

Posttranslational Events Leading to the Assembly of Photosystem II Protein Complex: A Study Using Photosynthesis Mutants from *Chlamydomonas reinhardtii*

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Abstract. We studied the assembly of photosystem II (PSII) in several mutants from *Chlamydomonas reinhardtii* which were unable to synthesize either one PSII core subunit (P6 [43 kD], D1, or D2) or one oxygen-evolving enhancer (OEE1 or OEE2) subunit. Synthesis of the PSII subunits was analyzed on electrophoretograms of cells pulse labeled with [¹⁴C]acetate. Their accumulation in thylakoid membranes was studied on immunoblots, their chlorophyll-binding ability on nondenaturing gels, their assembly by detergent fractionation, their stability by pulse-chase experiments and determination of in vitro protease sensitivity, and their localization by immunocytochemistry.

In *Chlamydomonas*, the PSII core subunits P5 (47 kD), D1, and D2 are synthesized in a concerted manner while P6 synthesis is independent. P5 and P6 ac-

cumulate independently of each other in the stacked membranes. They bind chlorophyll soon after, or concomitantly with, their synthesis and independently of the presence of the other PSII subunits. Resistance to degradation increases step by step: beginning with assembly of P5, D1, and D2, then with binding of P6, and, finally, with binding of the OEE subunits on two independent high affinity sites (one for OEE1 and another for OEE2 to which OEE3 binds). In the absence of PSII cores, the OEE subunits accumulate independently in the thylakoid lumen and bind loosely to the membranes; OEE1 was found on stacked membranes, but OEE2 was found on either stacked or unstacked membranes depending on whether or not P6 was synthesized.

PHOTOSYSTEM II (PSII)¹ is a major protein complex of the photosynthetic apparatus in oxygen-evolving species. Light-harvesting chlorophyll-protein complexes (LHCs) transfer excitons to PSII cores where primary photochemistry occurs. PSII complexes (PSII cores with oxygen-evolving enhancer [OEE] subunits) are able to carry out the oxidation of water.

The PSII core comprises five main intrinsic chloroplast-encoded subunits P5, P6, D1, D2, and cytochrome *b*₅₅₉ (59). Their molecular masses vary slightly from one species to another. Two subunits of 47–50 and 43–47 kD, called P5 and P6 in *Chlamydomonas reinhardtii* or by their molecular mass in higher plants (respectively encoded by *psbB* and *psbC* genes), bind most of the PSII core chlorophylls (58) and form the core antenna (9, 44). The chlorophyll-P5 and chlorophyll-P6 complexes—called, respectively, CPIII and CP47 in *C. reinhardtii* or CP47 and CP43 in higher plants—can be separated by electrophoresis at 4°C (13, 21). D1 and D2 of 32–35 kD (encoded, respectively, by *psbA* and *psbD* genes [18, 48, 64]) cooperate in the binding of the primary

reactants (44) and show sequence homologies with the subunits L and M of the reaction center from purple bacteria (39, 53). Three extrinsic polypeptides encoded by nuclear genes (12, 60) are involved in oxygen evolution; OEE1 (29–33 kD) stabilizes the association of manganese ions with thylakoid membranes, whereas OEE2 (20–24 kD) and OEE3 (16–18 kD) sequester calcium and chloride ions next to the oxygen-evolving site (for review see 42). Additional PSII subunits of low molecular mass have been identified (11) and some of them might have a role in oxygen evolution (35). Thus, a functional oxygen-evolving PSII complex requires the assembly of numerous polypeptides (for review see 47), which are encoded either in the chloroplast or in the nucleus, and the binding of ~50 cofactors, among which are chlorophylls, carotenoids, pheophytins, plastoquinones, a non-heme iron, and manganese ions. Once inserted and processed in the unstacked thylakoid membrane regions, the PSII subunits undergo a lateral translocation to the stacked regions where most of the mature PSII complexes accumulate (36).

In this work we studied PSII assembly using several mutants of *C. reinhardtii*. This unicellular green alga can grow heterotrophically on acetate and is thereby appropriate for

1. *Abbreviations used in this paper:* LHC, light-harvesting complex; OEE, oxygen-evolving enhancer; PSII, photosystem II; WT, wild type.

the isolation and growth of photosynthesis mutants (for review see 46). PSII mutants offer the opportunity to analyze how various polypeptide deficiencies alter PSII assembly. Such an approach has been used mainly to study pretranslational and translational events in the process of assembly (28, 47). In this work, we focus more specifically on posttranslational events. We have undertaken a comparative analysis of three types of PSII mutants: mutants unable to synthesize either one reaction center subunit (D1 or D2), one core antenna subunit (P6), or one OEE subunit (OEE1 or OEE2). We characterized PSII biogenesis in each strain by the synthesis, insertion into the membrane, turnover, partial assembly, binding of cofactors, phosphorylation, and translocation to the granal regions of the various PSII subunits. This mutant-based analysis led us to distinguish four main steps in the process of PSII assembly in the thylakoid membranes.

Materials and Methods

Cell Culture Conditions and Mutants

Wild-type (WT) and PSII mutants of *C. reinhardtii* were grown at 300 lux in Tris-acetate-phosphate medium, pH 7.2, to a density of $2-4 \times 10^6$ cells/ml. Partial characterization of the mutants has been previously reported for (a) the PSII core nuclear mutant F34 (12); (b) the PSII core chloroplast mutants FUD34 (20), FUD7 (4), and FUD47 (19); (c) the OEE nuclear mutants BF25 (3, 37) and FUD44 (38); and (d) the F54-14 double mutant lacking photosystem I and chloroplast ATP synthase (15). The nuclear mutant FUD39 lacking OEE2, isolated and generously provided by P. Bennoun (Centre National de Recherche Scientifique, Paris, France), is characterized for the first time in this paper.

Protein Isolation

Thylakoid membranes were isolated as described (10). They were either prepared for electrophoresis by solubilization in 1% SDS at a chlorophyll concentration of 1 mg/ml or solubilized in 1.25% digitonin/1% Triton X-100 at a chlorophyll concentration of 1.2 mg/ml to purify PSII particles. Control PSII core particles were isolated from mutant F54-14 and purified by sucrose density gradient centrifugation (15); an identical treatment was performed on the PSII mutants to test the state of PSII assembly.

PAGE

SDS-PAGE was run according to Laemmli (32) as modified (13) using 7.5-15% polyacrylamide gradients or 12-18% polyacrylamide gradients containing 8 M urea (45). Study of the binding of chlorophyll to newly synthesized P5 and P6 was performed on two-dimensional gels (first, 7.5-15% polyacrylamide gradient gel at 4°C; second, 12-18% polyacrylamide gradient gel with 8 M urea at room temperature). Polypeptides were stained by Coomassie brilliant blue R-250 or detected by autoradiography of the dried gels using industrial P films (Agfa-Gevaert, Rueil-Malmaison, France).

Antibody Preparation, Electroblothing, and Hybridization

To obtain α -OEE2 and α -OEE3 antisera, we purified the proteins as follows. Thylakoid membranes from the F54-14 mutant were incubated in 50 mM Tris, pH 11, at 4°C with continuous stirring for 30 min. Polypeptides released in the supernatant were precipitated in presence of 10% TCA, and the resulting pellet was washed twice in ice-cold 80% acetone, dried, and solubilized in the presence of 1% SDS for two successive preparative SDS-gel electrophoresis (first gel, 12-18% polyacrylamide gradient containing 8 M urea; second gel, 7.5-15% polyacrylamide gradient). OEE2 and OEE3 were electroeluted from the second preparative gel (27). Rabbits were given subcutaneous injections of the purified proteins in Freund's complete adjuvant; after 4, 8, and 16 wk, each rabbit was given booster injections of purified OEE2 or OEE3 with poly(A), poly(U) as adjuvant (25). α -P5, α -P6, and α -OEE1 antibodies were obtained by courtesy of N. H. Chua (Rockefeller University, New York). Antiserum prepared against PSII parti-

cles of maize, kindly provided by R. Bassi (University of Padova, Padova, Italy), was tested by immunoblotting technique with *C. reinhardtii*: it reacted with D1 and the two forms of D2 (unphosphorylated and phosphorylated [12]), but not with P5 and P6 (see Fig. 2 A). Overexposure of the immunoblots revealed some cross-reaction of the α -D1/D2 antiserum with the LHC subunits (not shown). Therefore this antiserum was used in immunoblotting experiments but could not be used for the immunocytochemistry. The specificity of all antibodies can be judged from the immunoblots of the WT thylakoid proteins in Fig. 1 A and Fig. 7 A.

Electrophoretic transfers were carried out as described (52). Protein saturation of the nitrocellulose sheets was performed using 5% low-fat milk in PBS. Binding of the antibody was detected using radioiodinated protein A (6).

In Vivo Labeling and Pulse-Chase Experiments

The pulse labeling was carried out for 45 min as described in (11), but under illumination with 1,000 lux and cycloheximide (20 μ M) as inhibitor of cytoplasmic translation. For the pulse-chase experiments, cells were pulse labeled for 5 min only in the presence of 10^{-4} M [14 C]acetate (specific activity 1.85 GBq/mM). The cells were then chased by addition of an excess of unlabeled acetate (50 mM) for 0, 30, 90, and 240 min. The pulse-chase experiment was performed in the presence of cycloheximide. This prevents rapid recycling of 14 C-labeled products during synthesis of nuclear-encoded polypeptides, which otherwise would obscure detection of the chloroplast-encoded polypeptides. However, a prolonged incubation with cycloheximide alters cell metabolism. In particular, cell division (generation time is 10 h in *C. reinhardtii*) is blocked. Therefore, we limited the chase length to 4 h to remain close to physiological conditions.

Trypsin Treatment

Thylakoid membranes were washed in Hepes buffer (20 mM Hepes-KOH, pH 7.5, 10 mM NaCl, 100 mM sucrose) and resuspended in the same buffer at a chlorophyll concentration of 0.5 mg/ml. Trypsin treatment (10 μ g/ml) was performed at 4°C in the dark for 30 min. The proteolytic digestion was stopped by the addition of an equal volume of buffer containing a protease inhibitor (2 mM PMSF). When incubated with a high concentration of trypsin (50 mg/ml), the membranes were used at a chlorophyll concentration of 1 mg/ml.

Immunocytochemistry

Broken cells were prepared in presence of 10 mM $MgCl_2$ as described (62). Broken cells were fixed in 0.5% glutaraldehyde, 2% paraformaldehyde, 4 mM phosphate buffer, pH 7, containing 10 mM $MgCl_2$, rinsed with phosphate buffer containing 0.1 M glycine, dehydrated in alcohol-water solutions, and embedded in Lowicryl K4M (7). Thin sections were immunolabeled and stained as described (55) using protein A labeled with colloidal gold particles (10 nm) instead of labeled goat anti-rabbit IgG. Labeling densities were estimated from several micrographs (taken at 60 kV in an electron microscope [400; Philips Electronic Instruments, Inc., Mahwah, NJ]) by counting gold particles on unstacked and stacked membrane regions (300-1,500 particles counted per antibody and mutant). A coordinate analyzer and computer (Tektronix, Inc., Beaverton, OR) were used to measure the membrane lengths. To determine the mean and standard deviation, the micrograph measurements, which include labeling both in stacked and unstacked regions, were grouped in packs of equivalent statistic significance, each containing ~ 30 particles counted in the regions of lower density. In view of the low labeling densities in the mutants, the results of those with similar characteristics (F34 and FUD34 on one hand and FUD7 and FUD47 on the other hand) were pooled to increase sample size.

Results

Pleiotropic Polypeptide Deficiencies in Mutants Lacking Synthesis of One PSII Core Subunit

A striking feature of the thylakoid membranes in most mutants defective in PSII primary photoactivity is their deficiency in the whole set of subunits engaged in the formation of the PSII protein complex (4, 12, 19, 20).

The differential level of accumulation of the PSII core

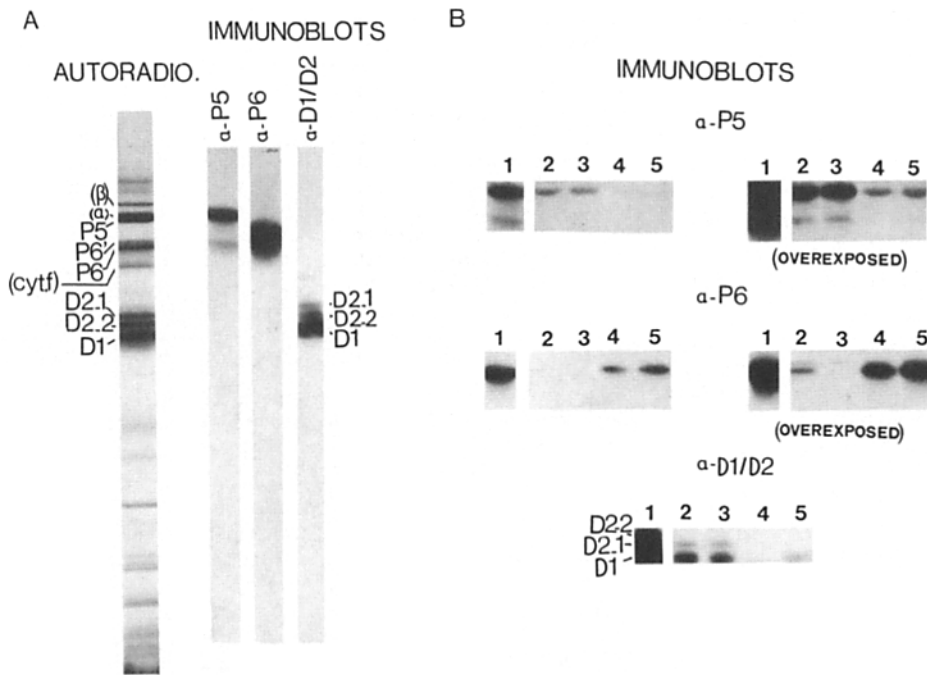


Figure 1. Accumulation of PSII core subunits in thylakoid membranes. Polypeptides were separated by SDS-urea PAGE, transferred to nitrocellulose, and probed with antibodies. (A) Newly synthesized polypeptide pattern of WT thylakoid membranes (45-min pulse labeling) and immunoblots of WT thylakoid membranes showing antibodies specificity. (B) Immunoblots of thylakoid membranes of WT and PSII core mutants: (lanes 1–5) WT, F34, FUD34, FUD7, and FUD47, respectively.

subunits in the thylakoids of such mutants can, however, be assessed on immunoblots. In Fig. 1 B the amounts of PSII core subunits accumulated in the WT of *C. reinhardtii* (lane 1) are compared with PSII mutants lacking synthesis of either P6 (lanes 2 and 3), D1 (lane 4), or D2 (lane 5). About 10% of the WT amount of P5 was found in the thylakoid membranes of the two mutants lacking P6 (lanes 2 and 3) whereas it was barely detectable in the mutants lacking D1 or D2 (lanes 4 and 5). We note that the α -P5 antibody recognized a main band in the position of P5 and a minor one slightly below P6 (Fig. 1 A). That the latter band is due to a genuine product of P5, probably a degradation product, and not to a faint cross-reaction with P6 is supported by its absence in the mutants showing little P5 but rather high P6 accumulation (see overexposed immunoblots in Fig. 1 B, lanes 4 and 5).

About 10% of the WT amount of P6 accumulated in the mutants lacking D1 or D2 (lanes 4 and 5). Although P6 was totally undetectable in the FUD34 mutant (lane 3), trace amounts of P6 could still be detected in the F34 mutant (lane 2). Some accumulation of D1 and D2 (the unphosphorylated form D2.2, but not the phosphorylated form D2.1) could be detected in the thylakoid membranes of the two mutants lacking P6 (lanes 2 and 3). In contrast, D2 was undetectable in the mutant lacking D1 (lane 4) but trace amounts of D1 were detectable in the mutant lacking D2 (lane 5). Another PSII reaction center subunit, cytochrome b_{559} (polypeptide L8), could be observed on SDS-urea gels after silver staining; some accumulation of cytochrome b_{559} was detected in the absence of the PSII core antenna subunit P6 (F34 mutant), but not in the absence of the reaction center subunit D2 (FUD47 mutant) (data not shown).

Synthesis of the PSII Core Subunits: Independence of P6 Synthesis

The rates of synthesis of the polypeptides encoded in the chloroplast were detected in 5-min pulse-labeling studies performed in the presence of [14 C]acetate after addition of

an inhibitor of cytoplasmic translation. Such experiments are shown in Fig. 2 A, lane 0, where one can compare the rates of synthesis of the PSII core subunits in three PSII mutants (panels 3–5) and in the WT (panel 1). As previously reported: (a) synthesis of P6 was totally absent in the chloroplast mutant FUD34 (panel 3) and hardly detectable in the nuclear mutant F34 (data not shown; 14, 20) (The conflicting claim of Jensen et al. [28] that F34 showed high levels of P6 synthesis but lacked D1 arose from additional mutations in the strain used by these authors [Girard-Bascou, J., Y. Pierre, and P. Delepelaire, manuscript in preparation]); (b) there was no synthesis of D1 in the FUD7 mutant (panel 4) which has a deletion in the *psbA* gene (4); and (c) there was no synthesis of D2 in the FUD47 mutant (panel 5) which is inactivated in the *psbD* gene (19). That some synthesis of D1 still occurred in this mutant is better seen in 45-min pulse-labeling experiments (Fig. 2 B, panel 5).

Thus, each of these PSII mutants was lacking synthesis of only one of the PSII core subunits. However, examination of the rates of synthesis of the other PSII core subunits indicated additional modifications in some mutants. In the absence of D1, the rate of D2 synthesis was unaffected while most of P5 synthesis was prevented (FUD7 mutant; panel 4). The control of D1 over P5 synthesis and conversely of P5 over D1 synthesis was reported in other *C. reinhardtii* mutants by Jensen et al. (28). In the absence of D2, most of the D1 and P5 synthesis was prevented (FUD47 mutant; panel 5) as noted by Erickson et al. (19). In this case, the decreased rate in P5 synthesis might be a consequence of the lower synthesis of D1.

In contrast, P6 synthesis occurred independently. This was indicated by similar rates of P6 synthesis in the WT and in mutants lacking D1 or D2 (cf. panels 4, 5, and 1) and conversely by similar rates of synthesis of P5, D1, and D2 in the WT and in mutants lacking P6 (cf. panels 3 and 1). The synthesis of polypeptide L8, which is probably a cytochrome b_{559} apoprotein (59), was unaffected in all mutants (data not shown).

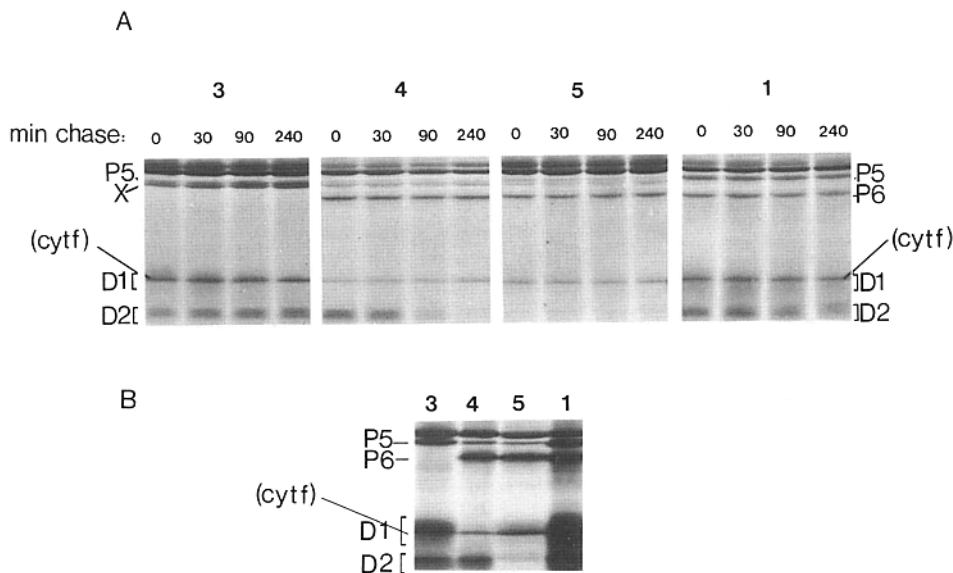


Figure 2. Analysis of the chloroplast-encoded polypeptides from WT and PSII core mutants on SDS-7.5–15% polyacrylamide gel. (A) Autoradiogram of cell polypeptides. Cells were pulse labeled for 5 min and then chased for 0, 30, 90, and 240 min. (Lanes 1 and 3–5) WT, FUD34, FUD7, FUD47, respectively. The polypeptide X presenting a slightly lower molecular mass than P5 is a cellular soluble protein (absent in thylakoid membranes; data not shown). (B) Autoradiogram of thylakoid membrane polypeptides (45-min pulse labeling).

Assembly of the PSII Core Subunits: A P5/D1/D2/L8 Step

To determine the state of assembly between the PSII core subunits remaining in these mutants, we followed the procedure for PSII particle purification from *C. reinhardtii* as described (15). Detergent-solubilized thylakoid membranes from cells pulse labeled in the presence of an inhibitor of cytoplasmic translation were loaded on a sucrose gradient. The distribution of the PSII subunits was analyzed among the gradient fractions after ultracentrifugation. The content of chloroplast-encoded polypeptides in such fractions is shown for the FUD34 mutant in Fig. 3 A. Most of the α and β subunits of the ATP synthase were found at the bottom part of the gradient (lane 5) whereas cytochrome *f* and cytochrome *b₆* were located in the middle of the gradient (lanes 15–19). PSII subunits P5, D1, D2, and L8 (a cytochrome *b₅₅₉* subunit) were found in the fractions immediately above those containing the ATP synthase subunits (lanes 7–10). P6 is totally absent in the FUD34 mutant (Fig. 1). Densitometric scanning of labeled PSII core subunits in each fraction from the various PSII mutants and from the control strain are shown in Fig. 3 B. The codistribution of the PSII core subunits in the control gradient illustrates their assembly in a complex. We observed that each of the PSII core subunits from the mutants lacking in P6 (FUD34 and F34) showed the same distribution and were found at sucrose densities close to that of the control PSII particles. In addition, the PSII subunits from such fractions could be coprecipitated by α -P5 antibodies (data not shown). We attribute the slight displacement towards lower densities of the PSII fractions in the F34 gradient to the higher contamination by LHCs (visible on Coomassie blue-stained electrophoretograms; data not shown), which is of lower density than PSII particles (15). These results indicate that the PSII subunits can assemble in a P5/D1/D2/L8 complex in the mutants lacking P6.

In contrast, the mutants lacking D1 or D2 (FUD7 and FUD47) displayed an erratic distribution of the remaining PSII core subunits, suggesting the absence of PSII assembly. However, we cannot exclude weak interactions between the

remaining PSII subunits in such mutants, which would be disrupted by detergent solubilization.

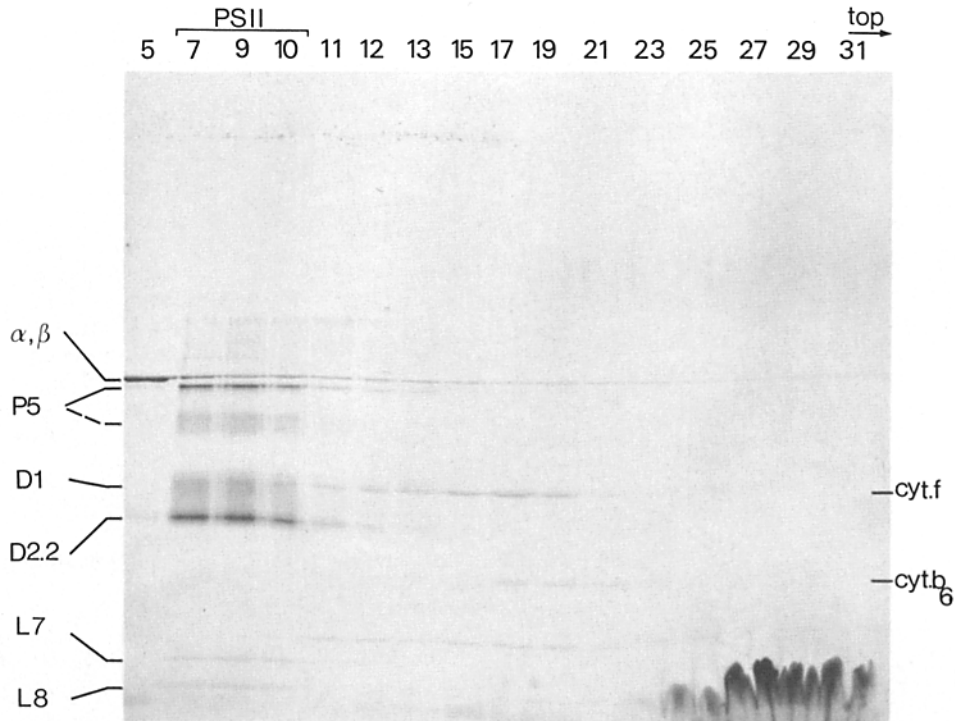
The Binding of Chlorophyll to the PSII Core Subunits: An Early Event

To examine the binding of chlorophyll to the PSII core antenna subunits, we looked for the presence of the chlorophyll-binding forms of P5 and P6 (CPIII and CPIV, respectively). In the WT (Fig. 4), CPIII and CPIV can be detected as spots off the diagonal in two-dimensional gel electrophoresis (arrows); newly synthesized products encoded by the chloroplast were selectively detected using cells pulse labeled for 45 min in presence of an inhibitor of cytoplasmic translation. CPIII and CPIV were heat sensitive and were not detected when the sample was heated before the first dimension electrophoresis (compare WT and WT heated). In the mutant lacking P6 (F34), only CPIII was detected. In the mutants lacking D1 or D2 (FUD7 and FUD47), CPIV was easily detected; CPIII could be detected in overexposed autoradiograms only (Fig. 4, *FUD47 inset*) owing to the impaired synthesis of P5 (Fig. 2). These results indicate that the binding of chlorophyll to P5 and P6 occurred in <45 min and independently of PSII core assembly. We similarly detected CPIII and CPIV in these mutants in shorter pulses (5 min); a subsequent chase of 50 min performed in the absence of protein synthesis was not accompanied by an increased conversion of P5 or P6 in CPIII and CPIV (data not shown). Thus binding of chlorophyll to P5 and P6 is an early event in the process of insertion into the membrane of the core antenna subunits.

Also detectable in Fig. 4 is the existence of a native form of D2 (*D2n*; an “in” diagonal spot indicated by arrows at the center of two-dimensional gels) in addition to its denatured form (*D2d*). *D2n* shared three characteristics with chlorophyll-binding complexes: (a) a modified electrophoretic mobility compared with the denatured form; (b) a heat sensitivity (compare WT and WT heated or FUD7 and FUD7 heated); and (c) an acetone sensitivity (data not shown). Whether *D2n* contains chlorophyll or another cofactor requires additional study.

A

SDS FUD34 gradient fractions



B

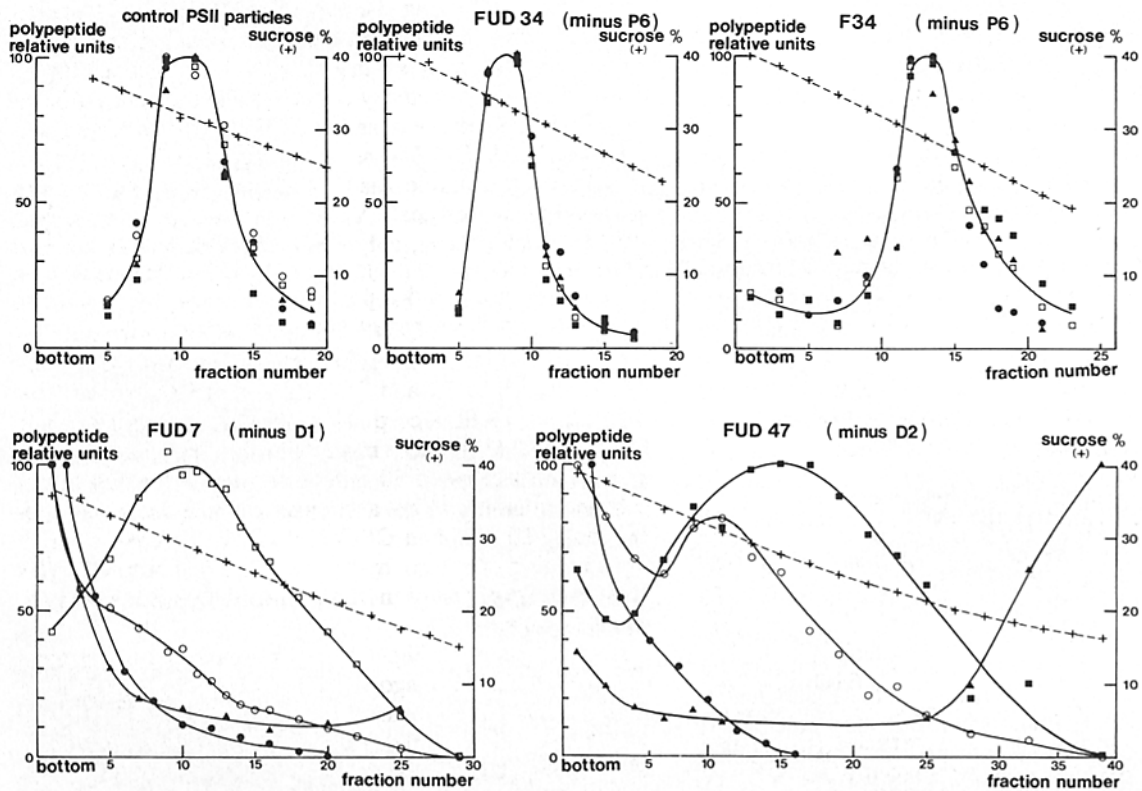


Figure 3. Newly synthesized polypeptide pattern of fractions after sucrose gradient centrifugation of solubilized membranes of PSII core mutants (45-min pulse labeling). (A) Autoradiogram of FUD34 mutant fractions. α and β are ATP synthase subunits. Cytochromes of the b_6/f complex are shown (*cyt.b₆* and *cyt.f*). Dashed line points to degradation product of P5 (as shown in Fig. 1). Analysis was by SDS-7.5–15% polyacrylamide gradient gel electrophoresis. (B) Same as A after densitometric scanning of autoradiograms of pulse-labeled fractions for P6 (○), D1 (■), D2 (□), and L8 (▲) and of autoradiograms after immunoblotting for P5 (●).

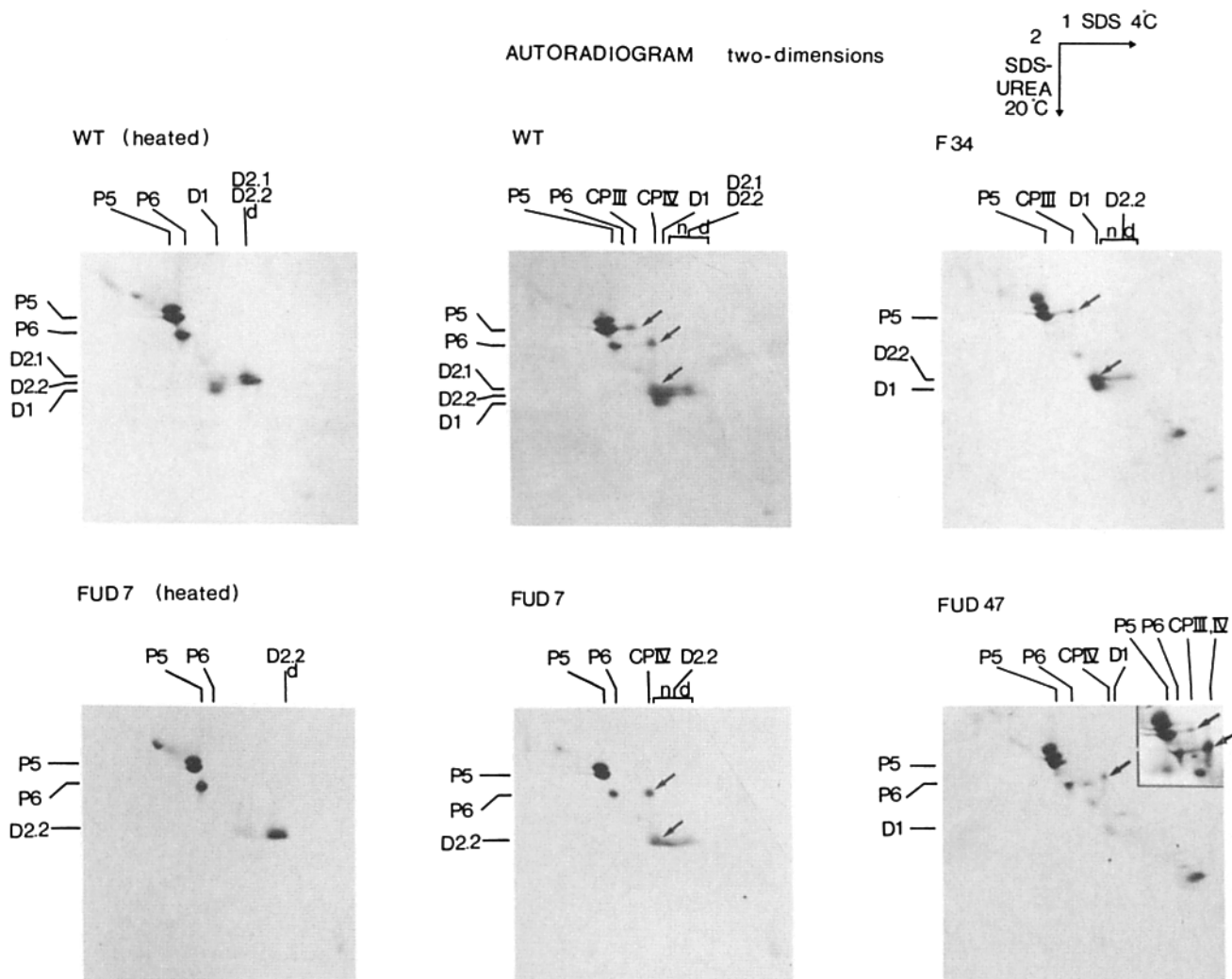


Figure 4. Autoradiogram of chloroplast-encoded polypeptides (45-min pulse labeling) of WT and PSII core mutants (use of thylakoid membranes in WT and FUD7 mutant and cells in F34 and FUD47 mutants). Analysis was by two-dimensional electrophoresis (first, 7.5–15% polyacrylamide gradient gel electrophoresis at 4°C; second, 12–18% polyacrylamide gradient containing 8 M urea, gel electrophoresis at 20°C). CPIII and CPIV are spots off the diagonal (*arrows*) whereas P5 (obscured by comigration with α subunit of the ATP synthase) and P6 are spots in the diagonal. Note that chlorophyll binding to P5 and P6, shown for reference in WT, occurs also in the mutants (*F34*, *CPIII*, *FUD47*, *FUD47 insert*, *FUD7*, and *CPIV*). D2 exists in a native form (*D2n*, *arrow*) and in a partially denaturated form (*D2d*) in F34 and FUD47 mutants as in WT. CPIII, CPIV, and D2n are heat sensitive (see their absence in heated WT and FUD7).

The Sensitivity of PSII Core Subunits to Proteases Is Increased in the Mutants

That several PSII subunits showed unchanged rates of insertion but no accumulation in the mutants' membrane suggested that they would be more susceptible to proteolytic degradation. Therefore we assessed the trypsin sensitivity of the newly synthesized PSII core subunits. In the WT (Fig. 5 A), a fraction of P5 and D1 was cleaved by trypsin whereas P6 and D2 showed little trypsin sensitivity. In contrast, P5, P6, and D2 were all trypsin sensitive in the FUD7 mutant lacking D1. As shown by immunoblotting, the whole pool of P6 accumulated in the FUD7 mutant was trypsin sensitive (Fig. 5 B). In the FUD34 mutant lacking P6 (Fig. 5 A), D1 and D2 were more sensitive to trypsin than in the WT. However, when the protease concentration was increased up to 50 mg/ml, P6, D1, and D2 were all trypsin sensitive. In addition, whereas P5 was totally digested by trypsin in the

FUD34 mutant, it was only converted to a slightly lower molecular mass form in the WT (Fig. 6 C). Thus we observed in vitro an increasing sensitivity to proteases along with a decreasing ability of the PSII core subunits to assemble in the thylakoid membranes.

Turnover of the PSII Core Subunits: Turnover of D2 Increases in the Absence of D1

We then addressed the question as to whether the above results could be correlated in vivo with an increased turnover of the PSII core subunits in the absence of assembly. A 4-h pulse-chase experiment is shown in Fig. 2. The lack of accumulation of D2 in the mutant lacking D1 correlated well with the increased turnover of D2 in this mutant as compared with that in the WT (cf. panels 4 and 7).

Surprisingly, in all the other instances the lack of accumulation of the PSII subunits, which showed rates of synthesis

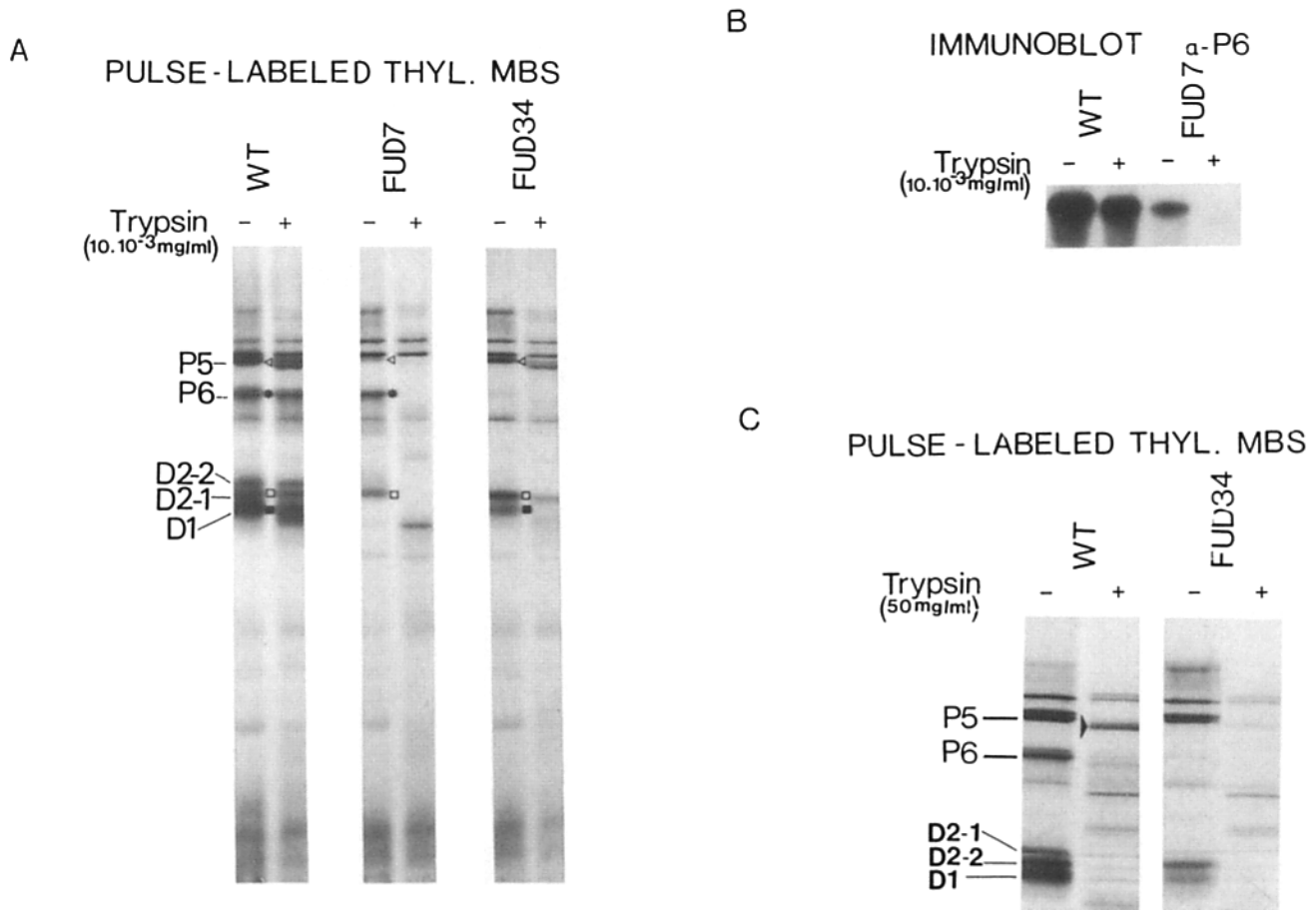


Figure 5. Polypeptide analysis of thylakoid membranes, after trypsin treatments, of WT and PSII core mutants on SDS-urea polyacrylamide gels. (A) Autoradiogram of pulse-labeled thylakoids treated or untreated with a low concentration of trypsin (symbols point to electrophoretic migration position of the intact PSII core subunits). (B) Immunoblot analysis of P6 in trypsin treated (as in A) and untreated membranes from WT or FUD7 mutant. (C) Autoradiogram of pulse-labeled thylakoids untreated or treated with a high concentration of trypsin (the arrowhead points to a P5 trypsinized form).

similar to that in the WT, could not be correlated with an increase in turnover within the time course of our chase experiment.

PSII Core Subunits Are Localized Predominantly in the Stacked Regions of the Thylakoid Membranes in the Mutants

We have previously used postembedding immunogold labeling on thin sections from broken cells to show that most of the mature PSII protein complexes were found in the stacked regions of the thylakoid membranes of the WT (55). In addition, significant labeling of the thylakoid membranes of PSII mutants could be obtained with antibodies directed against PSII subunits (54). Therefore, it was possible to investigate whether unassembled or partially assembled PSII core subunits had the property of segregating in the stacked membrane regions. The localization of P5 and P6 was assessed by this technique. As shown in Fig. 6, the labeling of the thylakoid membranes was much weaker in the mutants (B and D) than in the WT (A and C). The unstacked membrane regions were limited on one side by swollen lumen (clear to electrons) and on the other side by stroma (dense to electrons) while the stacked membrane regions were localized in the piles of membranes limited by two swollen lumen. For

P6, the densities of labeling in the stacked membrane regions were 1.4 particles/ μm in the mutants lacking D1 or D2 vs. 8.1 in the WT, with a background labeling of 0.1 particles/ μm estimated on the thylakoid membranes of the FUD34 mutant which is totally deficient in P6. For P5, the densities of labeling in the stacked membrane regions were 0.7 particles/ μm in the mutants lacking P6 vs. 7.5 in the WT, with a background labeling of 0.3 particles/ μm estimated on the thylakoid membranes of the FUD47 mutant which is highly deficient in P5. A quantitative estimation of the extent of segregation of P5 and P6 between the stacked and unstacked membrane regions is given in Table I. According to the representation we have used, a uniform distribution would yield values of about one, whereas distributions favoring stacked or unstacked membrane regions would give values, respectively, below and above one. Table I shows that P5 and P6 were predominantly in the stacked membrane regions in the mutants but not sequestered as strictly as in the WT case.

Localization of the OEE Subunits on the Thylakoid Membrane

As reported previously in *C. reinhardtii* (22, 30), mutant cells, unable to accumulate the PSII core because of the absence of synthesis of P6, D1, or D2, displayed an unaltered

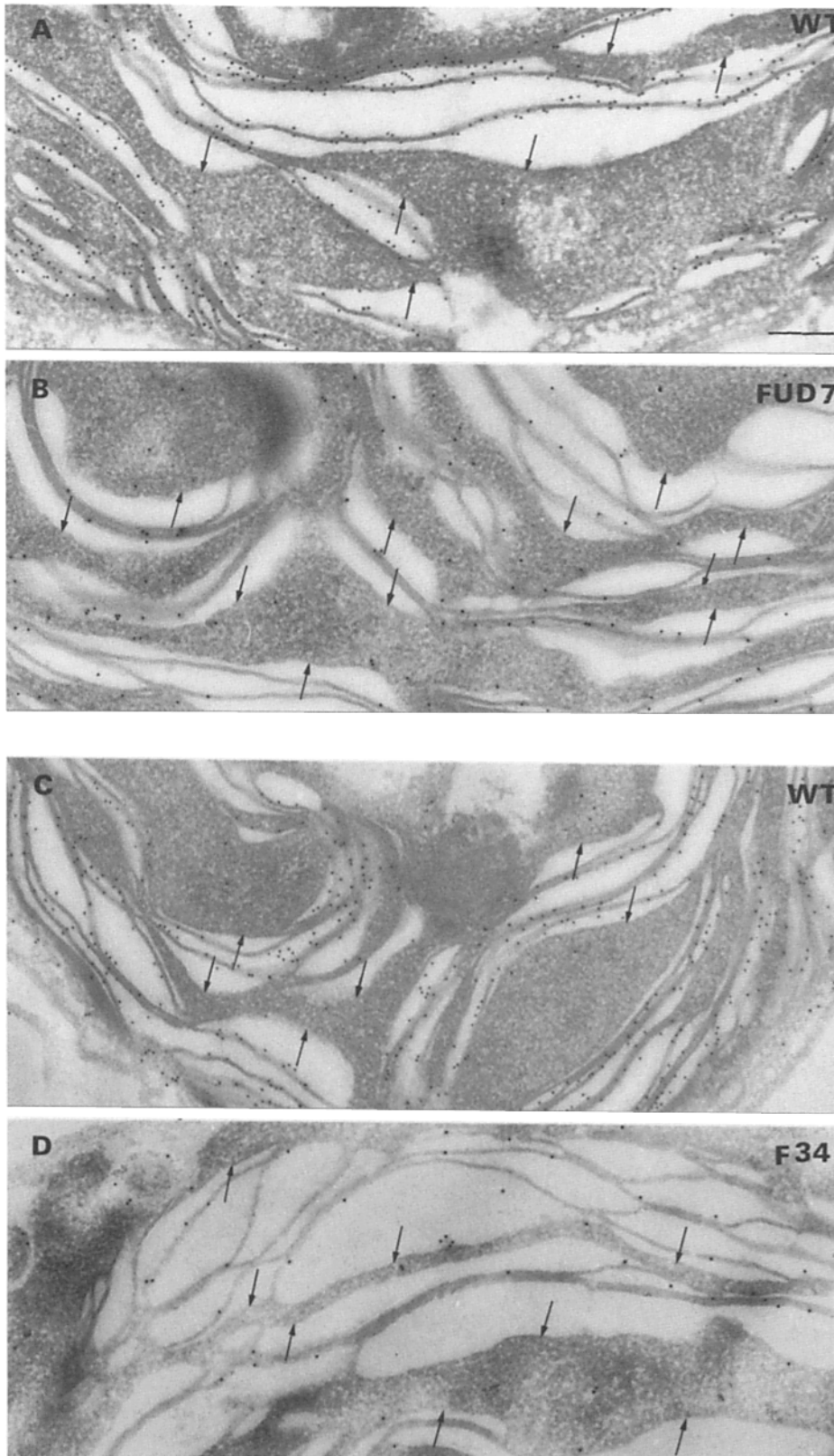


Figure 6. Immunogold labeling of α -PSII core antenna subunit antibodies in WT and PSII core mutants. α -P6 antibody (1/200) on WT (A) and FUD7 mutant (B) and α -P5 antibody (1/50) on WT (C) and F34 mutant (D). Arrows indicate unstacked membranes. Bar, 0.25 μ m.

accumulation of the OEE subunits in their mature form (Fig. 7 B). However, we observed that purified thylakoid membranes from these mutants were depleted of OEE subunits but had the same content of P6 as whole cells. This was in-

dicative either of a nonthylakoidal localization of the OEE subunits detected in whole cells or of their loose binding to the thylakoid membranes, causing loss during the purification.

Table I. Immunogold Labeling Densities

Subunit	WT	F34 and FUD34	FUD7 and FUD47
	d_u/d_s	d_u/d_s	d_u/d_s
P5	0.26 ± 0.13	0.49 ± 0.11	—
P6	0.18 ± 0.02	—	0.61 ± 0.15
OEE1	0.15 ± 0.03	0.21 ± 0.07	0.25 ± 0.08
OEE2	0.34 ± 0.07	1.65 ± 0.59	0.54 ± 0.09

Ratio of immunogold labeling densities in the unstacked over the stacked regions (d_u/d_s) in WT and PSII mutant thylakoid membranes.

Immunocytochemical analysis of the OEE subunits showed that they were chloroplast localized in the mutants as in the WT. This is exemplified for OEE2 at low magnification in Fig. 8 A: most of the gold particles were found in the chloroplast, while the cytoplasm, nucleus, and mitochondria were devoid of labeling. However, the α -OEE2 antiserum produced some extrachloroplastic labeling which was next to the plasma membrane in the mutants as in the WT (not shown). At higher magnification (Fig. 8, B-E), one observes that α -OEE1 and α -OEE2 antisera labeled the thylakoid membranes of the mutants as heavily as in the WT. We then calculated the labeling densities in the stacked and unstacked regions of the thylakoid membranes in each mutant. Table I shows that OEE1 was found mainly in the stacked regions. Surprisingly the localization of OEE2 depended on the synthesis of P6: OEE2 was largely retained in the unstacked membrane regions in the two mutants lacking P6, whereas it was found

in the stacked membrane regions in the two mutants showing P6 synthesis but lacking either D1 or D2.

Subunits OEE1 and OEE2/OEE3 Bind at Two Different Sites on the PSII Core Complex; Stabilization of PSII Core by OEE Binding

We compared three low-fluorescent nuclear mutants deficient in oxygen evolution that were partly characterized previously (37, 38). As illustrated by immunoblots of cells in Fig. 9 A, mutants BF25 and FUD39 specifically lacked synthesis of OEE2, whereas the FUD44 mutant lacked synthesis of OEE1.

Examination of the content of OEE subunits in thylakoid membranes of these mutants (Fig. 9 A) revealed that there was no membrane binding of OEE3 in the absence of OEE2, whereas OEE1 binding still occurred. On the other hand, the absence of OEE1 in the FUD44 mutant did not prevent membrane binding of OEE2 and OEE3. These results clearly demonstrate the existence of two binding sites: one for OEE1 and another for OEE2 to which OEE3 binds.

The amounts of OEE2 and OEE3 retained on the thylakoid membranes from the FUD44 mutant were, however, smaller than in the WT (Fig. 9 A) and may be accounted for by the decreased content of PSII core subunits in this mutant (Fig. 9 B). The OEE2-lacking FUD39 mutant also showed such a decrease in P6, D1, and D2, whereas the OEE2-lacking BF25 did not. Spectroscopic estimation of PSII centers, based on the PSII charge separation detected at 515 nm (63), confirmed that the FUD44 and FUD39 mutants contained

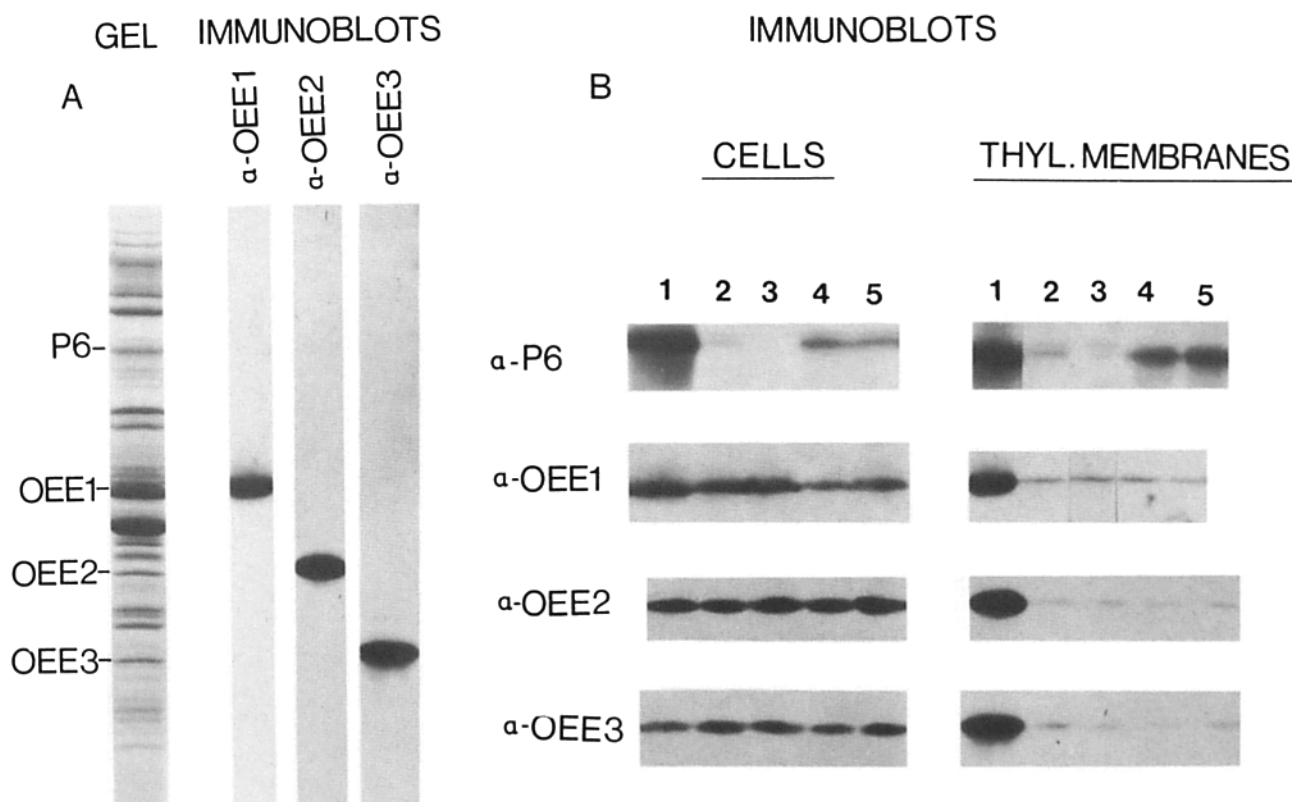


Figure 7. Accumulation of OEE subunits in cells or in thylakoid membranes of WT and PSII core mutants. (A) WT thylakoid membranes showing antibodies specificity. (B) Cells and thylakoid membranes of (lanes 1-5) WT, F34, FUD34, FUD7, and FUD47, respectively. Conditions are the same as in Fig. 1.

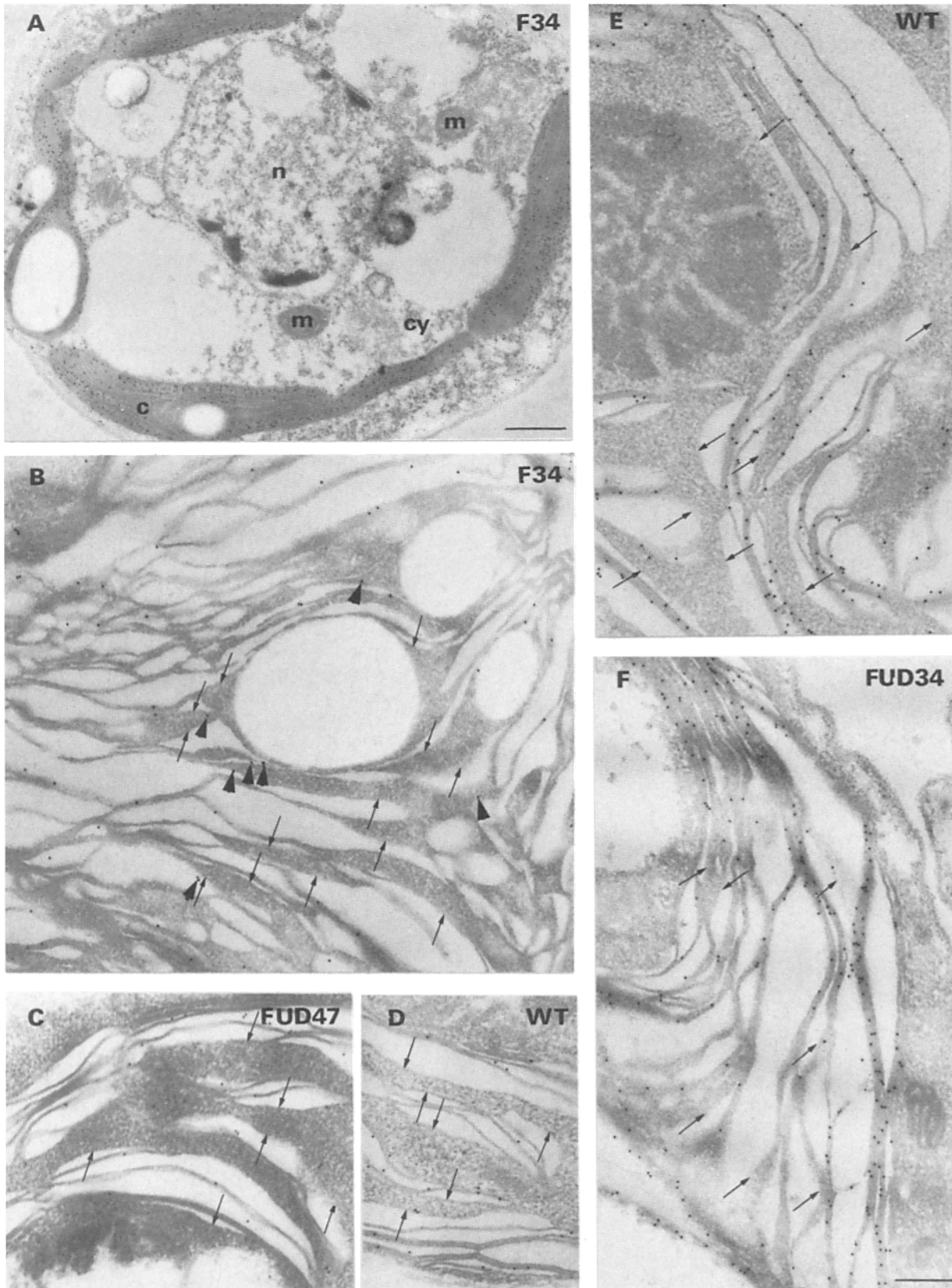


Figure 8. Immunogold labeling of α -OEE subunit antibodies in WT and PSII core mutants. (A) α -OEE2 antiserum 1/25 in F34. Most of the gold particles are concentrated on the chloroplast (c) and are absent from the nucleus (n), mitochondria (m), and cytoplasm (cy); some extrachloroplastic labeling appears near the plasma membrane. (B–D) α -OEE2 antiserum 1/25. Most of the gold particles are on the stacked membranes in FUD47 (C) as in WT (D), while in F34 (B) most of them (arrowheads) are present on the unstacked membranes (arrows). (E and F) α -OEE1 antibody 1/100 in WT (E) and FUD34 (F). Most of the gold particles are on the stacked membranes. For quantification, see Table I. Bars: (A) 0.5 μ m; (B–F) 0.3 μ m.

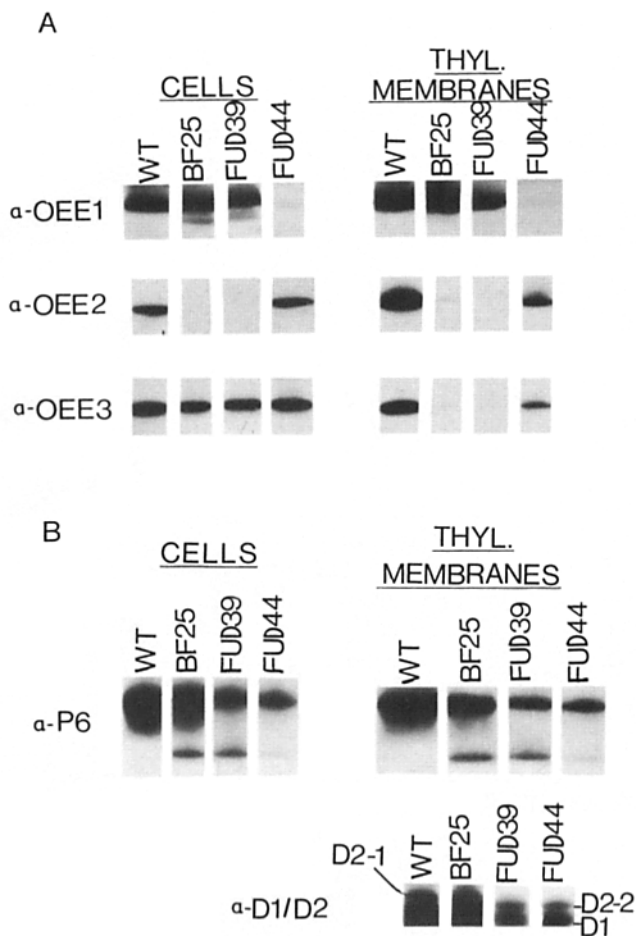


Figure 9. Immunoblot analysis of the OEE subunits (A) and PSII core subunits (B) in cells or thylakoid membranes of mutants lacking one OEE subunit. Conditions were as in Fig. 1.

only 30% of the PSII primary activity found in the WT on a chlorophyll basis as compared with ~65% in the BF25 mutant. Since the rate of synthesis of the PSII core subunits in these mutants was similar to that in WT (data not shown), we conclude that the decrease in their PSII content is indicative of a lower PSII core stability.

The α -P6 immunoblots of Fig. 9B revealed a lower molecular mass form of P6 in cells and in purified thylakoid membranes of OEE-lacking mutants, particularly in the absence of OEE2. It was taken as indicative of an interaction between P6 and OEE2 (and to a lesser extent OEE1), in the absence of which partial cleavage of P6 may occur.

Discussion

The comparative analysis of several PSII mutants unable to synthesize either a reaction center subunit, a core subunit, or an OEE subunit gave new insights on the processes of synthesis, accumulation, and assembly of the subunits of the PSII protein complex in the thylakoid membranes. The main characteristics of these PSII mutants are summarized in Tables II and III.

Translational and Posttranslational Control of the Accumulation of the PSII Core Subunits

We have observed *in vitro* that the trypsin sensitivity of several PSII core subunits was increased in the thylakoid membranes of PSII mutants that failed to accumulate the PSII core complex. This was indicative of the exposure of proteolytic sites on the PSII subunits, which were masked in the WT thylakoid membranes. Indeed, in the absence of D1 synthesis, we observed an increased turnover of D2 *in vivo* in the time range of our pulse-chase experiments (4 h). A similar increase in D2 turnover in the absence of either D1 or P5 synthesis was reported by Jensen et al. (28). However, we failed to observe an increased turnover of P5 and D2 in the absence of P6 synthesis as well as of P6 in the absence of D1 or D2 synthesis. An increased turnover of P6 has been reported in other PSII mutants from *C. reinhardtii* or maize (28, 33). However, in both cases the contrast between the amounts of labeled P6 in the mutants and in the WT was visible only 8–16 h after the end of the pulse labeling. Our pulse-labeling conditions, in the presence of an inhibitor of cytoplasmic translation, limited the chase length to 4 h (see Materials and Methods).

Still, we observed that exogenous, and in some instances endogenous, proteases have an increased access to the PSII core subunits remaining in the thylakoid membranes of the mutants that fail to accumulate the complex. A change in the interactions between the PSII subunits could contribute in two ways to an increased protease sensitivity: polypeptide regions normally engaged in (a) the interactions with cofactors or (b) between the subunits of the mature complex may become accessible to proteases in the absence of proper complex assembly. Indirect effects are also conceivable, resulting for instance from conformational changes or enhancement of molecular movements in the absence of assembly.

Besides this posttranslational control on the accumulation of the PSII subunits, it should be mentioned that, in several *C. reinhardtii* mutants unable to synthesize one PSII subunit, deficiencies in some of the other PSII subunits may be caused by a decrease in their rate of synthesis. Such a translational

Table II. Characteristics of Mutants Unable to Synthesize a PSII Core Subunit

Strain	Detected synthesis						Accumulation						Detected assembly	Chlorophyll binding
	P5	P6	D1	D2	L8	OEE	P5	P6	D1	D2	L8	OEE*		
F34	+	(ϵ)	+	+	+	+	(+)	(ϵ)	ϵ	ϵ	ϵ	+	Yes	Yes
FUD34	+	–	+	+	+	+	(+)	–	ϵ	ϵ	ND	+	Yes	Yes
FUD7	(+)	+	–	+	+	+	ϵ	(+)	–	–	ND	+	No	Yes
FUD47	(+)	+	(+)	–	+	+	ϵ	(+)	(ϵ)	–	–	+	No	Yes

Polypeptide synthesis and accumulation were indicated: (–) absent; (ϵ) traces; (ϵ) very low; ([+]) impaired; (+) level similar to the WT; and (ND) not determined. The ability of the PSII core subunits to assemble (Detected assembly) and of P5 or P6 to bind chlorophyll (Chlorophyll binding) are presented.

* OEE subunits are almost totally lost after thylakoid purification.

Table III. Characteristics of Mutants Unable to Synthesize an OEE Subunit

Strain	Detected synthesis				PSII	Accumulation			Membrane bound*		
	PSII [‡]	OEE1	OEE2	OEE3		OEE1	OEE2	OEE3	OEE1	OEE2	OEE3
FUD44	+	-	+	+	(+)	-	+	+	-	(+)	(+)
BF25	+	+	-	+	(+)	+	-	+	(+)	-	-
FUD39	+	+	-	+	(+)	+	-	+	(+)	-	-

Polypeptide synthesis and accumulation are indicated: (-) absent; ([+]) impaired; and (+) level similar to WT.

* OEE subunits when membrane bound are proportional to PSII accumulation.

[‡] Similar for the PSII core subunits P5, P6, D1, D2, and L8.

control of one PSII subunit over another has been reported for D2 over D1 by Erickson et al. (19) and for D1 over P5 by Jensen et al. (28). Translational control is also apparent in our study of the FUD7 mutant, where the absence of D1 synthesis impaired synthesis of P5 (4), and the FUD47 mutant, where the absence of D2 synthesis impaired that of D1 and probably as a consequence that of P5. Interestingly, P6 synthesis was unaltered in these two mutants as was the synthesis of P5, D1, and D2 in the F34 and FUD34 mutants which were unable to synthesize P6. The independence between P6 and D2 synthesis indicates that their respective genes *psbC* and *psbD*, which are well separated in the plastid genome of *C. reinhardtii* (46), are expressed independently. It is also the case in the cyanobacterium *Synechocystis* (56) although these two genes overlap as in most plant genomes (2, 8, 24). Therefore this independence of P6 synthesis seems to be a general feature in PSII biogenesis.

Biogenesis of D1 and D2 Subunits

The sequence homologies between D1, D2, and the L and M subunits of the bacterial reaction center (39, 53) and, more recently, the purification of D1/D2 photoactive particles (44) have demonstrated that D1 and D2 are the two subunits of the PSII reaction center that bind the primary reactants. The lack of assembly of the PSII core subunits in the mutants unable to synthesize either D1 or D2 suggests that assembly of the PSII reaction center is a prerequisite for PSII core assembly. Our study points out some differences between D1 and D2 in the process of PSII core biogenesis. Traces of D1 accumulated in the mutant unable to synthesize D2, whereas no D2 accumulated in the mutant unable to synthesize D1. Indeed there was an increased proteolytic disposal of D2 in the absence of D1. However, as noted above, D1 synthesis was drastically decreased in the absence of D2 whereas the reverse situation did not occur. Thus stoichiometric accumulation of the PSII reaction center subunits appears to depend both on a translational control of D1 by D2 and on a posttranslational control of D2 by D1. It has been reported that there was an increased turnover of D1 during photoinhibition without significant changes in the turnover of D2 (31). Presumably, in this case, rapid replacement of D1 occurs through insertion into the membrane of the new polypeptides triggered by the D2 present in the thylakoid membranes with which they would subsequently assemble.

Biogenesis of P5 and P6

The two core antenna subunits P5 and P6 are quite homologous with ~30% conserved amino acids in their membrane-spanning regions (2, 24). They bind the same amounts of chlorophyll and the resulting chlorophyll-protein complexes

have very similar spectral characteristics (9). We have observed in vivo that binding of chlorophyll is an early event in the biogenesis of these subunits. It was detected within the first 5 min of insertion into the membrane of P5 and P6 and occurred independently of PSII core assembly. This is much faster than other posttranslational modifications, such as PSII phosphorylation which occurs with a half-time of ~30 min (12). In barley, photoconversion of protochlorophyllide into chlorophyll paralleled the synthesis of PSII core antenna subunits, suggesting that binding of chlorophyll is concurrent with polypeptide synthesis and controls the translation (29). We conclude from the early binding of chlorophyll that the low stability of P5 and P6 in the PSII mutants has to be accounted by another process than the lack of binding of chlorophyll.

We reported previously that P6 can be more easily detached than P5 from the rest of the PSII core (58). Our present study reveals additional differences between the two core antenna subunits. Whereas we, and others (4, 28), observed a translational control of P5 by D1, it is apparent from our experiments that synthesis of P6 occurs totally independently. In addition, we have shown that P5 can assemble with D1 and D2 (and L8) in the absence of P6. Such a partial assembly could probably allow the primary charge separation to occur as suggested by the detection of some primary charge separation in a *Synechocystis* mutant inactivated in *psbC* (56). Assembly of the PSII core therefore appears to occur in two steps: (a) assembly of P5, D1, and D2 and then (b) assembly with P6. Since we observed that the two building blocks, P6 and P5/D1/D2, can segregate independently in the stacked membrane regions, it is likely that the second step of assembly occurs in the stacked regions whereas the first step might occur in the unstacked regions where the subunits are inserted cotranslationally (23).

Biogenesis of the OEE Subunits

The OEE subunits were processed to their mature form and accumulated in the PSII core mutants as in the WT. Such an observation was reported in *C. reinhardtii* (22, 47) and (for OEE1) in maize, grown in the dark or under far red light, which failed to accumulate PSII cores (51). In agreement with the conclusion that OEE1 was accumulated in the lumen of far red-grown maize thylakoids because it was protected from trypsin in right side out vesicles (51), we observed by immunogold labeling that the three OEE subunits were found on the thylakoid membranes of the PSII mutants as in the case of the WT. They were not floating in the lumen of the thylakoids, but stuck to the membranes. However, they interacted only loosely with the membranes since they were lost after purification of the thylakoids. These observations

suggest that the proteolytic activity on the luminal side of the membranes is lower than that on the stromal side; it has been shown, for instance, that in absence of the rubisco large subunits, the unassembled small subunits are rapidly degraded in the stroma (49).

Surprisingly, OEE1 and OEE2 were not uniformly distributed along the membranes of the mutants. They were found in the stacked membrane regions in mutants lacking synthesis of either D1 or D2, whereas OEE2, but not OEE1, was retained in the unstacked membrane regions in the two mutants lacking P6 synthesis. This observation excludes that the apparent membrane binding of the OEE subunits viewed after immunogold labeling would originate from an artificial cross-linking between membrane proteins and the OEE subunits floating in the lumen because of the embedding procedure. On the other hand, it points to the existence of binding sites of low affinity for OEE1 in the stacked membrane regions. These may be some of the purported OEE binding proteins proposed by Ljungberg et al. (34) or some lipids specifically found in the stacked membrane regions (43). The localization of OEE2 in the stacked membrane regions in the mutants lacking D1 or D2, and not in the mutants lacking P6, suggests a specific interaction between OEE2 and P6 which is, however, difficult to understand owing to the substoichiometric amounts of P6 in these mutants. Such an interaction is, however, consistent with the protection of P6 against proteolytic degradation in the presence of OEE2 (a degradation product of P6 was particularly abundant in mutants lacking OEE2).

Analysis of nuclear mutants lacking synthesis of either OEE1 or OEE2 gave further insight on the organization of the OEE subunits on the thylakoid inner surface. The immunoblots performed on the thylakoid membranes from these mutants revealed a stoichiometric binding on the PSII core of OEE2 and OEE3 in the absence of OEE1. In contrast, OEE3 binding was lost in the absence of OEE2 whereas OEE1 stoichiometric binding still occurred. Thus OEE1 and OEE2 have distinct binding sites on the PSII core, and OEE3 interacts with the PSII core by binding to OEE2. Such an interaction between OEE2 and OEE3 has been observed in reconstitution experiments (40). However, similar experiments have led to the opposite conclusion that the binding site of OEE2 is located on OEE1 (41). This latter view is clearly not supported by the present study.

The binding sites of the OEE subunits on the PSII core may involve several of its subunits. A cross-link between P5 and OEE1 was observed by two groups (5, 17) whereas experiments based on protease sensitivity were consistent with an interaction between OEE1 and P6 (26). In a mutant of *Scenedesmus*, which accumulates PSII cores but lacks the COOH-terminal processing of D1, there was an impaired binding of OEE2 and OEE3 (16). Therefore the OEE2 binding site may implicate D1, in addition to P6, as we discussed above.

Mayfield et al. (37, 38) reported that OEE1, but not OEE2, was required for PSII core stability in the thylakoid membranes. We have shown here that the amounts of PSII cores accumulated by the two mutants lacking synthesis of OEE2 were widely different, but always below that in the WT. These observations indicate that OEE2 also contributes to PSII stability. The mechanism by which the absence of an OEE subunit decreases the stability of the PSII cores in the membrane remains to be elucidated.

Conclusion

We have focused on various factors which may control the stability of the PSII subunits in *C. reinhardtii*. Among these parameters were (a) their ability to assemble in the thylakoid membranes; (b) the binding of chlorophyll or of phosphate groups, which may stabilize their proper conformation; and (c) their change in lateral distribution in the thylakoid membranes, which may control the exposure of the polypeptides to endogenous proteases. These aspects of PSII biogenesis were compared in several PSII mutants that failed to accumulate the PSII protein complex. All of them still showed binding of chlorophyll to the residual PSII core subunits. Therefore the lack of accumulation of the PSII protein complexes in the PSII mutants cannot be accounted for by a change in binding of chlorophyll to the PSII core subunits. We can also exclude that the instability of the PSII subunits was due to the absence of PSII phosphorylation since nonphosphorylated PSII core does accumulate in an LHC-deficient mutant (57, 59). A change in lateral distribution could be involved in a decreased stability of the PSII cores since we observed that P5 and P6 were less strictly sequestered in the stacked membrane regions in these mutants than in the WT. But the major contribution to the lower stability of PSII subunits in the mutants seems to arise from their failure to assemble properly. Indeed, we have observed an increased sensitivity to trypsin of the PSII core subunits with the decrease in PSII assembly in the mutants. In some instances, turnover studies demonstrated that endogenous proteases have an increased access to the PSII subunits in such mutants. The results discussed in this paper led us to distinguish four main steps in the process of PSII unit assembly (Fig. 10) that correspond to an increased stability of the PSII subunits.

In step one, the PSII core subunits, which are translated on thylakoid-bound ribosomes in the unstacked membrane regions (36), are synthesized in a concerted manner for P5/D1/D2 and independently for P6, with early binding of chlorophyll to P5 and P6. The OEE subunits are imported in the chloroplast, processed, and translocated independently in the thylakoid lumen where they can interact loosely with the membrane.

In step two, which occurs probably soon after step one, presumably in the unstacked membrane regions, partial stabilization of the PSII core subunits occurs by assembly of P5, D1, and D2.

In step three, further stabilization occurs by assembly of P6 with the P5/D1/D2 moiety of the PSII core, probably in the stacked membrane regions. The PSII core then associates with the OEE subunits through two independent binding sites of high affinity: one for OEE1 and another for OEE2 that may involve P6 (OEE3 binds via OEE2). This association (PSII complex) increases the stability of the PSII core.

Step four describes the assembly of the active PSII complex with its peripheral antenna, the LHC, leading to the formation of the PSII unit. The rationale for this step relies on previous studies. It is known that the PSII complex and the LHC are accumulated independently in the thylakoid membranes. However, some posttranslational modifications of PSII subunits seem to involve the presence of the LHC. Delepelaire has shown that there are slow posttranslational modifications of P6, D2, and two small integral membrane PSII subunits, L5 and L6 (12), that are due to polypeptide phosphorylations (14, 59). We observed previously that such

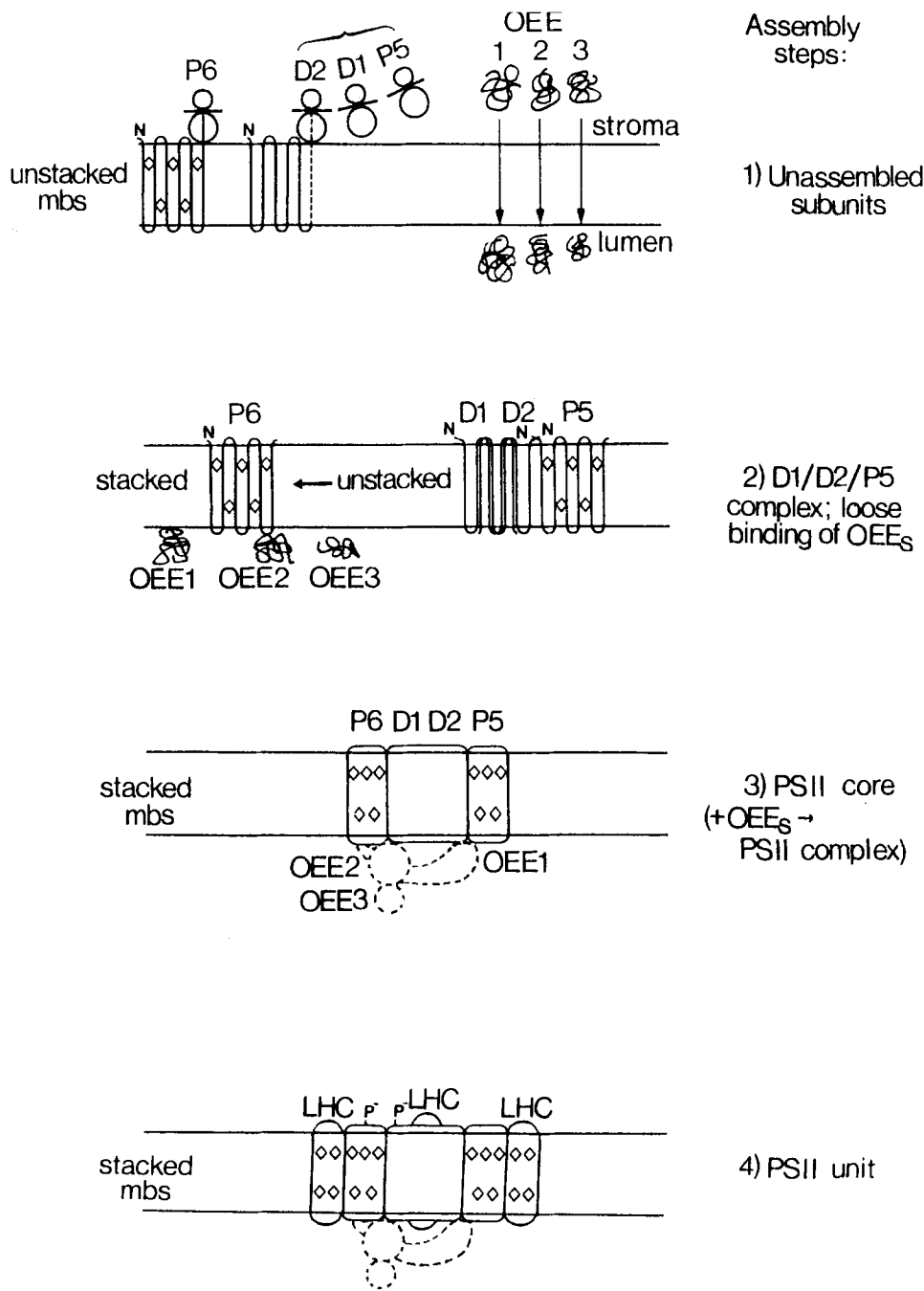


Figure 10. Proposed model of PSII assembly. (Step one) Synthesis of PSII core subunits is concerted for D2, D1, and P5 and is independent for P6. OEE subunits are translocated independently. (Step two) PSII core assembles in two steps: assembly of P5/D1/D2 (in stroma lamellae?) and assembly of (P5/D1/D2)/P6 (in stacked regions?) which stabilizes the reaction center. (Step three) Fully active PSII complex (PSII core and OEE subunits) assembles with further stabilization. (Step four) PSII unit (PSII complex and LHC) assembles with concomitant PSII phosphorylation. Schematic polypeptide structures are used in steps one and two; they are based on the predicted membrane-spanning segments and on the NH₂-terminal localization. The bracket indicates the concerted synthesis of D2, D1, and P5. The chlorophyll bound to the PSII core antenna and to the LHC is symbolized by diamonds. Interaction sites between OEE subunits and the PSII core are drawn taking into account the various reports on OEE binding sites (5, 16, 17, 26). Proper OEE binding is not a prerequisite for PSII core (as well as for PSII unit) assembly and is therefore drawn in dashed lines.

a PSII phosphorylation was lacking in the absence of PSII core accumulation (14) as well as in the absence of LHC accumulation (57, 59). We have then suggested that this phosphorylation is associated with the formation of the PSII unit. Such a PSII phosphorylation process may, in addition, facilitate the regulation of LHC-PSII interactions during state transitions (1, 50, 59, 61).

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