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Supramolecular structure of stacked and unstacked regions of the photosynthetic membranes of *Prochloron* sp., a prokaryote

(photosynthesis/thylakoids/chlorophyll a/b protein/freeze-fracture)

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ABSTRACT **Freeze-fracture replicas of the photosynthetic prokaryote *Prochloron* sp., collected at Coconut Island, Hawaii, show that the thylakoids are differentiated into stacked and unstacked regions much like the thylakoids of green algae and higher plants. On the exoplasmic (E) fracture face, the particle density is greater in stacked regions (≈3100 particles/μm^2) than in unstacked regions (≈925 particles/μm^2). On the complementary protoplasmic (P) fracture face, the particle density is lower in stacked regions (≈2265 particles/μm^2 than in unstacked regions (≈4980 particles/μm^2). The histogram of the diameters of E face particles in unstacked regions shows a single broad peak centered at 80 Å. In stacked regions, four distinct peaks, at 75, 105, 130, and 160 Å, are observed. These size classes are virtually identical to those found on E faces of thylakoids of the green alga *Chlamydomonas* and of greening pea chloroplasts. In the latter systems, the different size classes of E face particles are believed to represent photosystem II units surrounded by variable amounts of chlorophyll a/b light-harvesting complex. We propose that the same interpretation applies to the thylakoids of *Prochloron*, which contain a similar chlorophyll a/b complex. Our results add to the evidence supporting the view of the chlorophyll a/b complex as both a light-harvesting complex and a membrane adhesion factor. The similarity of the architecture of the thylakoids of *Prochloron* to that of green algal and plant chloroplasts also provides additional evidence of an evolutionary relationship between *Prochloron* and the chloroplasts of green plants.**

Prochloron, a unicellular photosynthetic prokaryote, is found in association with didemnid ascidians in marine environments (1–8). Members of the genus *Prochloron* and the cyanobacteria are prokaryotes that carry out photosynthesis in a manner similar to that of eukaryotic plants. This type of photosynthesis employs two reaction centers in noncyclic electron flow and evolves oxygen as a consequence of the splitting of water. Although the cyanobacteria contain high levels of phycobiliproteins for light harvesting, *Prochloron* lacks these pigments. Instead it contains a chlorophyll a/b pigment protin that is spectrally and electrophoretically indistinguishable from the chlorophyll a/b protein found in green algae and higher plants (7, 9). On the basis of these differences in light-harvesting pigment composition, Stanier and Cohen-Bazire (10) have supported the following version of the hypothesis of the endosymbiotic origin of chloroplasts: *Prochloron* and the cyanobacteria were derived from a common precursor cell capable of oxygen-evolving photosynthesis, and in turn, the cyanobacteria gave rise to the chloroplasts of red algae and *Prochloron* to those of green algae and higher plants.

Although previous studies have led to some controversi about the organization of the photosynthetic membranes in *Prochloron* (4–6, 8), virtually all of the published results support the following conclusions: The photosynthetic lamellae are typical closed thylakoids. As in the cyanobacteria, the thylakoids are the only observable membrane system in the cytoplasm and they are not enclosed by an envelope membrane. The thylakoids of *Prochloron* differ from those of the cyanobacteria in that they lack detectable phycobilisomes, large (≈300 Å) aggregates of phycobiliproteins localized on the thylakoid surface. Most significantly for the present work, thin sections of *Prochloron* suggest that there are some areas of close appression ("stacked regions") between adjacent thylakoids. The freeze-fracture electron microscopy presented here provides evidence that morphologically differentiated stacked and unstacked regions similar to those found in the chloroplasts of green algae and higher plants occur in the thylakoids of *Prochloron*.

MATERIALS AND METHODS

Prochloron was collected at Coconut Island, Oahu, Hawaii, as described (8). Green colonies of didemnid ascidians, *Diplosoma virens*, were collected from shallow waters. Green spherical cells, identified as *Prochloron*, were released from the ascidians by gently pressing on the colonies with a pasteur pipette while flushing the surface with sea water. Cells were centrifuged, then rinsed in filtered sea water and prepared on location for freeze-fracturing in one of three ways: (*i*) Cells were sedimented and samples of the pellet were applied to copper support discs. The disc and sample were plunged into liquid Freon 22 and quickly transfered to liquid nitrogen. (*ii*) Five milliliters of 70% glycerol/30% sea water (vol/vol) was added dropwise over a 2-hr period to 5 ml of cells in sea water. After equilibrating for 30 min, cells were centrifuged and frozen as above. (*iii*) Cells were fixed for 30 min in 1.4% (wt/wt) glutaraldehyde in sea water. An equal volume of 70% glycerol/30% sea water was added dropwise over 1 hr. Cells were then frozen as above. After transport of the samples in liquid nitrogen to Colorado, replicas were prepared by using a Balzers BA 360 freeze-etch device. Samples were fractured at −110°C. Replicas were examined in a Jeol 100C electron microscope.

Material was prepared for thin sectioning as follows: Didemnid colonies containing *Prochloron* were fixed on location for 2 hr in 1.5% glutaraldehyde in sea water, rinsed, and stored in sea water for 3–4 weeks. Samples were then postfixed in 2% aqueous $KMnO_4$ for 24 hr at room temperature, dehydrated with an acetone series, and embedded in Spurr's resin. Thin sections were stained with aqueous uranyl acetate and lead citrate.

Freeze-fracture replicas are mounted so that the direction of shadow is from bottom to top. The terminology of Branton *et al.* (11) as modified by Staehelin (12) is used for designating

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Abbreviations: LHC, light-harvesting complex. Freeze-fracture membrane nomenclature follows refs. 11 and 12. P, protoplasmic; E, exoplasmic; F, internal fracture face; $_u$ and $_s$, unstacked and stacked.

the thylakoid fracture faces. P refers to the protoplasmic leaflet and E to the exoplasmic, or luminal, leaflet of the thylakoid membranes. F stands for internal fracture faces of membranes. The subscripts $_u$ and $_s$ designate unstacked and stacked membrane regions, respectively. Particle size and density determinations were made as described by Staehelin (12).

RESULTS

The samples of *Prochloron* consisted of bright green spherical cells 8–10 μm in diameter. Light microscopic examination indicated that in many cases the central region of the cell was transparent. The samples appeared to be essentially free of contaminating organisms, although a few diatoms were found in some of the freeze-fracture replicas. Our electron micrographs of thin sections closely resemble those published previously (4–6, 8). The center of the cell is often occupied by scattered floculent material (Fig. 1*a*) that seems to be contained in a region delimited by (modified?) thylakoid membranes (see also ref. 6). Although they resemble some micrographs of vacuoles in eukaryotic plants, such images do not unequivocally demonstrate the presence of a genuine membrane-limited vacuole in *Prochloron*. The thylakoids and densely stained cytoplasm are concentrated toward the cell periphery. In freeze-fractured cells (Fig. 1*b*), the cytoplasm has a fairly rough texture, whereas the intrathylakoid lumen has a smooth appearance.

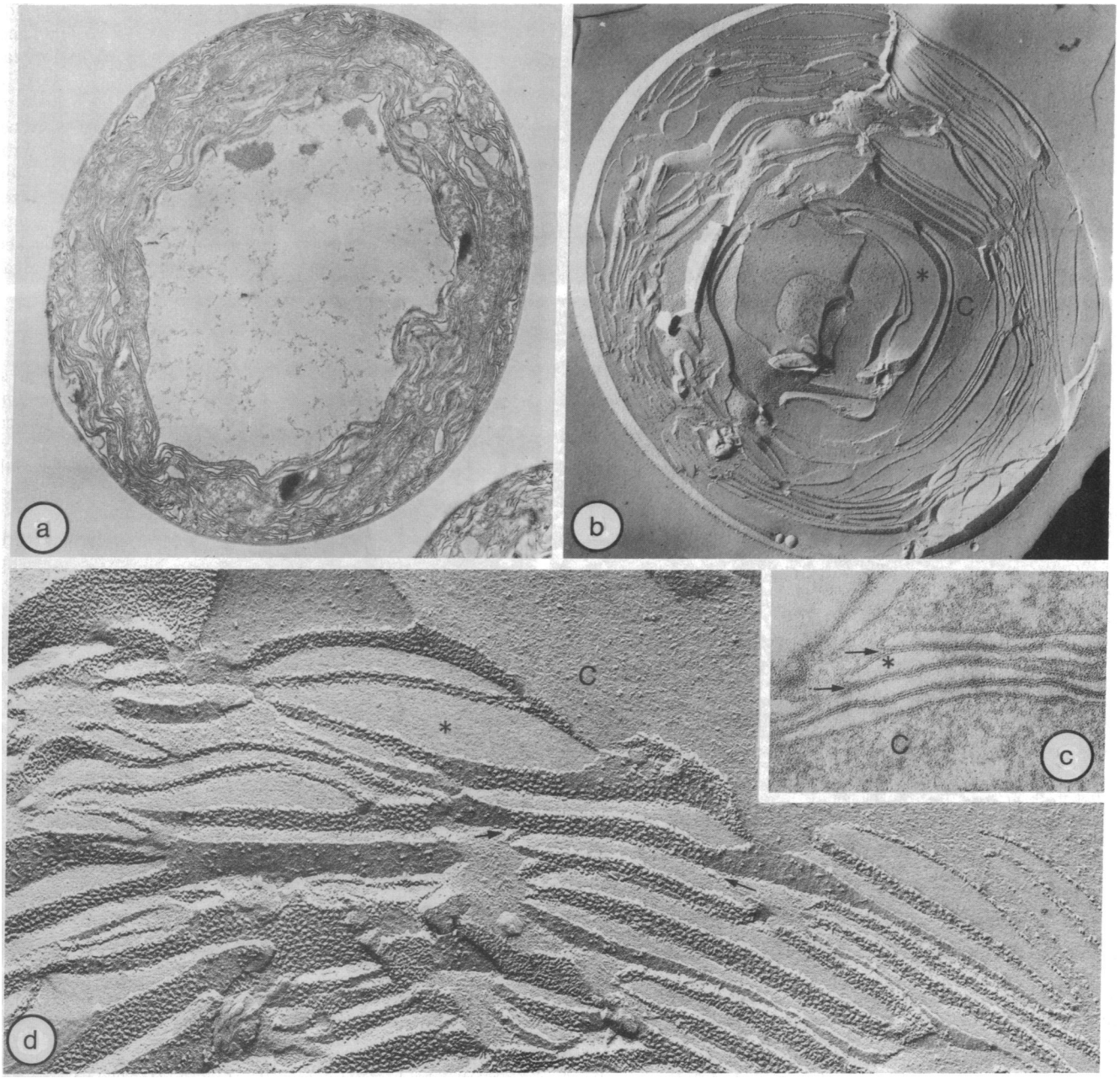

FIG. 1. (*a*) Thin section micrograph of a *Prochloron* cell. (×7500.) The central region, which contains some floculent material, appears to be delimited by membranes. The thylakoids and dense cytoplasm occupy the peripheral region. (*b*) Freeze-fracture replica of a *Prochloron* cell. (Glutaraldehyde, glycerol; ×11,000.) The lumen (*) within the thylakoids can be distinguished from the cytoplasm (C) by its smoother texture. (*c*) Thin section showing extensive regions of contact between adjacent thylakoids (arrows). (×92,000.) The thylakoid lumen (*) is relatively free of stained material, in contrast to the cytoplasm (C). (*d*) Freeze-fracture micrograph showing appression of adjacent thylakoid membranes. Areas of membrane contact (stacked regions) are marked by a greater density of particles on the E fracture face (arrows). (Glutaraldehyde, glycerol; ×51,000.)

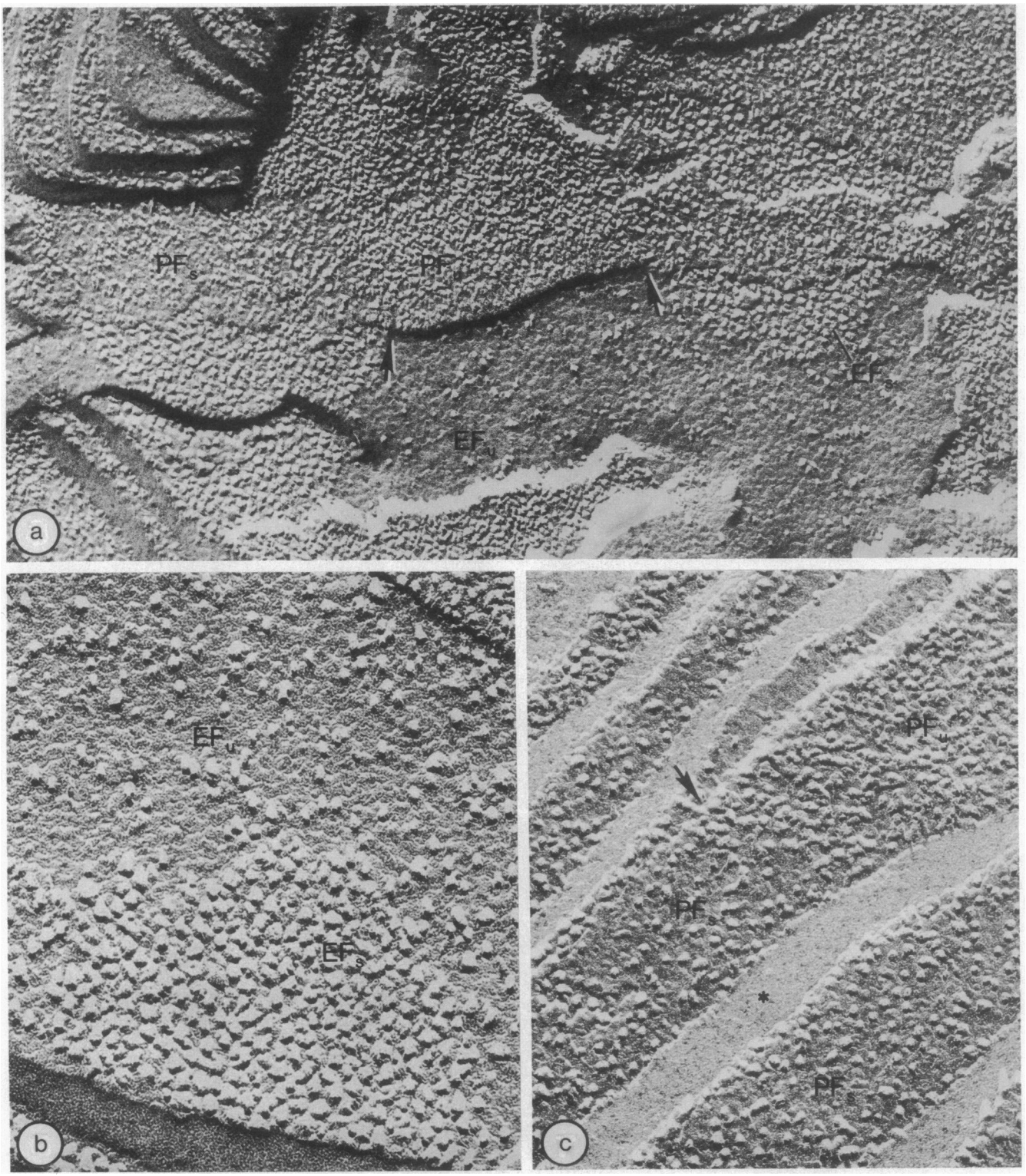

FIG. 2. (*a*) Micrograph showing large fracture faces of adjacent thylakoids. (Glycerol; ×76,000.) An unstacked region is visible in the center, where a high ridge (between arrows) indicates that there is space between the thylakoids. Where the ridge is small, the thylakoids are in contact with one another, forming stacked regions. Note the differences in morphology on both fracture faces between stacked and unstacked regions. Freeze-fracture terminology is explained in *Materials and Methods*. (*b*) High-magnification view of the thylakoid E face. (Glutaraldehyde, glycerol; ×150,000.) Particles are tightly packed in the stacked regions (EF_s). The density of particles in the unstacked region (EF_u) is higher than usual, but still noticeably less than in stacked regions. (*c*) P face of several thylakoids. (Glutaraldehyde, glycerol; ×150,000.) A transition from stacked (PF_s) to unstacked (PF_u) morphology coincides with the point where adjacent thylakoids separate (arrow). In some cases, it was difficult to distinguish PF_s regions from PF_u or even EF_u regions. *, Thylakoid lumen.

Electron micrographs of freeze-fracture replicas and thin sections of *Prochloron* sp. show that the photosynthetic thylakoids of this organism are differentiated into stacked and unstacked regions similar, but not identical, to those found in the chloroplasts of green plants. Close apposition of variable numbers of adjacent thylakoids is observed in thin sections (Fig.

Table 1. Particle density in thylakoids

Thyalkoid region	Particles per μm^2 of thylakoid fracture face*		Ratio of PF to EF particles	
	E face	P face	*Prochloron*	Spinach†
Stacked	3106 ± 43	2265 ± 57	0.7	2.3
Unstacked	925 ± 35	4979 ± 80	5.4	6.3

* Mean ± SEM.
† From ref. 12.

1*c*) and in freeze-fracture replicas (Fig. 1*d*). Morphological differences between stacked and unstacked regions are most clearly demonstrated when extensive fracture faces of adjacent thylakoids are obtained (Fig. 2*a*). The thickness of the ridge between these thylakoids gives an indication of how close together they are. In the center of the micrograph, a fairly high ridge indicates that there is space between the thylakoids. On both sides of the micrograph, the ridge becomes small, indicating appression between the adjacent membranes. The transition point between unstacked and stacked morphology coincides with the point where the thylakoids come together. Additional examples of the E face and the P face are shown at higher magnification in Fig. 2 *b* and *c*, respectively. On the E face, the stacked regions (EF_s) contain a high concentration of relatively large particles with little space discernible between particles. In contrast, the unstacked regions (EF_u) contain a much lower density of particles (Table 1). On the P face, the reverse is true, the density of particles being lower in stacked regions (PF_s) than in unstacked regions (PF_u). In Fig. 2*c*, the change in density can be seen to coincide with the transition from separate to appressed thylakoids. There is no obvious difference in the size of the PF particles between stacked and unstacked regions. Due to a degree of variability in the appearance of the fracture faces, positive identification was sometimes difficult, especially in identifying PF_s regions. Statistical determinations were made only from areas that could be unambiguously identified.

Histograms of particle diameters for each of the four types of thylakoid fracture face in *Prochloron* are shown in Fig. 3. EF_s particles fell into four discernible size categories centered at 75, 105, 130, and 160 Å. The histogram of EF_u particle diameters shows a single broad peak centered at 80 Å, with a shoulder at 105 Å. The mean particle size was 82 Å in unstacked and 114 Å in stacked regions. On the P face, the average diameter was slightly greater (88 Å) in stacked regions than in unstacked regions (84 Å).

Cells that had been briefly fixed and treated with glycerol were chosen for detailed analyses because these exhibited the best overall preservation with minimal osmotic damage or distortion resulting from ice crystal formation. However, samples treated only with glycerol (as in Fig. 2*a*) or given no pretreatment prior to freezing showed the same basic morphology.

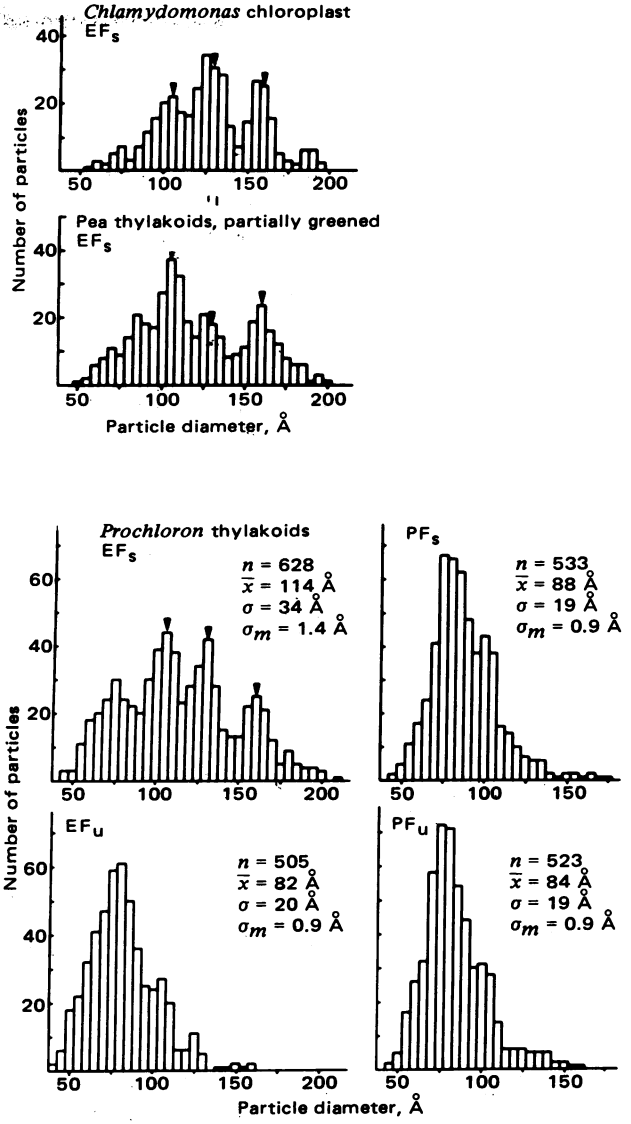

FIG. 3. (*Upper two histograms*) Particle diameters in EF_s regions of chloroplasts from *Chlamydomonas reinhardtii* (19) and from partially greened pea leaves (see *Discussion* and ref. 14). Arrowheads at 105, 130, and 160 Å emphasize the similarity in the positions of the maxima in the histograms of EF_s particle diameters in *Prochloron*, *Chlamydomonas*, and partially greened pea leaves. (*Lower four histograms*) Particle diameters on the four types of thylakoid fracture faces in *Prochloron*. n = number of particles measured; $\bar{x}$ = mean diameter; σ = standard deviation; σ_m = standard error of the mean.

DISCUSSION

The architecture of the thylakoids in *Prochloron*, as revealed by freeze-fracture electron microscopy, is strikingly similar to that found in the chloroplasts of green plants. The concentration of EF particles into areas of appression of adjacent thylakoids (stacked regions) is a feature characteristic of green algae and higher plants (15) and, as demonstrated here, of *Prochloron*. The thylakoids of all of these organisms contain a chlorophyll a/b light-harvesting complex (LHC). Withers *et al.* (7) have reported that the LHC of *Prochloron* is spectrally and electrophoretically indistinguishable from that of green plants. The observation of thylakoid stacking in *Prochloron* is therefore in agreement with considerable evidence (16–18) that links the presence of LHC to the ability of thylakoids to form membrane–membrane adhesions. The cyanobacteria, prokaryotes capable of oxygen-evolving photosynthesis, but lacking the chlorophyll a/b LHC, do not exhibit stacked thylakoids (13, 15).

Our results demonstrate the occurrence of four distinct size categories of particles, 75, 105, 130, and 160 Å in diameter, on the E face of stacked thylakoids in *Prochloron* (Fig. 3). Virtually identical size categories were observed in the thylakoids of *Chlamydomonas reinhardtii* (Fig. 3 *Upper*, from ref. 19) and in partially greened chloroplasts of pea seedlings (Fig. 3 *Upper*, from ref. 14). By examining plants that had been grown under intermittent light and subsequently exposed to 0–48 hr of continuous light, Armond *et al.* (14) were able to correlate the occurrence of an increasing proportion of EF_s particles in the

larger size categories with the incorporation of larger amounts of LHC into the thylakoids. These authors conclude that the 80-, 105-, 130-, and 160-Å EF_s particle size categories correspond to photosystem II reaction center complexes with 0, 1, 2, or 4 aggregates of associated chlorophyll a/b LHC. Because we find exactly the same size categories of EF_s particles in *Prochloron*, it seems reasonable to propose that the same types of complexes are present in this prokaryote. However, because *Prochloron* thylakoids contain a greater proportion of EF_s particles in the smaller size categories, we suggest that the average photosystem II unit size is smaller relative to higher plants. This is in agreement with the hypothesis that the smaller photosynthetic unit size of *Prochloron* (≈240 chlorophyll molecules per P700) relative to green plants (≈400 chlorophyll molecules per P700) is due primarily to the reduced level of chlorophyll a/b LHC (7, 9).

Differences between the thylakoids of *Prochloron* and higher plant chloroplasts are also observed in histograms of P face particle diameters. Whereas fully greened pea and spinach chloroplasts have two distinct size classes of PF particles at 80–85 Å and 105 Å (12, 14), *Prochloron* has only one broad size class centered at ≈80 Å. The larger size class in pea and spinach chloroplasts may represent an association of a fraction of the photosystem I particles with an as-yet-undefined LHC because the formation of this category of particles is also light dependent (14). The absence of the larger particles on the P face of thylakoids in *Prochloron* could represent another manifestation of the smaller photosynthetic unit size in this organism.

In addition, the ratio of PF_s particles to EF_s particles in the thylakoid membrane in *Prochloron* is 0.7 (Table 1), whereas that of the higher plant chloroplasts is about 2–3 (12, 14). At present we do not know whether this difference results from different cleaving properties of similar membrane constituents or from the presence of different types of constituents.

This study of the freeze-fracture morphology of the thylakoids of *Prochloron* represents a significant addition to a previous survey of thylakoid morphology among oxygen-evolving photosynthetic organisms (15). *Prochloron* resembles green algae and higher plants in that large size categories of EF particles, presumably representing photosystem II units, are concentrated into areas of thylakoid stacking. Both the cyanobacteria and red algae, however, have only unstacked thylakoids. In these groups, a homogeneous population of EF particles, measuring ≈100 Å in diameter, has been tentatively identified as consisting of the active photosystem II units (13, 20). Thus, the morphological studies agree with the evolutionary hypothesis based on pigment content (10) that two types of oxygen-evolving prokaryotes could have given rise to modern day photosynthetic systems employing two fundamentally different types of light-harvesting elements, the chlorophyll a/b LHC and the phycobiliproteins.

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