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Studies on the cytochrome b_6/f complex. II. Localization of the complex in the thylakoid membranes from spinach and *Chlamydomonas reinhardtii* by immunocytochemistry and freeze-fracture analysis of b_6/f mutants

Jacqueline Olive [a], Olivier Vallon [a], Francis-André Wollman [b], Michel Recouvreur [a] and Pierre Bennoun [b]

[a] *Laboratoire de Microscopie Electronique, Institut Jacques Monod du CNRS, Université Paris VII, 2 Place Jussieu, 75251 Paris Cedex 05, and* [b] *Service de Photosynthèse, Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75231 Paris Cedex 05 (France)*

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The distribution of the b_6/f complex among stacked and unstacked thylakoid membranes was studied by immunocytochemistry and freeze-fracture analysis of mutants of *Chlamydomonas reinhardtii* lacking the complex. Immunogold labeling demonstrates the presence of b_6/f complex in both regions of the thylakoid membrane in spinach and in *C. reinhardtii*. Numerous modifications were observed in the ultrastructure of the thylakoid membranes of mutants from *C. reinhardtii* lacking the complex. These modifications are consistent with the presence of b_6/f complexes in different states of association in the stacked and unstacked regions of the thylakoid membrane. In particular we present evidence for an association of some b_6/f complexes with the reaction centers of Photosystem I and II in large PFu and EFs particles, respectively.

Introduction

In recent years, it has been shown that the differentiation of the thylakoid membranes in stacked and unstacked regions corresponds to an asymmetry in the lateral distribution of the proteins associated with each photosystem: Photosystem I is restricted to the unstacked regions, whereas Photosystem II is located mainly in the stacked regions of the thylakoid membranes (for a review, see Ref. 1).

The location of the b_6/f complex, which contains electron carriers connecting the two photosystems, is more controversial. Its purported location has been alternatively in the unstacked membrane regions [2], in the regions interfacing the stacked and unstacked membranes [3,4] or in both the stacked and unstacked regions of the thylakoid membranes [5,6]. The latter hypothesis has received recent support from immunocytochemical studies on higher plant chloroplasts [7,8].

In the present paper, we investigated the distribution of the b_6/f complex among stacked and unstacked membranes by immunogold labeling of thylakoids from spinach and from the green algae *Chlamydomonas reinhardtii*. In addition, we used mutant strains from this algae, lacking the b_6/f complex, in a comparative freeze fracture study. This approach gives new insights on the possible

Abbreviations: EF, exoplasmic fracture face; PF, protoplasmic fracture face; s, stacked; u, unstacked; PS, Photosystem; Cyt, cytochrome; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

association of some b_6/f complexes with the reaction centers in the thylakoid membranes.

Material and Methods

The wild-type FuD8, F18 and F34F18 strains of *C. reinhardtii* were grown in Tris-acetate phosphate medium, under cool fluorescent light (500 lx). Cells were broken in the presence of $MgCl_2$, as previously described [9].

- Spinach leaves were purchased at the local market and chloroplasts were prepared according to Avron [10].
- SDS polyacrylamide gel electrophoresis (12–18%) was performed in the presence of 8 M urea, according to [11]. TMBZ staining was performed according to Ref. 12.
- For freeze-fracture experiments, the cells were frozen in Freon 22 and fractured in a Balzers' apparatus at −150°C.
- For immunocytochemical analysis, samples were fixed in 1% glutaraldehyde in 4 mM potassium phosphate buffer (pH 7.0) rinsed in 0.1 M glycine and embedded in lowicryl K4M, according to Carlemalm et al. [13]. Immunogold labeling and staining were performed as in Ref. 14, using goat serum (1 : 10 diluted) instead of bovine serum albumin.
- Thin sections and replicas were examined in a Philips EM 400 electron microscope and a Tektronix computer was used for quantitative measurements.

TABLE I

LABELING OF SPINACH AND *C. REINHARDTII* CHLOROPLASTS BY THE α-RIESKE ANTIBODY

	Proportion of label over stacked membranes (%)	Labeling densities (gold granules/μm)		Ratio of labeling densities unstacked/stacked
		unstacked membranes	stacked membranes	
Spinach	65.0	1.27	0.81	1.6
C. reinhardtii:				
wild type	69.8	0.24	0.18	1.3
F18	70.0	0.04	0.04	1
wild type-F18	68.7	0.20	0.14	1.4

Results

We performed an immunocytochemical study on the location of the b_6/f complex with an antibody raised against the Rieske protein from spinach [15]. As shown in the companion paper [16], the antibody cross-reacted only weakly with the Rieske protein from *C. reinhardtii*. Therefore we observed a rather low density of immunogold labeling on Lowicryl thin sections of the broken cells from the wild-type strain of *C. reinhardtii* (Table I). However, Fig. 1a shows unambiguous labeling both on the stacked and unstacked regions of the thylakoid membranes. In order to assess the specificity of this labeling we performed the same experiment on the F18 mutant which contains no Rieske protein [16]. This gave an estimate of the background labeling in our procedure. As shown on Fig. 1b and Table I the background labeling was evenly distributed between stacked and unstacked membrane regions and accounted for 15–20% of the total labeling on wild-type thylakoids. The corrected values for the wild type (wild type − F18 in Table I) show that the density of specific labeling was 40% higher in the unstacked regions than in the stacked regions. Taking into account the proportion of 75% stacked membranes in our preparation, we are led to conclude that a majority of the labeled b_6/f complexes, 68%, were located in the stacked regions of the thylakoid membranes.

In contrast to the case of *C. reinhardtii*, Lowicryl sections of spinach thylakoids were heavily labelled with the antibody (Fig. 1c and Table I). A significant labeling was observed both on unstacked and stacked thylakoid regions with no particular accumulation of gold granules at the junction of the two domains. The density of labeling was 60% higher in the unstacked than in the stacked membranes. The latter accounting for 65% of the total labeling. Negligible labeling occurred with a non-immune serum (Fig. 1d).

In order to get further information on the organization of b_6/f complexes in the thylakoid membranes of *C. reinhardtii*, we undertook a comparative freeze-fracturing study on thylakoids from the WT and from mutant strains lacking the b_6/f complex. Two such mutants were anlysed: the F18 mutant of nuclear origin and the FuD8 mutant of

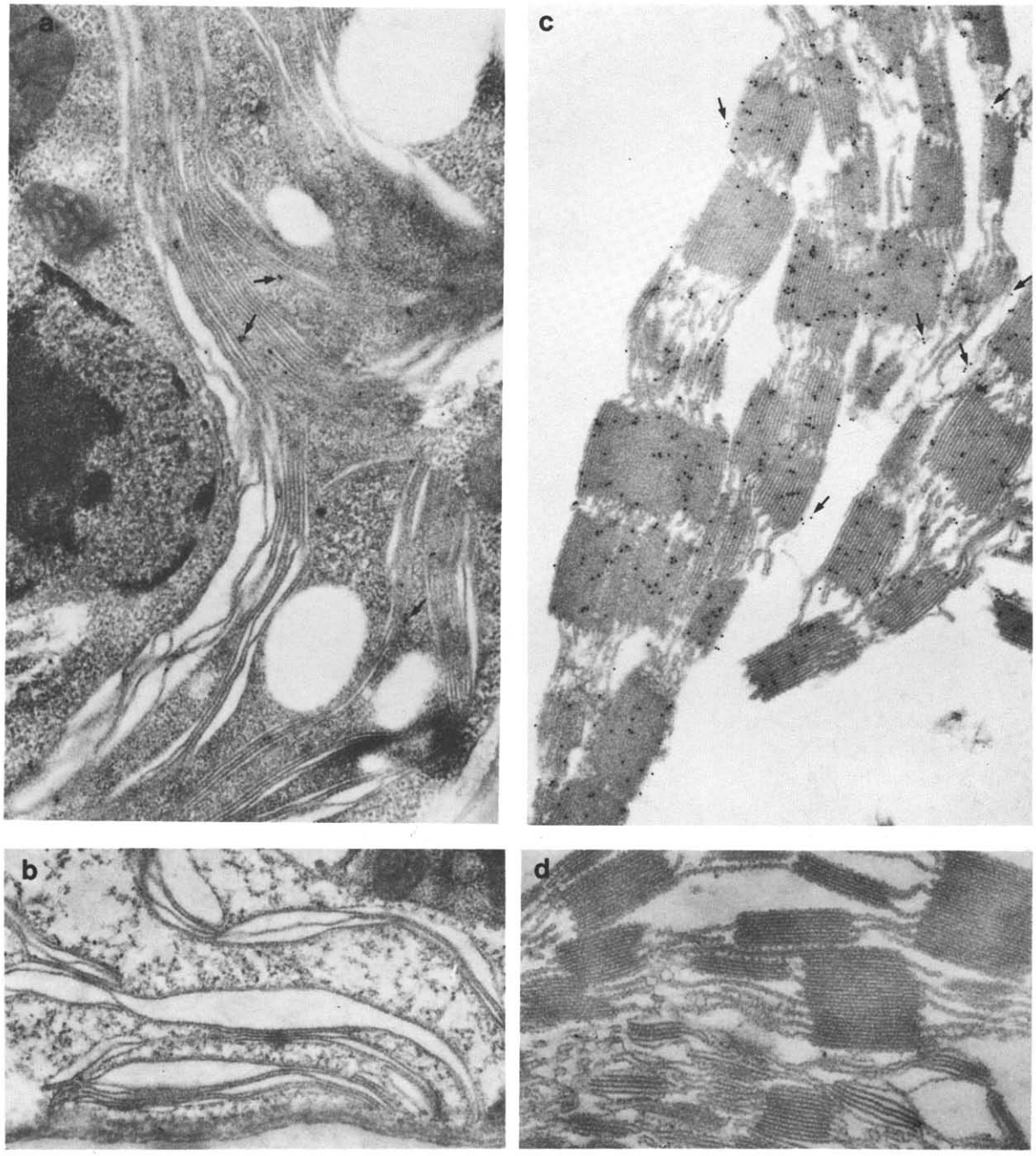

Fig. 1. Immunolabeling of the α-Rieske antibody on *C. reinhardtii* wild-type (a) and spinach (c) thylakoid membranes. Both stacked and unstacked membranes are labeled. Arrows indicate gold granules in unstacked membranes. In (b), F18 mutant lacking Cyt b_6/f complex, with the α-Rieske antibody and in (d) spinach thylakoids incubated with a non-immune serum, show negligible labeling. Magnification: 30000.

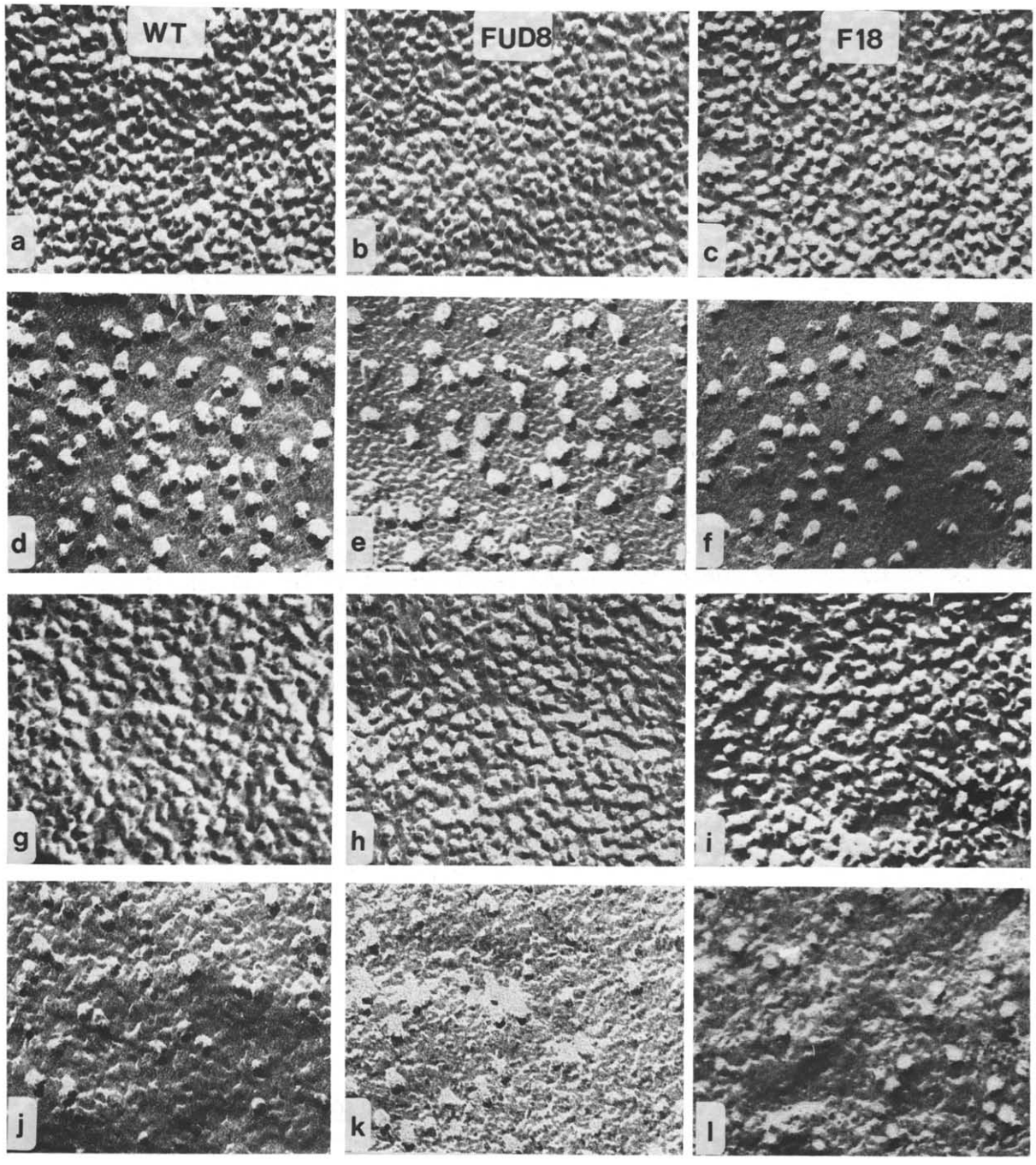

Fig. 2. Fracture faces of the wild-type (WT) FuD8 and F18 strains: PFs in (a)–(c), EFs in (d)–(f), PFu in (g)–(i) and EFu in (j)–(l). Magnification: 200000.

chloroplastic origin. These two strains have similar functional and polypeptide deficiencies as described in the accompanying paper [16].

As usually observed with chloroplasts from higher plants and green algae when broken in the presence of $MgCl_2$, there are distinct sets of par-

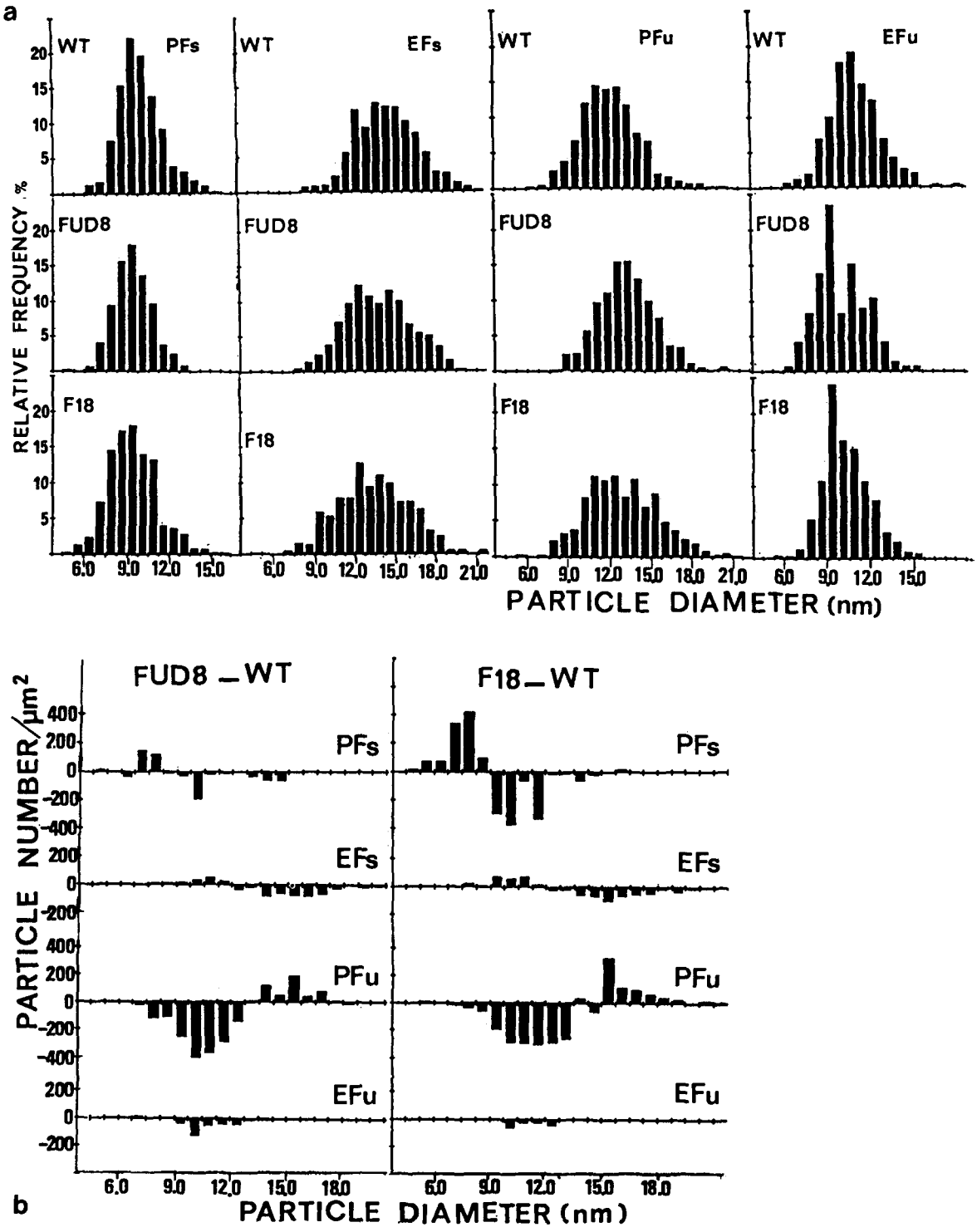

Fig. 3. (a) Histograms of the particle sizes of the wild-type (WT), FuD8 and F18 fracture faces. (b) Histogram of differences in densities of particles in each size class between the mutants and the wild type.

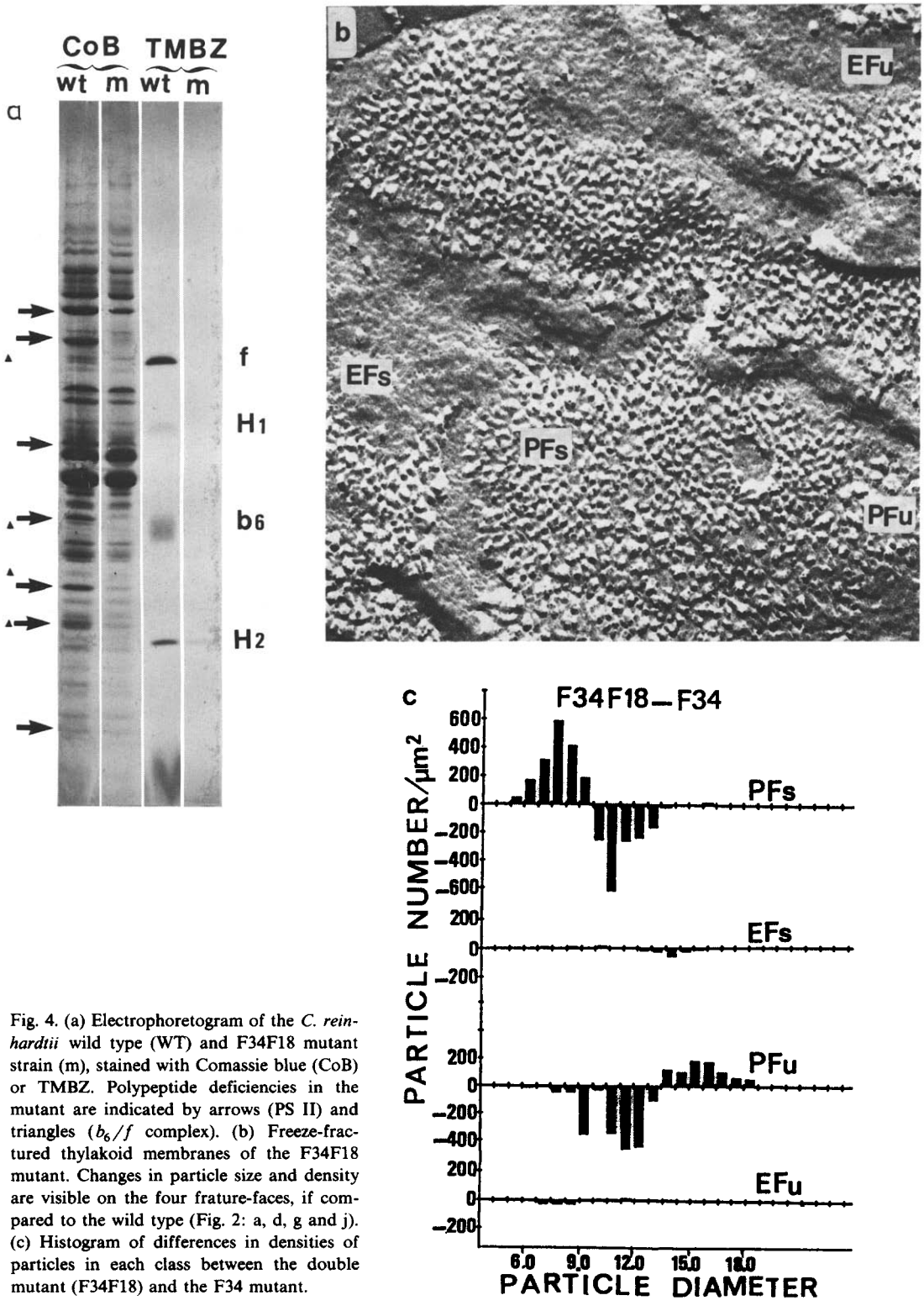

Fig. 4. (a) Electrophoretogram of the *C. reinhardtii* wild type (WT) and F34F18 mutant strain (m), stained with Comassie blue (CoB) or TMBZ. Polypeptide deficiencies in the mutant are indicated by arrows (PS II) and triangles (b_6/f complex). (b) Freeze-fractured thylakoid membranes of the F34F18 mutant. Changes in particle size and density are visible on the four frature-faces, if compared to the wild type (Fig. 2: a, d, g and j). (c) Histogram of differences in densities of particles in each class between the double mutant (F34F18) and the F34 mutant.

TABLE II

PARTICLE DENSITY

Values are measured in numbers per $\mu m^2 \pm$ S.E.

Strain	PFs	PFu	EFs	EFu
Wild type	6300∓180	5280∓270	1680∓310	790∓160
FUD8	6260∓545	4000∓400	1250∓260	420∓200
F18	6120∓430	4060∓160	1360∓170	570∓140

ticles on the freeze-fracture faces corresponding to either the stacked (EFs and PFs) or unstacked (EFu and PFu) thylakoid membranes. Although the overall aspect of the freeze fractured membranes was very similar in the three strains (Fig. 2a–i), a close examination of the densities and diameters of the particles on each type of fracture face revealed several modifications in the mutants.

A clear modification in the densities of particles (Table II) was observed on the PFu faces: it corresponds to a 20% decrease in density, equivalent to the loss of about 1000 particles/μm^2 in the two mutants. A smaller but significant decrease in particle density ($\approx$ 300 particles/μm^2) was also observed on the EFs and EFu faces of the mutants.

The histograms of particle sizes on the four fracture faces are shown on Fig. 3a. The shape of the histograms showed several modifications in the mutants as compared to the wild type. On PFs, EFs and EFu, the proportion of particles in the classes of larger diameter was reduced, whereas a shift towards the larger diameters was observed on PFu. In order to disclose which classes of particles were missing in the mutants, we analysed our results using the following rational: the number of particles in each size class was calculated in the three strains on the basis of the density of particles measured on the corresponding fracture face. We then constructed the histograms of the differences in densities of particles in each size class between the mutants and the wild type. These histograms are shown on Fig. 3b. The negative and positive values indicate loss and gain, respectively, of particles in the corresponding size class in the mutants as compared to the wild type. The modifications were qualitatively the same in the two mutants. They were the following.

– On the PFs faces: a decrease in the number of particles 9.0–12.0 nm in diameter which is balanced by an increase in the population of particles 6.0–9.0 nm in diameter.

– On the EFs faces: a decrease in the number of particles larger than 11.0 nm, only partly balanced by an increase in the number of particles 8.0–10.0 nm in diameter. This results in the net loss of EFs particles described above.

– On the PFu faces: a large decrease in the number of particles 7.5–13.0 nm in diameter and a smaller increase in the number of particles larger than 13.5 nm. The difference in amplitude of the two opposite changes explains the net decrease in density on the PFU faces as described above.

– On the EFu faces: an unbalanced decrease in particles larger than 10.5 nm.

In view of the multiplicity of the changes observed in freeze-fractured thylakoid membranes in single mutants lacking the b_6/f complexes we looked for a situation where the control membrane would be of a simpler composition. This is the case with photosynthetic mutants lacking PS II centers and consequently devoid of most but not all the EF particles observed in freeze-fractured thylakoid membranes [17–19].

A double mutant, the F34F18 strain, lacking both PS II reaction centers and b_6/f complexes, was compared with the single mutant F34 lacking the PS II centers only. On Fig. 4a are shown the polypeptide and heme deficiencies in the double mutant.

We have extensively characterized the ultrastructure of the F34 thylakoids previously [17,20]. In this strain, the disappearance of most of the EFs particles was accompanied by an increase in the density of the PFs particles due to some LHC complexes no longer associated with the PS II centers on the EF faces. The double mutant F34F18 had the same characteristics when compared with the wild type strain. However, it showed some additional modifications when compared with the single mutant F34 (Fig. 4b and c and Table III). There were about 1200 particles/μm^2 less on the PFu faces of the double mutant. In addition the histograms of differences in particle densities per size class (Fig. 4c), constructed from the histograms of particle sizes in the F34 and F34F18 (not shown) revealed a loss of approx. 1600 PFs particles/μm^2 of diameters larger than 10.0 nm balanced by an equivalent increase in the

TABLE III

PARTICLE DENSITY

Values are measured in numbers per $\mu m^2 \pm$ S.E.

Strain	PFs	PFu	EFs	EFu
F34	6950∓165	4770∓450	650∓130	300∓60
F34 F18	7000∓224	3550∓400	460∓150	290∓65

number of particles of smaller diameters. The density of EFs particles was somewhat smaller in the double mutant. It was, however, difficult to assess the significance of this latter modification owing to the small number of particles encountered on this fracture face. We note that the changes on the PFs and PFu faces are of same characteristics in the double mutant F34F18 and in the single mutants FuD8 and F18 (compare Tables II and III and Figs. 3b and 4c).

Discussion

Using two different approaches, immunocytochemistry and freeze-fracture analysis of thylakoid membranes from photosynthetic mutants, we obtained results consistent with the localization of b_6/f complexes in both the stacked and unstacked regions of the thylakoid membranes.

The present immunocytochemical study was performed with an antibody directed against the Rieske protein. As reported in the accompanying paper, the insertion of this protein in the thylakoid membrane probably requires the accumulation of cytochromes b_6 and f. The distribution of the Rieske protein must then parallel that of the whole complex. We observed labeling on the stacked and unstacked regions of the thylakoid membranes both in *C. reinhardtii* and spinach. This conclusion is in agreement with the observations recently reported on higher plant thylakoids using α Cyt f antibodies [8] or α b_6/f antibodies [7]. Although the present antibody showed very different affinities in *C. reinhardtii* and in spinach, we end up with a similar proportion of 65–70% of the labeling in the stacked regions. Unless some selective decrease in antigenicity in either of the two thylakoid regions occurred during fixation or embedding, this result indicates that a majority of the b_6/f complexes were located in the stacked regions of the thylakoid membranes in our experimental conditions.

Besides being an independent tool to investigate the distribution of b_6/f complexes along thylakoid membranes, freeze-fracture studies on mutants deficient in b_6/f complexes may provide additional information on the interactions between b_6/f complexes and some other membrane proteins.

Independent of a particular mutant, we consistently observed the same set of modifications in the ultrastructure of the thylakoid membranes. These modifications affected both the stacked and unstacked regions of the thylakoid membranes. However, the multiplicity of the changes prevents a straightforward answer as to which class of freeze-fracture particles represents the b_6/f complex.

In the unstacked membrane regions there was a drastic reduction in the density of PFu particles: more than 1000 particles/μm^2 of diameters ranging from 8.0 to 13.0 nm, disappeared in the three mutants, FuD8, F18 and F34F18. Some of these particles can accomodate individual b_6/f complexes which give rise to particles 8.5 nm in diameter upon reconstitution into liposomes and freeze-fracture [21]. The larger PFu particles which disappear in the mutants have diameters similar to that of PS I containing particles, 10.5–13.0 nm [22–24]. Therefore a fraction of the b_6/f complexes might be associated with PS I centers in these 10.5–13.0 nm large PFu particles. In this hypothesis, we expected the appearance in the mutants lacking the b_6/f, of a new population of individual PS I centers in 8.0–10.0 nm large PFu particles [22], originating from the b_6/f-PS I complexes present in the wild type and F34 strains. However, this phenomenon is masked by the parallel disappearance of the individual b_6/f complexes 8.5 nm large.

The increased number in PFu particles with diameters larger than 13.0 nm in the FuD8, F18 and F34F18 mutants is puzzling. We note that most of these large particles were rather flat and displayed a rod-like shape. They might arise from a reorganization of membrane structures involving modified lipid–protein interactions.

Still another modification in the unstacked region is the decrease in the density of EFu particles (about 300 particles/μm^2) in the F18 and FuD8 mutants as compared to the wild type. No such difference was observed in the comparison be-

tween the F34 and F34F18 mutants. Therefore the changes on the EFu faces might depend on the presence of PS II centers which were previously reported to correspond to part of the EFu particles [25]. The possible association of some b_6/f complexes with PS II centers gains additional support when the EFs faces are examined as discussed below.

In the stacked regions, the population of EFs particles which was mainly affected in the b_6/f single mutants, had a size distribution analogous to that of the particles which we [17,19,20] and others [18,26–28] have previously identified as containing the PS II reaction centers. There was no reduction in the relative amount of polypeptides associated with PS II in the FuD8 and F18 mutants as shown by SDS-polyacrylamide gel electrophoresis (see Fig. 2 in the accompanying paper). In addition, we observed a similar F_v/F_0 ratio and sigmoidicity of the fluorescence induction curves in the presence of DCMU in these mutants and in the wild type of *C. reinhardtii* (data not shown). Therefore we can exclude a significant effect of these mutations on the density of PS II centers in the thylakoid membrane.

Thus our freeze fracture analysis favours an association of some b_6/f complexes and PS II centers in the thylakoid membranes. The question is then raised as to why there was a change in the density of the EFs particles (and similarly in that of the EFu particles) in the mutants, since the absence of b_6/f complexes should preserve the PS II part of the particles. This should result in an increase in the number of particles in the 8.0–10.0 nm size classes corresponding to individual PS II reaction centers [29–31]. Such an increase was indeed observed on EFs (Fig. 4b) but to a smaller extent than expected, since it represented 1/4 and 1/3 of the EFs particles missing in the FuD8 and F18 mutants, respectively. As suggested above for the PFu faces, the presence in the wild type of individual b_6/f complexes in 8.5 nm large EFs particles, in addition to those associated with PS II in larger particles, accounts for this observation: the absence of individual b_6/f complexes will partly mask the appearance of PS II centers in the 8.0–10.0 nm size classes in the mutants.

Owing to their size distribution, the EFs particles remaining in mutants lacking the PS II centers [17,20] were good candidates to represent these individualized b_6/f complexes. Unfortunately most of the EFs particles present in the F34 single mutant, lacking in PS II centers, remained in the double mutant F34F18, lacking both b_6/f complexes and PS II centers. In addition the small number of EFs particles specifically lacking in the double mutant were not of about 8.5 nm but were larger than 10.0 nm.

We then considered the possibility that the partition coefficient of b_6/f complexes between PFs and EFs, varied in thylakoids having or not the PS II centers as is the case for LHC complexes [20,22]. In the hypothesis where more b_6/f complexes were retained on the PFs faces in the absence of PS II centers, we should observe an increased modification of the PFs characteristics in the F34F18 as compared to the case of the single mutant F18. In the three mutants lacking the b_6/f complexes, a fraction of the PFs particles underwent a shift in size from 10.0–14.0 nm to 75–10.0 nm. In agreement with our hypothesis, comparison of the F18 and F34F18 showed that the shift involved a larger number of PFs particles in the double than in the single mutant.

The interpretation of the shift of sizes on PFs remains unclear. One possibility would be that some LHC complexes, located on the EFs faces in the wild type were transferred on PFs in the F18 and FuD8 mutants. In this hypothesis the effect of the b_6/f mutation on the EFs particles would be an indirect effect through LHC displacement. In the mutant strains, these LHC complexes would correspond to the additional PFs population of smaller sizes whereas the absence of b_6/f complexes would cause the loss of the larger PFs particles. Then the shift on PFs would not have been observed in the comparison between F34 and F34F18, since LHC is no longer located on EFs faces in the absence of PS II centers [17,20]. Contrarily to this prediction, the size shift on PFs was still observed in the latter case. Therefore we must consider that an actual association of b_6/f complexes with some other protein in particles 10.0–14.0 nm in diameter exists on the PFs faces of the wild type and F34 thylakoids.

Conclusions

The changes in the organization of the particles on the EFs and PFu faces are consistent with an association of some b_6/f complexes with the PS II

and PS I reaction centers. Such a possibility has been recently discussed by Lam and Malkin [32,33]. It is also supported by our observation that purified PS I or PS II particles from *C. reinhardtii* frequently show b_6/f contamination upon heme staining with TMBZ (Wollman, F.-A., unpublished data). However, our results cannot be interpreted without the existence of a still larger proportion of b_6/f complexes located independently from the reaction centers on each fracture face. Such multiple locations strongly suggest that the relative proportion of b_8/f complexes in each type of location may vary upon changes in the physiological state of the chloroplast. For instance we note that the shift on the PFs faces involves less particles in the FuD8 than in the other mutants. In addition, complementary studies (unpublished data) on freeze-fractured intact cells of a mutant strain of *Chlorella sorokiniana,* S14, lacking the b_6/f complexes, showed changes qualitatively similar to those in broken cells of *C. reinhardtii*, but of different distribution. In particular the density of PFu particles showed little changes in the mutant as compared to the wild type but a significant shift towards smaller sizes was observed. This exemplifies a situation where a stronger association of b_6/f complexes with PS I centers could occur. The putative association of b_6/f complexes with some other protein on the PFs faces in *C. reinhardtii* and *C. sorokiniana* needs further investigation. Were it associated with some LHC, it would provide a transport system from stacked to unstacked regions upon phosphorylation of this complex [34].

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