Synthesis and Assembly of the D1 Protein into Photosystem II: Processing of the C-Terminus and Identification of the Initial Assembly Partners and Complexes during Photosystem II Repair[†]

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ABSTRACT: In previous studies [van Wijk, K. J., Bingsmark, S., Aro, E.-M., & Andersson, B. (1995) *J. Biol. Chem. 270*, 25685−25695; van Wijk, K. J., Andersson, B., & Aro, E.-M. (1996) *J. Biol. Chem 271*, 9627−9636], we have demonstrated that D1 protein synthesized in isolated chloroplasts and thylakoids is incorporated into the photosystem II (PSII) core complex. By pulse−chase experiments in these *in vitro* systems, followed by sucrose gradient fractionation of solubilized thylakoid membranes, it was shown that this assembly proceeded stepwise; first the D1 protein was incorporated to form a PSII reaction center complex (PSII rc), and through additional assembly steps the PSII core complex was formed. In this study, we have analyzed this assembly process in more detail, with special emphasis on the initial events, through further purification and analysis of the assembly intermediates by nondenaturing Deriphat-PAGE and by flatbed isoelectric focusing. The D2 protein was found to be the dominant PSII reaction center protein initially associating with the new D1 protein. This strongly suggests that the D2 protein is the primary "receptor" or stabilizing component during or directly after synthesis of the D1 protein. After formation of the D1−D2 heterodimer, cyt b_{559} became attached, whereas the *psbI* gene product was assembled as a subsequent step, thereby forming a PSII reaction center complex. Subsequent formation of the PSII core occurred by binding of CP47 and then CP43 to the PSII rc. The rapid radiolabeling of a minor population of a PSII core subcomplex without CP43 indicated that an association of newly synthesized D1 protein with a preexisting complex consisting of D2/cyt b_{559}/*psbI* gene product/CP47 was possibly occurring, in parallel to the predominant sequential assembly pathway. The kinetics of synthesis and processing of the precursor D1 protein were followed in isolated chloroplasts and were compared with its incorporation into PSII assembly intermediates. No precursor D1 protein was found in PSII core complexes, indicating either that incorporation into the PSII core complex facilitates the cleavage of the C-terminus or, more likely, that processing is more rapid than the assembly into the PSII core.

Photosystem II (PSII),[1] located in the thylakoid membranes of chloroplasts, contains over 25 different proteins of both plastid and nuclear origin (Andersson & Franzen, 1992; Erickson & Rochaix, 1992). Despite this complexity, only the D1−D2 proteins are needed to carry out primary photochemistry, and the D1−D2 heterodimer can be isolated as a functional complex (Tang et al., 1990). The D1 and D2 proteins are in close association with the *psbI* gene product as well as the α and β subunits of cyt b_{559}, forming so-called PSII reaction center (rc) particles (Nanba & Satoh, 1987). These particles can be isolated by detergent solubilization and liquid chromatography (Satoh, 1993; Roobol-Boza & Andersson, 1996). Oxygen-evolving PSII core complexes contain additionally three extrinsic subunits (33, 23, and 17 kDa) exposed to the lumenal thylakoid surface and two chlorophyll *a* binding antenna proteins (CP47 and CP43) as well as several low molecular mass subunits (≤10 kDa). Recent structural observations suggest that the PSII complex has a dimeric organization in its functional state (Santini et al., 1994; Boekema et al., 1995).

An intriguing property of PS II is that one of its rc subunits, the D1 protein, is turning over with an unusually high rate (Mattoo et al., 1984; Ohad et al., 1985). This phenomenon is a consequence of light-induced inactivation of PSII electron transport and concomitant oxidative damage to the PSII rc in a process which is referred to as photoinhibition [reviewed in Prasil et al. (1992) and Aro et al. (1993)]. In order to repair the damaged PSII complex, the photodamaged D1 protein is proteolytically degraded and replaced with *de novo* synthesized D1 protein.

Very little is known about this repair process of damaged PSII complexes. A general concept has been proposed, involving PSII complexes of different (antennae) size and redox activities [e.g., Neale & Melis, 1990; Adir et al., 1990; see Aro et al. (1993)]. The process is complicated since it requires lateral migration of PSII components within the

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[1] Abbreviations: chl, chlorophyll; DM, *n*-dodecyl β-D-maltoside; IEF, isoelectric focusing; LHCII, light harvesting complex; pD1, precursor of D1 protein; PMSF, phenylmethanesulfonyl fluoride; PSII, Photosystem II; rc, reaction center.

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heterogeneous membrane and (partial) disassembly of the PSII complex as well as religation of probably most of the cofactors and pigments bound to the PSII rc [see Prasil et al. (1992) and Aro et al. (1993)]. Furthermore, synthesis and insertion of the D1 protein into the membrane must somehow mechanistically be adjusted to the rate of damage to the PSII rc, since the amount of the D1 protein in the thylakoid membranes remains rather constant at different rates of light-induced damage to the D1 protein *in vivo* (Schuster et al., 1988; Kettunen et al., 1991; Schnettger et al., 1994). It still remains unclear to what extent the PSII complex is disassembled during D1 protein damage and degradation [see Adir et al. (1990), Barbato et al. (1992) and Hundal et al. (1990)] and, accordingly, how reassembly of PSII complexes occurs during the repair cycle. Only recently have the incorporation of newly synthesized D1 protein into existing PSII and reactivation of the electron transport reactions been addressed at a more detailed molecular level (Adir et al., 1990; van Wijk et al., 1994, 1995, 1996).

The D1 protein is synthesized with a nonconserved C-terminal extension of 8−16 amino acids consisting mainly of neutral and hydrophobic residues [see, e.g., Erickson and Rochaix (1992)]. The functional significance of the D1 precursor (pD1) and its processing has been a considerable issue of debate. The processing is catalyzed by a lumenal peptidase which has been partially isolated and characterized (Taylor et al., 1988; Inagaki et al., 1989; Bowyer et al., 1992; Fujita et al., 1995; Anbudurai et al., 1994). Studies on the mutant LF-1 of *Scenedesmus obliquus* mutant, which lacks the processing peptidase, demonstrated that the pD1 protein could be assembled into the PSII complex, allowing normal primary photochemistry to occur (Metz et al., 1985, 1986; Rutherford et al., 1988; Taylor et al., 1988). In contrast, water oxidation was impaired, because the unprocessed C-terminus obscures the manganese ligation of the water-splitting system (e.g., Nixon et al., 1992). Recent construction of mutants of the green algae *Chlamydomonas reinhardtii* (Lers et al., 1992) and the cyanobacterium *Synechocystis 6803* (Diner et al., 1988; Nixon et al., 1992), lacking the C-terminal extension, produced cells with a growth rate and photosynthetic activity equal to the wild-type.

Despite these mutant studies, it still remains unclear why this C-terminal extension of the D1 protein is present in all photosynthetic organisms studied, except for the green flagellate *Euglena gracilis* (Karabin et al., 1984). Possibly, the need for retaining this extension throughout evolution is to be found in the control of the assembly of the D1 protein into a functional PSII complex.

It has been possible to follow the replacement of the D1 protein into existing, damaged PSII complexes during translations in isolated chloroplasts and thylakoid membranes from mature leaves (van Wijk et al., 1995, 1996). In all experiments, the chloroplasts and thylakoids were isolated during the light period of the diurnal light/dark cycle. Since the D1 protein is turning over constantly in the light with a half-life of about 8 h under normal growth conditions, the preparations always contained a small pool of damaged PSII complexes requiring the synthesis and insertion of D1 protein for repair. Indeed, during the *in vitro* translations, the (p)-D1 protein was the most prominently labeled polypeptide, reflecting the relatively short lifetime of the D1 protein and the need for its insertion into damaged PSII complexes. The newly synthesized D1 protein assembled with high efficiency into existing PSII complexes and allowed analysis of the kinetics of the PSII repair process as well as the identification of various assembly intermediates (van Wijk et al., 1996). These PSII assembly intermediates, containing radiolabeled D1 protein, were isolated by fractionation of solubilized [with *n*-dodecyl β-D-maltoside (DM)] thylakoid membranes on sucrose gradients.

The aim of the present study is to further analyze PSII assembly during D1 protein turnover, to identify the initial assembly partner(s) of the D1 protein, immediately after termination of translation, and to investigate the function of the C-terminal extension. After runoff translation, followed by solubilization in DM and sucrose gradient fractionation, the PSII assembly intermediates in the gradient fractions were further purified by nondenaturing Deriphat-PAGE followed by SDS−PAGE. The PSII assembly intermediates smaller than 120 kDa were analyzed in more detail by flatbed isoelectric focusing (IEF). We show that the D2 protein is the primary assembly partner for the newly synthesized D1 protein, and subsequently cyt b_{559} and the *psbI* gene product are added to the complex. After formation of the PSII rc in a sequential manner, the chlorophyll *a* binding protein CP47 is attached, followed by binding of the CP43 protein.

In order to address the role of the C-terminal extension of the D1 protein in assembly, translations were carried out in intact chloroplasts isolated from older leaves. This resulted in translation of the D1 protein, with only very low amounts of radiolabeled D2 protein, which allowed the quantification of D1 protein in both its precursor and its mature form. The results reveal that in isolated chloroplasts, the precursor form of the D1 protein is processed after, as well as prior to, assembly, in agreement with our previous observations in isolated thylakoids (van Wijk et al., 1996). However, no precursor D1 protein was found in PSII core complexes, perhaps indicating that incorporation into the PSII core complex facilitates the cleavage of the C-terminus, but more likely indicating that processing of pD1 is more rapid than assembly of pD1 into the PSII core. Our findings will be further discussed in perspective of the physiology of the PSII repair cycle.

MATERIALS AND METHODS

Plant Material, Isolation of Chloroplasts and Thylakoid Membranes, and in Vitro Runoff Translation. Spinach was grown hydroponically in a growth chamber at a photon flux density of 400 μmol m^{-2} s^{-1} at 20 °C with a light/dark cycle of 12 h/12 h. Fully developed leaves, harvested during the first half of the light period, were used for isolation of intact chloroplasts on percoll gradients (van Wijk et al., 1995). *In vitro* translation in isolated chloroplasts was carried out at 50 μmol of photons m^{-2} s^{-1} at 23 °C (van Wijk et al., 1995). The chloroplasts (0.5 μg of chl μL^{-1}) were incubated for 5 min at 23 °C in a translation mixture composed of 330 mM sorbitol, 50 mM Hepes−KOH (pH 8.0), 10 mM dithiothreitol, 10 mM MgATP, and 40 μM of each amino acid except methionine. After this preincubation, carrier-free ^{35}S-labeled methionine was added to a concentration of 0.5 μCi μL^{-1}. After 4 min pulse-labeling, the radioactive methionine was chased by the addition of 10 mM cold methionine for 0−60 min. Translation was stopped by dilution with a 10-fold

volume of ice-cold medium containing 330 mM sorbitol, 50 mM Hepes−KOH (pH 8.0). Thylakoid membranes were isolated from the intact chloroplasts by osmotic breakage of the chloroplasts into RNase-free lysis medium, as described in van Wijk et al. (1995). *In vitro* runoff translation with isolated thylakoids was performed principally according to Klein et al. (1988b) with modifications presented in van Wijk et al. (1995). After a pulse-labeling period of 3 min (for pD1 processing), 5 min (for Deriphat-PAGE), or 10 min (for IEF experiments) with carrier-free [^{35}S]methionine (Amersham), an excess of nonradioactive methionine was added, and radioactivity was chased for 5, 10, or 30 min. Translation was terminated by adding a 10-fold volume of ice-cold lysis buffer. After translation, the thylakoids were washed several times in the same lysis buffer to remove nonincorporated radioactivity. Chlorophyll concentrations were determined in 80% acetone, using the absorption coefficients of Lichtenthaler (1989).

Separation of PSII Subcomplexes and Unassembled Proteins by Analytical Sucrose Gradient Centrifugation. After *in vitro* translation and careful repetitive washings in 10 mM Tricine (pH 7.8), the thylakoid membranes (0.5 mg of chl mL^{-1}) were solubilized with 1% (w/v) DM in 10 mM Tricine (pH 7.8) containing 2 mM phenylmethanesulfonyl fluoride (PMSF) on ice for 50 min and centrifuged on sucrose gradients (0.1−1 M sucrose in 10 mM Tricine, pH 7.8, and 0.03% DM and 2 mM PMSF) at 180000*g* for 20 h, 2 °C. Nineteen fractions of equal volume were collected from the bottom to the top of the centrifuge tube.

Deriphat-PAGE. Fractions 7−15 from the sucrose gradient centrifugations, earlier shown to contain various PSII complexes and unassembled proteins (van Wijk et al., 1995, 1996), were concentrated in Centricon tubes (Amicon) with a 3 kDa cutoff filter. The protein complexes were separated on nondenaturing Deriphat-PAGE (5−16% acrylamide gradient), essentially according to Barbato et al. (1995). No detergent was present in the gel, but 0.1% Deriphat-160 (Henkel Corp.) was added to the upper running buffer. After electrophoresis, the gels were photographed, fixed, and washed for 2 days in 30% ethanol (to prevent formation of salt crystals in the gels upon drying) and subsequently incubated for 15 min in Amplify (Amersham), dried, and exposed to X-ray films.

2D Electrophoresis. After Deriphat-PAGE, gel strips were cut and incubated in solubilizing buffer [2.5% SDS, 2.5% β-mercaptoethanol, 0.2 M Tris-HCl (pH 8.3), 0.2 M sucrose, and 6 M urea] for 3 h at room temperature and then heated for 5 min at 70 °C prior to loading on a second-dimension SDS−PAGE. Proteins were separated at 12 ° C on 14% linear gels or 12−22% gradient gels, containing 6 M urea using the Laemmli (1970) system. Either the gels were dried for autoradiography, or proteins were blotted on a poly(vinylidene difluoride) membrane. PSII polypeptides were immunodecorated with different polyclonal antibodies raised against isolated PSII proteins or synthetic peptides and detected using chemiluminescence (goat anti-rabbit IgG horseradish peroxidase conjugate; Bio-Rad). Antiserum against the *psbI* gene product was a kind gift of Prof. M. Ikeuchi (University of Tokyo, Japan), antisera against CP47 and CP43 were kindly provided by Dr. R. Barbato (University of Paduva, Italy), and antiserum raised against the C-terminus of the D2 protein was a gift from Dr. P. Nixon (Imperial College, University of London, U.K.). N-Terminal D1 antiserum was directed against V58−S70 in the lumenal loop between helices 1 and 2, and antiserum against cyt b_{559} was raised against purified protein.

Isoelectric Focusing. Sucrose gradient fractions of radiolabeled, solubilized thylakoid membranes (after runoff translation) were concentrated in Centricons with 3 kDa cutoff filters and applied to flatbed isoelectric focusing, essentially according to Dainese et al. (1990). Ampholytes (Pharmacia) forming the pH gradient from 4.0 to 6.5 were used in the isoelectric focusing bed (28 × 10 cm). Samples, mixed with isoelectric focusing bed, were loaded close to the cathode, and the gels were run at 2 °C for 12 h at constant power not exceeding 8 W. After termination of the run, the IEF beds were divided into 13 fractions. A small proportion of each fraction was used for pH determination, and proteins were eluted with 10 mM Tricine (pH 7.8), 0.03% DM, and 2 mM PMSF. After concentration and solubilization, the IEF samples were analyzed by SDS−PAGE with subsequent autoradiography and immunodetection of different PSII core polypeptides as described above.

RESULTS

Deriphat-PAGE Separation of PSII Complexes after Runoff Translation and Sucrose Fractionation of Thylakoid Membranes. In all experiments, the chloroplasts and thylakoids were isolated during the light period of the diurnal light/dark cycle. Since the D1 protein is turning over constantly in the light with a half-life of about 8 h under our growth conditions, the preparations always contained a small pool of damaged PSII complexes, which required the synthesis and insertion of D1 protein. Based on several observations and reports in the literature, we estimate that the time from damage to D1 degradation is about 10 min under conditions where damage and repair are in equilibrium. Thus, we can expect that about 1−3% of the PSII population in our preparations require replacement of the D1 protein. During the *in vitro* translations, the D1 protein and its precursor form were the most prominently labeled polypeptides (Figure 1), reflecting the relatively short lifetime of the D1 protein and the need for its insertion into damaged PSII complexes. The radiolabeled D1 protein was earlier shown to replace damaged D1 protein into existing PSII complexes (van Wijk et al., 1995). To analyze this incorporation process in detail with respect to the initial events and to separate the different assembly intermediates with high resolution, thylakoid proteins were radiolabeled during runoff translations (5 min pulse followed by 5, 10, and 30 min chase with cold methionine) and fractionated after sucrose gradient centrifugation. Nineteen fractions of equal volume were collected from the bottom (fraction 1) to the top (fraction 19) of the sucrose gradient [Figure 1B; for detailed characterization of the fractions, see van Wijk et al. (1995)]. Fractions 15−7, containing PSII complexes and proteins in the molecular mass region from 40 to 360 kDa, were each concentrated and further fractionated by nondenaturing Deriphat-PAGE (Figure 2). The major protein complexes were identified on the basis of the polypeptide composition after a second-dimension SDS−PAGE followed by immunoblotting (data not shown) and also on the basis of previous work (Peter & Thornber, 1991; Dreyfuss & Thornber, 1994; Bassi et al., 1995).

Figure 2A shows the chlorophyll-containing green bands in the unstained nondenaturing Deriphat gel. The slowest

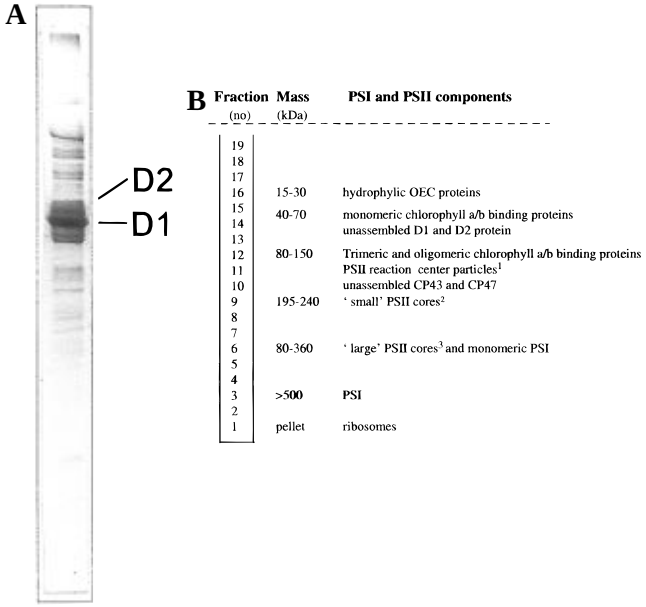

FIGURE 1: Analysis of membrane-bound ^{35}S-labeled translation products after runoff translation in isolated thylakoids. (A) Autoradiogram of thylakoid proteins separated by SDS−PAGE. Runoff translation for 5 min was followed by a chase of 10 min with an excess of unlabeled methionine. (B) Analysis of PSII subcomplexes and proteins in sucrose gradients of *n*-dodecyl *β*-D-maltoside solubilized thylakoid membranes. The assignment was determined by SDS−PAGE, followed by staining and immunodetection on Western blots, 77 K chlorophyll fluorescence, and calibration of molecular mass by standard PSII preparations and marker proteins [for details, see van Wijk et al. (1995)]. Fraction number and molecular mass distribution are indicated. [1]Reaction center particle containing D1−D2−cyt b_{559}, psbI; [2]small PSII core particles containing D1−D2−cyt b_{559}−*psbI*−CP43−CP47; [3]large PSII core particles also contain variable amounts of OEC33 (where OEC is oxygen-evolving complex), OEC23, OEC17 + CP29, and low molecular mass polypeptides.

migrating complex in the nondenaturing Deriphat-PAGE, the PSI−LHCI complex (Figure 2A), was only located in sucrose gradient fractions 7 and 8. PSII dimers, which migrate in Deriphat-PAGE just in front of the PSI−LHCI complexes (Dreyfuss & Thornber, 1994; Bassi et al., 1995), were only present in minor amounts probably due to the relatively long solubilization time [for a discussion on monomerization, see Santini et al. (1994)].

Monomeric PSII core complexes were detected in fractions 9 and 10 in agreement with earlier assignments based upon 77 K fluorescence and other assays (van Wijk et al., 1995). A faint green band, migrating slightly faster than the monomeric PSII cores in fractions 9 and 10, was identified as CP43-depleted PSII monomeric core complex as judged by direct blotting of the Deriphat gels and after a second dimension SDS−PAGE [data not shown; compare Bassi et al. (1995) and Komenda and Masojidek (1995)]. LHCII trimers, as the most intense green band, were found in fractions 11 and 12 and to a lesser extent also in fraction 13. LHCII monomers were concentrated in sucrose gradient fractions 13 and 14, and some free pigment was present in sucrose gradient fractions 15 and 16. Distribution of the chlorophyll−protein complexes in the different sucrose gradient fractions clearly followed the molecular mass of the complexes with a general overlap between two to three adjacent fractions (compare Figures 1B and 2A)

The combination of sucrose gradient fractionation and nondenaturing Deriphat-PAGE resulted in a good resolution of the different chlorophyll−protein complexes. The autoradiograms of the dried Deriphat gels after the different chase times (5, 10, and 30 min) were employed to follow the incorporation process of the newly synthesized D1 protein into PSII (Figure 2B). After 5 min pulse and 5 min chase, the radioactivity was concentrated in sucrose gradient fractions 12−14 in PSII rc complexes and two smaller PSII assemblies (ca. 40−120 kDa) (Figure 2B). At this initial stage in the assembly process, only minor amounts of radioactivity were found in CP43-less monomeric PSII core complexes, indicated by arrowhead 3 in Figure 2B (in fractions 9-11).

After 10 min chase (Figure 2B), PSII monomers (±CP43) in fractions 9−11 were among the most heavily radiolabeled chlorophyll−protein complexes, indicating that the chase time of 10 min was long enough to allow newly synthesized D1 protein to be incorporated into PSII core complexes (arrowheads 2 and 3 in Figure 2B). High amounts of

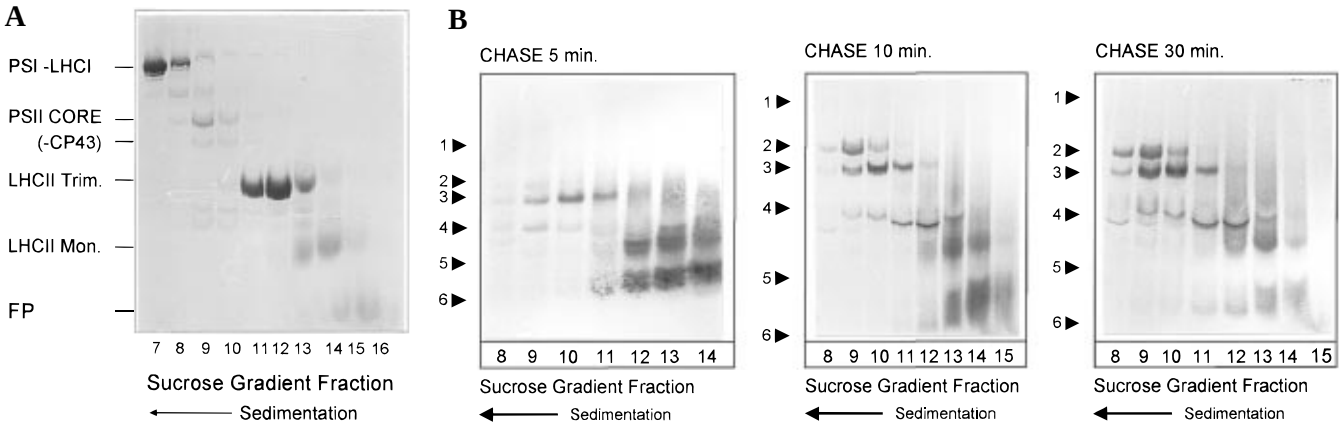

FIGURE 2: Deriphat-PAGE analysis of ^{35}S-labeled thylakoid−protein complexes isolated by sucrose gradient fractionation. Thylakoid membranes were pulse-labeled for 5 min, followed by different chase times (5, 10, and 30 min) with excess unlabeled methionine. Subsequently, nonincorporated radiolabel was removed, and thylakoids were solubilized in 1% (w/v) *n*-dodecyl *β*-D-maltoside and fractionated on sucrose gradients. Sucrose gradient fractions 7−15, containing complexes and proteins in the range of 280 kDa (fraction 7) to 30 kDa (fraction 15), were each concentrated and loaded on nondenaturing Deriphat-PAGE. The gels were dried and exposed to film. (A) Photograph of an unstained native gel; the visible bands are "green". (B) Autoradiograms after 5, 10, and 30 min chase time as indicated. The autoradiogram after 30 min chase time was obtained from the "green" gel of panel A. FP = free pigment; LHCII Mon. and LHCII Trim. are monomeric and trimeric forms of LHCII. Arrowheads indicate the position of the "green" bands shown in panel A. (1 = PSI core−LHCI, 2 = PSII core, 3 = PSII core without CP43, 4 = LHCII trimer, 5 = LHCII monomer, 6 = free pigment).

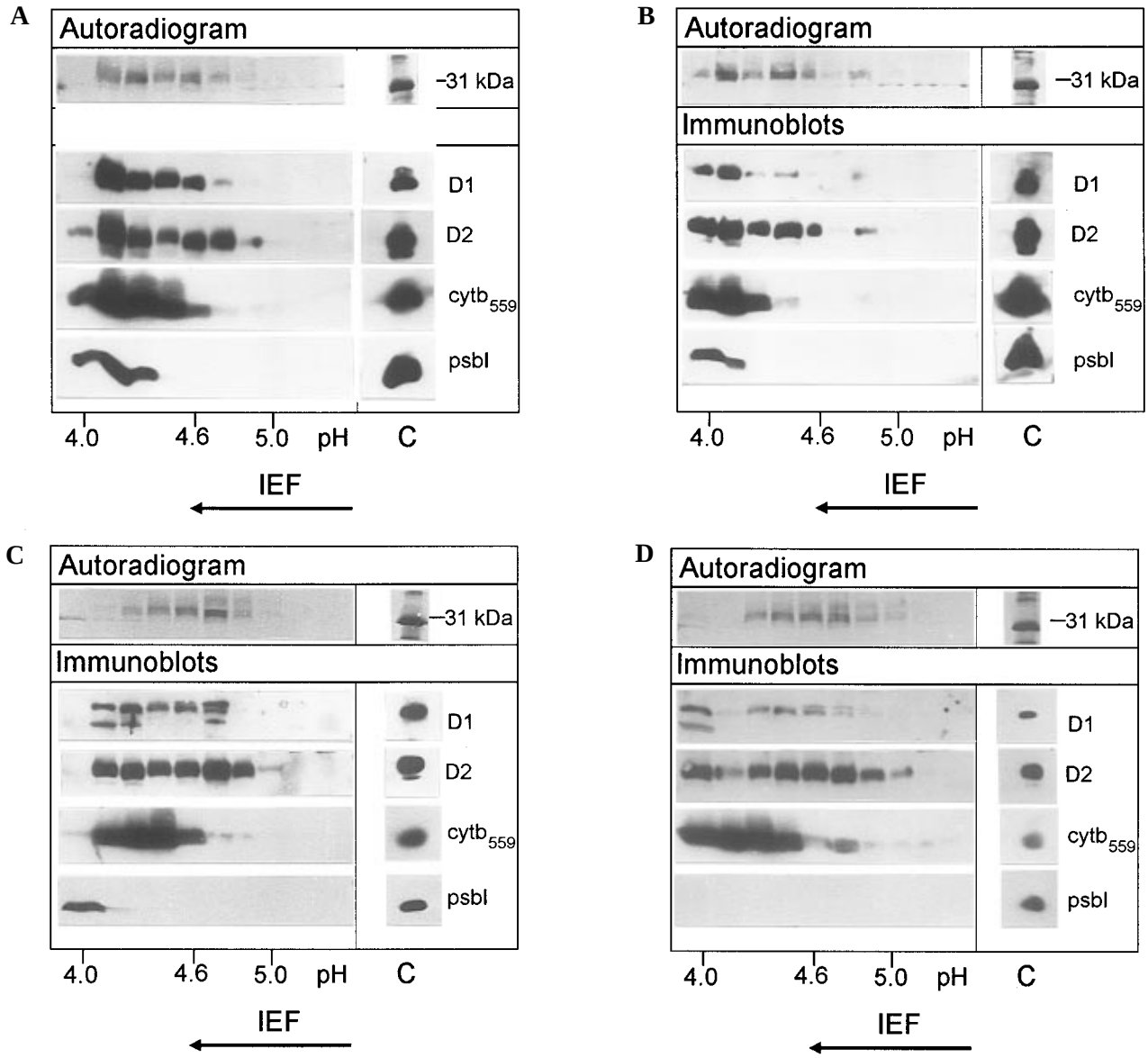

FIGURE 3: Analysis of radiolabeled PSII complexes by flatbed isoelectric focusing. Thylakoids were radiolabeled for 10 min during runoff translation followed by a 5 min chase in the presence of excess unlabeled methionine. After fractionation of radiolabeled thylakoids on sucrose gradients (see Figure 1B), sucrose gradient fractions 12, 13, 14, and 15 were each concentrated and further analyzed by flatbed isoelectric focusing with a pH gradient of 4−6.5. After focusing, each bed was divided into 13 fractions, and the proteins were eluted from the beds, concentrated, precipitated, and run on SDS−PAGE and blotted. Blots were first exposed on film to obtain the autoradiogram (top panels) and subsequently sequentially immunodecorated with four different monospecific antisera against PSII core proteins (D1, D2, *psbI* gene product, and the α subunit of cyt b_{559}). Blots and autoradiograms of the IEF analysis of sucrose gradient fractions 12 (A), 13 (B), 14 (C), and 15 (D) are shown. To normalize the immunoresponse of the IEF fractions for each antiserum, a control lane (C) loaded with 0.5 μg of thylakoids is shown on the right-hand side of each figure. The specific radioactivity (in arbitrary units) of the D1 protein present in each IEF fraction can be estimated by comparing the immunoresponse against the D1 antiserum with the intensity of the D1 band on the autoradiogram. On the right-hand side of each top panel, the position of a 31 kDa radioactive marker (carbonic anhydrase) is shown.

radioactivity were also found in sucrose gradient fractions 12−14, but clearly in much smaller (and nongreen) complexes than in fractions 9−11; two distinct radioactive bands could be distinguished migrating between LHCII trimers and monomers. A considerable amount of radioactivity was also found to migrate in front of the LHCII monomers in fraction 13, but especially in fraction 14, most likely representing unassembled radiolabeled proteins.

After 30 min chase, the distribution of radioactivity between different PSII complexes and assembly intermediates in Deriphat-PAGE had altered dramatically (Figure 2B). Most radioactivity appeared now in the PSII monomers with or without CP43 (arrowheads 2 and 3, respectively, in Figure 2B, fractions 8−11). Some radioactivity remained in rc-type complexes in fractions 11−12 and in a so-far unidentified complex in fraction 13 of about 60−90 kDa, migrating just below the PSII rc type complex. Only residual amounts of unassembled proteins remained in fractions 13−14.

These results corroborate earlier analyses revealing a stepwise assembly of PSII complexes during D1 protein turnover (van Wijk et al., 1996).

Separation of PSII Subcomplexes by Isoelectric Focusing. In order to analyze the initial assembly partners, the composition of the smallest PSII complexes detected by Deriphat-PAGE in fractions 12−15 was analyzed. To this end, the runoff translations were scaled up 4-fold, and 10 min radiolabeling (instead of 5 min) was applied to incorporate a greater amount of radioactivity. The chase time

was reduced to 5 min in order to accumulate the early PSII assembly steps. After the sucrose gradient centrifugation, fractions 12−15 containing the PSII rc complexes and smaller PSII assemblies were each subjected to a second analytical separation step by isoelectric focusing (IEF) (Figure 3). Thirteen IEF fractions focusing between pH values 3.9 and 6.5 were collected from each IEF run, and their polypeptide composition was determined by SDS−PAGE and immunoblotting (antisera against D1, D2, α-cyt b_{559}, and *psbI* gene product). The same blots were also exposed to X-ray film to localize the radiolabeled polypeptides in the IEF bed. For each gel, also a lane with a standard amount of thylakoids (0.5 μg of chlorophyll) was loaded, to serve as a basis for normalization of the different PSII polypeptides. Using the immunoresponse of these control lanes, the stoichiometry of the different PSII polypeptides in each IEF fraction could be estimated.

Figure 3A−D shows four panels with the analysis of the four sucrose gradient fractions (12−15) by IEF. In the upper part of each panel, the autoradiogram of the 30 kDa region (where the D1 and D2 proteins migrate) is shown, while below the autoradiogram immunoblots against the four rc proteins (D1, D2, α-cyt b_{559}, and *psbI* gene product) are depicted. In sucrose gradient fraction 12 (110 ± 30 kDa), at least 50% of the labeled D1 protein was assembled with the D2 protein, cyt b_{559}, and the *psbI* gene product to form PSII rc complexes, focusing at pH 4.1−4.3 (Figure 3A). All reaction center proteins were present in approximately similar stoichiometry as in the thylakoid membranes, shown in the control lane (C) on the right-hand side of the figure. In the two adjacent fractions, at higher pH values (4.4−4.5) the *psbI* gene product was present in substoichiometric amounts, indicating the presence of PSII rc subcomplexes without the *psbI* gene product. These subcomplexes were even more abundant in fraction 13 (see below, Figure 3B). Some newly synthesized D1 protein also focused at pH 4.7, where the D1 protein was present together with the D2 protein and with substoichiometric amounts of cyt b_{559} and in the absence of the *psbI* gene product, likely representing a D1−D2 heterodimer.

^{35}S-Labeled D1 protein in sucrose gradient fraction 13, containing even smaller complexes (80 ± 30 kDa) (Figure 3B), was predominantly divided into two populations with a p*I* of 4.1 (approximately 30% of the radiolabel) and 4.5−4.6 (approximately 70% of the radiolabel). Radiolabeled D1 protein at the higher pH value (4.5−4.6) had clearly the highest specific activity (amount of radiolabeled D1 protein normalized to amount of immunodetected D1 protein). No *psbI* gene product was present at this pH, and only residual amounts of the α subunit of cyt b_{559} could be detected. Based on the molecular mass determination (80 ± 30 kDa), it is most likely that the radiolabeled D1 protein with this higher p*I* value is forming a dimeric complex with the D2 protein. The radiolabeled D1 protein focusing at pH 4.1 (about 30% of the radiolabeled D1 protein of fraction 13) was present in a PSII rc complex without the *psbI* gene product. Thus, two D1 protein containing PSII subcomplexes had sedimented in sucrose gradient fraction 13, in agreement with the Deriphat-PAGE analysis (compare Figure 2C and Figure 2D). We conclude that both the D1−D2 heterodimer (at higher p*I* values) and the PSII rc complex without the *psbI* gene product (at lower p*I* values) were two early assembly stages.

Notably, in fraction 14 (60 ± 30 kDa), the newly synthesized, radiolabeled, D1 protein was equally present in both its processed and its unprocessed forms. Both forms were indistinguishable with respect to their p*I* value. Radiolabeled D1 protein focused predominantly at a pH value of 4.7. Immunobloting revealed that the newly synthesized D1 protein was not associated with cyt b_{559} nor with the *psbI* gene product, since they were both absent at this pH value (Figure 3C). The *psbI* gene product, although present in this sucrose gradient fraction, focused at the most acidic pH value, 3.9, completely separated from the other PSII rc proteins. Thus, PSII rc complexes (D1/D2/cyt b_{559}/*psbI*) were completely absent from sucrose gradient fraction 14. Radiolabeled D1 protein with a p*I* of 4.7 was accompanied by a relatively high amount of D2 protein. In the Deriphat-PAGE, we observed both a radiolabeled D1−D2-type complex migrating slower than monomeric LHCII as well as radiolabeled protein migrating faster than LHCII. Thus, it is most likely that both unassembled D1 and D2 proteins as well as a D1−D2 heterodimer were present.

In addition to the precursor and mature D1 protein, also a 27 kDa N-terminal fragment of the D1 protein was observed in sucrose gradient fraction 14 after IEF (Figure 3C). Since this protein fragment was not radiolabeled (note that the 27 kDa N-terminal fragment has about nine methionine residues), it does not represent a translation intermediate but rather a degradation product of the D1 protein. Interestingly, this degradation product was not present in sucrose gradient fractions 12 and 13 containing intact rc complexes. Therefore, the production of this specific D1 degradation fragment seems to be highly dependent on the size of the PSII subcomplex [see also van Wijk et al. (1995) for effect of protease inhibitors].

In the IEF analysis of sucrose gradient fraction 15, containing mainly proteins with a molecular mass of around 30 kDa, the newly synthesized D1 protein (both precursor and mature forms) focused at pH values 4.3−5.0. The highest specific activity was found at pH 4.6−4.8, as in fraction 14. Also small amounts of radiolabeled D2 protein were detected (weak bands migrating slower than for the D1 protein) at pH values 4.6−5.0, and peaking at pH 4.7. Estimated from the sedimentation in the sucrose gradient and in agreement with the Deriphat-PAGE (chase 10 min, Figure 2B), we conclude that unassembled D1 protein sedimented in this sucrose gradient fraction.

Relation between Processing and Assembly of the D1 Protein. The conserved C-terminus of the D1 protein is processed by a lumenal-specific protease (e.g., Inagaki et al., 1996). However, the function of this C-terminal extension is unclear (see the Introduction). Recently, we have shown that in isolated thylakoids, the unassembled pD1 protein forms a substrate for the lumenal processing peptidase (van Wijk, 1996). In addition, we observed that pD1 and D1 were equally distributed over the PSII reaction center and PSII core complex after short chase times. It is possible that in isolated thylakoids the peptidase operates under suboptimal conditions, leading to a relative slow processing activity. In the present paper, we aimed at studying the relation between processing and assembly further in isolated chloroplasts. To allow quantification of radiolabeled D1 and pD1, it is important to reduce the labeling of the D2 protein, since it migrates very closely to pD1 in SDS−PAGE. We had observed that with increasing leaf age, the D1 protein

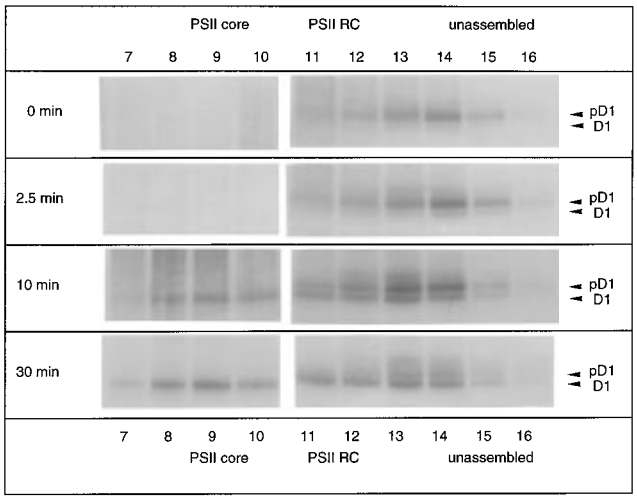

FIGURE 4: Kinetics of synthesis, processing, and assembly of the precursor D1 protein in isolated chloroplasts. Intact chloroplasts were pulse-labeled for 4 min, and the label was subsequently chased with an excess of cold methionine for 0−30 min. Subsequently, the thylakoid membranes were released from the chloroplasts, washed and solubilized in *n*-dodecyl β-D-maltoside, and fractionated by sucrose density centrifugation, as described under Figure 1.

becomes increasingly the predominant radiolabeled protein (this is a reflection of the decreased synthesis of completely new PSII complexes; synthesis in these older leaves is mainly occurring to compensate for damage of subunits). Thus, we isolated chloroplasts from older leaf tissue to study the relation between processing and assembly. Chloroplasts were pulse-labeled for 4 min and subsequently chased for up to 30 min (Figure 4). After each chase time (0, 2.5, 10, and 30 min), thylakoids were isolated and subjected to analysis by a same type of sucrose gradient fractionation as after the runoff translation experiments described above. Directly after the pulse-labeling (Figure 4), prior to the chase, more than 90% of the D1 protein was still unprocessed and was found to be unassembled (fractions 14−16) or in small PSII subcomplexes (fractions 12−13). Already after 2.5 min of chase, the total amount of D1 protein had tripled, but most of the D1 protein still remained unprocessed and unassembled or located in the small PSII reaction center assemblies. At this early chase time, no labeled D1 protein was incorporated into PSII core complexes.

After 10 min chase, when elongation was practically completed, more than half of the processing had occurred. As in case of the isolated thylakoids, the processed and unprocessed D1 protein were equally distributed over fractions 11−16 (Figure 4). This demonstrates that processing could occur on the unassembled protein but also that the unprocessed form could be efficiently assembled into PSII reaction centers. However, in contrast to the situation in isolated thylakoids, all D1 protein present in the PSII core complex (fractions 8−10) had already been processed. In fact, unprocessed D1 protein could never be detected in PSII core complexes isolated from radiolabeled intact chloroplasts. Finally, after 30 min chase time, more than 90% of the total D1 protein was processed, and about 50% was assembled into PSII core complexes. A small fraction of the D1 protein remained unprocessed and present in small (less than 100 kDa) subcomplexes or as unassembled protein in fractions 13−16. Also in the IEF analysis, pD1 protein (visible on the autorads and on the immunoblots) clearly accumulated at higher p*I* values in gradient fractions 14 and 15.

DISCUSSION

Synthesis of the D1 protein in mature, fully developed leaves occurs mainly to compensate for the light-induced damage and degradation of the D1 protein in the PSII complex. We have demonstrated that the synthesis and replacement process of the D1 protein can take place in isolated chloroplasts and, with lower efficiency, during runoff translations in isolated thylakoids (van Wijk et al., 1995, 1996). Three predominant assembly steps—unassembled D1 protein, PSII rc complexes, and PSII core complexes—were characterized by fractionation of pulse-labeled chloroplasts and thylakoids on analytical sucrose gradients. In this study, we present a detailed analysis of the early stages of the assembly process in order to identify the initial assembly partner(s) of the newly synthesized D1 protein and to further address the role of the C-terminal extension of the D1 protein.

It is important to emphasize that the chloroplasts and thylakoids were isolated during the light period of the diurnal light/dark cycle. Since the D1 protein is turning over constantly in the light with a half-life of about 8 h under our growth conditions, the thylakoids and chloroplasts always contained a small pool (estimated to be 1−3% of total PSII) of damaged PSII complexes, which required the synthesis and insertion of D1 protein. The translations were carried out under low intensity room light, since such light intensities have been shown to be optimal for repair *in vivo* (e.g., Greer et al., 1986). No photoinhibitory treatments were needed to create a small pool of damaged PSII complexes.

The improved resolution of the assembly intermediates on Deriphat-PAGE in combination with sucrose gradient fractionation resulted in several novel observations. Several small PSII assembly intermediates were detected after 5 min chase and disappeared after longer chase times. Thus, these smallest complexes formed transient assembly stages prior to formation of PSII rc and were not just a consequence of the solubilization procedure. The Deriphat-PAGE analysis also clearly demonstrated that after formation of the PSII rc, CP47 became attached and only subsequently CP43 associated with the CP47−PSII rc.

IEF analysis of the smaller complexes sedimenting in sucrose gradient fractions 12−15 excluded both the *psbI* gene product and cyt b_{559} as the initial assembly partner of the newly synthesized D1 protein. IEF analysis of fraction 13 appeared particularly crucial in the understanding of the initial assembly steps: About 50% of the radiolabeled D1 protein in fraction 13 focused at pH 4.5. The D2 protein was the only PSII rc protein present at this pH value which, in combination with the estimated mass of approximately 80 kDa of the complexes in this fraction, strongly suggests that a D1−D2 heterodimer was formed as the first step in the PSII repair process. The other subpopulation of the radiolabeled D1 protein in this sucrose fraction focused at pH 4.2. This complex contained also cyt b_{559} and only minor amounts of *psbI* gene product, implying the formation of a D1/D2/cyt b_{559} complex. The D1/D2/cyt b_{559} complexes were also present in sucrose gradient fraction 12, confirming the relevance of this complex as a transient assembly step in the repair process.

The *psbI* gene product was clearly the last assembly partner to be incorporated into the PSII rc complex. This observation is consistent with *psbI* gene inactivation mutants in *Chlamydomonas reinhardtii* and in *Synechocystis* sp. PCC6803 [see Künstner et al. (1995) and Ikeuchi et al. (1995) respectively]. These studies revealed that the *psbI* gene product is not an absolute requirement for the assembly of functional PSII, although the stability of the PSII complex was reduced. Thus, it seems that after termination of translation, D1 associates with the D2 protein and subsequently with cyt b_{559} and the *psbI* gene product.

The D1−D2 stoichiometry in fractions 14−15 was much lower than the stoichiometry in the thylakoid membranes. This indicates that a surplus of unassembled but stable D2 protein was present in the thylakoid membranes. Such a surplus of the D2 protein is in line with the IEF analysis of fraction 13 and implies that the D2 protein functions as a "receptor" for newly synthesized D1 protein. Such function requires that the D2 protein is more stable than the D1 protein and that D2 is preferentially under less stringent translational control than the D1 protein. The observation in etioplasts that the D2 protein can accumulate in the absence of chlorophyll, in contrast to the D1 protein, CP47, CP43, and other chlorophyll binding proteins (Klein et al., 1988a), provides further support for such a "receptor role" of the D2 protein. Also in fully developed chloroplasts, the translational control of the D2 protein seems to be more "relaxed"; it was shown that both synthesis and accumulation of the D2 protein, but not D1 or CP43, occur relatively independent of light, provided that ATP and a reducing agent are added (van Wijk & Eichacker, 1996). Finally, experiments with various *psb* gene inactivation mutants have assigned a key role for the D2 protein in accumulation of the D1 protein into the thylakoid membrane (Erickson et al., 1986; Jensen et al., 1986; de Vitry et al., 1989; Yu & Vermaas 1990). It is conceivable that the D2 protein already starts interacting and stabilizing the D1 protein during cotranslational insertion of the D1 protein via thylakoid membrane-bound ribosomes.

The stable accumulation of an unassembled thylakoid membrane protein with a possible role in translational regulation is not unprecedented: in *C.* reinhardtii, stable accumulation of a significant population of unassembled cyt *f* (about 10% of total) has been proposed to play a regulatory role in the synthesis of new cyt *f* copies (Kuras et al., 1995).

Since the discovery that the lifetime of the D1 protein is so much shorter than that of the other PSII proteins, the question has been raised to what extent the PSII core complex needs to be disassembled during PSII repair in order to allow removal of the damaged D1 copy and to allow insertion of a new D1 protein [see Hundal et al. (1990), Adir et al. (1990, Barbato et al. (1992), and Aro et al. (1993)]. From the analysis presented in this study, it has become clear that a complete disassembly of the PSII core during repair of photodamaged PSII complexes can occur. Thus, to facilitate the replacement of damaged D1 protein, the PSII dimers dissociate into PSII monomers (Barbato et al., 1992), the peripheral antennae is released, and an extensive disassembly of the PSII core complex may follow.

However, the rapid radiolabeling of a small population of PSII cores without CP43 indicates that the D1 protein can possibly also be inserted into a complex consisting of D2/cyt b_{559}/*psbI* gene product/CP47, in agreement with earlier

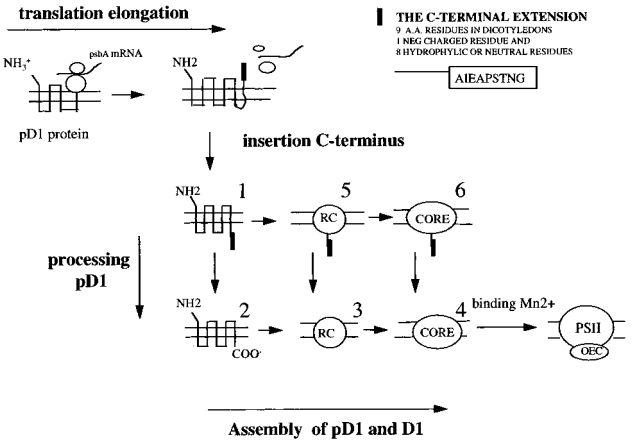

FIGURE 5: Schematic representation of the potential pathways of processing and assembly of the precursor D1 protein (RC = PSII reaction center assembly, core = PSII core complex, OEC = oxygen-evolving complex).

suggestions by Ohad and co-workers (Adir et al., 1990). It has been speculated (Ikeuchi et al., 1992) that the D1 protein is located at the periphery of the monomeric PSII core complex, whereas cross-linking studies of the *psbI* gene product revealed only cross-linking with the D2 protein and the α subunit of cyt b_{559} (Tomo et al., 1993). Therefore, several levels of disassembly could be envisioned in parallel to the extensive disassembly observed in this study. Different possibilities for the sequence of processing and assembly of the pD1 protein are summarized in Figure 5. After termination of translation on thylakoid-bound ribosomes, the apolar C-terminus is somehow maneuvered through the lipid bilayer. Since the processing peptidase is located exclusively on the lumenal side of the membrane (Fujita et al., 1995), the C-terminal extension has to arrive on the lumenal side before it can be processed. Based upon our earlier observations in isolated thylakoids (van Wijk et al., 1996), different pathways for assembly and processing are possible (1−2−3−4; 1−5−3−4; 1−5−6−4) (Figure 5). However, processing is a prerequisite for the final assembly and activation of the Mn cluster (e.g., Nixon et al., 1992).

For isolated chloroplasts as well as for thylakoid membranes, it can be concluded that mechanistically both the precursor and mature forms of the D1 protein can be incorporated into PSII reaction center assemblies. The proportion between PSII reaction center assemblies containing the precursor or the mature D1 protein was about the same after termination of elongation. Thus, there is no kinetic preference of pathway 1−2−3 over 1−5−3 (Figure 5), demonstrating that the C-terminal extension does not play an obvious mechanistic role in the initial assembly of the PSII reaction center. In the case of intact chloroplasts, no unprocessed D1 protein was found in the final PSII core complexes. Thus, pathway 1−5−6−4 is not likely *in vivo* (Figure 5). A possible explanation is that once the D1 protein is assembled into the PSII reaction center (fractions 11 and 12), processing becomes more rapid since the D1 protein is kept in an optimal configuration for the peptidase. This suggestion is supported by the observation that residual amounts of unprocessed D1 protein remained unassembled after prolonged chase times. Alternatively, processing and assembly are unrelated events, and only the difference in the kinetics of the two processes determines the presence of pD1 in the different complexes.

We conclude that under optimal conditions there is no obvious mechanistic relationship between processing of the D1 protein and its assembly into PSII core complexes. It remains, however, possible that under unfavorable conditions, such as low temperatures, the C-terminal extension may facilitate the translation of the charged, hydrophilic stretch preceding the extension, through the thylakoid bilayer. Alternatively, regulation of the activity of the processing peptidase could provide an additional option of posttranslational control of accumulation of active, water-splitting PSII centers. Investigation of the regulation of the processing peptidase under different environmental conditions should reveal such a role.

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