

# Evidence for Thylakoid Membrane Fusion during Zygote Formation in *Chlamydomonas reinhardtii*

Barbara Baldan,\* Jacqueline Girard-Bascou,‡ Francis-André Wollman,‡ and Jacqueline Olive§

\*Dipartimento Biologia, Università di Padova, 35100 Padova, Italy; ‡Laboratoire de Photosynthèse, Institut de Biologie Physico-Chimique, 75005 Paris, France; §Laboratoire de Microscopie Electronique, Institut Jacques Monod, Université Paris VII, 75005 Paris, France

**Abstract.** To understand whether fusions of thylakoid membranes from the parental chloroplasts occurred during zygote formation in *Chlamydomonas reinhardtii*, we performed an ultrastructural analysis of the zygotes produced by crossing mutants lacking photosystem I or II protein complexes, in the absence of de novo chloroplast protein synthesis. Thylakoid membranes from each parent could be distinguished on thin sections due to their organization in "supergrana" in mutants lacking photosystem I centers, by freeze-fracturing due to the absence of most of the exoplasmic-face

(EF) particles in mutants lacking photosystem II centers, by immunocytochemistry using antibodies directed against photosystem II subunits. We demonstrate that a fusion of the thylakoid membranes occurred during zygote formation ~15 h after mating. These fusions allowed a lateral redistribution of the thylakoid membrane proteins. These observations provide the structural basis for the restoration of photosynthetic electron flow in the mature zygote that we observed in fluorescence induction experiments.

THE unicellular green algae *Chlamydomonas reinhardtii* displays a well-characterized sexual cell cycle (see Harris, 1989, for review). Haploid vegetative cells differentiate in gametes upon nitrogen starvation which, after mating, yield diploid zygotes that undergo meiotic division releasing a haploid progeny. During zygote formation a number of intracellular rearrangements take place including nucleus and chloroplast fusions. Zygote formation has been studied in detail by Friedman et al. (1968) and Cavalier-Smith (1970, 1974, 1975, 1976). Mixing of the gametes is rapidly followed by fecundation. Within 1 h, the zygotes display two cup-shaped chloroplasts, each containing a pyrenoid and an eyespot from the two parents. 6 h after mating, the outer membrane of the two chloroplasts has fused and 8 h after mating, only one pyrenoid and one eyespot are apparent in a single chloroplast. In most cases, a light phase of 48 h followed by a dark maturation of ~6 d is necessary for a good germination of the zygotes. During this step the chloroplast undergoes an extensive dedifferentiation.

Each chloroplast from the two mating gametes contains a dense array of inner membranes that define numerous flat sac-like structures called thylakoids. Thylakoid membranes accommodate the protein complexes of the photosynthetic apparatus, which converts light energy in reducing power and ATP. Whether one of the two thylakoid membrane systems brought about by the parental gametes was selectively destroyed during zygote formation or whether they simply mixed or even fused inside the single chloroplast of the mature zygote, remained to be determined. The detection of a

possible reorganization of these thylakoid membranes during zygote formation has been prevented up to now for at least two reasons: the lack of specific markers for each parental thylakoid membrane system and the high starch content in the chloroplast gametes (Martin and Goodenough, 1975) which obscures the resolution of thylakoid membranes on thin sections.

In this paper, our aim was to study the reorganization of thylakoid membranes during early zygote formation of *C. reinhardtii*. To have specific markers for the thylakoid membranes from the two parental gametes, we studied crosses between two distinct mutants deficient in photosynthesis: one was deficient in photosystem I (PSI)<sup>1</sup> activity (F15), the other was deficient in photosystem II (PSII) activity (FUD34); both were stringent mutants and displayed characteristic fluorescence induction kinetics of PSI or PSII mutants (Girard et al., 1980; Bennoun et al., 1981). Biochemical analysis has shown that in each mutant the whole PSI or PSII reaction center complex was missing (Girard et al., 1980; de Vitry et al., 1989). Thylakoid membranes from PSII mutants can be easily recognized either by the absence of the PSII-containing EF particles upon freeze-fracture (Olive et al., 1979; Wollman et al., 1980) or by an absence of membrane labeling with antibodies directed against PSII subunits, in Lowicryl-embedded thin sections (de Vitry et al., 1989). In addition, thylakoid membranes of PSI mutants can be distinguished

1. *Abbreviations used in this paper:* CAP, chloramphenicol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PSI, photosystem I; PSII, photosystem II.

from those of PSII mutants on thin sections due to their characteristic organization in supergrana (Goodenough and Levine, 1969). Thus, the parental thylakoid system originating from PSI and PSII mutants can be easily distinguished by several features accessible by electron microscopy.

Since we crossed two strains bearing mutations in different genes, a genetic complementation could occur in the young zygotes (Bennoun et al., 1980). To avoid such a complementation we chose PSI and PSII mutations blocking specifically the synthesis of one major subunit of the PSI or PSII complexes translated in the chloroplast (Girard-Bascou, 1988; de Vitry et al., 1989) and we crossed the gametes in the presence of chloramphenicol (CAP), an antibiotic inhibiting chloroplast translation. We then studied the fate of the parental gamete thylakoid membranes during a 48-h period of zygote formation under continuous illumination. Based on freeze-fracture and immunocytochemistry analysis, we provide evidence for a complete fusion of the parental thylakoid membranes from the two gametes during zygote formation.

## Materials and Methods

### Strains and Culture Conditions

Wild-type 137c (WT) and mutants of *C. reinhardtii* were grown at 500 lux, 25°C in Tris-acetate-phosphate (TAP) medium.

F15 is a PSI-recessive nuclear mutant (Girard et al., 1980) specifically blocked in the synthesis of the apoproteins of CP1, polypeptides 2a (P2a) and 2b (P2b) (Girard-Bascou, 1988). This mutant accumulates wild-type levels of *psaA* and *psaB* mRNA indicating that F15 mutation blocks *psaA* and *psaB* expression at a posttranscriptional level (Choquet, 1987). FUD34 is a PSII chloroplast mutant which does not synthesize one of the core antenna subunits, the polypeptide 6 (P6) that corresponds to the apo-CP43 (Girard-Bascou, 1988; de Vitry et al., 1989). The mutation has been characterized on the *psbC* chloroplast gene (Rochaix et al., 1989). The reversion frequencies for the two strains were below  $10^{-6}$ .

### Gametogenesis

Gametes were obtained by growth on TAP medium with a reduced content of nitrogen (N/10) and a reduced content in acetate (0.7 gr/l), to decrease their starch content. Vegetative cells in exponential growth were resuspended in gametogenesis medium at a concentration of  $4 \times 10^6$  cells/ml<sup>-1</sup>. The suspension was stirred during 24–28 h in low light until gamete differentiation was completed, i.e., after two mitotic divisions.

### Mating

Mating procedure was as described in Goldschmidt-Clermont et al. (1990). The cross studied was: F15 mt<sup>+</sup> × FUD34 mt<sup>-</sup>. Zygote formation was allowed to proceed for 1 h before counting mating (quadriflagellate cells: QFC) and unmating (biflagellate:BFC) cells in the mixture. The mating efficiency was estimated by the percentage of fusion and is calculated as:  $2 \text{ QFC} / (2 \text{ QFC} + \text{BFC})$ . For the F15 mt<sup>+</sup> × FUD 34 mt<sup>-</sup> cross, the mating efficiency was 85–90%. Zygotes were analyzed during the light maturation phase, 3, 6, 9, 15, 24, and 48 h after mating.

### Fluorescence Measurements

Fluorescence induction kinetics patterns of dark adapted cells were analyzed under continuous illumination as in Bennoun and Delepelaire (1982). 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), which inhibits electron flow, was added at a concentration of  $2 \times 10^{-5}$  M. Photosynthetic activity was estimated through  $\Delta F/F_{\text{max}}$ , where  $F_{\text{max}}$  was the maximum level of fluorescence in the presence of DCMU and  $\Delta F$  was the difference between  $F_{\text{max}}$  in the presence of DCMU and the stationary level of fluorescence without DCMU.

### Pulse Labeling Experiments

Pulse labeling experiments with [<sup>14</sup>C]acetate with gametes and zygotes

were performed as follows: 200 ml of gamete suspensions ( $2 \times 10^6$  cells/ml) or 15-h-old zygote pellicles obtained by crossing 100 ml of F15 mt<sup>+</sup> and 100 ml FUD34 mt<sup>-</sup> ( $10^7$  cells/ml), were collected by slow centrifugation (gametes) or filtration (zygotes), washed, resuspended in minimum-Tris liquid medium and stirred during 1 h at 25°C under 500-lux illumination. Depending on the experiment, gametes or zygotes were incubated in the presence of 100 µg/ml CAP or 8 µg/ml cycloheximide or with both inhibitors for 30 min before addition of 2 µCi/ml [<sup>14</sup>C]acetate. Labeling was performed for 2 h, then stopped by adding an excess of cold acetate. Zygote pellicles were homogenized in 0.3 M sucrose/25 mM HEPES-KOH (pH 7.5)/1 mM MgCl<sub>2</sub> with a motor-driven Teflon pestle before purification of thylakoid membranes.

### Analysis of Thylakoid Membrane Polypeptides

Purification and solubilization of thylakoid membranes were achieved according to Chua and Bennoun (1975). Thylakoid membrane proteins were separated by electrophoresis on 7.5–15% polyacrylamide gels (Laemmli 1970; Chua and Bennoun, 1975). When loaded with pulse-labeled polypeptides, gels were exposed for autoradiography for 2–7 wk with Agfa film Curix MR4.

### Embedding

Gametes and zygotes were collected by centrifugation at 5,000 rpm for 10 min. The pellet was resuspended in 1% glutaraldehyde in 0.02 M sodium phosphate buffer pH 7.0 (Cavalier-Smith, 1970); it was washed in phosphate buffer, postfixed in 1% OsO<sub>4</sub> in phosphate buffer, and embedded in Epon/Araldite resin. Thin sections were stained in 4% uranyl-acetate followed by lead citrate according to Reynolds (1963). Micrographs at 70,000 magnification were used to measure the proportion of thylakoid membranes stacked in gametes and in zygotes.

For immunocytochemistry, cells were broken in a Yeda press (Yeda Scientific Instruments, Rehovot, Israel) in the presence of 10 mM MgCl<sub>2</sub> as described in Wollman and Diner (1980). Broken cells were fixed with 2% paraformaldehyde–0.5% glutaraldehyde in 4 mM potassium phosphate buffer and 10 mM MgCl<sub>2</sub>, rinsed with the same buffer + 0.1 M glycine and embedded in Lowicryl K4M (Carlemalm et al., 1982).

### Freeze-Fracture

Broken cells processed as above, were frozen in freon 22 and stored in liquid nitrogen. Freeze-fracturing and platinum-carbon shadowing were performed with a Balzers apparatus (Balzers, Lichtenstein) at a temperature of –150°C.

Particle sizes and densities were calculated on micrographs at a magnification of 200,000 using a coordinate analyzer and computer as described in Wollman et al. (1980).

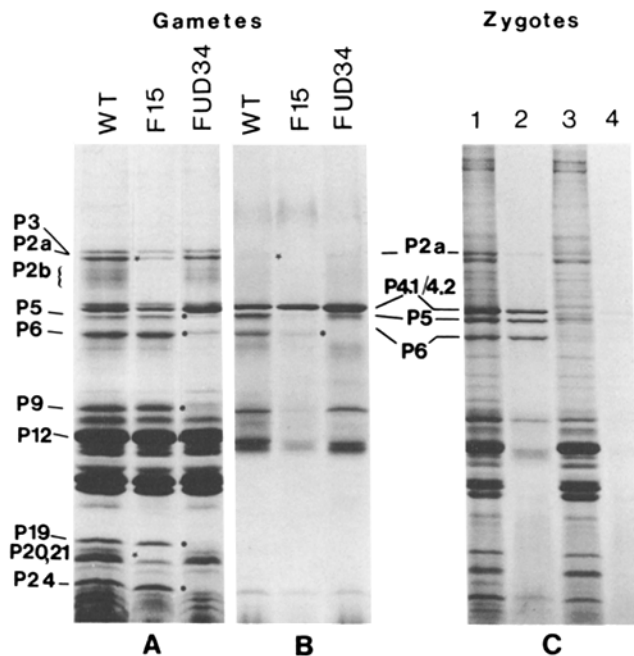
### Immunocytochemistry

α-P6 antibodies were obtained by kindness of Dr. N. H. Chua (Rockefeller University, New York). Lowicryl thin sections were labeled as described in Vallon et al. (1985, 1986), using colloidal gold-labeled Protein A instead of labeled goat anti-rabbit IgG.

## Results

### (a) Biochemical Composition of the Gamete and Zygote Thylakoid Membranes

We have chosen the F15 and FUD34 mutants because each was specifically altered in the synthesis of one of the major chloroplast-encoded subunits of either PSI (F15) or PSII (FUD34) protein complexes and are therefore completely deficient in each complex. These deficiencies in protein synthesis had to be assessed in the gametic state since gametogenesis is accompanied by a decrease in chloroplast translational accuracy due to renewal of the ribosomes (Siersma and Chiang, 1971; Martin and Goodenough, 1975; Bulté and Bennoun, 1990). A comparison of the thylakoid membrane polypeptides from the two mutants and the WT strains is



**Figure 1.** Electrophoretic gel pattern of thylakoid membrane polypeptides from WT and mutant gamete cells F15 and FUD34 of *C. reinhardtii*. The Coomassie blue staining patterns (A) and  $^{14}\text{C}$ -pulse-labeled thylakoid membrane polypeptides (B) are shown. Gamete cells were pulse labeled for 2 h with [ $^{14}\text{C}$ ]acetate (2  $\mu\text{Ci}/\text{ml}$ ) in the presence of cycloheximide (8  $\mu\text{g}/\text{ml}$ ). Stars indicate polypeptides missing in F15 and dots indicate polypeptides missing in FUD34. 15-h-old zygotes were pulse labeled for 2 h with [ $^{14}\text{C}$ ]acetate without translational inhibitor (C, lane 1), in the presence of 8  $\mu\text{g}/\text{ml}$  of cycloheximide (C, lane 2), in the presence of 100  $\mu\text{g}/\text{ml}$  CAP (C, lane 3), or in the presence of cycloheximide and CAP (C, lane 4). All thylakoid membranes were analyzed on 7.5–15% polyacrylamide gels. The time of exposure of the autoradiogram for the gametes was 3.5 times shorter than for the zygotes.

presented on Fig. 1 A. After Coomassie blue staining of this 7.5–15% polyacrylamide gel, we observed the same pleiotropic effect as in the vegetative cells for each mutation, leading to the deficiency of the whole set of subunits engaged in the formation of either the PSI (F15 gametes) or the PSII protein complex (FUD34 gametes) (Chua and Bennoun, 1975; Girard et al., 1980; Bennoun et al., 1981; Delepelaire, 1984). Further biochemical characterization by Western blotting confirmed that D1 and D2, the subunits of the PSII reaction center, were not accumulated in the FUD34 gametes (results not shown). In addition, we noted that P9, the apoprotein of CP29 (Bassi and Wollman, 1990), was missing from the thylakoid membranes in the gamete but not in the vegetative cells of the FUD34 mutant.

Chloroplast-encoded polypeptides synthesized in the gametes of the two mutants were analyzed in their gamete states by pulse-labeling in presence of [ $^{14}\text{C}$ ]acetate, after addition of an inhibitor of cytoplasmic translation (Fig. 1 B). The synthesis of P6 in the chloroplast mutant FUD34 and of P2a and P2b in the nuclear mutant F15 were totally absent in their gamete states as was the case in the vegetative state (Girard-Bascou, 1988; de Vitry et al., 1989). Thus we can exclude an exceptional synthesis of P2 (a and b) in F15 and of P6 in FUD34 during gametogenesis. Interestingly, we note that the rates of synthesis of P2a and P2b, the apoproteins of CP1, in the WT and FUD34 gametes were partic-

ularly depressed as compared with those observed in the vegetative state.

In the present study, we have used a F15  $\text{mt}^+$   $\times$  FUD34  $\text{mt}^-$  cross. F15 is a nuclear recessive mutation and FUD34 a chloroplast mutation. Since the chloroplast genome of the  $\text{mt}^+$  parent but not of the  $\text{mt}^-$  parent is preserved during zygote formation, WT alleles of each mutated gene could be expressed in these zygotes. Thus we checked zygote formation in the presence of CAP to maintain the deficiencies characterized in the parental gametes. We then checked whether de novo protein synthesis in the chloroplast could be efficiently cancelled by addition of CAP during zygote formation.

On Fig. 1 C are shown autoradiograms of protein translates associated with the thylakoid membranes in 15-h-old zygotes. As expected, P2 and P6 showed detectable rates of synthesis in the zygotes in the absence of translational inhibitors (lane 1) or in the presence of cycloheximide, an inhibitor of cytoplasmic translation (lane 2). In the presence of CAP used as an inhibitor of chloroplast translation (lane 3), the possible translation of P2 and P6 was obscured by electrophoretic comigrations of several nuclear-encoded polypeptides in the same region (Delepelaire, 1983). Therefore we pulse-labeled the zygotes in the presence of both CAP and cycloheximide (lane 4). In these conditions, only trace amounts of chloroplast translates were detectable, indicating that the synthesis of P2 and P6 remained CAP sensitive in the young zygote.

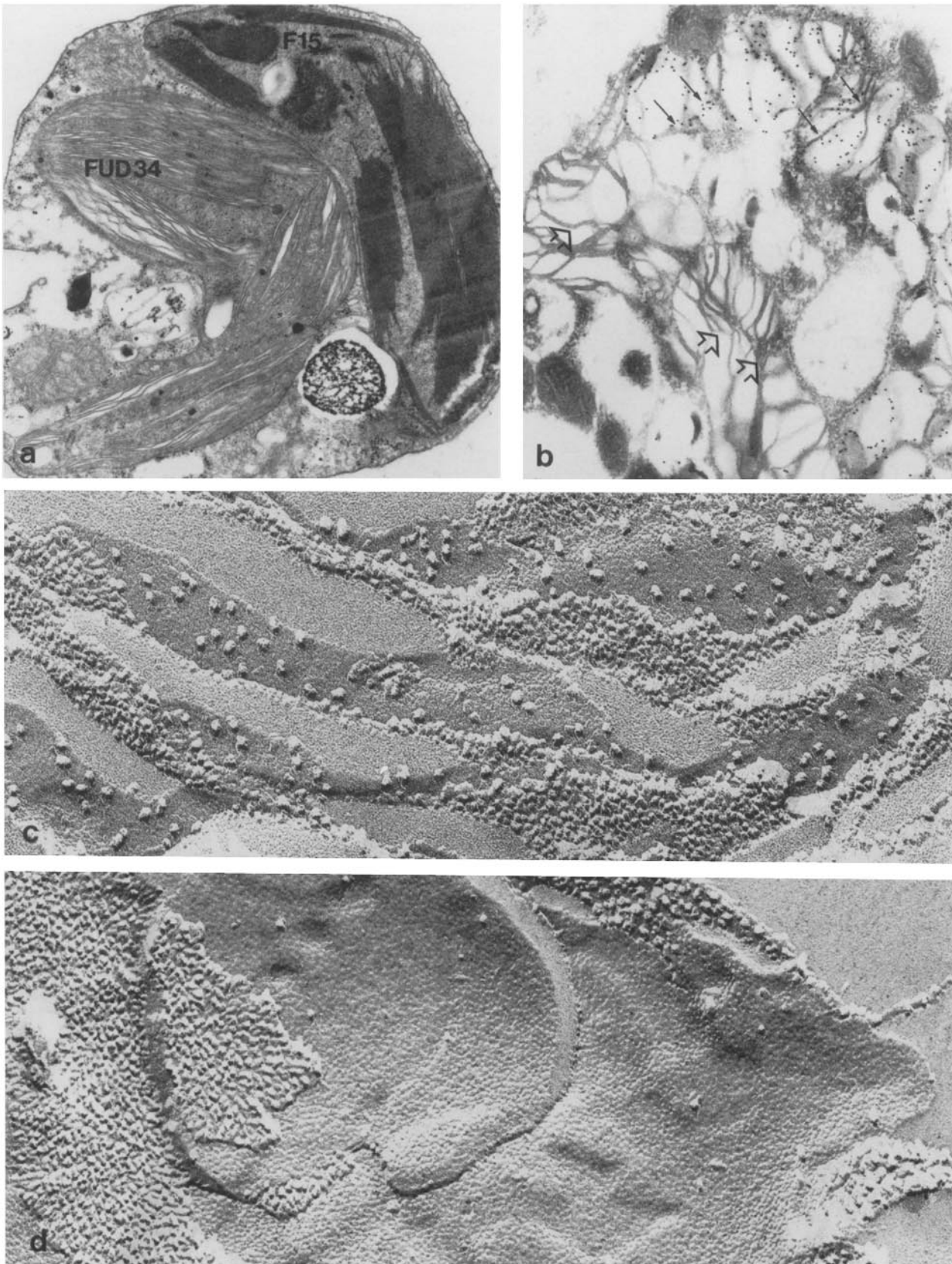
#### (b) Ultrastructural Analysis of the Thylakoid Membranes in the Zygotes Formed after Crossing F15 $\text{mt}^+$ $\times$ FUD34 $\text{mt}^-$ Gamete Cells

The ultrastructural analysis of the zygotes was then performed in the presence of CAP, during their light maturation phase 3, 6, 9, 15, 24, and 48 h after mating. In order to recognize thylakoid membranes originating from each parental gamete in these zygotes, we used several independent markers: (i) the stacking ratio, (ii) the labeling with the antibody against P6, and (iii) the size and density of the (EF) particles observed on freeze-fracture thylakoid membranes. In contrast with thin sections and immunolabeling techniques, which preserved much of the intracellular organization, freeze-fracturing techniques did not allow to establish easily whether the various fracture faces observed in the limited field of the electron microscope originated from the same cell. Thus we used the following rationale: the particle density was calculated on each EF face and the micrographs of similar densities were pooled to measure the particle size on each pack of equivalent density.

Even though maturation of the zygotes is a continuous process, we could distinguish three phases in the fusion of the parental chloroplasts.

**Phase A.** Phase A comprised the 3–6-h-old zygotes. It was characterized by the presence of the two individual parental chloroplasts, clearly visible on thin sections (Fig. 2 a). Using the percentage of stacked membranes as a marker, the parental origin of each chloroplast was easily established: the highly stacked membranes (which are darker in Fig. 2 a) were similar to those observed for the F15 gametes and the less stacked membranes were similar to those of the FUD34 gametes (results not shown).

Immunolabeling experiments (Fig. 2 b) confirmed the pres-



**Figure 2.** Morphological features of zygotes (F15 mt+  $\times$  FUD34 mt-) of *C. reinhardtii* in phase A. Thin sections showing two individual chloroplasts (a): one of them is similar to that observed in the F15 gamete whereas the other is similar to that in the FUD34 gamete (results not shown).  $\times 18,000$ .  $\alpha$ P6 immunolabeling (b): two membrane domains are distinguished, one of them labeled with the gold particles (thin arrows) and the other unlabeled (arrowheads).  $\times 28,000$ . EF faces of freeze fractured thylakoid membranes (c and d): EF faces showing

ence of two individual chloroplasts. One of them was labeled with the  $\alpha$ -P6 antibody and originated from the F15 gamete (filled arrows) and the other was not labeled with the  $\alpha$ -P6 antibody and therefore had a FUD34 origin (open arrows).

Two types of fracture faces were recognized in these zygotes. On the first one (Fig. 2 c), the particle density was of 1,080  $p/\mu m^2$ , which is similar to that observed on the EFs faces of the F15 parental gamete (result not shown). On the second type of fracture faces (Fig. 2 d), the particle density was 320  $p/\mu m^2$ . These fracture faces of low particle density should correspond to both EFs and EFu faces of the FUD34 parental gametes (380 and 150  $p/\mu m^2$ ) and to EFu faces of the F15 gametes (360  $p/\mu m^2$ ). Accordingly, we found a mean density of 320  $p/\mu m^2$  when the three sets of density values found in the gametes were pooled using a stacking ratio of 65% (which was observed in both mutants when the broken cells were incubated with 10 mM  $MgCl_2$ ). The high frequency of these fracture faces of low density (60–70%) in the zygotes in phase A was consistent with their mixed origin.

The histograms of the particle sizes on the EF faces of the zygotes in phase A can be compared on Fig. 3 with those obtained in the gametes of the FUD34 (open bars) and F15 mutants (hatched bars) on either EFs (histograms 1 and 2) or EFu (histograms 3 and 4). The particle size distribution on the EF faces of high density (histogram 5) in the zygotes in phase A was similar to that on the EFs from the gametes of the F15 mutant (histogram 2). The histogram of the particles on the EF faces of low density (histogram 6) matched the histogram constructed after pooling those from the EFs and EFu faces of FUD34 gamete (histograms 1 and 3) and from the EFu face of the F15 gamete (histogram 4) (results not shown).

**Phase B.** This phase comprised the 9–15-h-old zygotes and was characterized by significant morphological and functional changes.

On thin sections, the main observation was the presence of only one chloroplast (Fig. 4 a). In some sections, either two pyrenoids (py) or two eye-spots (not shown) were observed which indicated that the unique chloroplast resulted from the fusion of the chloroplasts from the two parental gametes rather than from the degeneration of one of them. Using the stacking ratio as a marker, it was no longer possible to distinguish the origin of the thylakoid membranes: the stacking ratio was similar to that observed in the WT (60–65% of stacked membranes).

Immunolabeling of P6 provided further information on the presence of both parental membrane systems in the unique chloroplast. The apparent homogeneity of the thylakoid membranes, observed on thin sections, was not reflected in their composition, as observed by immunolabeling. Densely labeled and unlabeled thylakoid membranes were present in two domains of a single cell, as already observed in phase A (result not shown). However, in several instances, new labeling patterns were observed (Fig. 4 b). The gold particles were either present only at one end of a thylakoid membrane

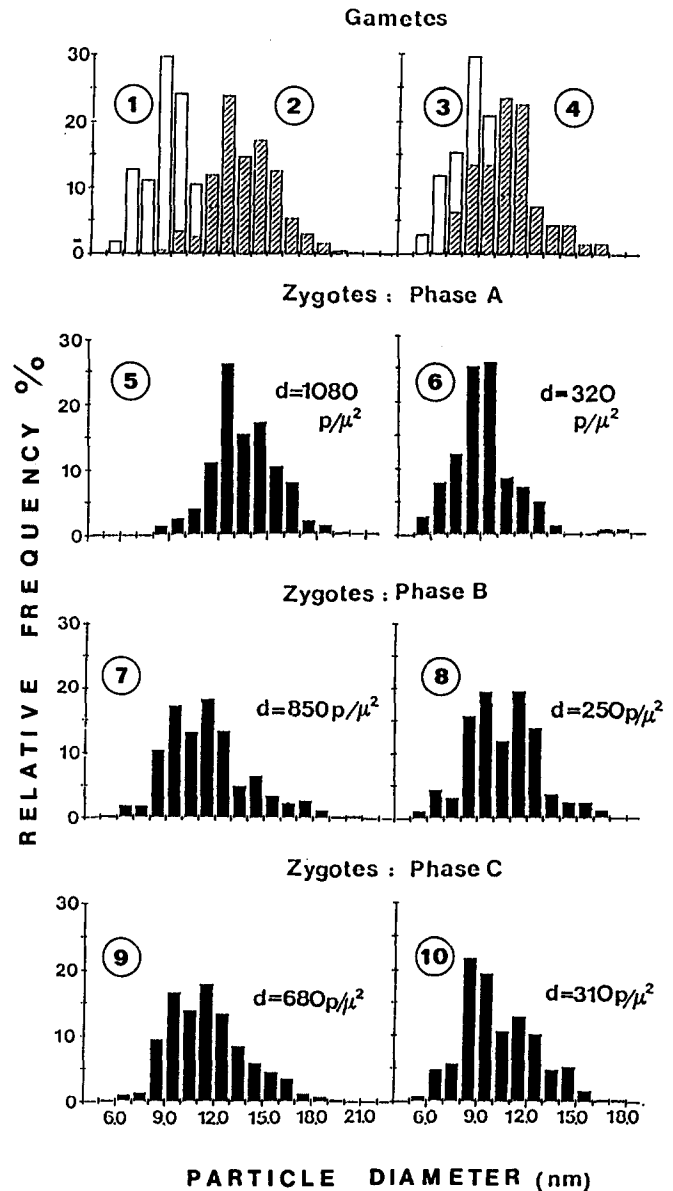
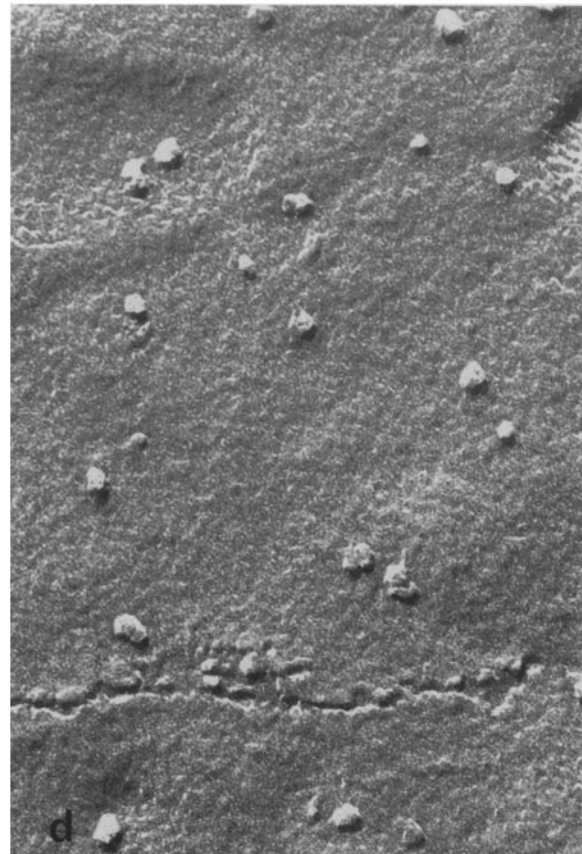
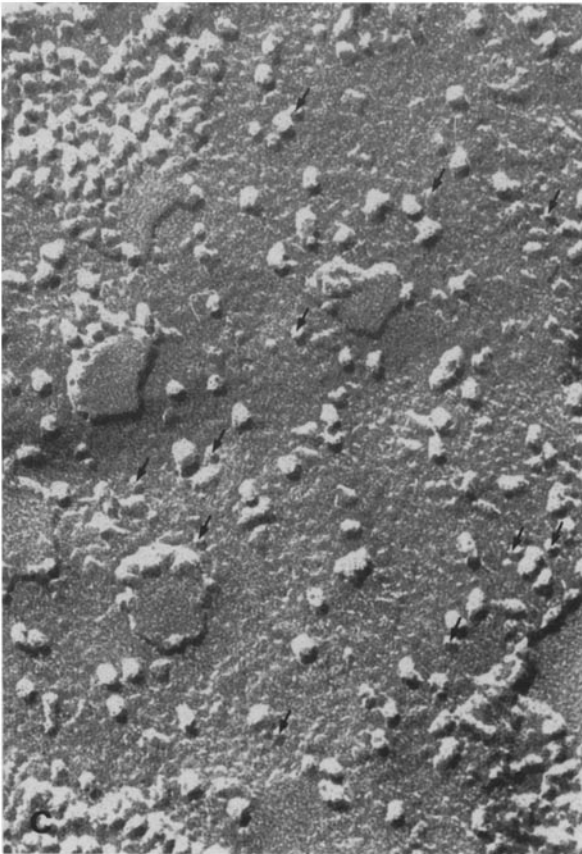
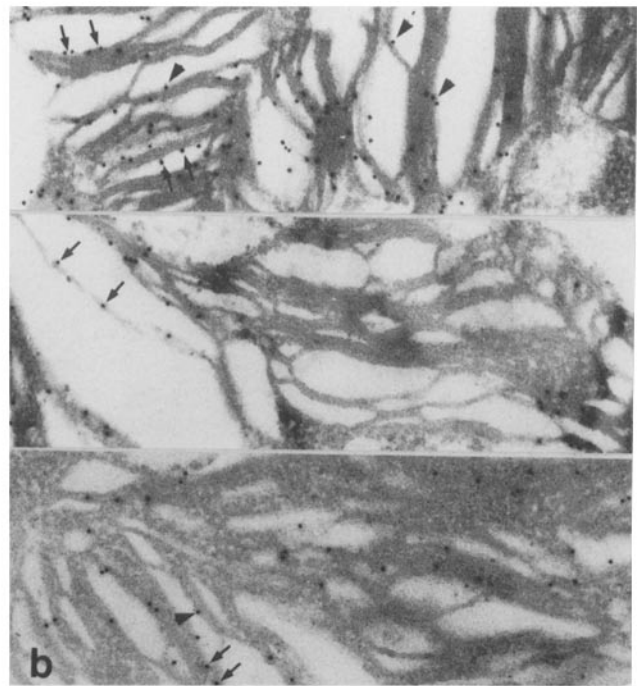


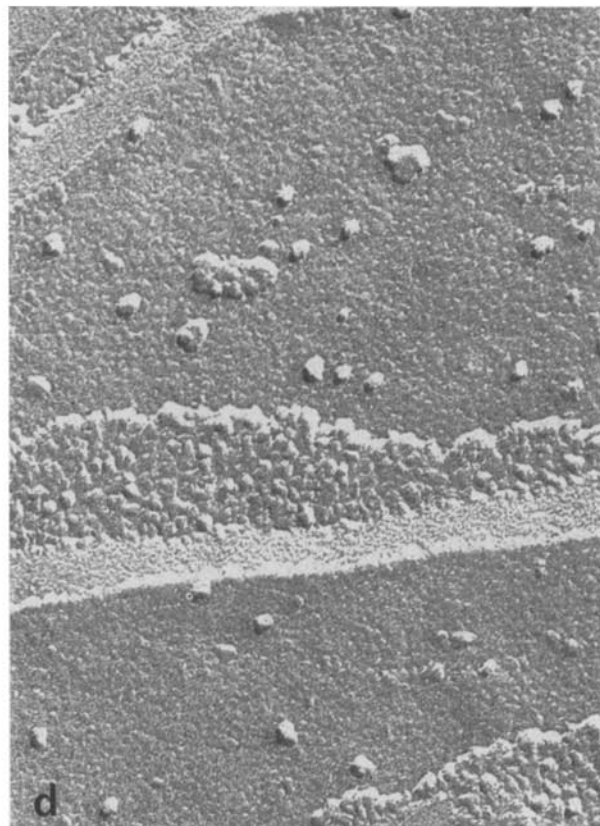
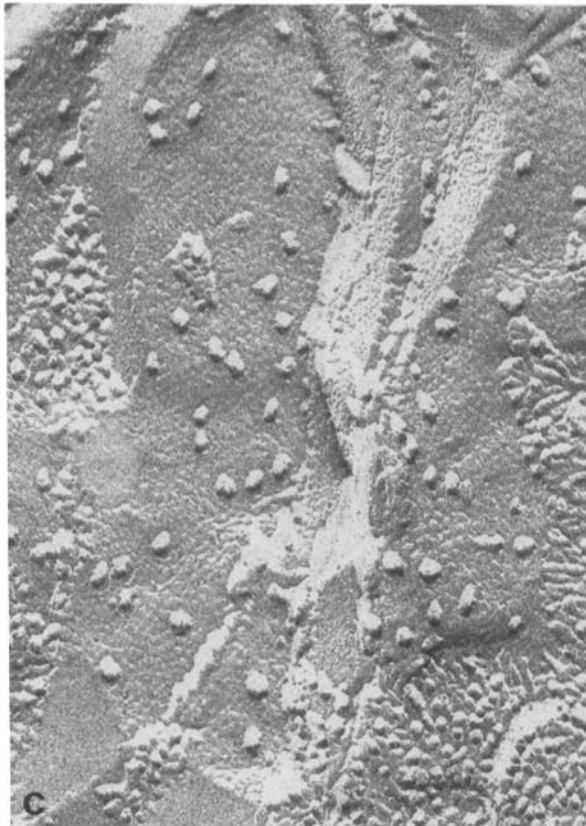
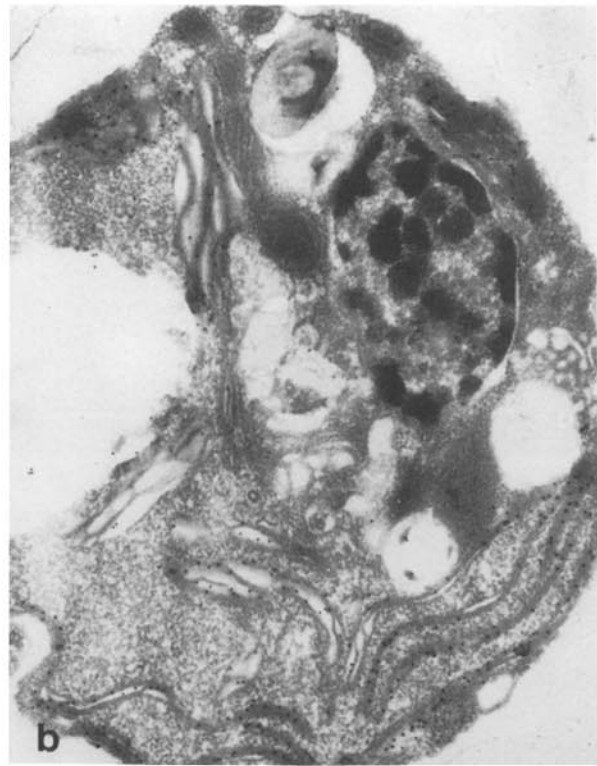
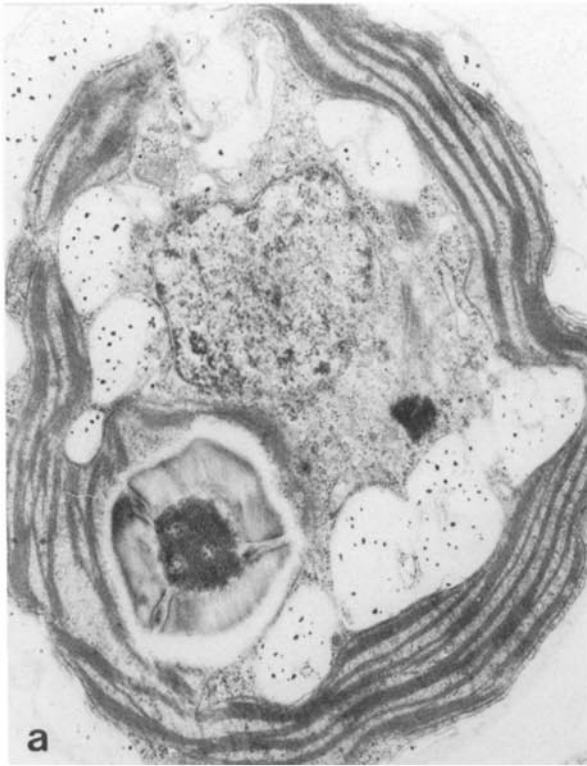
Figure 3. Particle size histograms of gametes and zygotes (F15 mt +  $\times$  FUD34 mt-) in phase A, B, and C. EFs and EFu particle sizes of FUD34 (1 and 3, open bars) and F15 gametes (2 and 4, hatched bars). Zygotes in phase A: (5) particle sizes on surfaces similar to that of Fig. 2 c; (6) particle sizes observed on surfaces similar to that of Fig. 2 d. Zygotes in phase B: 7 and 8 correspond respectively to particle sizes distribution on surfaces similar to that of Fig. 4, c and d. Zygotes in phase C: 9 and 10 correspond, respectively, to particle sizes on surfaces similar to that of Fig. 5, c and d.

(arrowheads), or they were distributed along a single membrane with a density lower than that observed in the F15 gamete (filled arrows). These distinct labeling patterns corresponded probably to fusing membranes and to the redistribution of the PSII centers in fused membranes respectively.

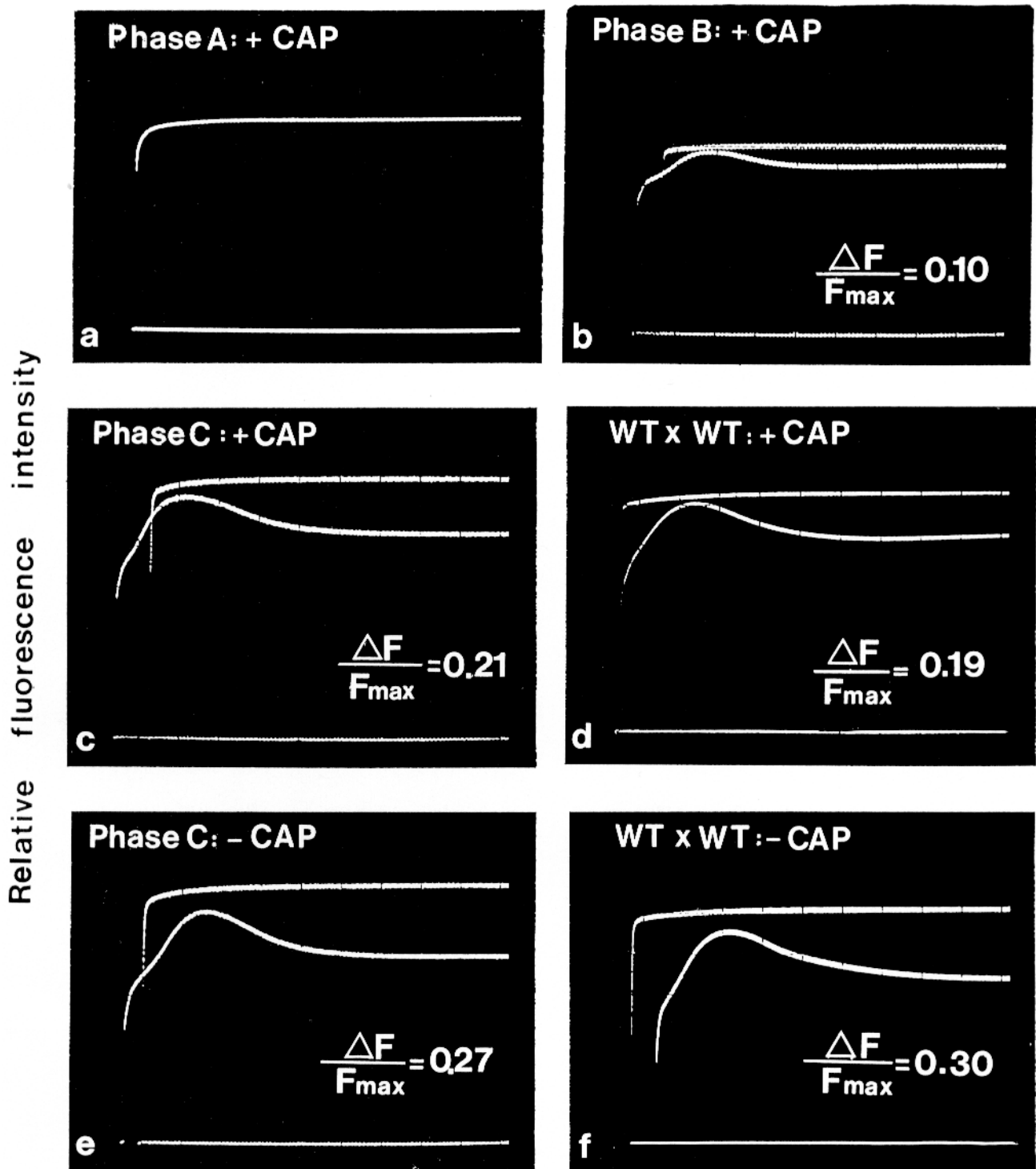
a particle density of 1,080  $p/\mu m^2$  (c), similar to that observed in the F15 gamete membrane, EF faces with low particle content of a mean density of 320  $p/\mu m^2$  (d); such a surface could originate either from the EFs or EFu of the FUD34 gamete, or from the EFu of the F15 gamete.  $\times 140,000$ .



**Figure 4.** Morphological features of zygotes (F15 mt+ × FUD34 mt-) of *C. reinhardtii* in phase B. Thin section (a): only one chloroplast is visible containing two pyrenoid residues (py), the membrane stacking ratio is similar to that observed in the FUD34 gamete (Fig. 3, c).  $\alpha$ P6 immunolabeling (b): the gold particles are either distributed along the membrane with a low density (arrows) or present in one extremity of the membrane (arrowheads).  $\times 30,000$ . Two EF faces of the thylakoid membranes (c and d). In c, the particle density is of  $850 \text{ p}/\mu\text{m}^2$ . The particle size is heterogeneous: small particles (filled arrows) are mixed with larger particles. In d, the particle density is  $250 \text{ p}/\mu\text{m}^2$ . As in (c) small and large particles are present.  $\times 230,000$ .



**Figure 5.** Morphological features of zygotes (F15 mt+ × FUD34 mt−) of *C. reinhardtii* in phase C. Thin sections (a): the appearance of the zygote is similar to that of a WT gamete or vegetative cell (unshown). ×17,000. αP6 immunolabeling (b): the gold particle distribution is homogenous on the thylakoid membranes. The gold particle density is lower than that observed on thylakoid membranes from the F15 gametes (Fig. 2 b, filled arrows). ×26,000. EF faces of the thylakoid membranes (c and d). The particle density is of 680 p/μm<sup>2</sup> on c and 310 p/μm<sup>2</sup> on d. ×150,000.



**Figure 6.** Fluorescence induction kinetics upon a dark-light transition: zygotes (F15 mt+ × FUD34 mt-) in phase A, B, and C in the presence of CAP (*a*, *b*, *c*, respectively) and in phase C in the absence of CAP (*e*). 48-h-old zygotes (WT × WT) in the presence of CAP (*d*) and in the absence of CAP (*f*). In all experiment zygotes were dark adapted in the presence of  $2 \times 10^{-5}$  M DCMU (*top curves*) or in the absence of DCMU (*bottom curves*). In *a* the two curves were similar.

The dilution of PSII centers in fused membranes was also apparent from the particle density observed on the EF faces. Besides surfaces similar to those of the parental gametes (data not shown), new EFs and EFu faces were characterized

by intermediate densities of 850 and 250 p/μm<sup>2</sup>, respectively (Fig. 4, *c* and *d*). It is clearly visible on Fig. 4 *c* that the particle sizes on such surfaces were highly heterogeneous (see *arrows* pointing to small EF particles on Fig. 4 *c*), as



confirmed by the corresponding histograms (Fig. 3, histograms 7 and 8). The histograms of these EFs (histogram 7) and EFu (histogram 8) particle sizes were both bimodal, with two maxima corresponding each to that of a particle population specifically found in either of the parental gametes (histograms 1 and 2 for EFs versus histograms 3 and 4 for EFu). Such a distribution was indicative of a lateral diffusion of the PSII centers in the fused membranes and of a dilution of the preexisting PSII centers leading to an EF particle density intermediate between those recorded in the parental gametes.

**Phase C.** This phase corresponded to the final step of the light period of zygote formation (from 15 to 48 h) when the diploid zygotes can be placed in darkness for further maturation before germination.

In thin sections (Fig. 5 *a*), the unique chloroplast had a cup-shape similar to that of the WT and the thylakoid membranes had a stacking ratio of 60–65% similar to that observed in the WT cells. On the immunolabeled sections, the distribution of P6 was homogeneous over all thylakoid membranes (Fig. 5 *b*). The gold particle density was however significantly lower than in the F15 gametes used for the cross (compare Fig. 5 *a* and Fig. 2 *b*, filled arrows). As noted above, such a decrease in density reflected the dilution of the PSII centers in the fused membranes. In the zygotes in phase C, it was no longer possible to find EF surfaces similar to those of the parental gametes. Two types of EF faces were observed with particle densities of 680 and 310 p/μm<sup>2</sup>, respectively (Fig. 5, *c* and *d*). The histograms of size distribution (Fig. 3, histograms 9 and 10) were indicative of the contribution of particles originating from both parental gametes.

### (c) Recovery of Photosynthetic Activity during Zygote Formation

We then looked for the possible recovery of linear electron transfer between PSII and PSI upon fusion of the thylakoid membranes from the PSI and PSII mutants during zygote formation. To this end we compared the fluorescence induction kinetics, which develop upon continuous illumination of dark adapted cells, at the various stages of zygote formation in F15 × FUD34 crosses and in 48 h old WT × WT zygotes (Fig. 6).

In the WT (Fig. 6, *d* and *f*, bottom curves), there was a biphasic rise in the fluorescence yield followed by a decrease to a steady state level. These fluorescence changes are known to reflect changes in the redox state of the primary quinone acceptor of PSII, Qa (Duysens and Sweers, 1963): the rise phases result from the photoreduction of Qa, whereas the decrease phase reflects its reoxidation by PSI. Therefore, when the reoxidation of Qa was prevented by the addition of DCMU, there was no decrease phase and the maximal fluorescence yield was higher than without DCMU (Fig. 6, *d* and *f*, top curves).

The fluorescence characteristics of the F15 × FUD34 zygotes must be analyzed with respect to the fluorescence induction patterns of the parental gamete cells. In both the vegetative and gamete cells of the FUD34 mutant, Qa is missing due to the absence of PSII centers, and consequently there is no variable fluorescence (Bennoun et al., 1981). In both the vegetative and gamete cells of the F15 mutant the fluorescence induction kinetics rise continuously due to continuous photoreduction of Qa within PSII, whereas its reoxi-

ation is prevented due to the absence of PSI activity (Girard et al., 1980). As expected from the preservation of each set of thylakoid membranes from the two mutant strains in phase A, the fluorescence induction kinetics of the zygotes indicated the absence of electron transfer between PSII and PSI. They rose continuously to a steady state level (Fig. 6 *a*) which was the same whether DCMU was added or not. As a control, mixing equal amounts of vegetative cells from the F15 and FUD34 mutants provided the same fluorescence induction patterns (results not shown).

A partial recovery of photosynthetic activity was observed during phase B. This is shown by the changes in the fluorescence kinetics on Fig. 6 *b*. The top curve recorded in the presence of DCMU, reached the  $F_{max}$  value corresponding to a total block in linear electron flow. The bottom curve, recorded in the absence of DCMU, showed characteristic increase and decrease phases in the fluorescence yield indicating a partial recovery of linear electron flow. As a consequence, the stationary level of fluorescence was lower than the  $F_{max}$  value obtained in the presence of DCMU. These changes in fluorescence characteristics of the zygotes were even more pronounced in phase C (Fig. 6 *c*), where we observed a complete fusion of the parental membranes. The fluorescence induction curves were indicative of the restoration of a highly efficient electron transfer between the PSII and PSI complexes: the ratio  $\Delta F/F_{max}$ , was similar to that obtained in WT zygotes, treated with the same concentration of CAP (compare Fig. 6, *c* and *d*). These  $\Delta F/F_{max}$  ratios were, respectively, of 0.21 and 0.19.

To further estimate the contribution of the membrane fusion process to the whole functional complementation observed in zygotes, we compared the fluorescence induction patterns in zygotes obtained by crossing either mutants F15 × FUD34 or WT × WT in the presence and in the absence of CAP. The efficiency of electron transfer in zygotes prepared without CAP was similar for WT × WT and F15 × FUD34 crosses:  $\Delta F/F_{max}$  were 0.27 and 0.30, respectively (Fig. 6, *e* and *f*). These values were slightly higher than those obtained when CAP was added (0.21 and 0.19, Fig. 6, *c* and *d*), indicating an additional restoration of photosynthetic activity due to translation of some chloroplast gene product in the zygotes.

## Discussion

This study demonstrates that the parental thylakoid membranes fused within the first 48 h of zygote formation in *C. reinhardtii*. This fusion was accompanied by a redistribution of the thylakoid membrane proteins along the fusing membranes and a restoration of an efficient linear electron flow. Since de novo synthesis of the chloroplast-encoded polypeptides lacking in the parental gametes was prevented by addition of CAP, the photosynthetic activity recovered during zygote formation was necessarily due to the electron transfer between the PSII protein complexes brought by the gametes of the PSI mutant and the PSI protein complexes brought by the PSII mutant. That the lateral redistribution of thylakoid membrane proteins in fused membranes restored their functional interactions is a further demonstration of the high fluidity of the thylakoid membranes which has already been implicated in the ultrastructural changes accompanying high salt to low salt transitions (Staehelin, 1976).

## Steps in the Fusion Process

Three subsequent steps could be distinguished within the 48 h time scale of our analysis. As a first step (phase A), fusion of gamete cells occurred within 6 h after mating. The early zygote is then a single cell containing two distinct chloroplasts with no detectable interactions as far as the chloroplast inner membranes are concerned.

In the following step (phase B), which lasted up to 15 h after mating, fusion of the chloroplast envelopes occurred, still preserving distinct features of the parental organelles, i.e., two eye-spots and two pyrenoids. During this phase, the thylakoid membranes were still heterogeneous in their protein content. Some had no PSII whereas others displayed a PSII content similar to that in the F15 mutant.

It should be noted however that the supergranular organization of the thylakoids, typical of the PSI mutant, disappeared. It has been shown previously (Wollman and Diner, 1980) that this supergranular organization was lost *in vitro* when cells from PSI mutants were broken in a low salt buffer. Moreover the stacking ratio then showed the same cation dependence as in PSII mutants. Therefore the supergranular organization observed *in vivo* in PSI mutants is not due to the absence of the PSI protein complexes *per se*. It probably originates from indirect metabolic changes altering the local cation concentration in the stroma. In this respect, fusion of the chloroplast envelopes in phase B lead to a homogeneous stromal cation composition surrounding the thylakoid membranes. Consequently, screening effects of the membrane surface charges (Barber, 1990) result now from a uniform stromal cation concentration and lead to a similar proportion of stacked membranes throughout the chloroplast.

The most remarkable change during phase B was the start of a fusion of the thylakoid membranes: in several instances we observed that gold granules, attached to the PSII core subunit P6, labeled discontinuous domains on continuous membranes. On the other hand, some thylakoid membranes were significantly labeled with the antibody but with a low density as compared with that of a F15-type of labeling. These latter two observations were taken as an evidence for developing fusions between thylakoid membranes from the parental gametes. Consistent with this view is our observation that in phase B, some EF surfaces had particle densities in between those of the parental gametes from the PSI and the PSII mutants and had particle sizes originating from both parental gametes. Moreover, the fluorescence induction curves in these zygotes indicated a partial recovery of linear electron flow from water to PSI acceptors. Thus, the diffusion of the PSII core complexes along fusing membranes elicited a functional interaction with the PSI centers on the same membranes.

During the last period of zygote formation (Phase C, 15–48 h), thylakoid membranes with either of the two parental gamete organizations disappeared. Instead, there was a homogeneous thylakoid membrane network having a lower content in PSII core complexes than in the F15 mutant. This dilution of the PSII cores reflected a total fusion between the two types of thylakoid membranes brought by the mating gametes. This fusion restored a full functional interaction between the membrane proteins originating from the two mutant strains: such a zygote displayed the same photosynthetic activity as a zygote resulting from a WT × WT cross in the presence of CAP.

## Genetic and Biochemical Complementation

We showed from the fluorescence analysis of the zygotes (F15 × FUD34) that there was a CAP-insensitive electron flow that could not be due to CAP insensitive chloroplast synthesis. However, as expected from previous studies (Bennoun et al., 1980), the efficiency of linear electron flow was higher when the cross between mutant gametes was performed in the absence of CAP. This indicated that, in the absence of CAP, *de novo* synthesis of some chloroplast-encoded polypeptides also contributed to the electron flow between the two photosystems. Thus the functional complementation observed in young zygotes resulting from crosses between mutants altered in different photosynthetic complexes has two distinct origins when no translational inhibitors are added: (a) biochemical complementation corresponding to the above described non translational complementation due to thylakoid membrane fusion, which is CAP insensitive; and (b) a genetic complementation due to the genetic expression of all protein components required for photosynthetic activity, which is CAP sensitive.

It has been recently shown that the b6/f complexes were selectively degraded during gametogenesis of WT cells and of most mutant cells, in the WT this *cyt b6/f* deficiency was accompanied by a decrease of photosynthetic activity (Bulté and Wollman, 1990). Thus, the biochemical complementation could also be dependent of the level of b6/f complexes in the gametes. This could explain the different levels of photosynthetic activity between WT zygotes in the presence of CAP and in the absence of CAP (Fig. 6, *d* and *f*). For the F15 × FUD34 cross, the restoration of photosynthetic activity in the presence of CAP was similar to that observed with the WT in the same condition (compare Fig. 6, *c* and *d*). These results suggest that the *cyt b6/f* concentration in the two mutant gametes could also play a role in the restoration of photosynthetic activity as well as the concentration of PSI and PSII complexes. Consistent with this view is our observation that crosses with "old gametes" which have lost most of their b6/f complexes, led to a very poor biochemical complementation. This could explain why this type of complementation has not been observed by Bennoun et al. (1980), who have also studied by fluorescence a cross between nuclear PSI and PSII mutants in the presence of CAP (F14 × F64).

Whereas our results clearly demonstrated that the parental thylakoid membranes fused during zygote formation, we still know very little on the fusion mechanism itself. We could not detect a phase at which either of the two sets of thylakoid membranes displayed a deep reorganization of its supra-molecular organization. Whether the two sets of thylakoid membranes fuse through numerous or a limited number of contact points deserves further ultrastructural investigations.

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## References

Barber, J. 1990. The fluid-mosaic nature of the thylakoid membrane. *In* Current

- Research in Photosynthesis. Vol. II. M. Baltscheffsky, editor. Kluwer Academic Press, Dordrecht/Boston/London. 715-724.
- Bassi, R., and F. A. Wollman. 1991. The chlorophyll a/b proteins of photosystem II in *Chlamydomonas reinhardtii*. *Planta (Berl.)*. 183:423-433.
- Bennoun, P., and P. Delepeleire. 1982. Isolation of photosynthesis mutants in *Chlamydomonas reinhardtii*. In *Methods in Chloroplast Molecular Biology*. M. Edelman, R. B. Hallick, and N. H. Chua, editors. Elsevier Biomedical Press. 25-38.
- Bennoun, P., A. Masson, and M. Delosme. 1980. A method for complementation analysis of nuclear and chloroplast mutants of photosynthesis in *Chlamydomonas reinhardtii*. *Genetics*. 95:39-47.
- Bennoun, P., B. A. Diner, F. A. Wollman, G. Schmidt, and N. H. Chua. 1981. Thylakoid polypeptides associated with photosystem II in *Chlamydomonas reinhardtii*: comparison of system II mutants and particles. In *Photosynthesis III. Structure and Molecular Organisation of the Photosynthetic apparatus*. George Akoyunoglou, editor. Balaban International Science Services. 885-899.
- Bulté, L., and P. Bennoun. 1990. Translational accuracy and sexual differentiation in *Chlamydomonas reinhardtii*. *Curr. Genet.* 18:155-160.
- Bulté, L., and F. A. Wollman. 1990. Mechanism and control of the inactivation of the cyt b6/f complexes during gametogenesis in *Chlamydomonas reinhardtii*. In *Current Research in Photosynthesis*. Vol. III. M. Baltscheffsky, editor. Kluwer Academic Press, Dordrecht/Boston/London. 715-718.
- Carlemalm, E., M. Garavito, and W. Villiger. 1982. Resin development for electron microscopy and an analysis of embedding at low temperature. *J. Microsc. (Paris)*. 126:123-143.
- Cavalier-Smith, T. 1970. Electron microscopic evidence for chloroplast fusion in zygotes of *Chlamydomonas reinhardtii*. *Nature (Lond.)*. 228:333-335.
- Cavalier-Smith, T. 1974. Basal body and flagellar development during the vegetative cell cycle and the sexual cycle of *Chlamydomonas reinhardtii*. *J. Cell Sci.* 16:529-556.
- Cavalier-Smith, T. 1975. Electron and light microscopy of gametogenesis and gamete fusion in *Chlamydomonas reinhardtii*. *Protoplasma (Berl.)*. 86: 1-18.
- Cavalier-Smith, T. 1976. Electron microscopy of zygospore formation in *Chlamydomonas reinhardtii*. *Protoplasma (Berl.)*. 87:297-315.
- Choquet, Y. 1987. Contribution à l'étude de la structure et de la biogenèse des centres photochimiques des végétaux supérieurs et de l'algue verte *Chlamydomonas reinhardtii*. Thèse de Doctorat Université, Université Paris VI, France.
- Chua, N. H., and P. Bennoun. 1975. Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii* wild-type and mutant strains deficient in PSII reaction center. *Proc. Natl. Acad. Sci. USA*. 72:2175-2179.
- Delepeleire, P. 1983. Characterization of additional thylakoid membrane polypeptides synthesized inside the chloroplast in *Chlamydomonas reinhardtii*. *Photobiochem. Photobiophys.* 6:279-291.
- Delepeleire, P. 1984. Partial characterization of the biosynthesis and integration of the photosystem II reaction centers in the thylakoid membrane of *Chlamydomonas reinhardtii*. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:701-706.
- de Vitry, C., J. Olive, D. Drapier, M. Recouvreur, and F. A. Wollman. 1989. Posttranslational events leading to the assembly of photosystem II protein complex: a study using photosynthesis mutant from *C. reinhardtii*. *J. Cell Biol.* 109:991-1006.
- Duysens, L. N. M., and H. F. Sweers. 1963. Mechanism of two photochemical reactions in algae as studied by means of fluorescence. In *Microalgae and Photosynthetic Bacteria*. The Japanese Society of Plant Physiologists, editors. Tokyo University Press, Japan. 353-372.
- Friedman, I., A. L. Colwin, and L. H. Colwin. 1968. Fine-structural aspects of fertilization in *Chlamydomonas reinhardtii*. *J. Cell Sci.* 3:115-128.
- Girard, J., N. H. Chua, P. Bennoun, G. Schmidt, and M. Delosme. 1980. Studies on mutants deficient in the photosystem I reaction centers in *Chlamydomonas reinhardtii*. *Curr. Genet.* 2:215-221.
- Girard-Bascou, J. 1988. Contribution à l'étude génétique de l'appareil photosynthétique chez l'algue verte *Chlamydomonas reinhardtii*. Thèse de Doctorat d'Etat. Université Paris XI, France.
- Goldschmidt-Clermont, M., J. Girard-Bascou, Y. Choquet, and J. D. Rochaix. 1990. Trans-splicing mutants of *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* 223:417-425.
- Goodenough, U. W., and R. P. Levine. 1969. Chloroplast ultrastructure of mutant strains of *Chlamydomonas reinhardtii* lacking components of the photosynthesis apparatus. *Plant Physiol.* 44:990-1000.
- Harris, E. H. 1989. The *Chlamydomonas* sourcebook. A Comprehensive Guide to Biology and Laboratory Use. Academic Press, Inc., Orlando FL.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Martin, N. C., and U. W. Goodenough. 1975. Gametic differentiation in *Chlamydomonas reinhardtii*. I. Production of gametes and their fine structure. *J. Cell Biol.* 67:587-605.
- Olive, J., F. A. Wollman, P. Bennoun, and M. Recouvreur. 1979. Ultrastructure-function relationship in *Chlamydomonas reinhardtii* thylakoids, by means of a comparison between the wild-type and the F34 mutant which lacks the photosystem II center. *Mol. Biol. Rep.* 5:139-143.
- Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17:208-213.
- Rochaix, J. D., M. Kuchka, S. Mayfield, J. Girard-Bascou, and P. Bennoun. 1989. Nuclear and chloroplast mutation affect the stability of the chloroplast psbC gene product in *Chlamydomonas reinhardtii*. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1013-1021.
- Siersma, P. W., and K. S. Chiang. 1971. Conservation and degradation of cytoplasmic and chloroplast ribosomes in *Chlamydomonas reinhardtii*. *J. Mol. Biol.* 58:167-185.
- Staelin, L. A. 1976. Reversible particle movements associated with unstacking and restacking of chloroplast membranes in vitro. *J. Cell Biol.* 71:136-158.
- Staelin, L. A. 1986. Chloroplast structure and supramolecular organization of photosynthetic membranes. In *Photosynthesis III: Photosynthetic Membranes and Light Harvesting Systems*. L. A. Staelin and C. J. Arntzen, editors. Springer-Verlag, Berlin. 1-84.
- Vallou, O., F. A. Wollman, and J. Olive. 1985. Distribution of intrinsic and extrinsic subunits of the PSII protein complex between appressed and non-appressed regions of the thylakoid membrane: an immunocytochemical study. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 183:245-250.
- Vallon, O., F. A. Wollman, and J. Olive. 1986. Lateral distribution of the main protein complexes of the photosynthetic apparatus in *Chlamydomonas reinhardtii* and in spinach: an immunocytochemical study using intact thylakoid membranes and a PSII enriched membrane preparation. *Photobiochem. Photobiophys.* 12:203-220.
- Wollman, F. A., and B. A. Diner. 1980. Cation control of fluorescence emission, light scatter, and membrane stacking in pigment mutants of *Chlamydomonas reinhardtii*. *Arch. Biochem. Biophys.* 201:646-659.
- Wollman, F. A., J. Olive, P. Bennoun, and M. Recouvreur. 1980. 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. *J. Cell Biol.* 87:728-735.