

Organization of the Photosystem II Centers and Their Associated Antennae in the Thylakoid Membranes: A Comparative Ultrastructural, Biochemical, and Biophysical Study of *Chlamydomonas* Wild Type and Mutants Lacking in Photosystem II Reaction Centers

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ABSTRACT We investigated the ultrastructure of thylakoid membranes that lacked either some or all of their Photosystem II centers in the F34SU3 and F34 mutants of *Chlamydomonas reinhardtii*. We obtained the following results: (a) There are no particles of the 160-Å size class on the EF faces of the thylakoids in the absence of Photosystem II centers (as in F34); the F34SU3 contains 50% of the wild-type number of PSII centers and EF particles. (b) The density of the particles on the PF faces of the thylakoids is higher in the mutants than in the wild type. (c) The fluorescence analysis shows that the organization of the pigments is the same regardless of whether 50% of the PSII centers are temporarily inactivated (by preilluminating the wild type) or are actually missing from the thylakoid membrane (F34SU3).

Our results, therefore, support a model in which: (a) each 160-Å EF particle has only one PSII center surrounded by light-harvesting complexes and (b) part of the PSII antenna is associated with 80-Å PF particles in both of the mutants and the wild type.

In 1964, Park and Biggins suggested that particles observed in the electron microscope correspond to photosynthetic units (36). We now have a better understanding of the structure of the thylakoid membrane and its relation to certain characteristics of the photosynthesis apparatus. This has led to three major assertions: (a) Photosystem I and photosystem II (PSI and PSII) activities are located on separate sites along the thylakoid membranes. PSI activity is uniformly distributed among grana and stroma lamellae, whereas PSII activity is almost completely restricted to the grana regions (4, 38, 41). (b) Freeze-fracturing of thylakoid membranes reveals particles on the EF faces having sizes that depend on the pigment composition of the PSII antenna (2, 29). (c) There is some evidence that the amount of PSII activity is related to the density of these EF particles (1, 30, 47).

More recently, attempts have been made to use photosynthetic mutants to correlate the presence of several proteins associated with specific functions and some of the particles revealed by freeze-fracturing (28, 32, 39, 43). This approach requires the use of mutants defective in only one particular function. Using genetic, biochemical, and biophysical methods, we have managed to characterize mutants of *Chlamydomonas reinhardtii* deficient in one function. These mutants are convenient for the study of the relationship of structure to function.

We have already reported in a previous paper (32) that the F34 nuclear mutant of *Chlamydomonas reinhardtii*, which is devoid only of photosystem II activity and which lacks the polypeptides associated with PSII reaction centers, shows an important reduction in the size and the density of particles associated with the EF faces of its thylakoids. A similar obser-

vation was also reported recently by Miller and Cushman, who used a pleiotropic mutant of tobacco greatly deficient in chlorophyll *a* and *b* (Chla and Chlb) which showed modifications in its chlorophyll-protein complex I and II (CPI and CPII) profile, together with an absent of PSII activity (28).

In this paper, we compare the ultrastructure of the thylakoids of the wild type (WT) of *Chlamydomonas*, the F34 mutant, and the F34SU3 mutant (a suppressed strain of F34 in which PSII activity has been partially restored). These experiments have led to new insights into the organization of the PSII centers and that of their associated antenna chlorophyll, which will be discussed.

MATERIALS AND METHODS

Materials

C. reinhardtii WT and mutants F34, F34SU3, FUD7, and F34SU4, isolated by P. Bennoun, were cultivated in Tris acetate-phosphate medium under continuous light of 4,000 lx (cool fluorescent light).

Fluorescence and spectroscopic measurements were made on intact cells in the culture medium. Purified thylakoids were obtained according to Chua and Bennoun (9) to perform SDS and lithium dodecyl sulfate (LDS) gel electrophoresis.

Freeze-fracture studies were performed on a broken-cell preparation, which has been described previously (32). Preparation of membranes was carried out at 2°C in the presence of 10 mM MgCl₂ to obtain stacked thylakoids. This feature was checked in thin sections.

Methods

Fluorescence experiments were performed in the presence of 10⁻⁵ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Continuous exciting light was filtered by two blue Schott BG38 (Schott, Inc., New York). Complementary red filters (four Ulanco Rubylith [Ulanco Products Co. Inc., N. Y.] plus two Kodak Wratten 70) were placed between the cuvette and the photomultiplier (EMI 9558 B/520 [EMI Electronics Ltd., Hayes, England]). The signal was displayed on a Tektronix 5103 storage oscilloscope (Tektronix, Inc., Beaverton, Oreg.). The shutter opening time was <1 ms.

Absorbance changes at 515 nm were measured with a differential spectrophotometer described by Joliot and Delosme (21). The detecting xenon flashes were filtered by an interference filter (Microphysic, 5150 Å, spectral bandwidth 34 Å at half height [Barnes Engineering Co., Stamford, Conn.]). Charge separation at the level of both the PSI and PSII centers contributes to the 515-nm absorbance change. This change is caused by a shift in the absorption spectrum of some pigments when an electric field is generated across the thylakoid membrane (electrochromic effect [23]). The contribution of each photosystem (22) can be detected by: (a) measuring the total change at 515 nm 430 μs after a saturating flash; (b) blocking PSII centers by a preillumination with several saturating flashes in the presence of 10⁻⁵ M DCMU and 10⁻² M hydroxylamine; (c) detecting the remaining signal in the same conditions as in a. This gives the PSI contribution. The PSII contribution is obtained by subtracting the signal in c from the signal in a. Electrophoresis of membrane polypeptides was carried out according to Laemmli (24) with a 7.5–15% linear gradient of acrylamide stabilized by a 5–17.5% sucrose gradient. Two types of gel electrophoresis were performed: (a) at 25°C with thylakoid membranes solubilized in SDS. This method permits seeing resolved polypeptides ranging from 70,000 daltons to ~15,000 daltons (9) after staining the gel with Coomassie Brilliant Blue; and (b) at 4°C with thylakoid membranes solubilized in LDS. This method optimizes the fraction of chlorophyll that remains associated with the pigment-protein complexes, mainly CPI and CPII (12).

Chlorophyll determinations were performed according to Arnon's method (3).

For freeze-fracture studies, the broken-cell preparation was impregnated directly in 20% glycerol, frozen in Freon 22, and stored in liquid nitrogen. Freeze-fracturing and platinum-carbon shadowing were then carried out with a Balzers apparatus (Balzers Corp., Nashua, N. H.) at -150°C. The replicas were examined in a Philips EM 400 electron microscope. The computation of the densities of particles and the histograms was performed on micrographs at 400,000 magnification with a digiplan Kontron computer. Micrographs were placed on the surface of this coordinate analyzer. A measuring cell allows counting of elements on the surface and measurement of length and area. The data are then stored in a computer. A histogram program gives the particle size distribution in classes 7.5 Å apart. Less spacing led to variations between adjacent classes that had no statistical significance. Between 250 and 500 particles were measured in each case.

RESULTS

The chlorophylls associated with the photosynthesis apparatus in the thylakoid membrane can be divided into two categories. Most of them are antenna chlorophyll, which harvest light energy for a few other chlorophylls located in the photochemical centers, which trap the excitons and perform the primary photoreaction. Variable fluorescence is controlled by the redox state of the primary acceptor Q of PSII. The fluorescence yield is low when Q is in an oxidized state (high quenching state, open centers) and high when Q is reduced (low quenching state, closed centers) (16).

The fluorescence rise under continuous illumination in the presence of DCMU (Fig. 1) reveals an interaction between several PSII centers. At the beginning of the illumination, all the primary acceptors are in an oxidized state. The excitons reaching the centers are efficiently trapped for photochemistry. In the course of the fluorescence rise, a growing number of Q is reduced. The decrease in the density of open centers results in an increase in the number of antenna chlorophylls available per open center (19). This occurs until the density of open

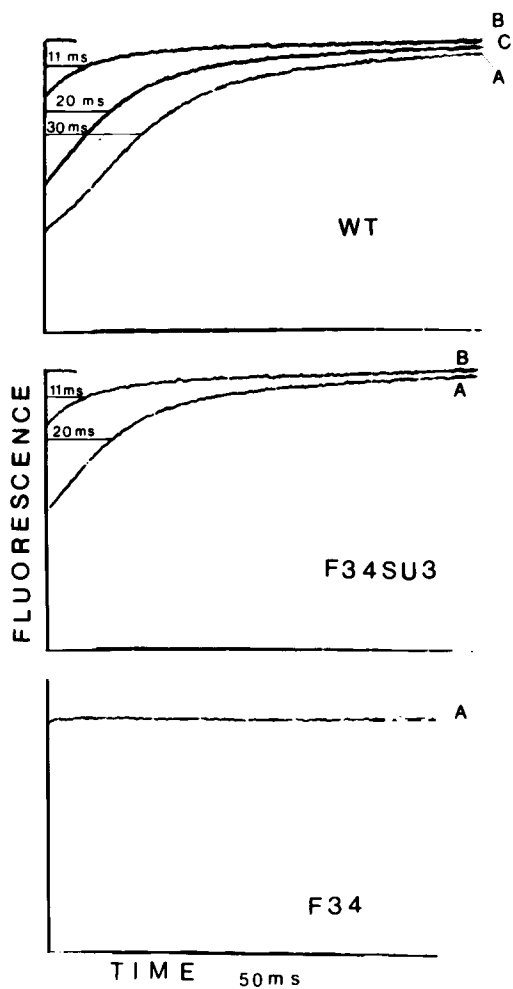


FIGURE 1 Fluorescence induction curve of the WT, the F34SU3, and the F34 mutants of *C. reinhardtii* in the presence of 10⁻⁵ M DCMU. A curves, dark-adapted cells (100% open centers). B and C curves, ~10% and 50% open centers, respectively) cells were preilluminated to reach F_{max} level and then placed in the dark for short periods (<1 s). This allows a fraction of the PSII centers to return to a high quenching state through back reactions between the reduced primary acceptors and the oxidized donors (5).

centers is so low that either no interaction remains between them or these interactions become negligible. In this situation, there is a maximum number of antenna chlorophylls available per open center. This number remains constant, even when the number of open centers further decreases. These characteristic features of the fluorescence rise are shown for the dark-adapted WT of *Chlamydomonas reinhardtii* (Fig. 1, curve *A*, which is sigmoid).

At a given light intensity, the half-time of the fluorescence rise in the presence of DCMU is a rough estimate¹ of the number of chlorophylls available per PSII center (i.e., an estimate of the "optical cross section" of the PSII centers) (15). A preillumination of the algae followed by short incubation times in the dark (see legend, Fig. 1) decreases the density of the open centers (high quenching state). Consequently, it decreases the half-time of the fluorescence rise. This is illustrated by comparing the WT in curve *A* (dark-adapted algae, 100% PSII centers in the quenching state *Q*) with those in curves *B* and *C* (preilluminated algae with, respectively, 10% and 50% of the PSII centers in the quenching state *Q* at the beginning of the induction curve). These percents of open PSII centers are estimated by measuring the area bounded by the induction curves and their F_{\max} asymptotes (13, 17). The decreasing $t_{1/2}$ value from curve *A* to curve *C* to curve *B* illustrates the decrease in the density of PSII open centers at the beginning of the corresponding fluorescence rises (i.e., the increase in the optical cross section of the PSII centers). Curve *B* displays a nearly exponential fluorescence rise. This indicates that the optical cross section of the PSII centers remains constant during the experiment. The interaction among the PSII centers is, therefore, negligible. According to the respective $t_{1/2}$ value, the optical cross sections of the centers in curve *B* is about three times larger than that shown in curve *A* when all the centers are open. This gives a relative estimate of the maximal optical cross section per PSII center in this strain.

The fact that mutant F34 displays no variable fluorescence (Fig. 1, F34, curve *A*) indicates the absence of detectable PSII active centers. The variable portion of the fluorescence induction pattern of the suppressed strain F34SU3 indicates that the PSII activity is partially restored. This mutant has about half as many PSII centers as the dark-adapted WT (Table I). It can be compared with the preilluminated WT (Fig. 1, curve *C*) in which 50% of the centers are closed by a preillumination and, thus, put in a low quenching state. The two curves (WT curve *C* and F34SU3 curve *A*) are identical with the same $t_{1/2}$ and the same shape. Moreover, the PSII centers have the same maximum optical cross section in the two strains, as shown by the fact that the two *B* curves in Fig. 1 (WT and F34SU3) also are identical. We thus conclude that there are no detectable modifications of the organization of the antenna pigments, as viewed by fluorescence studies, when 50% of the PSII centers are lacking in the thylakoid membrane (F34SU3).

Table I shows an estimate of the number of PSI and PSII active centers from the 515-nm absorbance change in the WT, the F34SU3, and the F34 mutants for the same amount of chlorophyll (see Materials and Methods). The PSI contribution is similar in the three strains, whereas there are <2% PSII centers in the F34 and ~50% in the F34SU3 as compared with

those in the WT. This result is consistent with the data obtained from our fluorescence measurements.

That no major changes occurred among the three strains either in parameters controlling the fluorescence yield other than the number of quenchers *Q* (secondary quenchers, trapping efficiency, etc.) or in the amplitude of the 515-nm change/center is supported by the good correlation observed in the relative number of centers measured by the two methods. This also confirms that it is correct to use the half-time of the fluorescence rise as a relative measure of the optical cross-section of the PSII centers in the three strains. The absence of changes in the parameters controlling the fluorescence yields in the WT and the F34SU3 is also supported by the identical fluorescence rises (curves *B*) obtained with these two strains when the same number of centers have returned to an active state after a preillumination.

The SDS electrophoretic gel pattern of the polypeptides of the thylakoid membranes in the three strains under discussion are shown in Fig. 2*A*. The F34 mutant lacks polypeptide 6 (47,000 daltons), is deficient in polypeptides 5 (50,000 daltons) and 12 (32,000 daltons), and in two smaller polypeptides, 19 and 24, in the 15–20,000 dalton region. F34SU3 partially recovers polypeptides 5, 6, and 12. The three strains have about the same *Chla/Chlb* ratio (Table I), and they contain similar amounts of CPI and CP II, as shown on the LDS electrophoretic gel pattern (Fig. 2*B*). This latter observation is consistent with the similar contribution of PSI to the 515-nm absorbance change in the three cases. It, then, also supports our assumption that no changes occurred between the three strains at 515 nm in the amplitude of the electrochromic effect produced by one charge separation. The Discussion includes a detailed comparison of the polypeptide patterns.

We compared the ultrastructure of the thylakoid membranes in the presence of 10 mM $MgCl_2$ using broken-cell preparations of the WT and the F34SU3 and F34 mutants. Extensive study of the stacking characteristics in F34 have been reported recently (48). These features are common to the three strains examined in this paper. In the presence of 10 mM $MgCl_2$, >80% of the thylakoid surfaces are in contact. There are almost no single thylakoids observed. The unstacked membrane regions are restricted to the outer thylakoid of each stack. Consequently, there is no separation between "grana" and stroma" regions, as is the case in higher plants where only 60% of the membrane are stacked in the presence of $MgCl_2$ (45). Although it was possible to use completely unstacked thylakoids, we chose the stacked preparation because it provides a better distinction between the large EF particles (160-Å size class) in the WT and the small ones, ~100 Å in F34. As described by others (18–31), the particles present on the EF_s and PF_s faces of freeze-fractured thylakoid membranes in the WT differ both in size and density. As Fig. 3 shows, the particle density is higher on the PF_s face than on the EF_s face and the particle sizes are larger on the latter. The EF_s particles in thylakoids of the F34 mutant are reduced in size and number as compared with those of the WT. The F34SU3 displays an ultrastructural pattern that is intermediate between that of the two other strains: the density of the EF_s particles is lower than in the WT, but their average size seems about the same.

We further characterized these changes by performing a detailed quantitative analysis of the size distributions and of the densities of the particles on both fracture faces in each strain.

As illustrated in Table II, we have found that there are 1,250

¹ This remains true in a comparison between different strains when no major changes occur in the quantum yield of photochemistry of PSII centers or in the number and properties of quenching species associated with these centers.

TABLE I

	Number of active centers			Half-time of the fluorescence rise in the presence of DCMU	Chl a/Chl b	Fraction of WT-like EF particles*
	a		b			
	PSI	PSII	PSII			
				<i>ms</i>		
				A‡ 30		
				C 20		
				B 11		
WT	180	100	100	A 20	2.75	100
				B 11		
F34SU3	159	47	46	A 20	2.65	50
				B 11		
F34	194	~3	<2	A —	2.57	0

Some characteristics of the WT, the F34SU3, and the F34 mutant strains of *Chlamydomonas reinhardtii*.

(a) Number of PSI and PSII centers obtained from the 515-nm absorbance changes. The contribution of each photosystem is given for a concentration of 20 $\mu\text{g}/\text{ml}$ of chlorophyll, assuming 100 PSII centers in the WT.

(b) Number of PSII centers obtained from the measurement of the area bounded by the fluorescence induction curve and its F_{max} asymptote using dark-adapted algae. We assume 100 PSII centers in the WT.

* WT-like EF particles belong to the type the distribution of which is illustrated by the WT histogram on the EF_s faces.

‡ Fluorescence rise A corresponds to dark-adapted algae, whereas B and C correspond to preilluminated algae (see legend, Fig. 1).

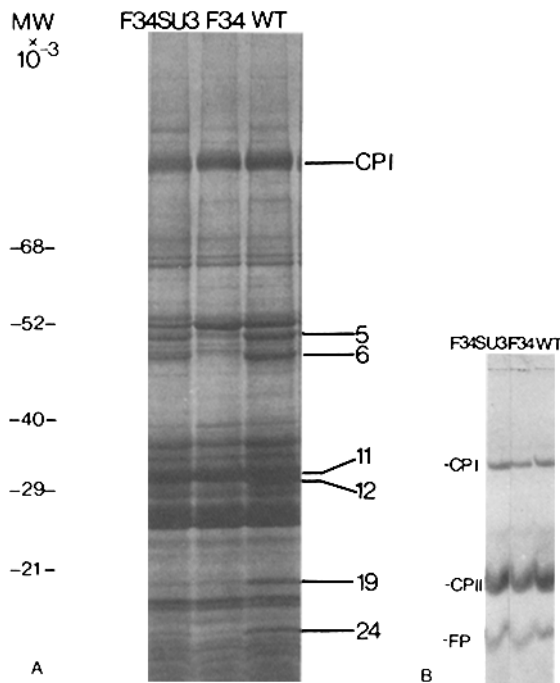


FIGURE 2 Electrophoretic gel patterns of the thylakoid membranes for the WT, the F34SU3, and the F34 mutants of *C. reinhardtii*. Both gels (A and B) contain a 7.5–15% linear gradient of acrylamide. 30 μg of chlorophyll was placed in each slot. (A) In the presence of SDS at 25°C: bands are numbered according to Chua and Bennoun (9). We use the same scale of molecular weights that these authors used. Polypeptides are stained with Coomassie Brilliant Blue. Bands 5, 6, and 12, associated with PSII centers (9). Bands 19 and 24, associated with PSII donors (7). Band 11, part of CPII. (B) In the presence of LDS at 4°C. This treatment enhances the fraction of pigments retained on the chlorophyll-protein complexes. The gel is unstained. CPI, chlorophyll-protein complex I containing the PSI reaction centers and part of PSI antenna. Contains Chl a only. CPII, chlorophyll-protein complex II, mainly associated with the PSII antenna, contains Chl a and b. For a brief review on CPI and CPII, see reference 46. FP, free pigments.

particles/ μm^2 on the EF_s face of the WT, whereas there are 787/ μm^2 in the F34SU3 mutant and only 350/ μm^2 in the F34. Thus, the number of EF_s particles decreases with the number of active PSII centers. However, the particle density on the PF_s

faces is higher in the F34SU3 (5,100/ μm^2) than in the WT (4,500/ μm^2) and is highest in the F34 (7,600/ μm^2). These changes cannot result from variations in the partition coefficient of one particular class of particles between the two faces from one strain to another, because there is a net increase in the total number of particles per micrometer squared from the WT (5,750/ μm^2) to the F34 (7,950/ μm^2).

Particle-size histograms of the EF_s faces of the three strains (Fig. 4a) clearly indicate important modifications in the particle-size distribution in the mutants as compared with that of the WT. Although the diameter of particles ranges from 80 to 200 Å in the latter, it is clear that the majority are ~165 Å. There are, however, populations of particles ~100, 120, and 135 Å in diameter. The F34 mutant has one major class of particles ~100 Å wide: almost no particles >120 Å wide have been recorded. Thus, the distribution of diameters represented on this histogram is considerably shifted toward the smaller values, as compared with that of the WT.

This pattern is not specific for the F34 nuclear mutation. Indeed, an EF histogram similar to that of F34 was obtained using the FUD7 chloroplast mutant of *Chlamydomonas reinhardtii*, which has no PSII activity. Thylakoids of this mutant display the same polypeptide pattern as the F34 mutant (6).

The size distribution of the EF_s particles in the F34SU3 mutant is a combination of those shown in the WT and F34 histograms. There are at least two distinct populations of particles: the major one is ~150 Å in diameter and the minor one ~80 Å.

Another suppressed strain derived from the F34 mutant, the F34SU4, has a small number of PSII centers. The histogram of the EF_s particles of its thylakoids appears to be similar to that of F34SU3. It is even easier to distinguish between small and large particles in this case.

Particle-size histograms from the PF_s faces (Fig. 4b) of the thylakoids in the WT and F34 strains are very similar and show that the majority of the particles are ~80 Å wide. In the F34SU3 mutant, however, the size distribution is slightly shifted toward the larger sizes.

DISCUSSION

The F34 nuclear mutant of *Chlamydomonas reinhardtii*, which lacks PSII centers, has been extensively characterized (8, 9, 11, 32). Two different methods were used in the present work to

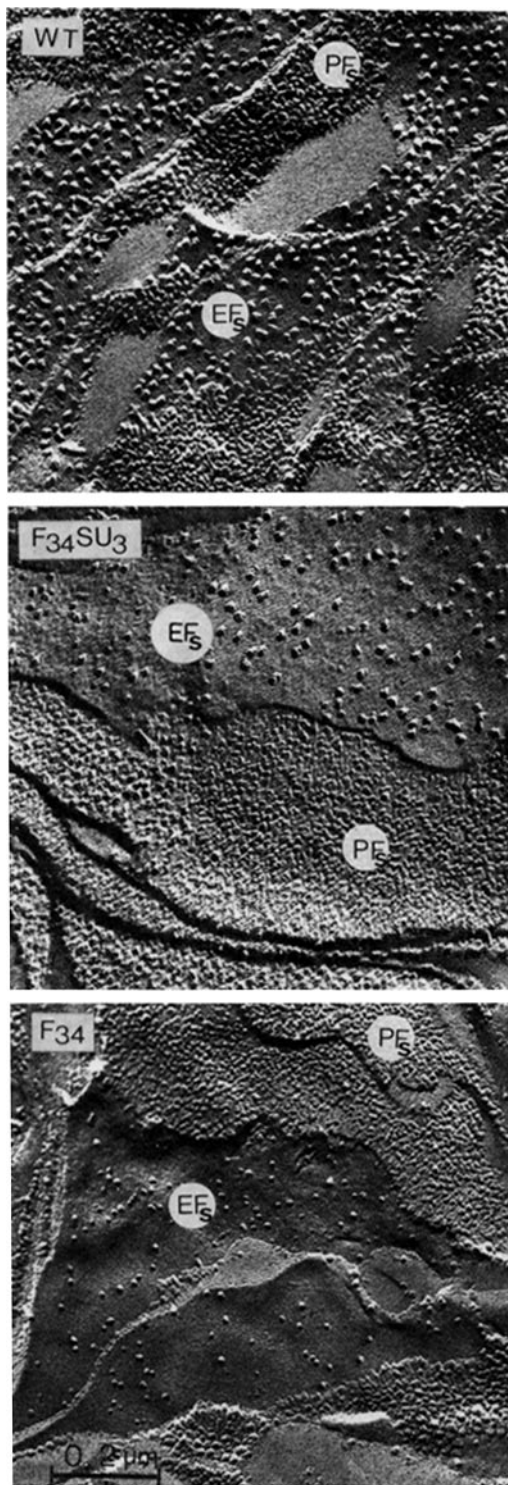


FIGURE 3 Thylakoid membranes freeze-fractured using broken cell preparations of the WT, the F34SU3, and the F34 mutants of *Chlamydomonas reinhardtii* in the presence of 10 mM MgCl₂. The WT is characterized both by small, closely packed intramembrane particles on the PF_s face and by less dense particles of larger diameter on the EF_s face. Fractured thylakoids in the F34 and F34SU3 also show an asymmetry in the distribution of particles between EF_s and PF_s faces. However, the EF particle density is considerably less in both mutant strains, particularly in the F34, than in the WT. Moreover, it clearly appears that the EF_s particle diameter is much smaller in the F34 mutant than in the WT. $\times 72,000$.

TABLE II
Number of particles per micrometer squared

	On the EF _s face		On the PF _s face	Total (EF _s + PF _s)
	Total	WT-like		
WT	1,250 \pm 92	1,250 (100%)	4,500 \pm 590	5,750
F34SU3	787 \pm 55	623 (50%)	5,100 \pm 648	5,887
F34	350 \pm 30	0 (0%)	7,600 \pm 409	7,950

Respective numbers of particles per micrometer squared on the EF_s and PF_s faces of the thylakoids in the WT, the F34SU3, and the F34 mutants of *Chlamydomonas reinhardtii*.

We assume two distinct types of EF particles, one ~ 100 Å in the F34 and one ~ 160 Å in the WT (see histograms). Both types, F34-like and WT-like EF particles, are present in the F34SU3.

detect the number of active PSII centers in each strain: the area bound by the fluorescence induction curve and its F_{max} asymptote in the presence of 10⁻⁵ M DCMU, and the contribution of PSII to the 515-nm absorbance change after one flash.

These two methods detect the primary photoact of PSII and are, thus, a measure of the number of PSII active centers. Both methods gave the same results: F34 mutant lacks PSII activity, whereas the F34SU3 contains $\sim 50\%$ of the WT number of PSII centers.

No changes have occurred in the CPI and CPII contents of the three strains, as shown by LDS gel electrophoresis. An analysis of the SDS electrophoretic gel patterns shows that the F34 mutant is deficient in three major polypeptides, 5, 6, and 12, at 50,000, 47,000, and 31,000 daltons. This mutant is also deficient in polypeptides 19 and 24 in the 15,000–20,000-dalton range. This pattern is typical of all the mutants of *Chlamydomonas* examined that are devoid of PSII photochemistry, regardless of whether they were of nuclear or chloroplast origin.² Because the charge separation occurs across the thylakoid membrane, we would expect the polypeptides that are directly related to the PSII reaction centers to be integral membrane proteins. It is also likely that the particles observed on the fracture faces include integral rather than peripheral membrane proteins. Among the five polypeptides in which F34 is deficient, only polypeptides 5 and 6 are not extractible with 6 M guanidine and, thus, are intimately associated with lipids (27). Interestingly enough, each of them binds four to five chlorophyll molecules and one β -carotene molecule (12). It has been shown that the two polypeptides in the range of 15,000–20,000 daltons (19 and 24) are missing in mutants unable to evolve oxygen, although they retain PSII photochemistry in the presence of artificial donors (7). The recovery of these two polypeptides varies in both mutant F34 and mutant F34SU3. This is because of their lability in PSII-deficient mutants: these two polypeptides are easily detached from the membrane during thylakoid purification.

Polypeptides 5 and 6 at 50,000 and 47,000 daltons can then be associated both with PSII centers and with intramembrane particles revealed by freeze-fracturing. It has been shown that these polypeptides are of chloroplast origin (10). The electrophoresis pattern of the thylakoid polypeptides of the F34SU3 mutant shows a partial restoration of polypeptides 5 and 6.

A comparison of the ultrastructure of the thylakoids of the WT, the F34SU3 mutant, and the F34 mutant leads us to

² Recent findings by Diner and Wollman show that purified PSII complexes containing the reaction centers are indeed composed of these very polypeptides.

conclude that the reduction in the number of PSII centers is accompanied by a decrease in the density of the EF_s particles: whereas the WT has 1,250 particles/μm², there were only 787/μm² in the F34SU3 mutant and 350/μm² in the F34 mutant. The particle-size histograms show that the EF_s particles remaining in the F34 mutant are only ~100 Å wide and represent structural entities other than the major class of EF particles (160 Å) present in the WT. Because there are only 162 particles/μm² <120 Å wide in the WT, we conclude that new particles are present on the EF_s faces of the F34 mutant. The density of EF_s particles resembling those of the WT in the F34SU3 (WT-like EF particle) is 623/μm², i.e., half of that present in the WT.³

Thus, the F34SU3, which partially recovers polypeptides 5 and 6 and displays 50% of PSII active centers, also has in its thylakoids 50% of the EF_s particles present in the WT. This further demonstrates, on a quantitative basis, the conclusion drawn in our previous paper (32) that EF particles in the WT are associated with PSII reaction centers and polypeptides 5 and 6.

Armond et al. (2) have shown that the light-harvesting complex containing Chl_a and Chl_b is probably a part of the large EF particles that are present in the WT. According to these authors, the light-harvesting complex would occupy about half of the volume of these particles. The LDS and SDS gel patterns show no detectable changes in the CPII region (light-harvesting complex (LHC) region) for the three strains we examined. In addition, the Chl_a/Chl_b ratio is even slightly lower in the F34 than in the WT. The localization of these light-harvesting complexes is, then, a very crucial point.

The F34 and the F34SU3 mutants show the same relationship between the numbers of F34-like EF particles present and the WT-like EF particles missing (28% and 26%, respectively). The small EF particles appearing in the mutants could then be part of the large EF particles that contain the PSII centers in the WT. However, there are too few small EF particles in the F34 to account for all the LHC still present in this mutant. Some of these might be located on the PF_s faces because the density of PF_s particles is higher in the mutants than in the WT. This leads to a model in which some LHC, when not associated with the EF particles containing the PSII centers, are located on both fracture faces of the thylakoid membranes in particles ~80 Å wide.

The identification of the large EF particles as complexes containing the PSII reaction centers raises the question of the number of PSII centers per EF particle. Our comparison of the ultrastructure of thylakoid membranes from the WT and the F34SU3 mutant shows that the latter possesses the same proportion of large EF particles and PSII centers. Thus, there is the same number of centers per large EF particle in the two organisms. Two types of models can be proposed: (a) Each particle contains several centers together with their associated antenna chlorophyll. The interactions between centers that we have described in our fluorescence study are then restricted to

³ Assuming a proportion of particles <120 Å wide (13% according to the WT histogram when *C. reinhardtii* is grown under 4,000 lx), we get a number *X* per micrometer squared of F34-like small EF particles in the F34SU3 mutant, which can be expressed as follows: $245 - 13 \times (787 - X)/100 = X$, $X = 164/\mu\text{m}^2$. 245 is the absolute number per micrometer squared of EF particles <120 Å in diameter in the F34SU3. Thus, the number of WT-like EF particles in the F34SU3 is $787 - 164 = 623/\mu\text{m}^2$, ~50% of the WT-EF particles.

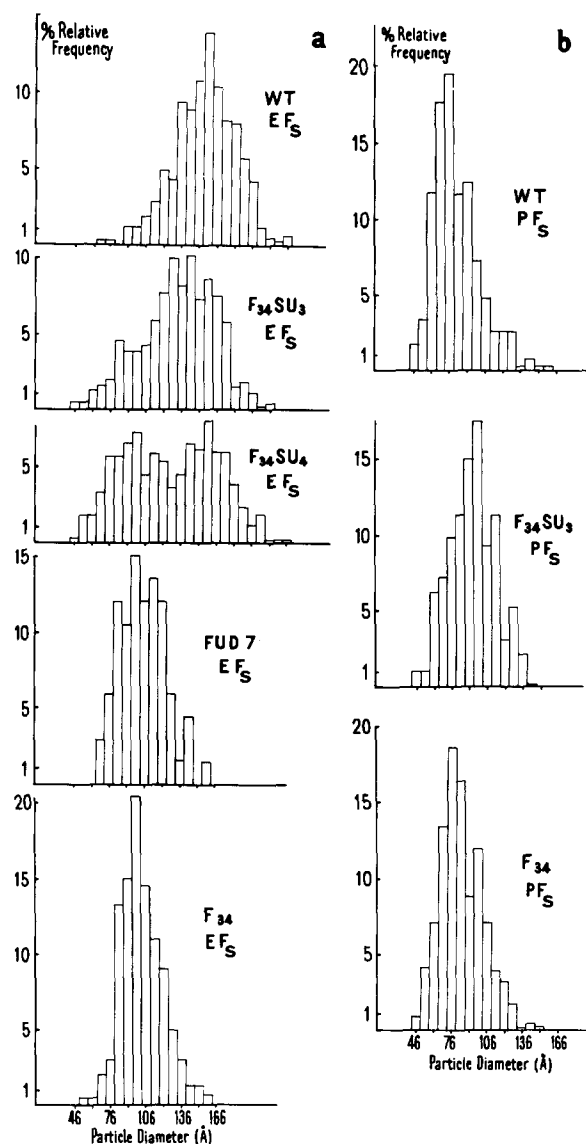


FIGURE 4 Particle size histograms from freeze-fractured thylakoids of *C. reinhardtii*. These were obtained from broken cell preparations in the presence of 10 mM MgCl₂. (a) Distribution of the sizes of the EF_s particles in the thylakoids of the WT, F34SU3, F34SU4, FUD7, and F34 mutants. The distribution is shifted to the smaller sizes from the WT to the F34 mutant. The majority of the particles in the WT is ~165 Å wide, whereas it is ~100 Å wide in the F34 and FUD7, which have no PSII centers. These two classes (165 Å and 100 Å) are both present in the F34SU3 and F34SU4, which recover a fraction of PSII centers. (b) Distribution of the sizes of the PF_s particles in the thylakoids of the WT, the F34SU3, and the F34 mutants. The particles are in the 80-Å range in the three strains. However, they are slightly shifted toward the larger sizes in the F34SU3.

a particle. (b) There is only one PSII center per particle. Interactions between centers must then be explained through interactions between EF particles.

The first model is strictly structural because a domain of energy migration is defined by a particular substructure in the membrane. The second model is more a statistical one because the domain of energy migration may include the whole of the thylakoid membrane.

According to model a, we expect homologous fluorescence-rise curves in the dark-adapted cells of the F34SU3 mutant and

the WT. They should both display the same $t_{1/2}$ and the same shape. As this is obviously not the case, this first model can be excluded.

We, therefore, favor model *b*. It suggests that the reduction in the density of quenchers (open centers) in a domain of energy migration should lead to the same modifications in the fluorescence-rise pattern (lower $t_{1/2}$, less sigmoid), regardless of whether part of the PSII centers are actually lacking in the thylakoid membrane (F34SU3) or are present in a nonquenching state (preilluminated WT). This is consistent with our observation that dark-adapted cells of the F34SU3 (Fig. 1, F34SU3, curve *A*) display the same fluorescence pattern as preilluminated cells of the WT (Fig. 1, WT, curve *C*).

According to model *b*, energy transfer between PSII units must then occur from one EF particle to another. Several hypotheses can be proposed.

(a) Some free chlorophyll, embedded in the lipid phase of the thylakoid membranes, channels energy from one EF particle to another. This is unlikely because electrophoresis studies (26) and Raman resonance spectroscopy (25) suggest that all the chlorophyll is organized in chlorophyll-protein complexes.

(b) EF particles of adjacent thylakoids, making contact in the grana regions, allow intermembrane energy transfer (44). Although we do not exclude the possibility that some energy transfer may occur through this path, we note that energy transfer occurs as efficiently in *C. caldarium*, in which no stacking occurs (14). In addition, assuming (a) one PSII center per EF particle and several centers visited during the lifetime of an exciton ($n > 3$ [20, 34]), energy transfer should also occur through the inner space of the thylakoids (150–200 Å wide), which seems rather unlikely.

(c) EF particles on the same fracture face are close enough to allow direct excitation transfer. Most of the theoretical models that account for energy transfer between PSII units use the Förster resonance transfer rates. The corresponding time constants evolve in $1/R_6$. According to Paillotin (33, 34) the time constant for energy transfer between two Chl_a, 15 Å apart, is 10^{12}s^{-1} . Such transfer between PSII units will occur only in periods shorter than the lifetime of the fluorescence in PSII inactive units (state Q^-/F_{max}) i.e., 10^{-9}s . This allows transfer between PSII units 50 Å apart, leading to a parameter of connection of ~ 0.5 (35), which is consistent with the value reported by Joliot and Joliot for *Chlorella pyrenoidosa* (19). As shown in Table III, only one-third of the particles have a neighbor in this distance range on the EF faces of the thylakoid membranes in the WT of *C. reinhardtii*. The domain within which all EF particles have at least one neighbor has a radius of 200 Å. Thus, the distances that we observe between particles on the EF_s faces do not allow direct excitation transfer between them. This could happen only in the organized arrays described in some thylakoid membranes (37). However, these regions do not seem to characterize the organization of the thylakoid membranes unless one assumes that the majority of these arrays is disrupted during the freeze-fracture process. The unusual conditions under which these arrays are present in significant amounts (upon treatment with low-salt buffers, after slow cooling [40, 42]) cast some doubt on this possibility.

(d) Indirect energy transfer occurs between EF particles within a single membrane. Part of the pigment antenna should then be organized in proteic complexes other than those contained in the EF particles. This is consistent with the fact that the increase in the number of antenna chlorophylls available per PSII center in dark-adapted cells of the F34SU3 as com-

TABLE III

$r = a + d^*$	$n \ddagger$				$\frac{n}{\Sigma n \neq 0}$
	0§	1	2	3	
Å					
130 ($d = 50$)	0.68	0.28	0.04	0	0.32
155 ($d = 75$)	0.20	0.44	0.19	0.17	0.80
180 ($d = 100$)	0.11	0.24	0.30	0.35	0.89
205 ($d = 125$)	0.01	0.16	0.21	0.72	0.99

Probabilities of finding (n) EF_s particles within a distance (r) of another EF_s particle. These probabilities were determined by drawing concentric circles of increasing diameter around 400 EF_s particles chosen at random in the 160-Å size class on several micrographs corresponding to different replicas of the WT freeze-fractured thylakoids.

* r is the radius of a circle concentric with the EF_s particle under study. Thus, it is the sum of a , radius of the particle (80 Å), and d , distance between the edge of the EF_s particle and the circumference of the circle. d corresponds to the maximal edge-to-edge distance registered in this distance class.

‡ n corresponds to the total number of particles contained in, or adjacent to, these circles.

§ This computation was performed without taking into account the overestimation by $\sim 15\%$ of the actual size of the chlorophyll protein complexes as a result of carbon platinum shadowing. The fraction of particles having no neighbors in each case is then a minimum value.

pared with those of the WT is not accompanied by an increase in the size of the corresponding EF particles. As judged by the distance between EF particles shown on Table III, the presence of only one 80-Å PF particle between two EF particles would allow sufficient interaction (< 50 Å) for energy transfer to occur. It could then easily be some of the particles on the PF faces that channel energy. We have shown that it is likely that some LHC are present on these faces in the mutants. Inasmuch as our fluorescence experiments reveal no differences in the organization of the antenna pigments in the WT and the F34SU3, it is unlikely that major changes occur in the membrane organization between the two strains. Thus, an appreciable amount of the PSII antenna should also be located on the PF_s faces of the thylakoids of the WT.

The present study, therefore, favors a model in which there is one PSII center per large EF particle (160 Å). Part of the associated antenna surrounds the center within the same particle whereas the rest of the chlorophyll, some of which allows energy transfer, is located in particles on the PF faces of the thylakoid membranes.

Addendum

Recent results published by D. J. Simpson (*Carlsberg Res. Commun.*, 1979, 44:305–336) using a mutant lacking in Chl_b support the presence of some LHC on the PF faces of the thylakoids.

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