Kinetic Resolution of the Incorporation of the D1 Protein into Photosystem II and Localization of Assembly Intermediates in Thylakoid Membranes of Spinach Chloroplasts*

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The chloroplast-encoded D1 protein of photosystem II (PSII) has a much higher turnover rate than the other subunits of the PSII complex as a consequence of photodamage and subsequent repair of its reaction center. The replacement of the D1 protein in existing PSII complexes was followed in two in vitro translation systems consisting of isolated chloroplasts or isolated thylakoid membranes with attached ribosomes. By application of pulse-chase translation experiments, we followed translation elongation, release of proteins from the ribosomes, and subsequent incorporation of newly synthesized products into PSII (sub)complexes. The time course of incorporation of newly synthesized proteins into the different PSII (sub)complexes was analyzed by sucrose density gradient centrifugation.

Immediately after termination of translation, the D1 protein was found both unassembled in the membrane as well as already incorporated into PSII reaction center complexes, possibly due to a cotranslational association of the D1 protein with other PSII reaction center components. Later steps in the reassembly of PSII were clearly post-translational and sequential. Different rate-limiting steps in the assembly process were found to be related to the depletion of nuclear encoded and stromal components as well as the lateral migration of subcomplexes within the heterogeneous thylakoid membrane.

The slow processing of precursor D1 in the thylakoid translation system revealed that processing was not required for the assembly of the D1 protein into a PSII (sub)complex and that processing of the un assembled precursor could take place. The limited incorporation into PSII subcomplexes of three other PSII core proteins (D2 protein, CP43, and CP47) was clearly post-translational in both translation systems.

Radiolabeled assembly intermediates smaller than the PSII core complex were found to be located in the stroma-exposed thylakoid membranes, the site of protein synthesis. Larger PSII assembly intermediates were almost exclusively located in the appressed regions of the membranes.

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The chloroplast-encoded D1 protein of photosystem II (PSII) is a large multisubunit protein complex in thylakoid membranes of oxygenic photosynthetic organisms containing at least 25 different subunits (see Refs. 1 and 2 for reviews). In eukaryotic organisms, nearly all proteins in the core of the complex are encoded by the chloroplast genome, while the more peripheral proteins are nuclear encoded, synthesized in the cytoplasm, imported into the chloroplasts, and subsequently targeted to the thylakoid membrane (see Refs. 2-6). It is assumed that most chloroplast-encoded membrane proteins are cotranslationally inserted into the thylakoid membrane since the ribosomes are bound to its stroma-exposed regions (7, 8). Synthesis of the chloroplast-encoded membrane proteins therefore takes place on these unstacked, stroma-exposed thylakoid regions (see Ref. 7), while the vast majority of the functional PSII complexes are located in the grana, the stacked thylakoid regions (see Refs. 9 and 10).

One of the chloroplast-encoded proteins is the multiple membrane-spanning D1 protein, which forms, together with the homologous D2 protein, the PSII reaction center (e.g. see Refs. 3 and 11). This heterodimer binds or contains all the essential redox components (P680, pheophytin, non-heme iron, quinones, the redox-active tyrosines, and accessory chlorophylls and carotenoids) that are needed to carry out the light-driven reduction of plastoquinone. Isolated PSII reaction center particles (11) consist of the D1-D2 heterodimer, two cytochrome b559 subunits, a low molecular mass protein (4.8 kDa; psbA gene product), and possibly a small nuclear encoded protein (6.1 kDa; psbW gene product) (12). The PSII core complexes are larger and are composed of the PSII reaction center surrounded by the chlorophyll a-binding proteins CP43 and CP47, an extrinsic 33-kDa protein, and a number of smaller proteins (<10 kDa) of unknown function. These particles contain manganese and can perform light-induced water oxidation (10).

A unique feature of PSII is that the turnover of the D1 protein is much higher than that of the other PSII proteins and is a consequence of photodamage of the PSII reaction center (13-16). To maintain the PSII complex in a functional state and to thereby avoid photoinhibition of photosynthesis, the damaged D1 protein must be replaced and is therefore synthesized at a much higher rate than all the other PSII proteins (e.g. Refs. 17-19). Degradation of some other PSII core proteins (especially the D2 protein) due to photodamage of the PSII complex has also been shown to occur, but the extent of this degradation is much lower than that of the D1 protein (13-15).

This unique replacement of the D1 protein allows the study of the mechanism of cotranslational insertion of a membrane protein and its assembly into a pre-existing multisubunit and

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† The abbreviations used are: PSII, photosystem II; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; PAGE, polyacrylamide gel electrophoresis.
membrane-bound complex. At the same time, the ligation of several cofactors during translation and assembly can be addressed. It is expected that several features of this complicated turnover process have a general significance for the assembly of other membrane-bound protein complexes.

The D1 protein, encoded by the psbA gene, is assumed to be cotranslationally inserted into the thylakoid membrane (see Ref. 7). Recently, it was shown that in the presence of ATP, this insertion does not require a proton-motive force or SecA (20).

Structural information on the organization of the PSII core complex is also important in understanding the possible ways in which the damaged D1 protein can be replaced. Several proposals have been made (e.g., Refs. 21 and 22) and are based on cross-linking studies (e.g., Refs. 23, 24, and 47), analysis of PSII deletion mutants (e.g., Refs. 2, 25, and 26), detergent treatments (11, 27), and in recent years, crystallization studies (28, 29). Most studies propose an organization of PSII in which the D1 protein is situated on the periphery of the core complex, with CP43 and CP47 primarily interacting with the D2 protein. A dimeric structure of PSII has been suggested in several studies (22, 28), and in this case, the dimer must dissociate into two monomers to allow replacement of the D1 protein. Such monomerization under strong light conditions has been observed in vitro (30). The monomers were found in the unstacked, stroma-exposed membranes, while the dimeric form was dominant in the stacked granal membranes (30).

The mechanism of replacement of the D1 protein in PSII has previously been studied in vivo in green algae (19, 31–33), Spirodela leaves (34), and recently in vitro using isolated spinach chloroplasts and thylakoids (35). It was proposed that the D1 protein associates in the stromal membranes with a PSII particle consisting of D2-cytochrome b559, CP47 (31) or D2-cytochrome b559 (34). However, it has been experimentally difficult to resolve assembly intermediates using in vivo systems. Therefore, no direct proof for these hypotheses has been given so far (for discussion, see Ref. 19).

In a recent study (35), we developed an experimental system based on analytical sucrose gradient centrifugation to follow the replacement of the D1 protein in isolated chloroplasts and to identify and to trap defined intermediate assembly stages. We have shown that in this in vitro system, the D1 protein could be assembled into PSII reaction center particles and core complexes. In contrast to the D1 protein, the other newly synthesized PSII core proteins (D2 protein, CP43, and CP47) accumulated in the thylakoid membranes predominantly as free unassembled proteins and in smaller (sub)complexes with a molecular mass of <100 kDa. It was also shown that the D1 protein could be synthesized and incorporated into existing PSII core complexes with a lower efficiency in a homologous run-off translation system (35) consisting of isolated thylakoids with bound ribosomes (see Refs. 7 and 36).

In this paper, we present a kinetic resolution of the assembly process of the D1 protein into existing PSII core complexes through application of pulse-chase experiments using our recently developed experimental system (35). The complete pathway of D1 protein incorporation is followed from translation elongation and termination and subsequent release of the newly synthesized product from the ribosomes to sequential incorporation into PSII subcomplexes. Moreover, the localization of the different PSII assembly intermediates and unassembled PSII core proteins in the heterogeneous thylakoid membrane is shown. A scheme summarizing the pathway of incorporation of newly synthesized PSII core proteins and its kinetics is presented.

EXPERIMENTAL PROCEDURES

Plant Material and Isolation of Intact Chloroplasts—Spinach was grown hydroponically at 23 °C in a light/dark cycle of 12 h/12 h. For all experiments, mature leaves were harvested in the first half of the light period. Isolation of intact chloroplasts was performed as described (35).

In Vitro Translation in Isolated Chloroplasts—In vitro translation in isolated chloroplasts was carried out in the light at 23 °C essentially as described (37). The chloroplasts (0.4–0.5 μg of chlorophyll μl−1) were incubated for 10 min at 23 °C with the light (~50 μmol photons m−2 s−1) in a translation mixture consisting of 330 mM sorbitol, 50 mM Hepes/KOH (pH 8.0), 10 mM dithiothreitol, 10 mM MgATP, and a 40 μM concentration of each amino acid except methionine. After this preincubation time, carrier-free 35S-labeled methionine was added to a final concentration of 0.4–0.5 μCi/ml−1. The labeled methionine was chased by the addition of 10 mM unlabeled methionine to the translation mixture. Translation was stopped by dilution with a 10-fold volume of ice-cold medium containing 330 mM sorbitol and 50 mM Hepes/KOH (pH 8.0). To separate the thylakoid-bound translation products from the stromal products, the chloroplasts were lysed in RNase-free lysis buffer containing 46 μM Hepes/KOH (pH 7.6), 118 mM potassium acetate, 7 mM magnesium acetate, 5 mM dithiothreitol, and 10 μg/ml heparin, and the thylakoid membranes were collected by a 3-min centrifugation at 5000 × g. Finally, the thylakoids were washed twice in the same buffer.

In Vitro Run-off Translation in Isolated Thylakoid Membranes—In vitro translation with isolated thylakoids was carried out essentially according to Refs. 35 and 36. Intact chloroplasts were lysed in RNase-free lysis buffer. The membranes were collected by centrifugation (3 min, 10,000 × g), washed twice with ice-cold buffer, and resuspended in the same buffer (10 mg of chlorophyll ml−1). In vitro translation was carried out in room light at 23 °C in lysis buffer supplemented with 2 mM ATP, 0.2 mM GTP, carrier-free [35S]methionine (0.4–0.5 μCi/ml−1), a 100 μM concentration of each amino acid except methionine, and thylakoids at a concentration corresponding to 0.4–0.5 μg of chlorophyll μl−1. The labeled methionine was chased by the addition of 10 mM unlabeled methionine to the translation mixture. Translation was stopped by the addition of a 10-fold volume of ice-cold lysis buffer. After translation, the thylakoids were washed twice with a large volume of the same buffer to remove unincorporated methionine.

Separation of (Sub)complexes by Sucrose Gradient Centrifugation—To separate the native PSII (sub)complexes and proteins after the in vitro translation experiments, we recently developed a fractionation system based upon sucrose density centrifugation of thylakoids solubilized with n-dodecyl β-D-maltoside (35). The distribution and identification of the proteins and protein complexes in the sucrose gradient were determined by several biochemical and biophysical methods and are described in Ref. 35. Thylakoids were washed twice in 10 mM Tricine (pH 7.8) and subsequently solubilized for 50 min on ice with n-dodecyl β-D-maltoside (3%, w/v) and 0.5 M sodium cholate (3% w/v) in the presence of 0.1–0.2 mM concentration of the protease inhibitor phenylmethylsulfonyl fluoride. The suspension was then centrifuged (20 h, 3 °C, 180,000 × g) on sucrose gradients (0.1–1 mM sucrose in 10 mM Tricine (pH 7.8) and 0.03% n-dodecyl β-D-maltoside), and 20 fractions of equal volume were collected from bottom to top. In the case of gradients to be analyzed by SDS-PAGE, proteins were precipitated in 10% ice-cold trichloroacetic acid (~30 min on ice), collected by centrifugation, and finally resuspended in SDS solubilization buffer (35).

Subfractionation of Thylakoid Membranes—Subfractionation of the thylakoid membranes was principally carried out as described (38). Thylakoids were solubilized for 30 min on ice in fractionation buffer containing 10 mM Tricine (pH 7.8), 10 mM NaCl, 10 mM MgCl2, 0.1 mM sodium cholate, and 0.5% (w/v) dodecyl β-D-maltoside at a chlorophyll concentration of ~0.4 mg/ml in the presence of 0.1–0.2 mM phenylmethylsulfonyl fluoride. Solubilization was stopped by a 30-fold dilution with ice-cold fractionation buffer. Stromal and crude granal membranes were separated by centrifugation for 30 min at 40,000 × g at 2 °C. The stromal membranes in the supernatant were collected by an additional centrifugation for 90 min at 100,000 × g at 2 °C.

Purity of the different subthylakoid fractions was tested by immunodetection with antibodies against the D1 protein and PSI reaction center proteins and by comparing the relative amount of LHCII and CF1 in both granal and stromal membranes by Coomassie Brilliant Blue-stained SDS-PAGE (data not shown). The chlorophyll a/b ratio was >6.5 for the stromal membranes.

Protein and Pigment Analysis—For SDS-PAGE, thylakoids or Triton-X100-solubilized samples were run on a 10% polyacrylamide gel and visualized with Coomassie Brilliant Blue stain. For SDS-PAGE, thylakoids or Triton-X100-solubilized samples were run on a 10% polyacrylamide gel and visualized with Coomassie Brilliant Blue stain.
**Mechanism of D1 Protein Incorporation**

**Fig. 1.** Membrane-bound radiolabeled proteins after translation in isolated chloroplasts and isolated thylakoids. Labeling with [35S]methionine was carried out for 4 min, followed by a 10-min chase with an excess of unlabeled methionine. After translation, the chloroplasts (C) were lysed, and thylakoids (T) were washed repeatedly to remove unincorpporated radioactivity. Pausing intermediates are indicated with i. Molecular mass standards are indicated in kilodaltons.

Prior to loading on the gels, SDS-PAGE was performed essentially according to Ref. 39, using 14% linear gels containing 6 M urea, which were run at 12 °C. Western blotting was performed essentially according to Ref. 40, using ECL (horseradish peroxidase-conjugated goat anti-rabbit IgG, Bio-Rad) for detection. In the case of 35S-labeled samples, gels were stained with Coomassie Brilliant Blue or silver nitrate, followed by incubation for 15–20 min in Amplify (Amersham Corp.) and drying. Quantification of proteins was performed by scanning the autoradiograms with a laser densitometer using the software package Image Quant (Molecular Dynamics, Inc.). Chlorophyll concentrations were determined spectroscopically in 80% acetone using the extinction coefficients described in Ref. 41.

**RESULTS**

**Translation Products in Isolated Intact Chloroplasts and Thylakoid Membranes—**Fig. 1 shows the synthesis products after translation in isolated intact chloroplasts and isolated thylakoids membranes. As previously shown (35), the translation products in the two translation systems were quite similar. Several chloroplast-encoded PSII proteins (pD1, D1, D2, CP43, and CP47) can be distinguished, but the D1 protein (observed both in the precursor (pD1) (see Refs. 42 and 43) and mature forms) was the most abundant PSII protein synthesized. In a recent study (35), we have shown that the elongation rates in the two translation systems were approximately equal (at least 40 amino acid residues/min) and that D1 translation intermediates were similar (see also Ref. 44).

**Kinetics of Protein Synthesis and Assembly in Isolated Chloroplasts—**In our previous study (35), we developed an analytical sucrose gradient fractionation system to study the assembly of the in vitro translated proteins into the PSII complex. The various subunits and (sub)complexes in the sucrose gradient fractions were identified by 77 K fluorescence emission spectra, pigment analysis, and immunodetection on Western blots (35). Further identification of the different complexes was achieved by subjecting defined PSII preparations (e.g. PSII cores and PSII reaction center particles) and molecular mass marker proteins to centrifugation (see Ref. 35). The assignment of sucrose gradient fractions with respect to these PSII (sub)complexes and proteins is summarized in Fig. 2. The fractions containing ribosomes were identified by immunodetection. We would like to emphasize that no unsolubilized material was discarded, allowing a total quantification of the assembly process.

Using this recently developed experimental system, we carried out a pulse-chase experiment in isolated chloroplasts in order to follow the elongation and termination of translation, the release of the nascent chains from the ribosomes, and the subsequent incorporation steps of the newly synthesized proteins into PSII (sub)complexes. The pulse time was kept as short as experimentally possible (5 min). After this pulse, followed by different chase times, the thylakoids were solubilized with n-dodecyl β-D-maltoside, and different PSII (sub)complexes were separated by sucrose density gradient centrifugation as described (35). To identify specific newly synthesized proteins and their time course of incorporation into complexes, autoradiograms of SDS-PAGE-separated proteins were prepared from the sucrose gradients of each pulse-chase sample. The autoradiograms of some of the gradients (2.5-, 10-, and 30-min chase times) are shown in Fig. 3A. The sucrose gradient fractions are numbered from the bottom (fraction 1) to the top (fraction 20) of the gradient.

**Synthesis and Assembly of Radiolabeled D1 Protein into PSII (Sub)complexes in Intact Chloroplasts—**To demonstrate the time course of assembly of the D1 protein into PSII (sub)complexes, the autoradiograms were scanned and quantified (Fig. 3B). Before starting the chase, the newly synthesized precursor and mature D1 proteins were predominantly located in fraction 15, as unassembled protein, and in fractions 12 and 13, incorporated into small complexes with molecular masses of 135-170 kDa, representing PSII reaction center particles (Fig. 3B). At this early stage, no radiolabeled D1 protein could be detected in fractions 6-10 (Fig. 3B), which contained the bulk of D1 protein located in PSII core complexes (Fig. 2).

After a 2.5-min chase, the total amount of radiolabeled (p)D1 protein had tripled, accumulating as unassembled protein (fraction 15) and in reaction center complexes (fraction 12) (Figs. 3, A and B). As can be seen in the autoradiogram (Fig. 3A), a small amount of labeled D1 protein could now also be detected in fraction 10, representing smaller PSII core complexes. In the five bottom fractions, many bands could be observed, representing nascent chains still attached to ribosomal (sub)complexes (Fig. 3A). The majority of these bands are nascent chains of the D1 protein as judged by immunoprecipitation.

**Fig. 2.** Assignment of PSII subcomplexes and proteins in sucrose density gradients of n-dodecyl β-D-maltoside-solubilized thylakoid membranes. Prior to loading on the sucrose gradients (0.1-1.0 M sucrose), the thylakoid membranes (120 μg of chlorophyll) were solubilized (at 0.5 mg of chlorophyll-mL-1) in 1% n-dodecyl β-D-maltoside for 50 min on ice. Sucrose gradients were centrifuged for 20 h at 180,000 × g and fractionated from bottom to top into 20 equal fractions. The assignment was determined by SDS-PAGE, followed by staining and immunodetection on Western blots, 77 K chlorophyll fluorescence, calibration of molecular mass by standard PSII preparations, and molecular weight mass proteins as described (35). Reaction center particles contain D1-D2-cytochrome b6-f-psb; small PSII core particles contain D1-D2-cytochrome b559-psb; CP43-CP47; and large PSII core particles are small core particles with variable amounts of OEC33 (where OEC is oxygen-evolving complex), OEC23, and OEC17 + CP29 and low molecular mass polypeptides.

**Table 1.** Fractionation of nascent D1 protein in isolated thylakoids membranes. The nascent protein was released from the ribosomes, and the assembly process was initiated by exposing the thylakoids to an excess of unlabeled methionine. After translation, the nascent chains were labeled for 2.5 min and chased for 10 min. The nascent protein was released from the ribosomes and subsequently assembled into PSII (sub)complexes. The nascent protein was detected in fractions 10-20, representing smaller PSII core complexes. The nascent protein was also detected in fractions 6-10, representing smaller PSII core complexes.
**Fig. 3.** Pulse-chase experiment in isolated chloroplasts. Translation in isolated chloroplasts was carried out for 5 min and was stopped directly (no chase) or was followed by a chase of 2.5, 5, 10, 30, or 60 min with unlabeled methionine. After translation, chloroplasts were lysed, and the thylakoid membranes were washed repeatedly, solubilized in 1% n-dodecyl β-D-maltoside on ice, and subjected to sucrose density gradient centrifugation. After fractionation of the sucrose gradient, the fractions were precipitated in 10% trichloroacetic acid and run on 14% SDS-polyacrylamide gels containing 6 M urea. The fraction numbers, 1–20, are indicated. 14C-Labeled marker proteins were run in the outer lanes of the gels. A, autoradiograms of trichloroacetic acid-precipitated proteins in the sucrose gradients after translation, followed by the indicated chase times (2.5, 10, and 30 min). Gels were dried prior to exposure to film. The sucrose gradient fractions are numbered from the bottom (fraction 1) to the top (fraction 20) of the tube. Molecular mass markers are indicated. Arrows indicate low molecular mass proteins. B, quantification of the distribution of 35S-labeled D1 protein (precursor and mature forms) (upper panel, black bars) and D2 protein (lower panel, shaded bars) in the sucrose gradients after 5 min of translation, followed by different chase times (0, 2.5, 5, 10, 30, and 60 min). Quantification of newly synthesized protein was carried out by scanning of the autoradiograms. To allow direct comparison of the quantity of the two proteins, the intensity was corrected for the number of methionine residues in each protein (the D1 and D2 proteins contain 11 and 8 methionine residues, respectively). rc, reaction center.
(data not shown).

After 5 min, the amount of radiolabeled D1 protein had nearly reached its maximum (data not shown). The newly synthesized D1 protein continued to accumulate predominantly as unassembled protein and in PSII reaction center complexes (in fractions 15 and 12, respectively) (data not shown).

After a 10-min chase, a clear incorporation of newly synthesized D1 protein into the "small" PSII core complexes and, to a lesser extent, into the "large" PSII core complexes (fractions 8–10 and 5–7, respectively) could be observed (Figs. 3, A and B). At the same time, the amount of unassembled labeled D1 protein (fractions 14 and 15) had diminished. With this increased chase time, several distinct translation intermediates (mainly of the D1 protein) between 15 and 24 kDa can be observed in the bottom fractions (Fig. 3B), but also in fractions 12–16. It should be noted that during these first 10 min of chase, the amount of nascent chains in the bottom fractions (fractions 1–5) decreased due to elongation, termination, and subsequent release from the ribosomes (Fig. 3A).

The process of incorporation of D1 protein into the smaller and larger PSII core complexes (fractions 8–10 and 5–7, respectively) continued for up to 30 min of chase. After a 30-min chase (Fig. 3, A and B), the bulk of the D1 protein had been incorporated into these PSII core complexes and into reaction center complexes of ~140–215 kDa (fractions 11 and 12). Only ~10% of the D1 protein remained unassembled (fractions 14 and 15). Nearly all nascent chains had been released from the ribosomes, with only some higher molecular mass proteins (>60 kDa) remaining on the ribosomes. A longer chase time of 60 min did not change the distribution of radioactivity significantly (data not shown), indicating that the incorporation process was finished after 30 min.

Synthesis and Assembly of D2 Protein, CP43, CP47, and Small PSII Core Proteins into PSII (Sub)complexes in Intact Chloroplasts—In principle, a similar process of synthesis and incorporation as for the D1 protein could also be observed for the D2 protein (Fig. 3, A and B), but with one very important difference: in the case of the D2 protein, a much smaller proportion of the newly synthesized protein could be incorporated into PSII reaction centers and core complexes (i.e. fractions 5–12) (Fig. 3B). Most of the radiolabeled D2 protein either remained as unassembled protein in fractions 14 and 15 or was to some extent incorporated into PSII reaction centers, found in fractions 11 and 12 (Fig. 3B).

These analyses were also carried out for CP43 and CP47. As can be observed from the autoradiograms (Fig. 3A), most of the synthesized CP43 and CP47 remained unassembled and accumulated in fractions 13 and 14. In the case of CP47, the synthesis rate was only half that of CP43, and no significant amount of CP47 was observed in the core complexes of fractions 5, 6, and 8–10.

The PSII core complex contains several low molecular mass chloroplast-encoded proteins (PsbE, PsbF, PsbH, PsbL, PsbK, PsbL, PsbM, and PsbN) (see Refs. 2 and 10), most of which contain one or more methionine residues. Indeed, at least three different, as yet unidentified, low molecular mass proteins below 10 kDa were found in sucrose density gradient fractions 5–16. Most of these labeled products appeared in fractions 15 and 16 in the case of shorter chase times (Fig. 3A), and with increasing chase times (30 and 60 min), the small proteins were quite effectively chased into complexes in fractions 8–10 (Fig. 3A). Strikingly, these small proteins did not show a (transient) accumulation in the reaction center complexes in fractions 12 and 13, but instead appeared to be chased directly into the PSII core complexes.

Incorporation Kinetics in Isolated Thylakoid Membranes of Radiolabeled D1 and Other PSII Core Proteins—To study the possible role of stromal factors for the D1 protein incorporation process into PSII (sub)complexes, a similar pulse-chase experiment, followed by analytical sucrose density gradient centrifugation and SDS-PAGE as described for the intact chloroplasts (Fig. 3) was carried out using isolated thylakoid membranes (Fig. 4). Since the processing of pD1 was found to be much slower in the isolated thylakoids than in the intact chloroplasts (see Fig. 1 and also Ref. 35), the role of processing in the assembly process could also be assessed.

The distribution of radiolabeled pD1 + D1, D2, and CP43 in different sucrose gradient fractions before the chase and after 2.5, 5, and 30 min of chase is shown in Fig. 4 (A and B). A correction for the number of methionine residues in the protein sequences was carried out in order to be able to directly compare the amounts of the three synthesized core proteins (Fig. 4B). No quantification could be done for CP47 due to very low levels of labeling (synthesis rate of CP47 was at least 10 times lower than that of the D1 protein).

Directly after the pulse (Fig. 4, A and B, upper panels), labeled pD1 + D1 accumulated predominantly as unassembled proteins (fractions 14 and 15) and in PSII subcomplexes of 130–160 kDa (fraction 12). A small amount of (p)D1 could also be found in the smaller PSII core complexes (fractions 8–10). On the other hand, CP43 and the D2 protein accumulated almost completely as "free" proteins, in fractions 13 and 14 and fractions 14 and 15, respectively. The total amount of synthesized D1 protein was more than twice that of the D2 protein and nearly six times that of CP43.

After a 2.5-min chase, (p)D1 protein could be observed in the small PSII core complexes (fractions 8 and 9) (data not shown), while very few other labeled products were apparent in fractions 1–10. The radiolabeled D2 protein was nearly exclusively located in fractions 14 and 15, while CP43 and CP47 were predominantly located in fractions 13 and 14 as unassembled proteins.

After a 5-min chase (Fig. 4, A and B, middle panels), D2, CP43, and CP47 continued to be located as unassembled proteins, while a considerable amount of D1 protein (17%) was integrated into the small PSII core complexes in fractions 8–10. Interestingly, the ratio between pD1 and D1 proteins in fractions 7–15 was quite constant (~0.5–0.6); thus, no specific accumulation of pD1 or D1 protein occurred in certain (sub)complexes, indicating that processing was not a prerequisite for incorporation into PSII (sub)complexes, in agreement with earlier studies using PSII mutants (29, 45).

After a 30-min chase (Fig. 4, A and B, lower panels), the distribution of these four PSII core proteins had changed. The radiolabeled D1 protein was now quite evenly distributed among PSII core complexes (fractions 8–10), reaction center particles (fraction 12), and free protein (fractions 14 and 15). No significant amounts of precursor D1 could now be detected, indicating that processing was completed. Newly synthesized D2 protein was predominantly found in reaction center complexes, while ~28% of this protein was incorporated into the smaller PSII complexes. Interestingly, radiolabeled D2 protein was nearly absent in fractions 10 and 11. Labeled CP43 (and CP47; data not shown) was chased to a limited extent into PSII core complexes, while the major portion remained unassembled in the membrane. No significant alterations occurred during longer chase periods (60 min) (data not shown), indicating that after 30 min of chase, the "end situation" for the assembly and incorporation of newly synthesized proteins had been reached.

Effect of Stromal Factors on Elongation, Synthesis, and In-
corporation of PSII Core Proteins—Although the general incorporation pattern of the various core proteins was quite comparable for the two translation systems (Figs. 3 and 4), incorporation of the D1 protein into complexes was restricted in the thylakoid system (Fig. 5). Fig. 5 shows a comparison of the disappearance of unassembled D1 protein and the kinetics of incorporation of labeled D1 protein into PSII core complexes in both translation systems. Initially, prior to the chase or with chase times of <10 min, ~35–50% of the radiolabeled D1 protein was unassembled in the membrane in both systems. In the case of the chloroplasts, the D1 protein was more effectively chased into complexes than in the case of the thylakoid system, and after 30 min, only 10% of the radiolabeled D1 protein was left unassembled in the chloroplasts, while in the thylakoid translation system, 30% remained unassembled. The percentage of radiolabeled D1 protein incorporated into PSII reaction center particles fluctuated around 30% in both translation systems (data not shown). After 30 min of chase, ~20% of the radiolabeled D1 protein was incorporated into the PSII core complexes in the thylakoid system, while ~30% was incorporated into the chloroplasts (Fig. 5). A direct comparison of the distribution of radiolabeled D2 protein and CP43 after a 30-min chase in both translation systems showed that the extent of incorporation of both proteins into PSII core complexes was quite limited in the two systems.

Assembly State of Newly Synthesized D1 Protein in Stroma-exposed and Granal Regions of the Thylakoid Membranes—The site of synthesis of the chloroplast-encoded thylakoid membranes is the stroma-exposed region of the thylakoid membranes (7, 8). The bulk (~80%) of the functional PSII complexes are located in the stacked granal membranes. To analyze the distribution of the different PSII assembly intermediates over the stromal and granal membranes, thylakoid membranes were fractionated into pure stromal and crude granal membranes by differential centrifugation after digitonin solubilization. The granal and stromal membranes were subsequently solubilized in n-dodecyl β-D-maltoside and then subjected to sucrose gradient fractionation analysis.

The autoradiograms of the trichloroacetic acid-precipitated sucrose gradient fractions from both the granal and stromal membranes after translation in isolated thylakoids (10-min pulse followed by a 60-min chase) are shown in Fig. 6A, while Fig. 6B specifically shows the quantitative lateral distribution
Mechanism of D1 Protein Incorporation

of the radiolabeled D1 protein. It can be observed that in the case of the granal membranes, the radiolabeled D1 protein was primarily located in PSII core complexes (fractions 5-10), while in the stroma-exposed membranes, most radiolabeled D1 protein was localized in smaller PSII complexes, such as PSII reaction centers (Fig. 6B). Furthermore, very little unassembled D1 protein was found in the granal membranes as compared with the stromal membranes, indicating that the D1 protein did only migrate from its site of synthesis in the stromal membranes to the granal membranes after it had been assembled together with other PSII subunits.

Radiolabeled D2 protein, CP43, and CP47 were found to accumulate predominantly as free proteins (fractions 13 and 14) in the stromal membranes, while only small amounts of D2 and CP43 could be detected in small and large PSII core complexes (fractions 8 and 9 and fractions 4 and 5, respectively) in the granal membranes. A similar lateral distribution of labeled PSII subcomplexes was observed after translation in isolated chloroplasts (data not shown). However, incorporation of labeled proteins into PSII core complexes and lateral migration to the granal regions were slightly more efficient than in the isolated thylakoids.

DISCUSSION

Mechanism of D1 Protein Replacement—In a recent study (35), we developed an analytical procedure based upon sucrose gradient centrifugation to follow the assembly of newly synthesized proteins into the PSII complex. This synthesis and assembly process was studied during translation in intact isolated chloroplasts as well as during run-off translation in isolated thylakoids. In the present study, we have used this experimental system to follow the kinetics of this incorporation process and determined the localization of the different reassembly steps in the heterogeneous thylakoid membrane. Also, we have now, for the first time, been able to obtain an initial general insight on the dependence of cytoplasmic and stromal components for D1 protein assembly. This had not been possible in the earlier in vivo studies of PSII assembly. Based on our experimental data and on previous observations including information from PSII mutant studies, we have summarized the events of D1 protein replacement in existing PSII complexes in Fig. 7A.

The initial incorporation step seems to involve both a slow, clearly post-translational incorporation and a faster, possibly cotranslational incorporation of D1 protein into existing PSII complexes (Fig. 7A). From the kinetics, we observe that both pathways occur in parallel (Figs. 3 and 4). In the slow, post-translational pathway, the precursor D1 protein accumulates first as a free unassembled protein after release from the ribosomes. Subsequently, the labeled protein associates with a PSII reaction center protein (e.g., the D2 protein) or with a small protein subcomplex (e.g., a D1-less PSII reaction center) to form transient PSII reaction center complexes, accumulating in sucrose gradient fraction 12. The involvement of a post-translational incorporation mechanism is particularly supported by the prominent accumulation of unassembled D1 protein within the first 10 min of chase time, followed by a relatively slow incorporation into the various PSII (sub)complexes. This post-translational mechanism is likely to occur if limited amounts of D2 protein or other PSII reaction center proteins are available for direct assembly of a PSII reaction center. The mechanism for the fast incorporation (Fig. 7A, "fast mechanism") could be a fast association of the unassembled D1 protein with a small PSII subcomplex, but could also be due to an interaction of the newly synthesized α-helices of the D1 protein during translation (a cotranslational assembly) with a PSII reaction center protein (e.g., the D2 protein) or a protein subcomplex (a D1-less PSII reaction center). After termination of translation, the D1 protein has already become part of PSII and is consequently directly recovered in the fractions of the sucrose gradients containing the reaction center particles. Preliminary experimental support for a cotranslational interaction of the D1 nascent chain with the mature D2 protein is found in the relatively high abundance of D1 translation intermediates.
in pseudopolysome preparations in addition to substantial amounts of D2 protein. Furthermore, this concept is supported by studies of PSII deletion mutants that have shown that the D2 protein has a translational control over the D1 protein (2, 25, 26).

With longer chase times, the D1 protein was progressively found in the small and large PSII core complexes in which also the extrinsic proteins of the oxygen-evolving complex and some minor chlorophyll a/b-binding proteins became bound; the step from the PSII reaction center to small PSII core complexes involved the lateral migration from stromal to granal regions. These later steps of assembly of PSII core complexes are post-translational and sequential as judged from the kinetics of

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The chase experiment also provided insight into the time scale of the elongation and incorporation process, although a precise estimation is difficult since the smaller complexes were only transiently present; elongation and release of the nascent chains from the ribosomes were completed within 10 min, while maximal accumulation of radiolabeled D1 protein as unassembled protein (Fig. 5, upper panel) or in the PSII reaction center complexes was reached after a 5-min chase (Figs. 3B and 4B). The appearance of the first small PSII core complexes took —10 min of chase. The assembly step from the reaction center (fraction 12) to these small PSII core complexes (fractions 8–10) seemed to be the rate-limiting step since only after maximal accumulation of radiolabeled D1 protein in the reaction center complexes had occurred was labeled D1 protein chased into PSII core complexes (see Fig. 3 on the lower panel). From the thylakoid subfractionation, it could be demonstrated that the core complexes were located in the granal membranes, while the smaller PSII (sub)complexes were located in the stromal membranes (Fig. 6). Thus, it seems that the rate-limiting assembly step from PSII reaction centers to PSII core complexes involved lateral migration from stromal to granal membranes. The most likely explanation is that the actual lateral migration of the newly assembled PSII reaction centers from stromal to granal membranes is slow and that the PSII cores are formed in the grana by connecting certain core proteins to the reaction center.

Requirements for Nuclear Encoded and Soluble Stromal Components—Although the composition of synthesized PSII proteins in the run-off translation system was rather similar to the composition in isolated chloroplasts (thus, no soluble stromal factors or biosynthesis of cofactors was needed for translation elongation in the light; see also Ref. 25), the absence of stromal factors restricted the incorporation of radiolabeled D1 protein into PSII (sub)complexes (Figs. 4 and 5). In the run-off translation system of isolated thylakoids, a significant proportion of synthesized D1 protein remained unassembled in the membrane even after a chase time of 30–60 min, indicating that stromal components are needed for incorporation of the D1 protein into PSII (sub)complexes. These stromal components can be either cytoplasmic components or soluble stromal factors assisting in the assembly process (chaperones) or stabilizing cofactors (pigments, heme) synthesized within the chloroplast. Reconstitution experiments will be needed to identify these stromal factors.

Processing of Precurso D1 Protein—The slow processing of precursor D1 protein in the case of translation in isolated thylakoids allowed us to obtain direct biochemical evidence that processing and incorporation of the D1 protein into PSII core complexes are unrelated events. This means that pD1 can be processed prior to assembly, thus as a free protein in the membrane, as well as after incorporation into a PSII (sub)complex. Incorporation of unprocessed D1 protein into PSII core complexes has been shown in the Scenedesmus LF1 mutant, in which the processing enzyme is missing (43), while site-directed mutagenesis in the green algae Chlamydomonas reinhardtii has suggested that the C-terminal extension has no function in the assembly of PSII (46).

Incorporation of D2 Protein, CP43, CP47, and Smaller PSII Core Proteins—Based on the kinetics of accumulation of the D2 protein and CP43 in the sucrose gradient fractions after translation in both chloroplasts and thylakoids, we concluded that incorporation of the D2 protein and CP43 into PSII (sub)complexes exclusively follows a post-translational and sequential pathway, as is summarized in Fig. 7B. Synthesis of D2 protein and CP43 is likely to be polycistrionic, with psbD (encoding the D2 protein) located upstream of psbC (encoding CP43), since only one transcript has been detected (see Ref. 2).

Although substantial amounts of the D2 protein, CP43, and CP47 were synthesized in both translation systems, only a small percentage of these proteins was incorporated into the PSII core complexes. Instead, they accumulated predominantly as free unassembled proteins and, in the case of the D2 protein, also in PSII reaction center complexes (Figs. 3B and 4B). During translation experiments with intact chloroplasts, slightly more D2 protein was incorporated into PSII cores and PSII reaction centers as compared with translation in isolated thylakoids, indicating that soluble stromal factors or chloroplast biosynthetic products slightly improve the incorporation of these two proteins. However, since the assembly of newly synthesized D2 protein, CP43, and CP47 is low even in isolated chloroplasts, the major limiting factor for their assembly process must be a cytoplasmic (nuclear encoded) component that is normally (in vivo) imported into the chloroplasts and that is freely available only to a limited extent in the chloroplast itself (see Fig. 7B). The accumulation of the D2 protein, CP43, and CP47 as unassembled subunits is therefore a reflection of their relatively low rate of light-induced damage and subsequent degradation (as compared with the D1 protein) (for discussion, see Ref. 35).

The PSII complex contains several small (<10 kDa) chloroplast-encoded proteins (2) containing up to three methionine residues. Several small proteins (especially of 4, 4.5, and 8 kDa) were synthesized in both the chloroplast (Fig. 3A) and, to more limited extent, thylakoid (data not shown) translation systems. The small radiolabeled proteins were abundant in fractions 15 and 16 in the case of the short chase times, and with increasing chase times (30 and 60 min), they were quite effectively chased into PSII core complexes. Thus, the three small PSII proteins seemed to be actively synthesized and incorporated into the PSII core, without transient accumulation in PSII reaction center complexes. This suggests the possibility that in addition to the D1 protein, some of these small proteins are also replaced. To our knowledge, no previous study has been reported concerning a high turnover of these small, chloroplast-encoded proteins. Further identification and assessment of their turnover are currently in progress.

The experimental system described in this paper has opened many new possibilities to study the assembly of PSII and possibly other thylakoid-bound complexes. Experiments are in progress to identify the nuclear encoded and stromal components needed for efficient incorporation of the D1 protein into the existing PSII core complexes and to characterize the initial assembly steps in molecular detail.

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Kinetic Resolution of the Incorporation of the D1 Protein into Photosystem II and Localization of Assembly Intermediates in Thylakoid Membranes of Spinach Chloroplasts

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