fig. 5 b). × 100,000.
TABLE I

Distribution of Large Particles on the Thylakoid Outer Surface

| Preparation                  | Particle density (per μm²)* |
|------------------------------|----------------------------|
| Untreated (see Fig. 1)       | 1.051 ± 143*               |
| Pyrophosphate washed (Fig. 2)| 710 ± 67I                  |
| Sucrose washed (Fig. 3)      | 26 ± 18                    |
| Reconstituted (Fig. 4)       | 674 ± 110                  |

* ± Standard error.
† A t test of these two values yields a result of t = 6.833. Probability of a larger value for the data is P < 0.001.

TABLE II

ATPase Activities of Various Membrane Preparations (Mean of Four Measurements)

| Preparation                           | Specific activity* (μmol of inorganic phosphate released per mg of chlorophyll per minute at 37°C) |
|---------------------------------------|--------------------------------------------------------------------------------------------------|
| Untreated (Fig. 1)                    | 5.15 ± 0.67                                                                                        |
| Pyrophosphate washed (Fig. 2)         | 5.33 ± 0.81                                                                                        |
| Sucrose washed (Fig. 3)               | 0.56 ± 0.08                                                                                        |
| Reconstituted (Fig. 4)                | 4.94 ± 0.90                                                                                        |
| Untreated + 10 mg immunoglobulin      | 0.08 ± 0.10                                                                                        |
| Untreated + 10 mg nonimmune serum     | 5.28 ± 0.94                                                                                        |

* Reaction mixture consisted of a membrane sample containing 10 μg of chlorophyll activated as described by Lien and Racker (20); 0.8 ml of a solution containing 40 mM Tricine, 10 mM CaCl₂, 10 mM ATP, pH 8.0; and Tricine-NaOH buffer, 0.02 M, pH 8.0, added to bring the final volume of the sample for assay to 1.0 ml.
† ± Standard error.

Grana regions obscure the outer surfaces of such regions. Particles covering the surface of an active membrane such as the thylakoid might be expected to have an enzymatic function, but of what kind? Strotmann, Hesse, and Edelmann (44) have reported a technique for washing spinach thylakoid membranes in such a way as to specifically remove certain proteins from the surface of the membrane. They found that both carboxydismutase and CF₁ could be removed from the membrane by washes of low osmotic strength, but the coupling factor could be removed from the membrane only if the washes were of low ionic strength. They then reasoned that these enzymes could be specifically removed from the membrane by developing a procedure where the membranes were first washed in a buffer of low osmotic (but relatively high ionic) strength, and then washing them in a buffer of high osmotic (but low ionic) strength. They report that repeated washes in 10 mM sodium pyrophosphate pH 7.6 removed carboxy dismutase (and a small amount of low molecular weight material) from the membrane, and that when this wash was followed by washes in 300 mM sucrose, 2 mM Tricine pH 7.5, nearly pure CF₁ could then be selectively removed from the membrane surface. We have used this procedure to confirm their observations, and have followed structural changes at the membrane surface as the removal takes place.

Fig. 2 shows the thylakoid outer surface after removal of carboxydismutase by repeated washes in sodium pyrophosphate. This procedure causes no loss in CF₁ activity (measured as Ca²⁺ dependent ATPase activity), but nevertheless there is a reduction in the number of large particles visible on the outer surface by nearly 30% (see Table II). The smaller particles on the membrane surface are more clearly visible now that some of the larger particles have been removed.

After the thylakoids have been washed in 300 mM buffered sucrose as described, the appearance of the outer surface is radically altered, as seen in Fig. 3. Virtually all of the large particles are removed by the treatment (see Table I and Fig. 5 c) and the measurable ATPase activity of the membrane preparation has been all but eliminated. Only the smaller particles (which we have not been able to remove from the membrane surface) and a very few large particles remain. With the removal of the large particles it is possible to easily perform particle size measurements of the small particles, which have an average particle diameter of 90 Å (Fig. 5 c). However, even in preparations where all large particles have been removed it is difficult to make accurate measurements of their distribution because of their small height, which makes them visible only at very low shadowing angles. By analyzing relatively small regions of the membrane, we have estimated their concentration at 1,500 particles/μm², but this is only a preliminary value. In summary, although approximately 30% of the large particles visible on the outer surface can be removed without loss of measurable ATPase activity, the removal of the remaining 70% is accomplished by the nearly total loss of ATPase activity.
FIGURE 3 Thylakoid outer surface after repeated washes in sodium pyrophosphate buffer followed by washes in 300 mM sucrose 2 mM Tris pH 7.5, a treatment which removes virtually all measurable coupling factor activity from the membrane. The large particles which were visible in Figs. 1 and 2 are now almost totally removed, and only the smaller 90 Å particles remain (compare with particle size histogram, Fig. 5c). × 100,000.

FIGURE 4 Thylakoid outer surface treated as described for Fig. 3 to remove all CF, activity and large particles, and then "reconstituted" by the addition of purified CF. ATPase activity is restored by this procedure, and the large particles reappear as well. × 100,000.
Changes Associated with Reconstitution of CF₁ Activity

Procedures for the complete purification of the CF₁ have been described (20) and we have prepared purified CF₁ according to this method. When thylakoid membranes from which the CF₁ has been removed are incubated with purified CF₁ in the presence of divalent cations, the protein becomes reassociated with the membrane, and membrane-bound ATPase activity reappears (21). Fig. 4 illustrates the appearance of the spinach thylakoid outer surface after a membrane preparation similar to that shown in Fig. 3 (that is, from which all ATPase activity had been removed) had been "reconstituted" by the addition of purified CF₁ in the presence of divalent cations. Clearly, the readdition of CF₁ causes a reappearance of the large particles on the thylakoid outer surface. The fact that the reconstitution of CF₁-depleted thylakoid results in a reappearance of the large particles has been reported by other workers (10, 35), and we regard these reports as good evidence for the actual identification of these large particles as the CF₁.

Histograms of particle sizes (Fig. 5), measurements of particle distributions (Table I), and assays of membrane-bound ATPase activity agree well with our observation that a subset (approximately 70%) of the large particles on the thylakoid outer surface can be identified as CF₁ molecules. Furthermore, the fact that both the specific activity and particle density of the CF₁ reconstituted membranes are nearly the same as those of the pyrophosphate-washed membranes indicates that the CF₁ molecules become reattached to specific binding sites.

Antibody Labeling Experiments

We have prepared an antiserum to the CF₁ by standard immunological techniques, and have established the specificity of the antiserum by diffusion tests against pure and mixed antigens (see Fig. 6). Addition of this antiserum to membrane preparations results in a complete inhibition of ATPase activity (see Table I), and in the agglutination of isolated chloroplasts (see Figs. 7 and 8) in agreement with the results of Kannangara, van Wyk, and Menke (16). In an effort to determine precisely the location of the CF₁ on the thylakoid membrane, we have localized the binding of the antiserum with a ferritin-labeled antibody. Fig. 9 illustrates the appearance of the thylakoid outer surface after application of the antiserum and the ferritin label. The entire membrane outer surface is covered with ferritin molecules, indicating clearly the presence of the CF₁ antigen on this surface of the membrane, but preventing any direct observation of what structures the label might be bound to on the membrane. The intensity of labeling is not surprising, since each individual CF₁ may present several available antigenic sites (19), and in addition the indirect labeling technique might cause a "piling up" of label on each CF₁ antibody.

A similar preparation examined in thin section is shown in Fig. 10. The ferritin label is clearly present on the external membrane surface throughout much of the area shown in the micrograph, but it is not present in regions of membrane stacking (grana). If the thylakoid membranes used for the experiment are first unstacked by repeated washings in a low-salt buffer according to the method of Izawa and Good (13), no regions of membrane contact are present, and the label is evenly distributed along the surface of the mem-

Figure 5 Histograms of particle size measurements made from the thylakoid preparations illustrated in Figs. 1-4. (A) Untreated; (B) Pyrophosphate washed; (C) Pyrophosphate and sucrose washed; (D) Reconstituted.
brane (Fig. 11). Berzborn et al. (2), in apparent contrast to these results, observed that an antiserum to the CFt caused a clumping of the large particles on the membrane outer surface. In our experiments, such aggregation was not observed, probably because a very high concentration of immunoglobulin was employed which may have precluded cross-linking by saturating antigenic sites on the CFt. At first glance, these results suggest that the CFt might be excluded from regions of membrane stacking. But it is also possible that molecules of CFt present in stacked regions are not accessible to the ferritin label, and can therefore only be labeled in unstacked preparations. In an effort to answer this important question concerning the distribution of CFt molecules on thylakoid membranes, we have examined the appearance of the thylakoid outer surface after unstacking the membranes in a low-salt buffer.

**Effects of Thylakoid Unstacking and Restacking on CF Distribution**

When chloroplast membranes are suspended in a buffer of low ionic strength such as Tris or Tricine, the characteristic regions of membrane contact are lost, and the membranes become unstacked (11, 13, 34). The process of unstacking is an important tool in analyzing the location of particles found on the thylakoid surface, because it makes observable those regions of the thylakoid surface which previously were involved in stacking. If CFt molecules are indeed present in stacked regions as well as in unstacked regions, then the process of membrane unstacking should have little or no effect on the distribution of particles observed on the membrane outer surface. If, however, the CFt particles are excluded from regions of membrane contact, then a reduction in the overall density of CFt particles should be observed.
as the surface components of stacked and unstacked regions become intermixed.

When membrane preparations are frozen for etching experiments, they are usually suspended in a very dilute (7 mM or less) buffer. For stacked preparations, this buffer contained 2 mM Mg++, which stabilizes both the stacked configuration of the membranes and the attachment of the CF₁ to the membrane surface. But the inclusion of divalent cations in the buffer of an unstacked preparation, while it would stabilize the attachment of the CF₁, would begin to bring about a rapid restacking of the membranes and would therefore not be suitable. Conversely, the suspension of unstacked membranes in a dilute buffer lacking divalent cations, while it would preserve the unstacked configuration, results in a loss of ATPase activity and a removal of large particles from the membrane surface (Our experiments indicate that routine unstacking experiments in 50 mM Tricine caused no loss of CF₁ activity, although suspension in a 5 mM Tricine buffer did cause a reduction of activity). To circumvent these problems we found it necessary for these experiments first to fix the membranes in glutaraldehyde, then to suspend the fixed preparation in a dilute buffer before freezing. This process preserves the unstacked configuration and also cross-links the CF₁ particles to the membrane surface, preventing their removal in dilute buffer. A series of control experiments examining the effect of this procedure on the sizes, number, and distribution of particles on the thylakoid outer surface showed that the fixation procedure had no effect on the appearance of the outer surface, and that the procedure protected the CF₁ molecules from removal by dilute low-salt buffer.

Fig. 12 shows the effect of unstacking on the thylakoid outer surface. The apparent density of the large particles on the outer surface is greatly reduced, although the ATPase activity of the membrane preparation is unaltered. The sizes of the particles are unchanged (Fig. 14 a), and the
FIGURE 10 Thin section of an isolated spinach chloroplast incubated with ferritin-labeled antibody against the CF₁. Membrane outer surfaces (arrows) are labeled intensively with ferritin, but where those surfaces come into contact in regions of stacking (grana) no label is present. × 87,000.

FIGURE 11 Thin section of an isolated spinach chloroplast, unstacked by washing in low-salt buffer, then incubated with ferritin-labeled antibody against the CF₁. The label is now spread evenly along the outer surface of the membrane, and no areas of label exclusion are apparent. × 87,000.
FIGURE 12 Thylakoid outer surface after unstacking of the membranes in low-salt buffer. This procedure causes no loss in CF$_1$ activity, but the apparent density of the large particles has been reduced to $261 \pm 33$ particles per $\mu$m$^2$. $\times$ 100,000.

FIGURE 13 Thylakoid outer surface, prepared as described for Fig. 12, but then treated with divalent cations to restore the stacked configuration. The particle density is increased by this procedure to $690 \pm 116$ particles per $\mu$m$^2$, and there is no change in CF$_1$ activity. $\times$ 100,000.
reduction in apparent density of particles is consistent with the idea that the entire complement of coupling factor is limited to the surfaces of unstacked membrane regions. When the entire thylakoid system is unstacked, these particles seem to possess sufficient lateral mobility within the plane of the membrane to diffuse into previously stacked regions, thereby lowering their apparent density.

The effect of unstacking on the distribution of CF\textsubscript{1} molecules is reversible, as shown in Fig. 13, which illustrates the appearance of the thylakoid outer surface after membrane restacking has taken place by the addition of 2 mM Mg\textsuperscript{2+} to the preparation. The original density of large particles on the outer surface is restored after complete restacking has been achieved (Table III), and the process of restacking brings about no change in ATPase activity.

Measurements made on thin sections of the percentage of total membrane surface area involved in stacking fit very well with the interpretation that the drop in apparent density of large particles on the outer surface is due to lateral movements of CF\textsubscript{1} particles into the stacked regions of the membrane system, from which they were previously excluded (see Table IV). Unstacking and restacking of spinach chloroplasts also causes no changes in membrane-bound ATPase activity (Table III).

**Exclusion of Coupling Factor from Specific Regions of Experimentally Unstacked Membranes**

Occasionally, certain unique surface structures are visible on the thylakoid membrane, as shown in Fig. 15. These take the form of regular striations occurring at a spacing of approximately 75 Å. These structures were not present in most of our preparations, but in others they appeared regularly, although only in unstacked material. These structures have been observed previously (37), and are related to structures within the membrane itself (25). Of particular interest to this study is the fact that CF\textsubscript{1} molecules are excluded from regions of

**TABLE III**

| Preparation  | ATPase specific activity (mean of four measurements)* | Particle density (per µm\textsuperscript{2})f | Percentage of membrane surface in stacked regions* |
|--------------|-----------------------------------------------------|---------------------------------------------|--------------------------------------------------|
| Stacked      | 5.33 ± 0.81                                         | 710 ± 67                                    | 64.6 ± 8.4                                       |
| Unstacked    | 5.78 ± 0.94                                         | 261 ± 33                                    | 0                                                |
| Restacked    | 5.44 ± 0.62                                         | 690 ± 116                                   | 59.1 ± 7.0                                       |

* Standard error.
† Large (greater than 120 Å in diameter) particles only.

**TABLE IV**

**Corrected Calculations of CF\textsubscript{1} Distribution**

| Preparation | Particle density (per µm\textsuperscript{2})* | Fraction of unstacked membranes† | Corrected particle density (per µm\textsuperscript{2})f |
|-------------|------------------------------------------------|---------------------------------|-------------------------------------------------------|
| Stacked     | 710                                            | ×                               | 251.34                                                |
| Unstacked   | 261                                            | ×                               | 261.00                                                |
| Restacked   | 690                                            | ×                               | 282.21                                                |

* Mean values for CF\textsubscript{1} particles, from Table III.
† Fraction of total membrane surface area in unstacked regions.
‡ Values in this column indicate the number of CF\textsubscript{1} particles per µm\textsuperscript{2} of total membrane surface area.
such arrays, indicating that the actual binding site for the CF₁ may be a discrete structure at the membrane surface which is displaced by the formation of these regular arrays. The conditions which cause the formation of these arrays are not understood.

Exclusion of CF₁ from Certain Regions of Partially Unstacked Membrane Preparations

Fig. 16 shows part of a thin section of isolated chloroplasts partially unstacked by a single, brief (30 min) wash in 50 mM Tricine buffer at 4°C. This sample was processed according to the method of Oleszko and Moudrianakis (35) and CF₁ molecules are visible as discrete particles on the surface of the thylakoids. Unstacking is not complete in this preparation, and in areas where stacked thylakoids have apparently just begun to separate (see Fig. 16), the newly unstacked membranes can be observed free of any surface particles. If particles were present in stacked regions, upon stacking, they might be expected to become at once apparent. Instead, these newly unstacked regions are apparently devoid of particles visible in thin section.

DISCUSSION

Identification of Carboxydismutase and CF₁ Molecules

The picture of the thylakoid outer surface presented here is in general a more complicated one than other studies have indicated. Two classes of large particles, each removable by a separate washing procedure, a complement of smaller particles, and occasionally small, regular arrays of ridges are each observable on the outer surface. The distribution of at least one of the classes of large particles seems restricted to the noncontacted regions of the thylakoid surface.

It seems quite likely that the subset of large particles removable by washing in sodium pyrophosphate may represent individual molecules of carboxydismutase. Particles of similar size have been reported in association with thylakoid mem-

![Figure 15](image_url)

**Figure 15** Thylakoid outer surface showing the rows of ridges which appear inexplicably in some preparations of chloroplast membranes. These ridges usually occur in rows as shown, and show a spacing of 75 Å. These structures protrude only slightly from the membrane surface, and are visible only at low shadowing angles. Arrows mark two such rows on the micrograph. × 100,000.
FIGURE 16 Thin section thru a preparation of isolated chloroplast membranes partially unstacked by brief suspension in low-salt buffer. Although CF₁ particles (cf) are clearly visible over much of the thylakoid surface, several regions where the membranes appear to be in the process of separation are devoid of particles (arrows). This indicates an absence of particles from stacked regions. × 120,000.

branes (7), other workers have presented immunological evidence for the presence of this enzyme on the thylakoid surface (16), and its removal by pyrophosphate washings is consistent with the findings of Strotmann et al. (44) that such washings remove carboxydismutase. Exactly why such a small proportion of the total carboxydismutase complement of the chloroplast should be bound to the thylakoid surface is unclear. Nevertheless, in contrast to other observations (10, 35), these results indicate that a fraction of the large particles on the thylakoid outer surface cannot be identified with CF₁, and is removable under conditions reported to remove carboxydismutase.

There are substantial differences between our values for particle density on the outer surface and those recently reported by Berzborn et al. (2). We find it likely that differences in washing procedures applied before fixation or freezing could remove different amount of carboxydismutase molecules from the membrane surface. As pointed out by others (16), only a small fraction of the total chloroplast carboxydismutase is membrane-bound and, depending on the ionic composition of the washing media, different proportions of the molecules could become detached from the membranes. Since the membrane-binding parameters of carboxydismutase are still poorly defined compared to those of the CF₁ (44), the actual extent of binding of carboxydismutase to thylakoids remains unresolved. Further elucidation of the functional significance of membrane-bound carboxydismutase is definitely needed.

On the basis of results presented here and
elsewhere, the CF\(\text{t}\) can be identified as the complement of large particles remaining after the removal of a subset of large particles (probably carboxydismutase) from the thylakoid outer surface. We regard the most conclusive evidence in this regard to be the fact that the readdition of purified CF\(\text{t}\) results in a reappearance of the large particles on the outer surface. Identical results were reported earlier by Oleszko and Moudrianakis (35) and Garber and Steponkus (10), although the former did not use purified coupling factor, and in neither case were both the ATPase level and particle density restored to control levels.

**Distribution of Coupling Factor Molecules**

If we consider the identification of CF\(\text{t}\) particles to be a settled question (reasonable, we think, in light of the evidence), interest then turns naturally to its distribution over the expanse of the thylakoid membrane outer surface. Ferritin labeling of stacked and unstacked thylakoids (see Figs. 10 and 11) seems to indicate that no coupling factor is present in regions of membrane stacking. But these results can also be interpreted as indicating that CF\(\text{t}\) is indeed present in stacked regions, but no label can penetrate in stacked systems because of the close appression of adjacent thylakoids. Indeed, some have suggested (35) that CF\(\text{t}\) present on the membrane surface may be pushed deeper into the membrane matrix by the stacking process, and therefore may be distributed over the entire expanse of the membrane system. These workers predict that such a process would alter the internal fracture faces of the thylakoid membrane, and suggest that searching for such alterations would be a useful area of inquiry.

We believe that the data presented in this paper, together with the observation of Ojakian and Satir (34) that the size categories of fracture face particles are preserved when thylakoids are experimentally unstacked, render such a point of view untenable. Our work, in agreement with that of others (16), shows that all measurable CF\(\text{t}\) activity of a stacked membrane preparation can be inhibited by the addition of specific antiserum (see Table II). This means that all active CF\(\text{t}\) molecules are at least accessible to the antiserum, if not to antiserum coupled with ferritin. We must consider the possibility, however, that CF\(\text{t}\) in stacked regions may be inactive, or, more importantly, may not be accessible to activation procedures used for the ATPase assay used to measure CF\(\text{t}\) activity.

If this were the case, then unstacking the thylakoid membranes should expose new sites for ATPase activity by making the enzyme molecules which previously were unavailable to either the antiserum or ATPase assay procedure. Repeated experiments comparing the measurable ATPase activity in stacked, unstacked, and restacked membranes, however, have failed to detect any differences in ATPase activity (Table III). We must conclude: (a) that all CF\(\text{t}\) molecules contribute to the measurement of ATPase activity, since no new activity appears upon unstacking; and (b) that all CF\(\text{t}\) molecules are accessible to the antiserum prepared against them, since total inhibition of ATPase activity by the antiserum is observed.

One possible difficulty with this finding is that our methods of measuring coupling factor activity involve incubation of the activated enzyme in a Ca\(^{++}\)-containing buffer solution (20). Ca\(^{++}\) promotes membrane restacking, and this fact casts doubt on the measurements of ATPase activity in unstacked membranes. However, the membranes are clearly unstacked when trypsin-activated, and therefore the accessibility of the large trypsin molecule to previously stacked regions is guaranteed. In addition, other results (Staehelin, unpublished results) indicate that restacking is a slow and gradual process which takes as long as one hour to complete, while our membrane samples were incubated in the Ca\(^{++}\)-containing buffer for only the 10 min required to measure ATPase activity (20).

Additional evidence to support this interpretation is provided by the analysis of surface changes accompanying unstacking and restacking. Our experiments show that thylakoid membranes exhibit a large apparent decrease in particle density on their outer surfaces when unstacked, a decrease not accompanied by any loss of ATPase activity. If CF\(\text{t}\) molecules were present in significant numbers in stacked regions, unstacking would not be expected to bring about such a dramatic decrease in particle density. The magnitude of the decrease correlates very well with measurements of the percentage of total thylakoid surface area involved in contact in stacked and restacked preparations (Table III), and can only be explained by assuming that the CF\(\text{t}\) particles are excluded from regions of thylakoid contact during stacking. Similarly, the fact that the restacking of thylakoid preparations brings about a renewed increase in apparent parti-
cle density leads to the same conclusion. In light of the fact that the washing and reconstitution experiments discussed earlier establish the identity of the large particles remaining after sodium pyrophosphate washes as the CF1, the behavior of these particles during unstacking and restacking experiments leads to the unavoidable conclusion that they are excluded from regions of membrane stacking.

This conclusion is further supported by the thin sections of chloroplasts subjected to a brief unstacking treatment followed by the fixation and staining treatment of Oleszko and Moudrianakis (35) to make CF1 molecules visible. Such images show the apparent coupling factor molecules to be absent from the freshly unstacked membrane surface (Fig. 16).

A concentration of CF1 in unstacked (stroma) lamellae is further suggested by the biochemical studies of Arntzen, Dilley, and Newmann (1), who demonstrated that chloroplasts which contained greater amounts of stromal membranes (young lettuce leaf chloroplasts, sorghum bundle sheath chloroplasts) have high photophosphorylation activities when compared to chloroplasts typified by a lesser proportion of stromal membranes. The capacity to accumulate protons, presumably through electron transport, shows a reverse distribution, however.

**Other Surface Structures**

The identity of the small particles which remain at the thylakoid surface after the removal of larger particles presents an interesting problem for further work. We consider it likely that this particle may correspond to protruding elements of the integral proteins visible on the fracture face formed by the outer leaflet of the thylakoid membrane (Cu-face in some literature: (11, 26), but at present we have no firm experimental support for this point of view. The arrays of regular structures occasionally visible on the outer surface (Fig. 15) have been shown elsewhere to correspond to elements of a large integral particle spanning the thylakoid membrane (25, 37).

**Structural Differentiation During Thylakoid Stacking**

The results reported here concerning exclusion of coupling factor from stacked membrane regions complement certain other observations made concerning similar rearrangements in particle distribution. Ojakian and Satir (34) have reported that the stacking of chloroplast membranes in *Chlamydomonas* brings about a migration of particles seen on the internal fracture faces of the thylakoid. Staehelin and Miller (43) have also reported that thylakoid stacking in spinach brings about a concentration of multisubunit particles on the inner membrane surfaces in stacked regions. When these observations are combined with what we have reported here, it becomes apparent that the seemingly random intermixing of components which occurs in unstacked membranes is abolished by the stacking process. The coupling factor is excluded from stacked regions, particles on the inner surface of the membrane are concentrated in such regions (43), and distinct particle migrations seem to take place among particles visible on the fracture faces (34). Mechanisms behind these movements may involve the mechanical exclusion of surface components, the existence of ionically mediated binding sites on adjacent membrane surfaces, or other interactions between membrane...
components which take place during stacking. The physiological alterations which attend this dramatic structural change induced by thylakoid stacking remain unclear, and present a challenging problem for future work.

Dr. Miller thanks Dr. Daniel Branton for the use of his laboratory facilities. Thanks are also due to Gayle J. Miller and Stephanie Krah for their expert technical assistance.

Portions of this work were supported by NIGMS Grant GM-18639 to L. A. S., and by U.S. Public Health Service grant GM-06637-15 to the the Electron Microscope Service Facility at Harvard University.

Received for publication 27 May 1975, and in revised form 18 August 1975.

REFERENCES

1. ARNTZEN, C. J., R. A. DILLEY, and J. NEWMANN, 1971. Localization of photophosphorylation and proton transport activities in various regions of the chloroplast lamellae. Biochim. Biophys. Acta. 245: 409–424.

2. BERZBORN, R. J., F. KOPP, and K. HEUHELLER, 1974. Mobility of chloroplast coupling factor I (CF₁) at the thylakoid surface as revealed by freeze-etching after antibody labelling. Z. Naturforsch. Teil. B. Anorg. Chem. Org. Chem. Biochem. Biophys. Biol. 29C: 694–699.

3. BISHOP, P. G. 1974. Lamellar structure and composition of chloroplasts in relation to photosynthetic electron transfer. Photochem. Photobiol. 20: 281–299.

4. BRANTON, D. 1966. Fracture faces of frozen membranes. Proc. Natl. Acad. Sci. U.S.A. 55: 1048–1056.

5. BRANTON, D. 1969. Membrane structure. Annu. Rev. Plant Physiol. 20: 209–238.

6. BRANTON, D., and D. W. DEAMER. 1972. Membrane structure. Protoplasmatologia 20: 193: 265–275.

7. BRANTON, D., and R. B. PARK. 1967. Subunits in chloroplast lamellae. J. Ultrastruct. Res. 19: 283–303.

8. DILLEY, R. A. 1971. Coupling of ion and electron transport in chloroplasts. Curr. Top. Bioenerg. 4: 237–271.

9. FAHEY, J. L., and E. M. MCKELVEY. 1965. Quantitative determination of serum immunoglobulins in antibody-agar plates. J. Immunol. 94: 84–90.

10. GARBER, M. P., and P. L. STEPONKUS. 1974. Identification of chloroplast coupling factor by freeze-etching and negative-staining techniques. J. Cell Biol. 63: 24–34.

11. GOODENOUGH, U. W., and A. STAHELIN. 1971. Structural differentiation of stacked and unstacked chloroplast membranes. J. Cell Biol. 48: 594–619.

12. HOWELL, S. H., and E. N. MOUDRIANAKIS. 1967. Hill reaction site in chloroplast membranes: non-participation of the quasarsome particle in photoreduction. J. Mol. Biol. 27: 323–333.

13. IWAMOTO, S., and N. E. GOOD. 1966. Effect of salts and electron transport on the conformation of isolated chloroplasts. II. Electron microscopy. Plant Physiol. 41: 544–552.

14. JAGENDORF, A. T., and M. SMITH. 1962. Uncoupling phosphorylation in spinach chloroplasts by absence of cations. Plant Physiol. 37: 135–141.

15. JAGENDORF, A. T., and E. URBE. 1966. ATP formation caused by acid-base transition of spinach chloroplasts. Proc. Natl. Acad. Sci. U.S.A. 55: 170–177.

16. KANNANGARA, C. G., VAN WYK, D., and W. MENKE, 1970. Immunological evidence for the presence of latent Ca⁺⁺-dependent ATPase and carboxydismutase on the thylakoid surface. Z. Naturforsch. Teil. B. Anorg. Chem. Org. Chem. Biochem. Biophys. Biol. 25B: 613–618.

17. KARU, A. E., and E. N. MOUDRIANAKIS, 1969. Fractionation and comparative studies of enzymes in aqueous extracts of spinach chloroplasts. Arch. Biochem. Biophys. 129: 655–671.

18. KIRK, J. T. O. 1971. Chloroplast structure and biogenesis. Ann. Rev. Biochem. 40: 161–196.

19. LIEN, S., R. J. BERZBORN, and E. RACKER, 1972. Partial resolution of the enzymes catalyzing photophosphorylation. IX. Studies on the subunit structure of coupling factor I from chloroplasts. J. Biol. Chem. 247: 3520–3524.

20. LIEN, S., and E. RACKER, 1971. Preparation and assay of chloroplast coupling factor CF₁. Methods Enzymol. 23: 547–555.

21. LIEN, S., and E. RACKER, 1971. Partial resolution of the enzymes catalyzing phosphorylation. VIII. Properties of silicotungstate-treated subchloroplast particles. J. Biol. Chem. 246: 4298–4307.

22. LOWRY, O. H., N. J. ROSENROUGH, A. L. FARR and R. J. RANDALL, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–275.

23. MACKINNEY, G. 1941. Absorption of light by chloroplasts. Curr. Top. Bioenerg. 4: 237–271.

24. McCARTY, R. E., and E. RACKER. 1966. Effect of a coupling factor and its antiserum on photophosphorylation and hydrogen ion transport. Brookhaven Symposia in Biology 19: 202–214.

25. MILLER, K. R. 1976. A particle spanning the thylakoid membrane. J. Ultrastruct. Res. In press.

26. MILLER, K. R., and L. A. STAHELIN. 1974. Fine structure of the chloroplast membranes of Euglena gracilis as revealed by freeze-cleaving and deep-etching techniques. Protoplasma. 77: 55–78.
28. Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev.* **41**: 445–502.

29. Moor, H., and K. Mühlthaler. 1963. Fine structure in frozen-etched yeast cells. *J. Cell Biol.* **17**: 609–628.

30. Moudrianakis, E. N. 1964. A particulate structure on the membranes of spinach chloroplasts. *J. Cell Biol.* **23**: 63 a. (Abstr.).

31. Mühlthaler, K. 1972. Freeze-etch studies on chloroplast thylakoids. Proceedings of the 11th International Conference of Photosynthesis Research. Stresa. The Hague. Vol. II: 1423–1429.

32. Mühlthaler, K., H. Moor, and J. W. Szarkowski. 1965. The ultrastructure of the chloroplast lamellae. *Planta (Berl.)* **67**: 305–323.

33. Oda, T., and H. Huzisige. 1965. Macromolecular repeating units in the chloroplast membrane. *Exp. Cell Res.* **37**: 481–484.

34. Ojakian, G. K., and P. Satir. 1974. Particle movements in chloroplast membranes: quantitative measurements of membrane fluidity by the freeze-fracture technique. *Proc. Natl. Acad. Sci. U.S.A.* **21**: 2052–2056.

35. Oleszkow, S., and E. N. Moudrianakis. 1974. The visualization of photosynthetic coupling factor in embedded spinach chloroplasts. *J. Cell Biol.* **63**: 936–948.

36. Ouchterlony, Ö. 1958. Diffusion-in-gel methods for immunological analysis. *Prog. Allergy.* **5**: 1.

37. Park, R. B., and A. O. Pfeifhofer. 1969. Ultrastructural observations on deep-etched thylakoids. *J. Cell Sci.* **5**: 299–312.

38. Park, R. B., and A. O. Pfeifhofer. 1969. The effect of ethylene diaminetetraacetate washing on the structure of spinach thylakoids. *J. Cell Sci.* **5**: 313–319.

39. Park, R. B., and P. V. Sane. 1971. Distribution of function and structure in chloroplast lamellae. *Ann. Rev. Plant Physiol.* **22**: 395–430.

40. Singer, S. J. 1974. The molecular organization of membranes. *Ann. Rev. Biochem.* **43**: 805–833.

41. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D.C.)* **175**: 720–731.

42. Slater, E. C. 1953. Mechanism of phosphorylation in the respiratory chain. *Nature (Lond.)* **172**: 975–978.

43. Staehein, L. A., and K. R. Miller. 1974. Particle movements associated with unstacking and restacking of chloroplast membranes in vitro. A freeze-cleave and deep-etch study. Eighth International Congress on Electron Microscopy. Canberra, Aust. II: 202–203.

44. Strotmann, H., H. Hesse, K. Edelmann. 1973. Quantitative determination of coupling factor CF$_1$ of chloroplasts. *Biochim. Biophys. Acta.* **314**: 202–210.