Co-translational Assembly of the D1 Protein into Photosystem II*

(Received for publication, February 2, 1999, and in revised form, March 15, 1999)

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Assembly of multi-subunit membrane protein complexes is poorly understood. In this study, we present direct evidence that the D1 protein, a multiple membrane spanning protein, assembles co-translationally into the large membrane-bound complex, photosystem II. During pulse-chase studies in intact chloroplasts, incorporation of the D1 protein occurred without transient accumulation of free labeled protein in the thylakoid membrane, and photosystem II subcomplexes contained nascent D1 intermediates of 17, 22, and 25 kDa. These N-terminal D1 intermediates could be immunoprecipitated with antisera directed against the D2 protein, suggesting co-translational assembly of the D1 protein into photosystem II complexes. Further evidence for a co-translational assembly of the D1 protein into photosystem II was obtained by analyzing ribosome nascent chain complexes liberated from the thylakoid membrane after a short pulse labeling. Radiolabeled D1 intermediates could be immunoprecipitated under nondenaturing conditions with antisera raised against the D1 and D2 protein as well as CP47. However, when the ribosome pellets were solubilized with SDS, the interaction of these intermediates with CP47 was completely lost, but strong interaction of a 25-kDa D1 intermediate with the D2 protein still remained. Taken together, our results indicate that during the repair of photosystem II, the assembly of the newly synthesized D1 protein into photosystem II occurs co-translationally involving direct interaction of the nascent D1 chains with the D2 protein.

Photosystem II (PS II) is a multiprotein membrane complex that catalyzes water oxidation and reduction of plastoquinone. The reaction center complex of PS II consists of the D1 and D2 proteins, the a and b subunits of cytochrome b$_{559}$, and the psbI and psbW gene products (1). The PS II reaction center binds all the components necessary for the primary charge separation (2). Oxygen-evolving PS II complexes additionally contain the intrinsic chlorophyll a binding proteins (CP43 and CP47), the oxygen-evolving complex, and several small proteins of unknown function (3). Recent structural studies indicate that the PS II core complexes can exist as a dimer (4).

The D1 protein has an unusually high turnover rate as compared with other chloroplast proteins (5, 6). The photodamaged D1 protein in the PS II centers is replaced constantly with newly synthesized D1 protein to maintain PS II in a functional state (7).

The D1 protein, encoded by the plastid psbA gene, is synthesized on membrane-associated ribosomes and inserted into thylakoid membrane during its synthesis (8, 9). Moreover, ribosomes have shown to pause at specific sites during translation of membrane-bound psbA mRNA, and this was hypothesized to facilitate the binding of cofactors to the D1 protein (10). It has been suggested that the first step of PS II repair is the association of newly synthesized D1 with the D2 protein, D2-cytochrome b$_{559}$ (11), or D2-cytochrome b$_{559}$-CP47 (12) in the stroma-exposed thylakoid membranes. Studies on different PS II gene deletion mutants of Chlamydomonas reinhardtii have shown that the protein synthesis of D1 and D2 is tightly coupled and that the D2 protein directly or indirectly regulates the synthesis of D1 protein (13). Contrary to the D1 protein, the synthesis and short term accumulation of the D2 protein are relatively independent of light and availability of chlorophyll (14). These results, together with our earlier studies on D1 synthesis and assembly into PS II (15–17), suggest a crucial role for the D2 protein in the stabilization of the D1 protein.

In this study, we have addressed the question whether the D1 protein assembly into PS II is occurring co-translationally. Reports on co-translational assembly of proteins into multiprotein complexes are scarce (18, 19). It has been hypothesized, however, that ribosome pausing, which is known to occur also during D1 translation (10), may control protein targeting and insertion into the membrane as well as the concomitant assembly into a protein complex (20). It can be envisaged that the efficiency and coordination of D1 replacement during PS II repair would be greatly facilitated by co-translational assembly of the D1 protein into PS II.

We show that the newly synthesized D1 protein cannot be trapped as a free protein after termination of translation but is directly associated with other PS II proteins. Isolation of ribosome nascent chain complexes further indicated that the D1 nascent chains interact already during translation elongation with the D2 protein. A scheme summarizing the assembly pathway of the newly synthesized D1 protein into PS II during the repair process is presented.

Experimental Procedures

Plant Material and Isolation of Intact Chloroplasts—Spinach was grown hydroponically at 23 °C in a light/dark cycle of 10 h/14 h. For all the experiments fully developed leaves were harvested 1 h after the lights were turned on.

Immediately after harvest, spinach leaves were briefly homogenized in 330 mm sorbitol, 5 mm ascorbate, 0.05% bovine serum albumin, 2 mM EDTA, 1 mM MgCl$_2$, 50 mM Hepes-KOH, pH 7.6, filtered through Miracloth and centrifuged for 1 min at 1000 × g. The pellets were resuspended in Medium A (330 mm sorbitol, 2 mm dithiothreitol, 50 mM Hepes-KOH, pH 8.0) and loaded on the top of Percoll step gradients (40
and 70% in Medium A) and spun for 5 min at 4500 \( \times g \) at 4 °C. Chloroplasts in 70% Percoll were diluted with Medium A, spun for 2 min at 1300 \( \times g \), and washed once with Medium A. The chloroplasts used in this study were more than 90% intact.

**In Vitro Translation in Chloroplasts**—In vitro translations in isolated chloroplasts were performed essentially as described in Ref. 21. After 5 min of preincubation (0.5 \( \mu \)g chlorophyll/\( \mu \)l) at 23 °C in the light (about 50 \( \mu \)mol of photons m\(^{-2} \) s\(^{-1} \)), carrier-free \(^{35}\)S-labeled methionine was added in a final concentration of 0.5 \( \mu \)Ci/\( \mu \)l, and chloroplasts were pulse labeled for 2.5 min, followed by an additional 5-min chase in the presence of 10 \( \mu \)M unlabeled methionine where indicated. The translation was stopped by adding a 10-fold volume of ice-cold lysis buffer (7 \( \mu \)M MgOAc, 50 mM KOAc, 250 mM Tris-HCl, pH 6.0 (Medium B). A mixture of protease inhibitors (antipain (2 \( \mu \)g/ml), leupeptin (2 \( \mu \)g/ml), phenylmethylsulfonyl fluoride (100 \( \mu \)g/ml)) was added to all solutions.

**Sucrose Gradient Fractionation of Thylakoid Membranes**—Thylakoids (0.5 mg of chlorophyll) were solubilized for 5 min on ice in medium B containing 1% n-dodecyl-\( \beta \)-d-maltoside (DM). Homogeneous solubilization was ensured and accelerated by using a Potter homogenizer. Subsequently the suspension was loaded on a linear sucrose gradient of 11 ml (5–35% sucrose in 5 ml MgCl\(_2\), 10 ml NaCl, 0.5 \( \mu \)M betaine, 0.03% DM, and 25 mM Mes-NaOH, pH 5.7) made with Gradient Master\(^{\text{TM}}\) (model 106, Biocomp Instruments, Inc., New Brunswick, Canada) and spun for 26 h at 180,000 \( \times g \) at 4 °C. After centrifugation, the sucrose gradients were divided into 20 fractions of equal volume using a Piston Gradient Fractionator (model 151, Biocomp Instruments, Inc.).

**Isolation of Ribosome Nascent Chain Complexes**—Chloroplasts (equivalent to 100 \( \mu \)g of chlorophyll) were pulse labeled with \(^{35}\)S-labeled methionine for 2.5 min and thereafter solubilized with 2% polyoxyethylene 10 toluene-\( \beta \)-methyl ether in Medium C (50 mM Hepes-KOH, pH 7.5, 5 mM MgOAc, 50 mM KOAc, 250 mM chlomophenicol, 0.5 mM mg/ml heparin, 2 mM dithiothreitol) for 10 min. Ribosomes were collected by centrifugation through a 1.0 M sucrose cushion in Medium C at 270,000 \( \times g \) for 1 h.

**Immunoprecipitation**—Immunoprecipitation of sucrose gradient fractions was performed by adding the antiserum to each fraction, and after overnight incubation the IgGs were collected using bovine serum albumin-saturated protein A-Sepharose. The beads were washed for five times with 10 ml Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, and the bound antigen was released in the sample buffer.

For immunoprecipitation of ribosome pellets under denaturing conditions, the ribosome pellets were solubilized with 1% SDS in 15 mM dithiothreitol, 100 mM Tris-HCl, pH 7.5, diluted with four volumes of 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, and immunoprecipitation was performed as above. For co-immunoprecipitation, the ribosome pellets were solubilized with 1% DM in 50 mM Tris-HCl, pH 7.5, before addition of antibodies.

**Protein Analysis**—Nondenaturing Deriphat-PAGE (5–16% acrylamide gradient) was used in this study. The ribosome pellet, together with subcomplexes in the sucrose gradients. The assignment was based on the immunoblot analysis (A), molecular mass calibration (data not shown), and previous studies (15).

**RESULTS**

**Insertion of Newly Synthesized D1 Protein into PS II Subcomplexes in Intact Chloroplasts**—In earlier studies we observed that the incorporation of the newly synthesized D1 protein into PS II core complexes proceeded in a stepwise manner (17). Immediately after pulse labeling (5 min), the newly synthesized D1 protein predominantly sedimented in the top of the sucrose gradient as unassembled subunits (16). Therefore, we postulated that the assembly of the D1 protein occurs mainly in a post-translational pathway in which the full-length D1 first accumulated as an unassembled protein, in parallel to a possible co-translational pathway in which D1 incorporates directly into PS II reaction centers (16).

In this study, we investigated these two possibilities in more detail. If co-translational assembly is the dominant mechanism, our earlier observation of a post-translational assembly route could be due to a detergent sensitivity of the initial assembly steps. To avoid this, we reduced the solubilization time to 5 min while still achieving complete solubilization of the membrane by homogenizing during solubilization. In addition we further optimized the analytical sucrose gradient fractionation earlier used to separate the different assembly steps and PS II subcomplexes (15). The pH of the solubilization and gradient buffers was lowered to about pH 6.0, and 0.5 \( \mu \)M betaine was included in the gradients to stabilize the water-soluble proteins associated with PS II (24). The distribution of PS II subcomplexes in the sucrose gradients of DM-solubilized thylakoid membranes was further studied by analyzing the sucrose gradient fractions 3–16 (of 20) on nondenaturing Deriphat gels, followed by Western blotting analysis (Fig. 1).

From the D1 immunoblot of Deriphat-PAGE (Fig. 1A, top panel) and previous analysis (25), it is clear that sucrose gradient fractions 3–9 contained dimeric PS II cores of about 400 kDa. Fractions 7–9 contained monomeric PS II cores. The assignment of PS II subcomplexes in the sucrose gradient (Fig. 1B) was based on the immunoblot analysis, molecular mass calibration (data not shown), and previous studies (25).

Comparisons of anti-D1 and anti-CP43 immunoblots of Deriphat-PAGE also revealed CP43-less PS II monomers and dimers (Fig. 1A). However, the presence of free CP43 in sucrose fractions containing PS II monomer and dimer suggest that CP43-less PS II core complexes were at least partially due to dissociation of CP43 from PS II complexes during the run of Deriphat gels.

Analysis of pulse labeled thylakoids (2.5-min pulse and no chase) by a combination of the optimized sucrose gradient...
fractionation and nondenaturing Deriphat-PAGE (Fig. 2) revealed a rapid assembly of nearly all newly synthesized proteins into PS II subcomplexes. Radiolabeled proteins appeared predominantly in PS II reaction center complexes (fractions 10 and 11) and in PS II monomers with and without CP43 protein (fractions 7–9) (Fig. 2A). Importantly, only traces of unassembled radiolabeled proteins were detected (fractions 12–16).

SDS-PAGE analysis of the same sucrose gradient fractions (Fig. 2B) showed that the precursor and mature D1 proteins ((p)D1) were the major radiolabeled proteins. After this short pulse, about 95% of all radiolabeled (p)D1 proteins were found to be incorporated into PS II reaction centers and monomers. Thus, with our optimized procedures only minor amounts of free newly synthesized (p)D1 proteins could be detected in fractions 12–15 (Fig. 2), in contrast to our earlier observations of a significant transient population of unassembled D1 protein (16). Therefore, it is likely that D1 assembly into PS II occurs in a co-translational manner via detergent-sensitive assembly stages.

Despite our improved protocols, stable assembly of D2 and CP47 still did not occur in our studies (Fig. 2B) and is therefore likely to depend on newly synthesized nuclear-encoded factors or biosynthesis of cofactors. Without exception, however, some of the newly synthesized CP43 protein was always found assembled into PS II core monomers (Fig. 2B). It is likely that after replacement of the D1 protein, a newly synthesized CP43 or the one released during earlier stages of PS II repair cycle (25, 26) can reassociate with PS II.

Direct Evidence for D1 Nascent Chains in PS II Subcomplexes—Because nearly all newly synthesized D1 protein was immediately (after a 2.5-min pulse) found associated with the PS II reaction center and monomeric complexes, we addressed the question of whether the nascent D1 protein interacts with other PS II polypeptides already during translation elongation. As a first approach, we searched for D1 protein synthesis intermediates in PS II monomers and reaction centers.

To allow the accumulation of more radioactivity into the D1 protein and its translation intermediates, a 5-min chase was applied after a 2.5-min pulse with cold methionine. After translation, chloroplasts were lysed, solubilized, and spun on a sucrose gradient. A, autoradiogram of the newly synthesized thylakoid membrane proteins after sucrose gradient fractionation. Fractions 7–16 were separated by SDS-PAGE. B, autoradiogram of sucrose gradient fractions immunoprecipitated with the D1 or D2 antibody. Sucrose gradient fractions 7–14 were immunoprecipitated with the antisera raised against the N-terminal residues (58–86) of the D1 protein and the residues 230–245 of the D2 protein. The precipitated products were separated by SDS-PAGE. C, D1 immunoblot of PS II subcomplexes. Sucrose gradient fractions 8–11 were concentrated and run in Deriphat-PAGE. The PS II monomer and reaction center complexes on Deriphat-PAGE were cut, rerun in SDS-PAGE, and immunodetected with the antiserum raised against the N-terminal residues (58–86) of the D1 protein.
proteins in ribosome pellets. After a 2.5-min pulse in intact chloro-
plasts, the thylakoids were isolated and solubilized in 2% polyoxyeth-
ylene 10 tridecyl ether for 10 min on ice. Thylakoid-bound polysomes
were then isolated by centrifugation through a 1.0M sucrose cushion.

The D2, or the CP47 protein and with the respective presera (panel on
the left). The identity of the labeled polypeptides precipitated with the
D2 antiserum was verified by Western blotting with the N-terminal D1
antiserum and with the D2 antiserum (panel on the right). Also, the
products precipitated by the D2 antiserum were released in the sample
buffer, diluted, and reprecipitated with the antiserum raised against
the N-terminal residues of the D1 protein (panel in the middle). B,
autodiragram of immunoprecipitated products from DM-solubilized
ribosome pellets. The ribosome nascent chain complexes were solubi-
ized in DM, and immunoprecipitation was performed as described for
panel A.

DISCUSSION

In this study we have addressed the initial assembly steps of the D1 protein into the PS II complex. The high natural turn-
over of the D1 protein and the experimental possibility to preferentially label the D1 protein during in organello
translations provides a unique model system to address the relation between translation, insertion, and assembly.

Because the elongation of the D1 protein in isolated chloro-
plasts is completed within 10 min (15), we applied only short
(2.5 min) pulse labeling to trap the newly synthesized D1 protein immediately after termination of translation. Despite
the short pulse, most of the labeled thylakoid proteins, which
almost exclusively consisted of the precursor and mature forms
of the D1 protein, had already been incorporated into PS II
reaction center and PS II core complexes. Not even a transient
pool of free D1 protein could be trapped in the thylakoid mem-
brane. This strongly suggests that the elongation and assembly
processes of the D1 protein are tightly coupled.

Labeled D1 synthesis intermediates were frequently found
in gradient fractionated DM-solubilized thylakoids. The 17-
and 22-kDa intermediates migrated mostly on the top of the
gradient as free polypeptides. The main D1 intermediate of 25
dkDa, however, was strongly associated with PS II core subcom-
plexes, suggesting that the D1 protein already during transla-
tion elongation interacts with other PS II core components (Fig.
3). Moreover, this interaction is likely to be tight because it was
not disrupted during the sucrose gradient centrifugation.

More direct evidence for co-translational assembly of the D1
protein into PS II was obtained by studying the interaction of
D1 nascent chains with other PS II core proteins in the ribo-
some nascent chain complexes. Immunoprecipitation with the
N-terminal D1 antibody revealed D1 intermediates of 15–30
kDa (Fig. 4). The labeled D1 intermediates in ribosome pellets
(and PS II core complexes) could be efficiently chased into the
mature form, and they could also be released from ribosomes by puromycin, indicating that they do represent D1 nascent
chains, not the degradation products of D1. The D1 intermedi-
ates corresponded to the ribosome pausing sites in membrane-
bound psbA mRNA that have been mapped through toe print
analysis (10). The 17-kDa D1 synthesis intermediate is likely to
originate from ribosome pausing occurring when two trans-
membrane domains (TMs) have been inserted into the mem-
brane, with the third TM having just emerged from the ribo-
some tunnel/groove. The 22-kDa D1 intermediate was, as
opposed to other studies (10), only faintly labeled in our exper-
iments. The other major D1 synthesis intermediate of 25 kDa
most likely results from ribosome pausing after four TMs have
been translated and inserted into the membrane.

Co-immunoprecipitation of D1 nascent chains from ribosome
pellets with the D2 antiserum strongly supports the idea of
cotranslational interaction of the D1 nascent chains with the
D2 protein (Fig. 4). After the D1 nascent chain has started

Interestingly, the 17-kDa (as well as the 25-kDa) labeled D1
nascent chain could be efficiently co-immunoprecipitated with
the D2 antibody from the ribosome pellets solubilized with
nonionic detergent DM (Fig. 4B). Likewise, different interme-
diates of the D1 protein could also be co-immunoprecipitated
with the CP47 antibody (Fig. 4B). However, as shown in Fig.
4A, incubation of ribosome nascent chain complexes with CP47
antibody under denaturing solubilization conditions did not
result in the precipitation of any labeled polypeptides.

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lished data.
insertion into the thylakoid membrane during its early synthesis, interaction with the D2 protein becomes obvious after two TMs have been inserted into the membrane (17-kDa pausing intermediate) (Fig. 5). This interaction, however, is not very tight and can easily be disrupted with SDS. With further elongation of the D1 nascent chain to 25 kDa the interaction with the D2 protein becomes more tight and can no longer be disrupted by SDS. Incomplete unfolding by SDS is typical for most hydrophobic membrane proteins (27), and hydrophobic interactions between TMs have been shown to persist even under denaturing conditions (SDS) (28). A similar tight protein–protein interaction, possibly due to the hydrophobicity of both interacting proteins, has also been observed in other co-immunoprecipitation studies under denaturing conditions (29). The co-immunoprecipitation of the 25-kDa D1 nascent chain with the D2 antibody was not due to any unspecific aggregation of membrane proteins or unspecific immunoprecipitation, because no D1 intermediates could be precipitated with D2 pre-serum and CP47 antiserum under similar denaturing conditions. Besides, no cross-reaction of the D2 antibody used in our experiments with the D1 protein has ever been observed.

Although the nascent D1 chains seems to interact co-translationally with the D2 protein, it is not known whether a translocon channel (i.e. SecY) is involved during D1 synthesis and membrane insertion. Studies on translocation of membrane proteins have indicated that the folded TMs can laterally exit the translocon channel and enter the lipid environment either before or after termination of translation (30–32). A possible lateral exit of TMs during synthesis might be important in repair of photodamaged PS II centers when only the D1 protein has to be replaced by de novo synthesis.

Structural studies have revealed close interaction between TM D and E of the D1 and D2 proteins, which may account for the tight association of the 25-kDa D1 nascent chain with the D2 protein. Moreover, most of the ligands for pigments and cofactors reside in the amino acid residues in the D and E helices of the D1 and D2 proteins (see Ref. 33). Therefore, the role of ribosome pausing is probably not only limited to D1 protein folding and assembly during the synthesis but may also ensure the co-translational ligation of cofactors to PS II under repair as suggested by Kim et al. (10).

Co-immunoprecipitation of D1 nascent chains under non-denaturing conditions occurred not only with the D2 antibody but also with CP47 antiserum, suggesting that CP47 may also interact with the synthesizing D1 protein during the repair process or, more likely, that D1 can assemble into a complex consisting of both the D2 protein and CP47. Structural studies of PS II core locate CP43 and CP47 on opposite sides of the D1/D2 reaction center (4, 34). Positioning of these two proteins adjacent to the D1 or the D2 protein is, however, not clear from the structural data (35). Our present results on co-immunoprecipitation of D1 nascent chains with the D2 and the CP47 antiserum lend strong support for a close association of D2 and CP47. CP43, on the other hand, is easily released from PS II during the repair process (25, 26) to make the replacement of the damaged D1 protein possible.

Taken together, our results indicate that during the repair of PS II, the assembly of the newly synthesized D1 protein into PS II occurs co-translationally and the nascent D1 chains start interacting with the D2 protein already during translation elongation. At this stage, CP47 may also be associated with the D2 protein, whereas CP43 reassembles with the PS II core complex only after termination of D1 translation.

**Acknowledgments**—The antibodies against the D1 protein and CP47 were kindly provided by Drs. J. E. Boynton and R. Barbato, respectively.

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