IDENTIFICATION OF CHLOROPLAST COUPLING FACTOR BY FREEZE-ETCHING AND NEGATIVE-STAINING TECHNIQUES

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ABSTRACT
Identification of chloroplast coupling factor particles, by the freeze-etching and negative-staining techniques, was made utilizing chloroplast thylakoids isolated from spinach leaves. Complete removal of particles, comparable in diameter to purified coupling factor particles, from the outer surface of freeze-etched thylakoids was achieved by treatment with 0.8% silicotungstate. Reappearance of particles, comparable in diameter to purified coupling factor particles, on the outer surface of freeze-etched thylakoids was demonstrated by combining silicotungstate-treated thylakoids with purified chloroplast coupling factor. Negative-staining results were in agreement with the freeze-etch data. The results demonstrate that the chloroplast coupling factor particles are exposed on the outer surface.

A striking, characteristic feature of negatively stained chloroplast thylakoids is the presence of particles ± 90 Å in diameter on their outer surface (3, 4). Particles of similar diameter and location have been observed on negatively stained (1) and thin-sectioned (14) submitochondrial vesicles also. Particles ± 90 Å in diameter have so far not been observed on thylakoids in conventional thin sections.

Identifying the chemical and functional nature of the ± 90-Å particles observed on negatively stained thylakoids has led to some controversy. At one point they were considered to be related to light-gathering and electron-transporting aspects of chloroplasts, the so-called “quantasomes” (11). However, particles of a similar size removed from thylakoids are associated with a cryptic, trypsin-activated ATPase (15, 3). Further work has indicated the relationship between the cryptic ATPase and the chloroplast coupling factor (7). Washing thylakoids with ethylenediamine tetraacetate (EDTA) at low ionic strength removes a large portion of the chloroplast coupling factor (CFt) and also of the ± 90-Å particles (7, 8, 15). When repeated EDTA washings and subsequent gradient fractionation were used to remove CFt entirely, the resulting thylakoids, which completely lacked the ± 90-Å particles, constituted only a small proportion of the original population (3).

Possibly more critical evidence is obtained with the use of silicotungstate (STA) which at low concentrations removes the coupling factor (F1)
from submitochondrial vesicles (13) and CF1 from subchloroplast vesicles (5) virtually quantitatively. Lien and Racker were able to demonstrate both the complete removal of the ± 90-A particles from the surface of thylakoids due to the STA treatment, and restoration of the ± 90 A particles when the STA-treated thylakoids were combined with purified CF1 (5). The present work extends this study by using not only negative staining procedures which show only surface particles, but also freeze-etch procedures which are able to demonstrate details of the surface and internal structures of membranes. The combination of these procedures enables us to associate the ± 90-A particles seen on negatively stained thylakoids more firmly than before with the chemical entity CF1, and to show that CF1 particles are exposed on the outer surface in frozen but unfixed thylakoids.

MATERIALS AND METHODS

Chloroplast thylakoids were isolated from greenhouse-grown spinach leaves (Spinacia oleracea L. 'Bloomsdale') according to Heber (2), except that the chloroplasts were osmotically ruptured by resuspending in 10 mM NaCl. Preparation of CF1-depleted thylakoids and reconstitution of CF1-depleted thylakoids with purified CF1 particles was done according to the procedure of Lien and Racker (5). The thylakoids were washed in 10 mM NaCl before negative staining and freeze etching. Purified CF1 particles were prepared according to Lien and Racker (4).

Thylakoids and purified CF1 particles were negatively stained with 2% K-PTA at pH 7.0. Freeze etching of the material was done according to the procedure of Moor and Mühlethaler (9), using a Balzers' BA-360 M freeze-etching unit (Balzers High Vacuum Corp., Santa Ana, Calif.). In this study, minimal etching indicates that the samples were shadowed as soon as possible after fracturing, and deep etching indicates that the samples were etched for 30 s before shadowing. The terminology used for labeling the two fracture faces and the two etched surfaces is after Mühlethaler (10). The replicas and negatively stained preparations were viewed and photo-
FIGURE 4 An untreated thylakoid that has been freeze fractured without subsequent etching. The two fracture faces, characteristic of single thylakoids that have been fractured without etching, can be seen. The outer fracture face has numerous small particles present, while the inner fracture face has fewer larger particles present. OFF, outer fracture face; IFF, inner fracture face. x 50,000.

RESULTS AND DISCUSSION

Negative-staining results indicate that thylakoids used in this work are not stacked as in intact chloroplasts but are swollen and vesiculate (Figs. 1–3). Untreated, negatively stained thylakoids have numerous ± 90-Å particles, apparently protruding from the edge of the thylakoid (Fig. 1). Since the thylakoids are flattened out after negative staining, the ± 90-Å particles appear very pronounced along the edges of the thylakoids but are indistinct on the flattened surface. Thylakoids treated with 0.8% STA show an almost complete removal of the ± 90-Å particles (Fig. 2). The particles removed from the surface of negatively stained thylakoids by STA treatment are in the same diameter range as purified CF₁ particles (Fig. 21).

Freeze fracturing of thylakoids without subsequent etching reveals two fracture faces (Fig. 4). The fracture faces are represented diagrammatically in Fig. 5. The inner fracture face, as described by Mühlethaler (10), has the larger particles of the two fracture faces (Fig. 6). The average particle size is about 140 Å with two size groups of ± 100 Å and ± 150 Å in diameter, respectively (Figs. 6 and 23). The particle concentration of the inner fracture face is approximately 1,650 particles/μm². A typical outer fracture face of untreated thylakoids is characterized by numerous small particles (Fig. 7). The size and concentration of particles on this face are such that it is difficult to make accurate measurements. Approximate measurements indicate a particle concentration of about 3,100 particles/μm².

Figs. 8 and 9 illustrate untreated thylakoids that have been fractured and deep etched. The etched outer surface reveals numerous ± 150-Å particles (Fig. 9). The etched inner surface also has numerous particles present (Fig. 8). Untreated thylakoids that are deep etched without fracturing reveal particles ± 150 Å in diameter on the outer surface (Fig. 10).

Thylakoids treated with 0.8% STA are devoid of any of the ± 150-Å particles observed on the outer surface of untreated thylakoids (Figs. 13 and 15). No particles are apparently removed from the other membrane surface, the inner surface, in STA-treated thylakoids (Fig. 14).

The inner fracture face of STA-treated thylakoids (Fig. 11) has a particle concentration of about 710 particles/μm² as compared to the inner fracture face of untreated thylakoids (Fig. 6) which has a particle concentration of about 1,650 particles/μm². The particle concentration on the outer fracture face of STA-treated thylakoids is similar to that of untreated thylakoids (compare Figs. 12 and 7). A particle diameter frequency distribution of the inner fracture face of STA-treated thylakoids suggests either that the ± 150-Å particles, observed on the inner fracture face of untreated thylakoids, are removed by STA treatment or that the membrane is altered such that they are no longer visible on the inner fracture face (Fig. 24). The particles absent from the inner fracture face after STA treatment are in the same diameter range as the particles removed from the etched outer surface by STA treatment.

Removal of particles comparable in diameter to purified CF₁ particles represents the first step in the conventional biochemical procedures of resolution and then reconstitution. To determine if the particles removed by STA treatment represent...
CF1 particles, purified CF1 particles must be added to CF1-depleted thylakoids. After reconstitution, reappearance of particles in the same diameter range and same position in the membrane structure as those removed by STA treatment should be shown. Also, particles removed by STA treatment and particles which appear after reconstitution should be in the same diameter range as purified CF1 particles. In addition, reconstituted thylakoids should show at least partial restoration of biological activity which was eliminated by STA treatment.

The above requirements for identification of CF1 particles were successfully carried out by combining the STA-treated thylakoids with purified CF1 particles. After reconstitution, the outer surface of negatively stained thylakoids show reappearance of the ± 90-Å particles (Fig. 3). These particles are in the same diameter range as particles removed by STA treatment, and both are in the same diameter range as negatively stained, purified CF1 particles (compare Figs. 3 and 21). Reconstituted thylakoids that have been deep etched have ± 150-Å particles present on the etched outer surface (Figs. 19 and 20) which are in the same diameter range as the particles removed by STA treatment, and both are in the same diameter range as purified CF1 particles that have been freeze etched (Fig. 22). A particle diameter frequency distribution of the inner fracture face of reconstituted thylakoids shows reappearance of particles ± 150 Å in diameter (Fig. 25). The number of particles in this size range is approximately 30% less than that present in untreated thylakoids. STA-treated thylakoids reconstituted with purified CF1 particles show partial restoration of the Ca++-dependent ATPase activity and cyclic photophosphorylation that was eliminated by STA treatment (5).

The inner fracture face of reconstituted thylakoids (Fig. 16) has a particle concentration of about 1,230 particles/μm². The outer fracture face (Fig. 17) and inner surface (Fig. 18) of reconstituted thylakoids are similar in morphology to those of untreated and STA-treated thylakoids.

A difference in the diameter of the outer surface particles between the thylakoids that are negatively stained and the thylakoids that are freeze etched is obvious (compare Figs. 1 and 3 with Figs. 9, 10, 19, and 20). This is to be expected, since a coating of the metal over the particles in freeze-etched preparations will result in particles that are larger in diameter than those visualized by the negative-staining technique. Thus the diameter of CF1 particles, for example, is ± 150 Å in freeze-etched preparations, and ± 90 Å in negatively stained material.

The inner fracture face of most deep-etched thylakoids is distorted as compared to the outer fracture face of deep-etched thylakoids (compare Figs. 9, 13, and 19 with Figs. 8, 14, and 18). The greater distortion of the inner fracture face after etching is possibly due to the fracture plane in the thylakoids being close to the inner surface (12).

The effect of negative staining on the morphology of membranes is a controversial subject, and this is one reason why the freeze-etch technique was utilized to identify CF1. Freeze etching involves "physical" fixation by rapid freezing in liquid Freon, and there is good reason to believe that membranes prepared in this manner approximate the membrane as it exists in its natural state (6). This paper has demonstrated that the ± 150-Å diameter particles, now identified as CF1 particles, are exposed on the outer surface of thylakoids (Figs. 9, 10, 19, and 20). This is in contrast to previous work with freeze-etched mitochondria which indicated a lack of particles on the outer surface. Freeze-etch data showing the CF1 particles exposed at the outer surface of freeze-etched thylakoids are supported by previous antibody work (7).

The data presented in this paper do not presently rule out the possibility that CF1 particles may be partially buried in the membrane. The ± 150-Å particles that are present on the inner fracture face in untreated thylakoids are not visible after STA treatment but are again visible after reconstitution. The possibility exists that the ± 150-Å particles on the inner fracture face represent partially buried CF1 particles, or, alternatively, that the membrane is changed by the STA treatment such that the particles are no longer visible, and then following reconstitution the membrane structure is reversed and the ± 150-Å particles are again visible. A comparison of the shadow cast by purified CF1 particles and membrane-associated CF1 particles (compare Fig. 22 with Figs. 9, 10, 19, and 20) indicates that the purified CF1 particles cast a longer shadow. The longer shadow cast by freeze-etched purified CF1 particles may be an indication that the CF1 particles are partially buried in the membrane.

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FIGURE 5 Illustrative representation of a single chloroplast thylakoid that has been fractured and deep etched. The two fracture faces, inner fracture face (IFF) and outer fracture face (OFF), are exposed by fracturing with minimal etching. The two etched surfaces, outer surface (OS) and inner surface (IS), are exposed when a fractured thylakoid is deep etched. The inner fracture face is exposed by a fracture which proceeds from the outer surface toward the inner surface, thus removing the outer fracture face. The inner fracture face lies next to the inner surface. The outer surface is then exposed if the fractured thylakoid is deep etched. The outer fracture face is exposed when a fracture proceeds from the inner surface toward the outer surface, thus removing the inner fracture face. The outer fracture face lies next to the outer surface. The inner surface is then exposed if the fractured thylakoid is deep etched.

FIGURE 6 The inner fracture face of an untreated thylakoid revealed by fracturing and minimal etching. It is characterized by particles of two size groups, ± 100 Å and ± 150 Å. The particle concentration is greater than that in STA-treated thylakoids (Fig. 11). IFF, inner fracture face; I, ice. x 100,000.

FIGURE 7 The outer fracture face of an untreated thylakoid revealed by fracturing and minimal etching. It is characterized by numerous small particles with a size and concentration such that accurate measurements are difficult. The particle concentration is greater than that on the inner fracture face of untreated thylakoids. OFF, outer fracture face; I, ice. x 100,000.

FIGURE 8 An untreated thylakoid that has been fractured and deep etched. The fracture plane is indicated by the arrow. The membrane below the fracture plane, the inner surface, was exposed by deep etching. The inner surface has numerous particles present and its surface morphology is different from that of the outer fracture face. The outer fracture face is fairly well preserved after deep etching. OFF, outer fracture face; FP, fracture plane; IS, inner surface; I, ice. x 100,000.

FIGURE 9 An untreated thylakoid that has been fractured and deep etched. The fracture plane is indicated by the arrow. The membrane surface below the fracture plane, the outer surface, was exposed by deep etching. The outer surface is covered with ± 150-Å particles. The inner fracture face has been distorted by deep etching. IFF, inner fracture face; RP, fracture plane; OS, outer surface; I, ice. x 100,000.

FIGURE 10 Untreated thylakoid exposed by deep etching without fracturing. The outer surface has numerous ± 150-Å particles present. No fracture plane is present since only deep etching was involved. OS, outer surface. x 100,000.
It becomes obvious in comparing negatively stained thylakoids and deep-etched thylakoids that the positioning of CF1 particles on the membrane is different in the two preparations. In negatively stained thylakoids there is a "stalk" between the 90-Å particles (CF1) and the membrane, whereas in deep-etched thylakoids the "stalk" is not observed (compare Figs. 1 and 3 with Figs. 9, 10, 19, and 20). This raises the possibility of an artifact created by one of the two techniques. Recognizing the possibility of artifacts due to chemical fixation, it is tempting to suggest that the "stalk" observed on negatively stained thylakoids is an artifact and that the deep-etch preparations more closely approximate the membrane in its natural state.

The current investigation is the first, to our knowledge, to demonstrate and identify CF1 particles with the help of the freeze-etching technique. The procedure followed in this investigation may also prove useful in the identification of other particles in chloroplast thylakoids and other biological membranes.

We wish to thank Drs. A. T. Jagendorf and M. V. Parthasarathy, Section of Genetics, Development, and Physiology, and Drs. R. E. McCarty, E. Racker, and J. D. Telford, Section of Biochemistry and Cell Biology, for their critical evaluation of the manuscript. Special thanks go to Dr. M. V. Parthasarathy for the use of his electron microscope facilities and many helpful suggestions offered during the course of this study and to Dr. E. Racker for his helpful and stimulating discussions. Also, we wish to thank Ms. Elizabeth Johnson for doing the chloroplast thylakoid illustration. Purified CF1 was a gift from Dr. E. Racker's laboratory.

Received for publication 29 October 1973, and in revised form 20 May 1974.

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**Figure 11** The inner fracture face of an STA-treated thylakoid revealed by fracturing and minimal etching. The particle concentration is less than that of untreated thylakoids (compare with Fig. 6). Most of the particles present are ± 100 Å in diameter. **IFF,** inner fracture face; **I,** ice. × 100,000.

**Figure 12** The outer fracture face of an STA-treated thylakoid revealed by fracturing and minimal etching. No detectable alteration in particle size or concentration has occurred. **OFF,** outer fracture face; **I,** ice. × 100,000.

**Figure 13** An STA-treated thylakoid that has been fractured and deep etched. The fracture plane is indicated by the arrow. The membrane surface below the fracture plane, the outer surface, was exposed by deep etching. The outer surface is devoid of the ± 150-Å diameter particles observed on the outer surface of untreated thylakoids. The inner fracture face has been distorted by deep etching. **IFF,** inner fracture face; **OS,** outer surface; **FP,** fracture plane; **I,** ice. × 100,000.

**Figure 14** An STA-treated thylakoid that has been fractured and deep etched. The fracture plane is indicated by the arrow. The membrane surface below the fracture plane, the inner surface, was exposed by deep etching. The inner surface has numerous particles present, indicating no apparent removal of particles by STA. The outer fracture face is fairly well preserved after deep etching. **OFF,** outer fracture face; **FP,** fracture plane; **IS,** inner surface; **I,** ice. × 100,000.

**Figure 15** An STA-treated thylakoid exposed by deep etching without fracturing. The outer surface is devoid of the ± 150-Å particles that were observed on untreated thylakoids. **OS,** outer surface; **I,** ice. × 100,000.

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**Figure 21** Purified chloroplast coupling factor particles that were negatively stained with 2% K-PTA at pH 7.0. Numerous ± 90-Å particles can be seen. CF₁, chloroplast coupling factor particles. × 100,000.

**Figure 22** Purified chloroplast coupling factor particles that were freeze etched. Numerous ± 150-Å diameter particles are visible. CF₁, chloroplast coupling factor particles; I, ice. × 100,000.

**Figure 16** The inner fracture face of a reconstituted thylakoid revealed by fracturing and minimal etching. The particle concentration is greater than that of STA-treated thylakoids (see Fig. 11). There are particles present in the ± 100-Å and ± 150-Å diameter range. IFF, inner fracture face; I, ice. × 100,000.

**Figure 17** The outer fracture face of a reconstituted thylakoid revealed by fracturing and minimal etching. Its morphology is similar to that of untreated and STA-treated thylakoids. OFF, outer fracture face; I, ice. × 100,000.

**Figure 18** A reconstituted thylakoid that has been fractured and deep etched. The fracture plane is indicated by the arrow. The membrane surface below the fracture plane, the inner surface, was exposed by deep etching. The inner surface has numerous particles present and is similar to the inner surface of untreated and STA-treated thylakoids. The outer fracture face is fairly well preserved after deep etching. OFF, outer fracture face; FP, fracture plane; IS, inner surface; I, ice. × 100,000.

**Figure 19** A reconstituted thylakoid that has been fractured and deep etched. The fracture plane is indicated by the arrow. The membrane surface below the fracture plane, the outer surface, was exposed by deep etching. The outer surface has numerous ± 150-Å diameter particles present. The inner fracture face has been distorted by deep etching. IFF, inner fracture face; FP, fracture plane; OS, outer surface; I, ice. × 100,000.

**Figure 20** A reconstituted thylakoid that has been deep etched without fracturing. The outer surface has numerous ± 150-Å diameter particles present. OS, outer surface; I, ice. × 100,000.
Figure 23 Particle diameter frequency distribution of the inner fracture face of untreated thylakoids. Two particle size groups are detectable.

Figure 24 Particle diameter frequency distribution of the inner fracture face of STA-treated thylakoids. Only the particle size group of $\pm 100 \, \text{Å}$ is detectable with very few of the larger $\pm 150\text{-Å}$ particles present.

Figure 25 Particle diameter frequency distribution of the inner fracture face of reconstituted thylakoids. Two particle size groups are detectable. The particle distribution is similar to that of untreated thylakoids except that there are fewer particles in the larger size group.

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