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Ultrastructural Comparison of *Cyanidium caldarium* Wild Type and III-C Mutant Lacking Phycobilisomes

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ABSTRACT

Cyanidium caldarium **wild type and III-C mutant lacking phycobilisomes were compared with respect to the ultrastructural organization of particles on the freeze-fractured thylakoid membrane.**

In the wild type, the particles on the exoplasmic fracture face were arranged in the same manner as that reported for the phycobilisomes on the membrane surface. The phycobilisomes constitute the major part of the photosystem II antenna and their absence in the III-C mutant was accompanied by a completely different arrangement of the particles on the exoplasmic fracture face.

The density of these particles was almost two times higher in the mutant than in the wild type while that of the particles on the protoplasmic fracture face was about the same.

The relative densities of the particles on the exoplasmic fracture face in the two organisms was consistent with the 2-fold higher photosystem II to photosystem I ratio in the mutant compared to the wild type as determined by measurements of the field indicating absorbance changes.

These particles were 100 Angstroms in both organisms.

It is concluded that the particles on the exoplasmic fracture face in *Cyanidium* are probably substructural units of the particles observed on the same surface in higher plants and green algae and attributed to photosystem II.

In recent years, freeze-fracturing and freeze-etching techniques have provided detailed ultrastructural characterization of the thylakoid membrane (18). Freeze-fracturing reveals two intramembranal surfaces, EF[1] and PF, which, in grana stacks are covered with particles which are to a first approximation of homogeneous size. In green algae and higher plants, the PF particles have a diameter of 80 Å while those on the EF surface are 160 Å (25, 26). Large particles (150 Å) have been described on the outer surface PS (23).

Attempts have been made to correlate these particles to functional entities. It has been demonstrated that 30% of the large PS particles correspond to carboxydismutase and the remainder to the coupling factor (17). Several authors have proposed that EF and PF particles correspond to protein complexes associated with PSII and I, respectively. This correlation is supported by the following.

EF and PF Particles Have the Same Distribution among Grana and Stroma Membranes as PSII and PSI. Digitonin treatment separates stacked and unstacked membranes (2), *i.e.* grana and stroma lamellae. The unstacked regions are rich in PSI activity lacking almost completely that of PSII. In these regions, the PF surface is rich in the 80 Å particles and the EF surface is almost bare. The PF surface of the grana regions shows the same density of 80 Å particles as in the stroma lamellae. The corresponding EF surface is covered with particles. These stacked regions show high activity of both photosystems.

Size of EF Particles Depends upon Pigment Composition of PSII Antenna. It has been shown that a Chl *b*-deficient mutant of *Chlamydomonas reinhardi* contained EF particles smaller than 160 Å (11). Armond *et al.* (1) showed that an illumination of plastids from previously flashed leaves produced an increase in the size of the EF particles (from 80 to 160 Å). During the same period, the Chl *a/b* protein was added to PSII antenna.

These results were all obtained from studies on green algae and higher plants. Few freeze-fracturing studies have been performed on blue-green or red algae (19, 26). They contain accessory pigments, phycocyanin and phycoerythrin respectively, which are organized in large complexes (phycobilisomes) attached to the outer surface of the thylakoid membrane. These are clearly linked to the PSII reaction centers (15) and are arranged in a regular manner on the membrane surface (8, 10). It is probable that PSII units are arranged in the same way within the thylakoid membrane.

We attempted to characterize the organization of the EF face particles of the phycocyanin containing algae, *Cyanidium caldarium*. We will attempt to show how the organization of the particles on the EF face relates to that of the phycobilisomes. To this end, we will show how a genetic mutation leading to the loss of the phycobilisomes affects the arrangement of the particles on the EF face.

MATERIALS AND METHODS

Cells were grown under conditions described in a previous paper (7).

For freeze-fracture studies, the cells were resuspended in distilled H_2O. They were neither fixed nor infiltrated with glycerol. Small amounts of cells were mounted on gold discs, rapidly frozen in Freon 22, and then stored in liquid N_2. Freeze-fracturing and platinum-carbon shadowing were performed with a Balzer's apparatus. During the whole operation, the specimen temperature was maintained at −150 C. The cleaned replicas were studied with a Philips EM 301 electron microscope. Direction of shadowing is indicated by a large black arrow on the micrographs. Shadows appear white.

Absorbance changes at 515 nm were measured using a differential spectrophotometer described by Joliot and Delosme (12). The actinic light was provided by a dye laser (Phase-R DL 1100; total pulse duration 600 nsec; dye: rhodamine 6G, 584 nm λ_{excit} < 625 nm. Detecting flashes were filtered by an interference filter (Microphysics, 5150 Å, spectral band width 34 Å at half-height).

RESULTS

Freeze-Fracture of *Cyanidium* Wild Type Chloroplast. Figure 1 shows an over-all view of a chloroplast of *Cyanidium* wild type.

[1] Abbreviations: EF: exoplasmic fracture face; PF: protoplasmic fracture face; PS: protoplasmic surface.

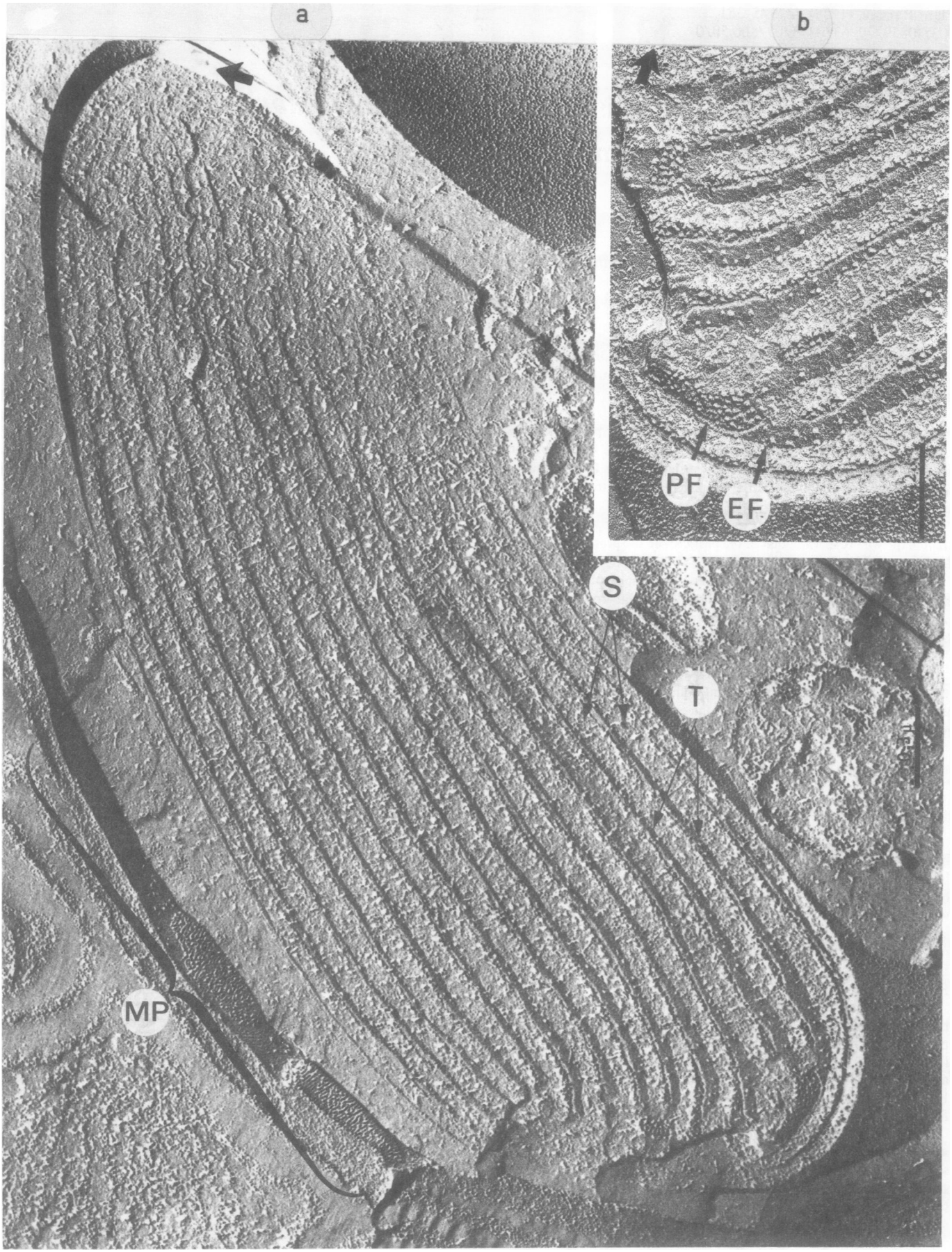

FIG. 1. a: General view of freeze-fractured wild type chloroplast of *C. caldarium* (× 65,000). T: thylakoid; MP: plasmic membrane; S: stroma. b: Lower right part of Figure 2a (× 100,000). Details of EF and PF faces of thylakoids are seen. Few particles are seen on EF faces. PF faces are covered with particles.

FIG. 2. EF and PF faces of thylakoids of *Cyanidium* wild type (× 100,000). There is a clear complementarity between the two faces; rows of particles appear on EF faces.

The fracture plane is perpendicular to that of the thylakoid membranes showing narrow ridges. The space between the thylakoids is the stroma. The freeze-fracture faces EF and PF of the cell membrane are apparent to the left of the micrograph. One of these shows a regular lattice arrangement of particles which is complementary to the depressions on the other face having the same lattice characteristics.

In the upper right corner is an enlargement of part of the same micrograph. The fracture face next to the stroma side of the thylakoid membrane (PF) shows closely packed arrangements of particles. The other fracture face (EF) shows a small number of particles as well as a number of depressions which appear to be complementary to the particle organization of the PF surface.

EF and PF Thylakoid Surfaces of *Cyanidium* Wild Type. Figure 2 shows several parallel thylakoids. Two of them, in the center of the picture, have been fractured in their plane showing their EF and PF surfaces. EF particles are shown in more detail in another micrograph taken at a higher magnification (Fig. 3).

The EF surfaces show linear rows of particles which are disorganized in some places. The distance between the rows is about 500 Å while the particle size appears homogeneous, about 100 Å (Fig. 4). Their density is about 1,500/μm^2 (Table I).

The PF faces shown in Figure 2 are covered with particles slightly smaller than those of the EF faces. Linear rows free of particles appear to complement the particle arrangements of the EF faces.

EF and PF Thylakoid Surfaces of *Cyanidium* III-C. In Figure 5 are seen linear arrangements about four particles wide which cover the EF surface. These rows are 1,000 to 1,300 Å apart and consist of particles whose size distribution is described in Figure 4 with a maximum at 100 Å. The particle density on the EF surface is about 2,600/μm^2 (Table I).

The PF surfaces (Fig. 6) are covered with particles distributed in two regions of different appearance. These domains are 600 and 1100 Å wide, respectively. They appear complementary to the two different types of areas apparent on the EF surfaces. This arrangement is clearly illustrated by close observation of Figure 5 where the complementarity of these two types of areas between the EF and PF surfaces is indicated.

Figure 6 shows in addition detached pieces of a neighboring thylakoid which have remained selectively stuck to the 600-Å-wide domain, further implying a heterogeneity in the surface composition.

PSI and PSII Contribution to 515 nm Absorbance Change. The 515 nm absorbance change is due to an electrochromic effect arising from the interaction between the transmembrane electric field and the thylakoid membrane pigments (14).

When measured 1.3 msec after an actinic flash (phase a, ref. 12), this absorbance change reflects the number of centers of both PSI and PSII which have undergone a charge separation.

To determine the relative contribution of the two photosystems to this absorbance change, PSII was blocked by a preillumination in the presence of 10^{-2} M hydroxylamine and 10^{-5} M DCMU (4). The remaining signal then corresponds to PSI alone. In order to determine the contribution of PSII we substracted the PSI signal from the 515 nm absorbance change when all photochemical centers were active, *i.e.* before the preillumination and the addition of hydroxylamine and DCMU.

The results are given in Table I.[2] The PSII to PSI ratio is about two times higher in the III-C mutant than in the wild type.

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[2] These spectroscopic measurements were performed at the same Chl concentration for the two organisms. It shows that there are more active centers in the III-C mutant than in the wild type for the same amount of Chl. This implies that: (*a*) either some Chl are loosely or not associated with functional centers in the wild type (this is consistent with the lower Fv/Fo ratio in the latter as compared with the former [7]); or (*b*) the size of PSI antenna is higher in the wild type than in the III-C mutant. At the same Chl concentration this is deduced from:

$$n_1S_1 + n_2S_2 = n_1'S_1' + n_2'S_2'$$

As $n_1 = n_1'$, $n_2' > n_2$ (Table I), $S_2 = S_2'$ (7); then $S_1 > S_1'$. n_1: number of active PSI centers; S_1: antenna size of PSI; x: wild type; x': III-C mutant.

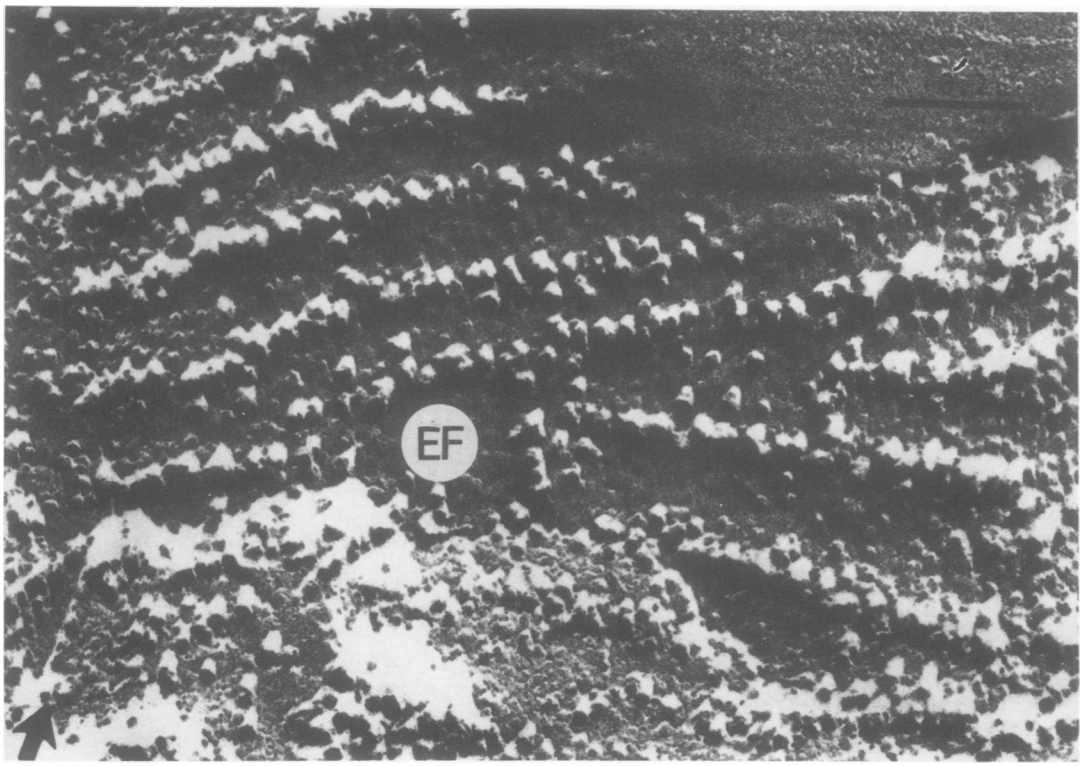

FIG. 3. EF face of a thylakoid of *C. caldarium* wild type (× 200,000). There is a regular 500 Å spacing between rows of particles. These are homogeneous in size.

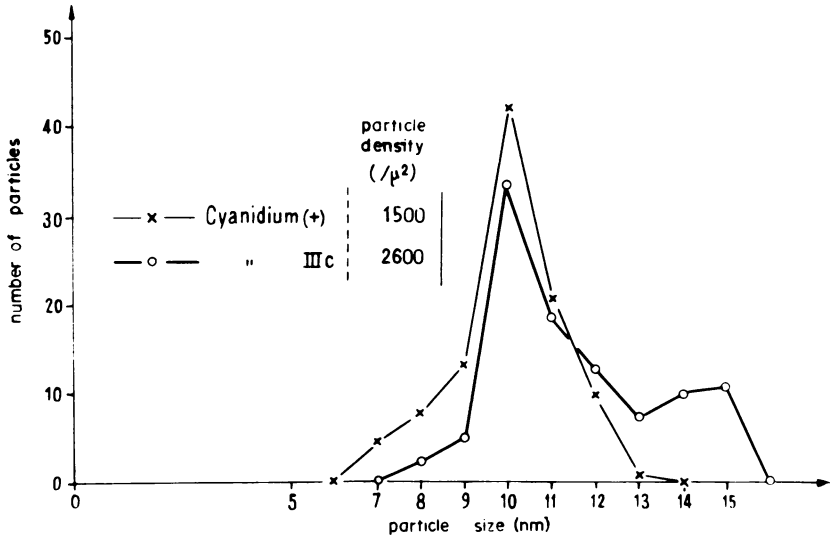

FIG. 4. Histogram of EF particles of III-C mutant and wild type of *C. caldarium*. Particles have an average size of 100 Å. Heterogeneity appearing in EF particle distribution of III-C mutant (maxima 100 and 140 Å) can be due to aggregation of these particles: during platinum shadowing overlapping of two neighboring particles may occur. This would give rise to particles of higher size.

DISCUSSION

All of the results presented here are interpreted through a comparison of the membrane characteristics of the III-C mutant and the wild type of *C. caldarium*.

A definite complementarity is shown by the EF and PF faces in both the III-C mutant and the wild type. Nevertheless one observes differences in their organization of the EF particles. Characterization of the III-C mutant (20) showed the absence of phycocyanin and consequently of the phycobilisomes containing this pigment. There is no difference in the Chl content of the PSII antenna, about 40 Chl (7).

The phycobilisomes are attached to the outer surface of the thylakoids in *Cyanidium* as shown by Seckbach (24). In other phycocyanin-containing algae, these have been reported to be highly organized in rows 400 to 500 Å apart on the thylakoid membrane surfaces (8). This arrangement is strikingly similar to the one reported here on the EF surfaces in the wild type. The EF particles, with an average diameter of 100 Å, are smaller than the 350 Å phycobilisomes (10).

The EF particles could be an intramembranal part of the phycobilisomes (5). This possibility is excluded as we still see particles having the same 100 Å size in the III-C mutant lacking phycocyanin. Our results show that the EF particles probably correspond to a structure associated with the phycobilisomes since the absence of the latter produces a restructuring of the geometrical

Table I. Particle densities and photosynthetic active centers in wild type and mutant thylakoid membranes.

Numbers of photosynthetic active centers were estimated from the amplitude of the 515nm absorbance change 1.2 ms after an actinic flash. The comparison between Cyanidium wild type and III-C mutant was performed at a chlorophyll concentration of 20µg/ml for the spectrophotometric measurements. The increase in particle density and in the PSII/PSI ratio between the wild type and III-C mutant is given with an uncertainty of 15%.

	wild type	III-C Mutant	Increase %
Number/µm² of			
PF Particles	3065 ± 245	3400 ± 272	--
FF Particles			
a) organized areas	1463 ± 117	2585 ± 207	77
b) non-organized areas	1472 ± 117	2280 ± 182	
Number[1] of			
PS I centers	72	82	
PS II centers	49	100	
Ratio, PS II/PS I	0.67	1.21	81

[1]The numbers of PSI and PSII centers are given relatively to the PSII contribution to the 515nm absorbance change in the III-C mutant.

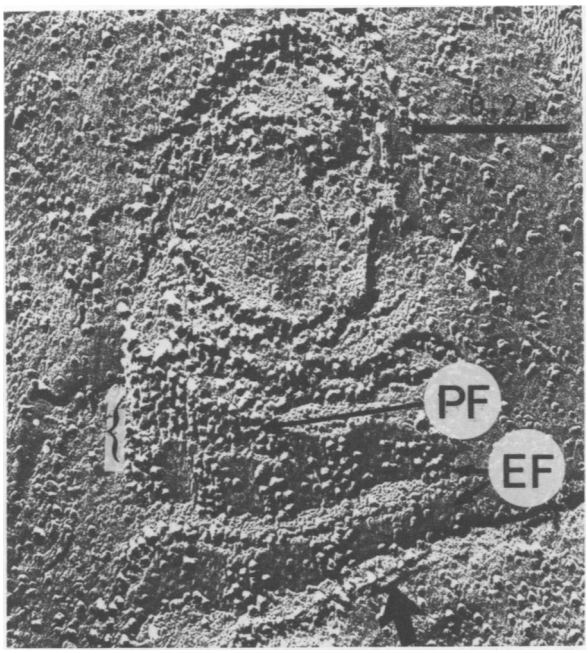

FIG. 5. EF and PF faces of thylakoids of *Cyanidium* III-C mutant (× 100,000). Complementarity between the two faces is indicated on the left of the micrograph. Aggregates of particles, 600 Å wide, on the EF faces are about 1,200 Å apart.

organization of the EF particles. As the phycobilisomes transfer more than 90% of their absorbed light energy to PSII (15), the EF particles could reasonably correspond to the Chl-containing units of this photosystem.

The EF particles are apparently able to aggregate once the phycobilisomes are detached (III-C mutant) which is consistent with the fluidity of the thylakoid membrane (21). Similar aggregation is observed in various membrane systems (3, 9) where integral proteins overcome electric repulsive forces and associate in closely packed arrangements forming hydrophilic zones in the membrane.

This aggregation leads to more contact between EF particles in the III-C mutant than in the wild type. As energy transfer between PSII units (13) occurs through contacts between the antennae, then the aggregation of EF particles may increase the transfer efficiency. In agreement, fluorescence induction curves show that the efficiency of energy transfer is higher in the mutant than in the wild type (7).

The density of the EF and PF particles was computed in both algae (Table I). There are about 1.8 times as many particles on the EF surface of the III-C mutant than on that of the wild type, while the number of PF particles is comparable. The ratio of PSII to PSI is also about 1.8 times higher in the III-C mutant than in the wild type. This indicates that both the EF particle density and the PSII centers (per PSI) increase by almost a factor 2 in the III-C mutant as compared to the wild type. This is consistent with assigning the EF particles to PSII.

The EF particle density in *Cyanidium* wild type is similar to that of spinach chloroplasts, 1,500/µm² as against 1,150/µm² (26). The higher EF particle density of 2,600/µm² in the III-C mutant could compensate for the reduced PSII activity due to the loss of the phycobilisomes.

The 100-Å average diameter of the EF particles described here is smaller than the size reported previously for these particles in green algae and higher plants (16, 25). It is similar to the size of the EF particles reported for blue-green and red algae (19, 26). These differences in size can be correlated to differences in pigment composition of the PSII antennae Chl *a/b* protein *versus* phycobilin. We demonstrated in a previous paper (7) that the PSII antenna of *C. caldarium* contains only 40 Chl instead of the 250 Chl found in green algae. The smaller the EF particles, the smaller the Chl containing antenna. In green algae and higher plants lacking the Chl *a/b* protein because of mutation or of particular conditions of greening (1, 11, 22), the EF particles are of reduced size, 80 to 100 Å. From these studies arose the concept of a core subunit of the EF particles (1) to which is added variable amounts of Chl *a/b* protein leading to particles of larger size (160 Å as a maximal value). The core subunit would then contain only a small part of the antenna. It is similar to the antenna core we described in a previous paper (7). The EF particles of *C. caldarium,* and more generally of red and blue-green algae, would then correspond to the core subunit of the EF particles seen in green algae or higher plants.

The number, the size, and the organization of the EF particles in the wild type and the III-C mutant of *C. caldarium* can be correlated with biophysical measurements of the PSII units. It is likely that these particles are an integral part of PSII and closely associated with the reaction centers. Further investigation using other mutants should provide additional information on this question.

Remarks on General Organization of EF Particles of *Cyanidium caldarium.* The EF particle organization described in this paper for both the mutant and the wild type is not visible on all of the thylakoid fracture faces found in the micrographs. However, the EF particle density is the same on the organized and nonorganized areas. This is true as well for the PF particles.

In the mutant, the few remaining phycobilisomes (7) could be responsible for the regions in which are observed the closely packed arrangements of EF particles described in Figure 5. It has

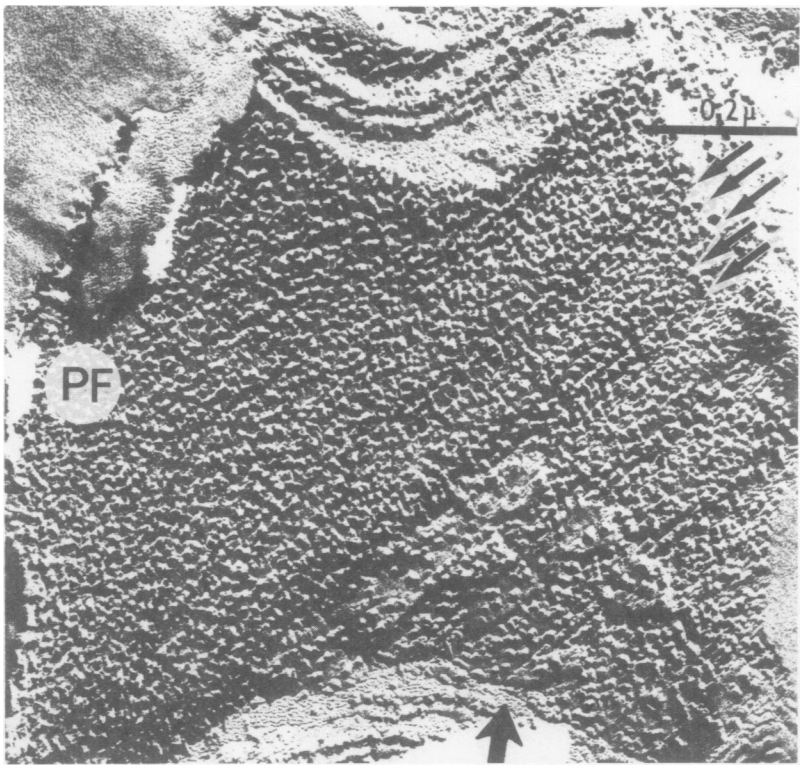

FIG. 6. PF face of thylakoid of III-C mutant (× 100,000). (⇒): zone 600 Å wide; (⇛): zone 1,100 Å wide. These two zones appear complementary with the organization of the EF face. Notice membrane fractions stuck on PF face.

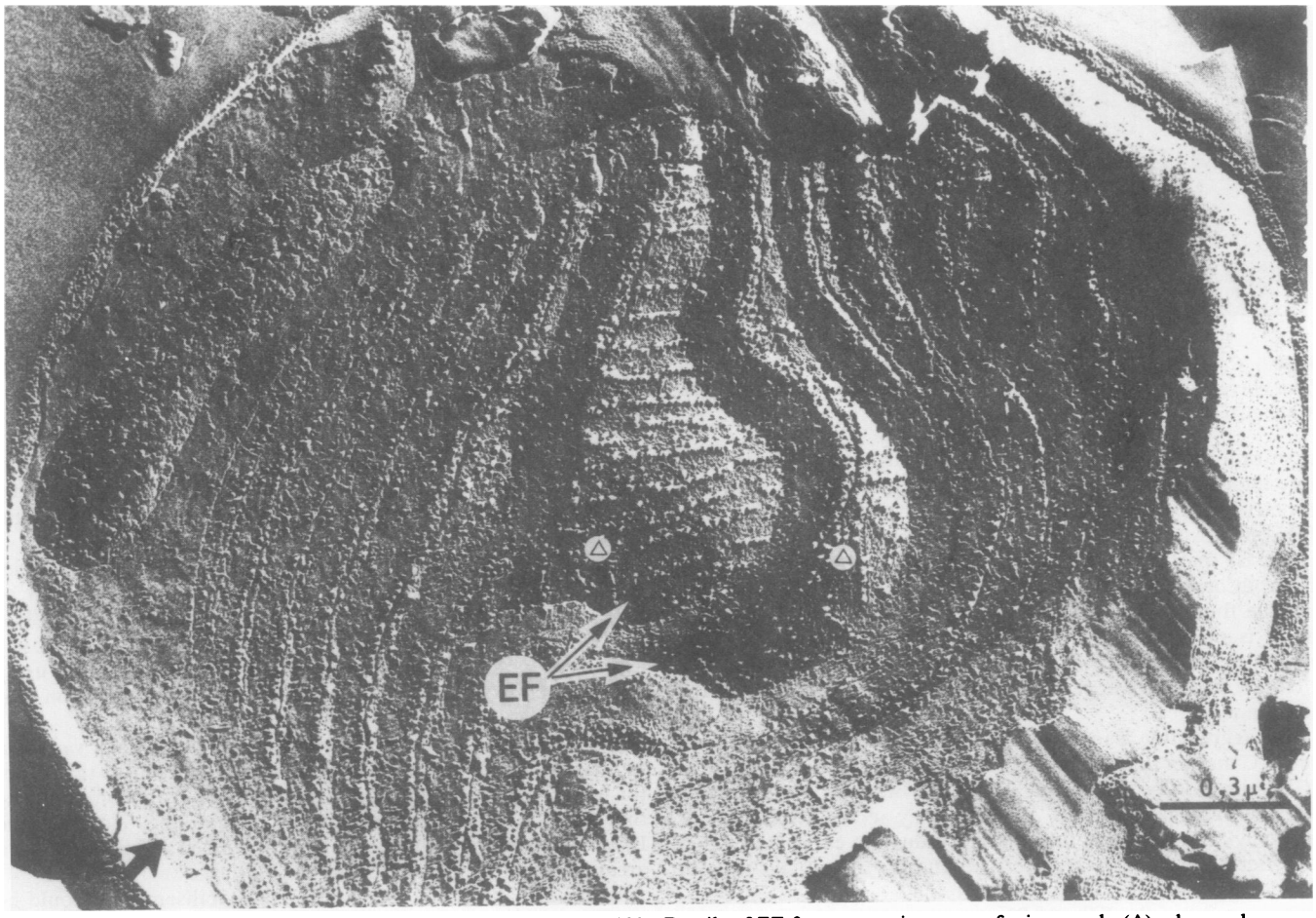

FIG. 7. Freeze-fractured chloroplast of *Cyanidium* wild type (× 65,000). Details of EF face appear in center of micrograph. (△): places where rows of particles are half-disrupted.

been previously reported (9) that when a few peripheral proteins (*i.e.* the phycobilisomes) remain linked to integral ones (*i.e.* the EF particles) the latter form clusters within the membrane.

This cannot explain why there are also organized and nonorganized areas on the EF faces of the wild type. Two explanations can be put forward: (*a*) the membrane is divided into two zones having different functional roles; (*b*) freezing and freeze-fracturing disrupt the membrane organization.

The first hypothesis is consistent with the fact that in *Cyanidium* wild type only half of the PSII units are attached to the phycobilisomes while the others show no contribution of phycocyanin to their antennae (6). The first fraction would correspond to the particles organized in linear rows and the second to the nonorganized areas.

However, partial contribution of the second hypothesis is likely as shown in Figure 7 where semiorganized regions can be seen on the EF faces of the wild type.

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