In Vitro Synthesis and Assembly of Photosystem II Core Proteins

THE D1 PROTEIN CAN BE INCORPORATED INTO PHOTOSYSTEM II IN ISOLATED CHLOROPLASTS AND THYLAKOIDS*

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The D1 reaction center protein of the membrane-bound photosystem II complex (PSII) has a much higher turnover rate than the other PSII proteins. Thus, the D1 protein has to be replaced while the other PSII components are not newly synthesized. In this study, D1 protein replacement into PSII complexes was followed in two in vitro translation systems: isolated chloroplasts and a homologous run-off translation system consisting primarily of isolated thylakoids with attached ribosomes. The incorporation of newly synthesized radiolabeled products into different (sub)complexes was analyzed by sucrose density gradient centrifugation of the nascent polypeptide chains from the ribosomes and identification of at least four assembly steps of the PSII complex, as shown below.

(i) Both in isolated chloroplasts and in thylakoids, newly synthesized D1 protein is predominantly incorporated into existing PSII subcomplexes, indicating that synthesis and import of nuclear-encoded factors is not needed for D1 protein replacement.

(ii) In chloroplasts, D1 protein incorporation into PSII core complexes is more efficient than during translation in isolated thylakoids. In the thylakoid translation system, a large percentage of radiolabeled D1 protein is found in smaller PSII subcomplexes, like PSII reaction center particles, and as unassembled protein in the membrane. This indicates that stromal factors are required in the replacement process of the D1 protein.

(iii) Both in isolated chloroplasts and in thylakoids, the other PSII core proteins D2, CP43, and CP47 are also synthesized and released from the membrane-bound ribosomes, but incorporation into PSII complexes occurs to a much smaller extent than the D1 protein. Instead they accumulate predominantly as unassembled proteins in the thylakoid membrane.

(iv) In chloroplasts, synthesis of the D1 protein seems to be adjusted according to the possibilities of incorporation into PSII complexes, while synthesis of the D2 protein, CP43, and CP47 is less regulated and their accumulation as unassembled protein in the membrane is abundant.

1 The abbreviations used are: PSII, photosystem II; Chl, chlorophyll; DM, n-dodecyl β-D-maltoside; OEC, oxygen evolving complex; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Mes, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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D1 Protein Incorporation into Photosystem II

Lakoid membranes (15, 16). This cotranslational insertion does take place in the stroma exposed thylakoid membranes. It is unknown by which mechanism the D1 protein and the other chloroplast-encoded membrane proteins are inserted into the membrane. However, it has recently been shown that in the presence of ATP, a proton-motive force and SecA are not required for translation and integration into the thylakoid membranes (17).

The replacement of the D1 protein into PSII has been addressed experimentally in vivo using green algae (9, 10, 18, 19) or using Spirodela leaves (20). In most of these studies, a cycling of the damaged PSII reaction center from grana to stroma lamellae was proposed to occur, but a “D1-less PSII” reaction center in the stroma lamellae has never been detected. Melis and co-workers (18, 19) proposed a repair cycle of PSII involving several putative assembly intermediates consisting of a PSII core with different sized antennae complexes and so-called QB-nonreducing PSII centers. In other studies it was proposed that the D1 protein associates in the stroma lamellae with a PSII subcomplex consisting of D2-cytochrome b559, CP47 (9) or with D2-cytochrome b559 (20).

The idea of cycling of a damaged PSII reaction center or core complex was supported by in vitro photoinhibition studies, in which isolated thylakoids were illuminated by strong light for extended periods. These studies indicated an apparent redistribution of PSII core proteins over the stroma and grana lamellae (21, 22). It was suggested that upon proteolysis of the D1 protein in absence of protein synthesis, a partial disassembly of the PSII core occurred and that a PSII subcomplex or individual PSII proteins migrated to the stroma lamellae.

Based on the proposed topology for the PSII core (23), one could hypothesize that the D2 protein, CP47, cytochrome b559, and possibly CP43 remain connected to each other, still allowing exchange of the D1 protein and relocation of the redox components. Studies using deletion mutants of PSII proteins have shown that mutants devoid in D2 protein synthesis do not show significant accumulation of the D1 protein. This indicates that the D2 protein might be involved in the elongation process of D1 translation or is needed for early stabilization of D1 (2, 11, 24).

The aim of this study is to establish an experimental system that allows investigation of the replacement of the D1 protein and to analyze the dependence of this process on import of nuclear-encoded products and synthesis of stromal factors. Moreover, we wanted to identify intermediate assembly steps in this process of D1 protein turnover and compare the synthesis and assembly of the D1 protein with the other plastid-encoded PSII core proteins.

Therefore, we applied in vitro translation in isolated chloroplasts to study the D1 synthesis and incorporation into PSII complexes (eg. Ref. 25; see also Ref. 16). In addition we used a homologous run-off translation system, consisting of isolated thylakoids with attached ribosomes (26). Synthesis of proteins can only take place through elongation of the nascent chains associated with the membrane-bound ribosomes; reinitiation is not possible (26). In both translation systems, good translation rates of the PSII core proteins have been shown. However, to our knowledge, no information has been presented on the extent of incorporation of the translation products into thylakoid-bound complexes. Nor have these systems been used in the study of the D1 protein replacement. Since biosynthesis of chlorophyll and carotenoids can take place in isolated mature chloroplasts but not in isolated thylakoids and since stromal factors are naturally present in chloroplasts, a comparison of the two translation systems allows us to look specifically into the importance of biosynthesis of PSII cofactors and stromal factors for the D1 replacement. The translation products and incorporation into complexes were analyzed by use of sucrose gradient centrifugation.

MATERIALS AND METHODS

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, leaves were harvested in the first half of the light period. Immediately after harvest, the leaves were homogenized in medium A (330 mM sorbitol, 5 mM ascorbate, 2 mM EDTA, 20 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 0.5 mM KH2PO4, 5 mM Mes-KOH (pH 6.1)), and centrifuged for 2 min at 10,000 × g. The pellet was resuspended in medium B (330 mM sorbitol, 5 mM ascorbate, 2 mM EDTA, 20 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 2 mM NaNO3, 50 mM Hepes-KOH (pH 7.6)) and loaded on a Percoll cushion (35% in medium B) and spun for 10 min at 5000 × g at 2 °C. The intact chloroplasts were collected and washed once in medium C (330 mM sorbitol, 50 mM Hepes-KOH (pH 8.0), 10 mM dithiothreitol). All steps were carried out as quickly as possible, keeping the samples at temperatures close to 0 °C in order to keep the chloroplasts competent for translation.

In vitro Translation in Chloroplasts—In vitro translation in isolated chloroplasts was performed essentially as described in Ref. 26. The chloroplasts (0.4–0.5 μg of Chl·ml–1) were incubated for 10 min at 23 ºC in the light (about 50 microeinstein·cm–2·s–1) in a translation mixture containing medium C, 10 mM ATP, 10 mM GTP, and 10 μM of each amino acid (minus methionine). After this preincubation time, carrier-free 35S-labeled methionine was added in a final concentration of 0.4–0.5 μCi/ml. The labeled methionine was chased by adding 10 μM cold methionine to the translation mixture. Translation was stopped by adding a 10-fold volume of ice-cold medium B. To separate the thylakoid-bound translation products from the stromal products, the chloroplasts were lysed in RNase-free lysis medium, containing 46 μM Hepes-KOH (pH 7.6), 118 mM potassium acetate, 7 mM magnesium acetate, 5 mM dithiothreitol, 10 μM·ml–1 heparin. Subsequently, the thylakoids were washed twice in the same buffer, directly frozen, and stored at –20 °C.

Run-off Translation in Isolated Thylakoids—Isolation of rough thylakoids and in vitro translation assays with such thylakoids were carried out essentially according to Ref. 27. Intact chloroplasts were lysed in the RNase-free lysis medium. The membranes were spun down, washed twice, and resuspended gently in the lysis medium.

In vitro translation was carried out in room light at 23 °C in the lysis medium supplemented with 2 mM ATP, 0.2 mM GTP, carrier-free [35S]methionine (0.4–0.5 μCi/ml), 100 μM of each amino acid (minus methionine), and thylakoids at a concentration corresponding to 0.4–0.5 μg of chlorophyll·ml–1. The labeled methionine was chased by adding 10 μM cold methionine to the translation mixture. Translation was stopped by adding a 10-fold volume of ice-cold lysis medium. After translation, the thylakoids were washed twice with a large volume of the same buffer to remove non-incorporated methionine.

Sucrose Gradient Centrifugation—For separation of thylakoid-bound complexes, thylakoids containing 60 or 120 μg of chlorophyll were washed twice and finally resuspended in a 10 mM Tricine buffer (pH 7.8) at 1 mg of Chl·ml–1, and a mixture of protease inhibitors (Boehringer Mannheim) was added. The final concentrations of the inhibitors were apronitin (10 μg/ml), phosphoramidon (0.2 mg/ml), leupeptin (0.02 mg/ml), E64 (0.1 mg/ml), NaEDTA (0.5 mg/ml). Alternatively, 110 μM phenylmethylsulfonyl fluoride was added.

An identical volume of the same buffer containing 2% n-dodecyl β-D-maltoside (DM; Sigma) was added, and the thylakoids were solubilized in the dark on ice for 50 min. The suspension was mixed gently every 10 min to ensure homogeneous solubilization. Subsequently, the suspension was loaded on a linear sucrose gradient of 13 ml (0.1–1.0 M sucrose + 0.03% DM) and spun for 20 h at 180,000 × g at 2 °C in a SW40 rotor.

After centrifugation, the sucrose gradient was divided into 20 fractions, of equal volume from bottom to top, with a syringe and frozen directly. In the case the gradient was analyzed by SDS-PAGE, proteins were precipitated in 10% ice-cold trichloroacetic acid (>30 min on ice), collected by centrifugation, and resuspended in a SDS-solubilization buffer.

When needed, sucrose gradient fractions were concentrated by spinning in 3-ml Centricron concentrators with 3- or 10-kDa cut-off filter (Amicon), following the manufacturer’s instructions.

In this study of the Molecular Weight and Location of PSII Complexes in the Sucrose Gradients—In order to estimate the molecular weight and identity of the different protein (sub)complexes in the sucrose gradient,
Isolated PSII-enriched membranes (BBY's; Ref. 27) were suspended in 10 mM Tricine (pH 7.8) containing 1% DM at 0.5 µg of Chl ml⁻¹ and were loaded directly or after 1 h of solubilization on ice on sucrose gradients. PSII core complexes (prepared according to Ref. 28) were resuspended at 0.5 µg of Chl ml⁻¹ in 0.5% or 1% DM in 10 mM Tricine buffer (pH 7.8) and directly (without solubilization time) spun on sucrose gradients as described above. To study in which fraction PSII reaction center particles were located, CP43, CP47, the extrinsic proteins from the oxygen evolving complex (OEC), and possible residual antennae proteins were removed from the PSII cores by solubilization (15 min, dark, on ice) in a 20 mM Bis-Tris buffer (pH 6.0), 1% Triton X-100, and 4 mM LiClO₄ (29). After solubilization, the suspension was desalted on a Sephadex G-25 column (Pharmacia Biotech Inc.), eluted by a 10 mM Tricine buffer (pH 7.8) including 0.03% DM and concentrated on Centricon concentrators (30-kDa cut-off filter, Amicon). The concentrate, containing the PSII reaction centers and stripped proteins, was spun on sucrose gradients.

To estimate the molecular weight of the various complexes distributed in the sucrose gradients, a set of native reference proteins (Phar- tronett, dark on ice) in a 20 mM Bis-Tris buffer (pH 6.0), 1% Triton X-100, and 4 mM LiClO₄ (29). After solubilization, the suspension was desalted on a Sephadex G-25 column (Pharmacia Biotech Inc.), eluted by a 10 mM Tricine buffer (pH 7.8) including 0.03% DM and concentrated on Centricon concentrators (30-kDa cut-off filter, Amicon). The concentrate, containing the PSII reaction centers and stripped proteins, was spun on sucrose gradients.

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D1 Protein Incorporation into Photosystem II

**Fig. 2.** Sucrose density gradient centrifugation of n-dodecyl β-o-maltoside-solubilized thylakoid membranes. Prior to loading on the sucrose gradient (0.1–1.0 M sucrose), the thylakoid membranes (120 μg of Chl) were solubilized in 1% DM for 60 min on ice. Sucrose gradients were centrifuged for 20 h at 108,000 × g from bottom to top into 10 or 20 equal fractions. a, photograph of a typical sucrose gradient of DM-solubilized thylakoid membranes. Bands are indicated by Roman numerals (I–VI, from bottom to top). b, Assignment and estimated molecular weight of the complexes and proteins in the sucrose gradients. Molecular weight distribution and assignment of the complexes were determined on the basis of the data shown in Figs. 3 and 4 and cited literature. At the bottom of the figure, additional information from literature used for the assignment is summarized.

This assignment is based on the analysis of the fractionated sucrose gradients by means of SDS-PAGE followed by Coomassie staining (Fig. 3a) or silver staining (not shown), immunodetection on Western blots (Fig. 3, b and c), 77 K chlorophyll fluorescence (Fig. 3d), and pigment analysis (Table I). Calibration of the molecular weight distribution was done using molecular marker sets (not shown) and isolated PSI preparations (BBYs, PSI cores, and PSI reaction center particles; not shown). Fig. 2b (bottom) summarizes additional information from literature (e.g. Refs. 3, 37, and 38) used for the assignment.

In the bottom fraction of the gradients (Fig. 2b), ribosomes and polyisomes (molecular mass of ribosomes about 2500 kDa) were found by immunodetection2 (not shown). Partial solubilization of the ribosomes occurred, and ribosomal subcomplexes were found predominantly in the bottom of the gradient (fractions 1–5).

Band I, close to the bottom of the tubes (Fig. 2b), contains predominantly PSI as judged from staining (Fig. 3a) and immunodetection (Fig. 3b) of the gels and the characteristic 77 K fluorescence peak at 735 nm (Fig. 3d; see Ref. 39). The complexes in band I have an estimated molecular mass of 550–700 kDa, suggesting that PSI is here in dimeric form (see Ref. 40).

Band II (molecular mass 300–400 kDa, as calibrated by molecular size markers; data not shown) contains the bulk of monomeric PSI complexes (estimated molecular mass is 340 kDa according to Ref. 40), which is concluded from the Coomassie-stained gels, immunoblots, and the 77 K fluorescence peak at 735 nm (Fig. 3, a, b, and d, respectively). PSIII complexes are also found in this band, judging from staining and immunodetection of the gels (Fig. 3, a–c) and the 77 K fluorescence peaks at 685 and 695 nm (typical for PSIII complexes; see Ref. 39) (Fig. 3d). These PSIII core complexes contained a variable amount of the extrinsic proteins of the OEC (clearly to be observed in Fig. 3 (a, stained gels; b and c, immunoblots) and some of the chlorophyll a/b antennae (eg. CP29). The core of DM-solubilized isolated BBYs migrates in this band (not shown).

Band III (Fig. 2b) (200–280 kDa) clearly contains the bulk of the PSI core complexes, as judged from the 77 K fluorescence peaks at 685 and 695 nm (Fig. 3d) and immunoblotting (Fig. 3, b and c). In the Coomassie-stained gels (Fig. 3a), all the main PSI core proteins (D1, D2, CP43, and CP47) are clearly detectable. Isolated PSI core complexes (28), containing D1/D2psbB gene product/CP43/CP47/cytochrome b599, and possibly some of the other small PSI proteins (<10 kDa), are recovered in this band. No LHCl proteins were found in this band, which is consistent with the high chlorophyll a/b ratio of around 50 (Table I).

Bands IV and V predominantly contain the chlorophyll a/b-binding proteins of the antennae of PSI (see also Refs. 38 and 41). This is also reflected in the low chlorophyll a/b ratio of around 1.5 (Table I) and the 77 K fluorescence peak at 677 nm. Band IV contains the bulk of 25- and 27-kDa chlorophyll-binding proteins of LHClI (Fig. 3, c and d), while band V is relatively enriched in the minor chlorophyll a/b proteins CP24, CP26, and CP29 (data not shown; see Fig. 3a). According to the estimation with the molecular size markers (not shown), band IV contains complexes of around 80–150 kDa while band V contains free proteins and complexes of around 40–70 kDa. Minor amounts of PSI core proteins (less than 5%) is also found in band IV, V, and VI (Fig. 3, b and c). Notably, PSI reaction center particles, isolated as reference material, did run in Band IV (not shown). Finally, band VI (Fig. 2b) was very much enriched in carotenoids trapped in DM micelles (Table I).

For the study of the mechanism of incorporation of the newly synthesized PSI proteins it is essential to know where “free” or unassembled D1, D2, CP43, and CP47 proteins are expected to sediment in the gradient. All four of these hydrophilic proteins are expected to bind detergent, adding 10–20 kDa to the molecular mass. In case of CP43 and CP47 a substantial number of proteins (see bottom Fig. 2b) could be bound (apoprotein versus holoprotein), adding another 15–25 kDa to the molecular mass. Based on the calibration with the 14C-labeled markers, unbound CP43 and CP47 was expected and did indeed run in the top of band IV and lower part of band V (not shown), while unbound D1 and D2 proteins are expected to run in band V and to a lesser extent in band VI (confirmed by preliminary experiments using separation by isoelectric focusing; data not shown).

The hydrophilic OEC proteins are expected to bind less detergent and were accordingly found in and above band VI (Figs. 2b and 3, a–c), in agreement with the soluble 14C-markers.

We can thus conclude that sucrose gradient analysis of solubilized thylakoid membranes provides a good tool to study the incorporation of the newly synthesized PSI core proteins into subcomplexes. The analysis makes it possible to follow the
release of nascent chains from the ribosomes and identification of at least four assembly steps: (i) free protein, (ii) PSII reaction center, (iii) PSII core complexes without chlorophyll a/b antennae and OEC, and (iv) PSII core complexes with some of the chlorophyll a/b antennae and OEC proteins bound.

Distribution of $^{35}$S-Labeled Translation Products in Sucrose Gradients

Translation in Isolated Chloroplasts—The sucrose gradient centrifugation described above was used to study the extent of
incorporation of the newly synthesized PSII proteins into PSII (sub)complexes. A pulse time of 30 min followed by a chase of 30 min was chosen since this pulse time gave optimal incorporation per chloroplast (not shown). After a 30-min chase, nearly all nascent chains were fully elongated and processing of pD1 was complete (see Fig. 1). Longer chase times were avoided, since proteolysis of translation products and rupture of the chloroplasts could be expected at the applied translation temperature of 23°C.

Fig. 4a shows the distribution of the trichloroacetic acid-precipitated [35S]methionine in a sucrose gradient after the translation in isolated chloroplasts. The corresponding autoradiogram of the 35S-labeled synthesis products is shown in the lower part of the figure. Most of the radioactivity can be found in the fractions 6–16, from bands II and IV. Very little radioactivity is found in the bottom fractions of the tubes (attached to ribosomes; Fig. 2b) or in the top of the gradient, where small fragments of nascent chains could be expected. The autoradiogram shows significant amounts of newly synthesized D1, D2, CP43, CP47, CF1$, and PSI-A, B. The four PSII core proteins are well separated and were quantified in each fraction (Fig. 4b and Table II). The normalized distribution for each of the proteins is shown in Table II.

Several important results can be found in Fig. 4 and Table II.

(i) In agreement with Fig. 1, it can be observed that the D1 protein accounted for more than 50% of the newly synthesized PSII proteins (Table II).

(ii) Most of the newly synthesized D1 protein was found in band III (41%) and band II (20%), representing different forms of PSII core complexes (Fig. 2b).

(iii) Substantial amounts (25%) of labeled D1 protein were found in band IV, representing complexes with molecular mass values from 80 to 190 kDa. The most likely composition of these smaller complexes is a D1-D2 heterodimer, a PSI reaction center consisting of D1-D2-cytochrome b$_{559}$-psbL, or PSI reaction centers with CP47 attached, with calculated molecular masses of 70, 100, and 180 kDa, respectively (Fig. 2b; see "Discussion"). A 10–20-kDa contribution to the calculated molecular weight from attached detergents or lipids must be added.

(iv) A small amount of radiolabeled D1 protein (12%) was found in bands V and VI, representing unassembled D1 protein (see Fig. 2b).

(v) In contrast to the D1 protein, the newly synthesized proteins D2, CP43, and CP47 accumulated predominantly (>40%) as unassembled proteins in bands V and VI (D2) and in the top of bands IV and in V (CP43 and CP47). Thus, incorporation of these three proteins into PSII core complexes is not as efficient as for the D1 protein.

(vi) A minor proportion of the newly synthesized D2 protein and CP43 was incorporated into PSII core complexes (bands II and III), while incorporation of CP47 was barely observed.

(vii) Several polypeptide fragments were found in fractions 11–16. In particular, a 17-kDa fragment was very abundant in fractions 11–14. This fragment could be attached to a PSI subcomplex (or to another complex) of 40–200 kDa. It is less likely that this fragment results from proteolysis during solubilization of the samples for SDS-PAGE, since no significant accumulation of other smaller fragments was found in these fractions.

Translation in Isolated Thylakoids—To study the possible role of stromal factors in the D1 protein incorporation and reassembly process of PSII, the distribution of the labeled proteins was analyzed after run-off translation in isolated thylakoids. The distribution of trichloroacetic acid-precipitated radioactivity in the sucrose gradient and the accompanying autoradiogram is shown in Fig. 5a. Quantification of the distribution of radiolabeled pD1+D2, D2, and CP43 is shown in Fig. 5b and Table II. Not much effect of longer chase periods (60 min) was observed (not shown), indicating that after 30 min of chase the final stage for the assembly and incorporation of newly synthesized proteins had been reached.

As is evident from Fig. 5a, much of the radioactivity accumulated in band IV and V, in contrast to the situation in isolated chloroplasts (Fig. 4a), where most radioactivity accumulated in band III. The radiolabeled D1 protein was quite evenly distributed over band III (PSII core complexes), IV (PSII reaction center particles), and V (unassembled protein) (Fig. 5, a and b and Table II) but was hardly detectable in band II.

Thus the absence of stromal components limited the incorporation of newly synthesized D1 into PSII core complexes.

Newly synthesized D2 protein was located either in band III (PSII core complexes) or in band V (as unassembled protein). Less radiolabeled D2 protein was found between band III and V, in contrast to the situation for the D1 protein (Fig. 5, a and b). Labeled CP43 was chased to a limited extent (about 30%) into PSII core complexes (band III), while the rest remained unassembled in the membrane. CP47 was very clearly confined to fractions 12–14 (Fig. 5a).

It can thus be concluded that components needed for incorporation of D1 protein, D2 protein, and CP43 into PSII core complexes in band III were limiting. In this respect it is interesting to note that the amount of synthesized D2 and CP43 in band III was approximately identical, suggesting that they may be located in the same complexes. Further purification experiments are in progress to verify this possibility.

### Table II

| Protein | Total | >280 kDaa | 195–240 kDa | 70–195 kDa | 5–70 kDa | Total |
|---------|-------|------------|-------------|------------|----------|-------|
| D1      | 53/62 | 22/1       | 41/33       | 25/36      | 12/30    | 100/100|
| D2      | 23/20 | 12/0       | 30/28       | 18/21      | 40/51    | 100/100|
| CP43    | 17/15 | 8/0        | 26/31       | 26/32      | 40/37    | 100/100|
| CP47    | 7/3   | 0/NDb      | 23/ND       | 46/ND      | 31/ND    | 100/100|

|       | %     | %       | %       | %       |         |       |
|-------|-------|---------|---------|---------|---------|-------|
| D1    | 100/100|         |         |         |         |       |
| D2    | 100/100|         |         |         |         |       |
| CP43  | 100/100|         |         |         |         |       |
| CP47  | 100/100|         |         |         |         |       |

- a Estimated molecular weight.
- b Band.
- c In chloroplasts.
- d Not determined (too little protein).
Fig. 4. Distribution of stable incorporated \[^{35}\text{S}\]methionine in proteins and (sub)complexes after translation in isolated chloroplasts and fractionation by sucrose density gradients. Translation in isolated chloroplasts was carried out for 30 min and was followed by a 30-min chase with cold methionine. After translation, chloroplasts were lysed and the thylakoid membranes were washed repeatedly, solubilized in 1% DM on ice, and spun on a sucrose gradient. After fractionation of the sucrose gradient, the fractions were precipitated in 10% trichloroacetic acid. a, quantitative distribution and autoradiogram of trichloroacetic acid-precipitated \[^{35}\text{S}\]methionine in the fractions of the sucrose gradient. Quantification of the distribution of radioactivity was done by scintillation counting of 1% of each fraction. Gels were dried prior to exposure to film. The fraction numbers (I–19) and bands (I–VI) are indicated. \(^{14}\text{C}\)-Labeled marker proteins were run in the outer lanes. b, quantification of the distribution of \[^{35}\text{S}\]-labeled D1, D2, CP43, and CP47 in the fractions from sucrose gradients. Quantification of the newly synthesized proteins was performed by scanning of the autoradiograms shown in Fig. 4a. In order to allow direct comparison of the quantity of the four proteins, the intensity was corrected for the number of methionine residues in each of the proteins (D1 and CP47 contain 11 methionine residues, while D2 and CP43 contain 8 methionine residues each).
Fig. 5. Distribution of stable incorporated [\(^{35}\)S]methionine after translation in isolated thylakoids and fractionation by sucrose density gradients. Translation in isolated thylakoids was carried out for 5 min and was followed by a 30-min chase with cold methionine. After translation, the thylakoid membranes were washed repeatedly, solubilized in 1% DM on ice, and subjected to a sucrose gradient. After centrifugation of the sucrose gradient, each fraction was precipitated in 10% trichloroacetic acid. a, quantitative distribution and autoradiogram of trichloroacetic acid-precipitated [\(^{35}\)S]methionine in the fractions of the sucrose gradient. For details, see legend to Fig. 4a. b, quantification of the distribution of \(^{35}\)S-labeled D1, D2 and CP43 in the sucrose gradients. For details, see legend to Fig. 4b.
Sensitivity toward Endogenous Proteases

A substantial amount of radioactivity in bands IV and V was lost during solubilization in the absence of protease inhibitors (not shown). The autoradiograms showed that in the presence of the protease inhibitors more labeled D1, D2, CP43, and CP47 were recovered in bands IV and V (not shown). Little effect of the inhibitors was observed, however, on newly synthesized D1 incorporated into PSII complexes (band II and III) (not shown), indicating that if the newly synthesized products D1, D2, CP43, and CP47 are not incorporated into PSII complexes, they are easily accessible for endogenous proteases.

**DISCUSSION**

In a recent study, the replacement of the D1 protein and the reactivation of PSII after light-induced damage in intact cells of the green algae Chlamydomonas reinhardtii was investigated (10). It proved difficult to resolve biophysically different reassembly stages of PSII by this in vivo system, because of the absence of significant accumulation of reassembly intermediates. In the present study, an in vitro setup was developed in which translation and reassembly could be more easily controlled. The idea was that such a system should allow biochemical identification of the reassembly stages of PSII and investigation of the mechanism of replacement of the D1 protein.

Since the D1 protein is encoded by the chloroplast genome and since insertion in the thylakoid membrane is cotranslational, the translation systems of isolated chloroplasts and thylakoids (16, 25, 26) was used. The D1 protein was the most abundant PSII protein synthesized in this material isolated from mature leaves (Fig. 1). During translation in both systems also many other chloroplast-encoded proteins were synthesized, and the composition of synthesized PSII proteins was rather similar for both translation systems. This indicated that with the short pulse time applied (5 min), no soluble stromal factors or biosynthesis of cofactors or pigments were needed for translation elongation (see also Ref. 42 for the role of chlorophyll in elongation). Synthesis of the D2 protein was more pronounced than synthesis of CP43 and CP47, in agreement with the previous in vivo labeling of C. reinhardtii cells after short light induced inactivation of PSII (10). In this respect, it is relevant to note that the psbD gene (encoding for D2) and the psbC gene (encoding for CP43) form only one transcript and consequently translation of CP43 and D2 is polycistronic (see Ref. 2). Apart from the shorter lifetime of the D2 protein as compared to CP43 (see Ref. 43), the upstream location of psbD from psbC might contribute to the faster synthesis rate of the D2 protein as compared to CP43 in translation systems. Synthesis of CP47 was less than synthesis of CP43 (and the D2 protein), especially in case of translation in isolated thylakoids. This may reflect a different regulation of translation of the psbB transcript encoding for CP47 (see Ref. 2).

In contrast to the synthesis pattern in chloroplasts and thylakoids isolated from mature leaves (as described in the present study), the PSII core proteins (D1, D2, CP43, and CP47) are synthesized in approximately equal amounts in young, developing leaves (17) or etioplasts (eg. Refs. 44 and 45). This difference reflects the synthesis and assembly of new PSII complexes in young leaves in contrast to what is predominantly a replacement of the D1 protein into existing PSII complexes of mature leaves, as illustrated in Fig. 6.

To analyze the incorporation of the synthesized D1 protein and other PSII core proteins into PSII complexes, we developed a fractionation of subcomplexes based upon sucrose density gradient centrifugation following solubilization of thylakoid membranes in the mild, non-ionic detergent DM (Figs. 2 and 3). At the chosen detergent/protein ratio, solubilization time, and temperature, the thylakoid membranes were optimally solubilized, while integrity of the hydrophobic PSII core was not affected. By application of 77 K fluorescence, pigment analysis, calibration with molecular size marker proteins, different standard PSII preparations, and SDS-PAGE, followed by staining or immunodecoration with antibodies, we have shown that it is possible to discriminate between different sized PSII (sub)complexes by this procedure as summarized in Fig. 2b.

The sucrose gradient analysis of solubilized thylakoid membranes allowed identification of at least four assembly steps during the incorporation of the newly synthesized D1 protein, including free, unassembled proteins, PSII reaction centers, and different PSII core complexes.

Analysis on the sucrose gradients of the newly synthesized proteins from the chloroplast and the thylakoid translation systems showed that all nascent chains were released from the

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3 K. J. van Wijk, B. Andersson, and E.-M. Aro, unpublished results.
protein was more effectively incorporated into the PSII core membrane with a lifetime of at least 30 min, after their assembly into PSII reaction center particles. This indicated that a proportion of the D1 protein remained as a free protein or was at least partially assembled into PSII core complexes and a significant fraction of these proteins were present in the isolated chloroplasts. Consequently, this indicates that nuclear- and chloroplast-encoded proteins within the PSII core complexes were recycled and it also means that in the chloroplasts isolated from the leaves during the light cycle, a certain percentage of the PSII complexes is depleted of their D1 protein (Fig. 6).

To further investigate whether the incorporation of the D1 protein and reassembly of the PSII complex was dependent on soluble stromal factors and biosynthesis of cofactors (such as pigments and quinones), we repeated the sucrose gradient analysis with a run-off translation system, consisting of isolated thylakoids with attached ribosomes (Fig. 5 and Table I). During this run-off translation, the biosynthesis of plastquinones (Q), pheophytin, heme, chlorophylls, and carotenoids could not take place and most of the soluble stromal proteins were absent since the thylakoids were washed twice prior to the translation.

Although the composition of synthesized PSII proteins in the run-off thylakoid translation system was rather similar to that in isolated chloroplasts (Fig. 1), the absence of stromal factors clearly restricted the incorporation of D1 into PSII (sub) complexes. This indicated that although no soluble stromal factors or biosynthesis of cofactors or pigments were needed for translation elongation (see also Ref. 42), these factors appeared essential for efficient incorporation of newly synthesized D1 protein into existing PSII complexes.

Two major differences in the extent of D1 incorporation into PSII complexes were observed between the two translation systems, as summarized in Table I.

(i) In thylakoids, synthesized D1 protein was only to a limited extent assembled into PSII core complexes and a significant proportion of the D1 protein remained as a free protein or was assembled into PSII reaction center particles. This indicates that stromal factors were rate-limiting for incorporation of the D1 protein into PSII complexes.

(ii) In thylakoids, no labeled D1 was incorporated into the PSII cores with extrinsic proteins (band II). This indicates that in addition to the nuclear factors (see previous sections), stromal factors are required for the assembly step from complexes in band III to band II, representing fully assembled PSII complexes.

The PSII core proteins D2, CP43, and CP47 were also synthesized in both the chloroplast and thylakoid translation systems, but it should be stressed that incorporation into PSII complexes did occur to a much lesser extent than for the D1 protein. Instead, these three proteins accumulated predominantly (Figs. 4b and 5b, Table II) as free proteins in the thylakoid membrane with a lifetime of at least 30 min, after their release from the membrane-bound ribosomes. In the chloroplast translation system, these three core proteins differed in the extent of incorporation into PSII subcomplexes. The D2 protein was more effectively incorporated into the PSII core complexes than CP43 and CP47. This difference in incorporation reflects a difference in life-time of the three subunits and the need for replacement in the PSII complex (see Fig. 6 and Ref. 43).

In the thylakoid translation system, newly synthesized D2 protein and CP43 remained unassembled in the membrane (band V) or accumulated distinctly in PSII core complexes (band III). Some D2 protein accumulated into PSII reaction center complexes (band IV), as was observed in the isolated chloroplasts. Interestingly, the amount of D2 and CP43 that was incorporated into the PSII core complexes was approximately identical (Fig. 5b), suggesting that in the thylakoid translation system the same component(s) limits the incorporation of CP43 and D2 protein into the PSII core complex. This component is likely to be a soluble stromal factor since more of these two proteins could be incorporated into PSII core complexes in isolated chloroplasts.

The inefficient incorporation of D2, CP43, and CP47 into PSII reaction center particles (D2 protein) and core complexes (D2, CP43, and CP47) in both translation systems is likely to be related to the possibility that they were synthesized to assemble complete new PSII complexes, since the turn-over rate of these proteins is not as high as for the D1 protein (see Fig. 6). To assemble these new PSII complexes it is most likely that nuclear-encoded proteins are required, which explains the substantial accumulation as free proteins in the membrane of these three newly synthesized core proteins. These nuclear-encoded proteins could either be factors "assisting" in the assembly (e.g. chaperones or ligases for chlorophyll, carotenoids, and heme) or components of the PSII core complex itself. Until now, it was believed that all PSII reaction center proteins and core proteins were chloroplast-encoded, except for the three extrinsic proteins of the oxygen evolving complex. However, recently it has been suggested that a nuclear-encoded 6.1-kDa protein is also part of the PSII reaction center (4). If this protein is indeed part of the PSII complex, its presence in the thylakoid membrane may be a prerequisite for the assembly of new PSII centers and therefore explain that the incorporation of newly synthesized D1, D2, CP43, and CP47 proteins into complexes is limited in the two experimental in vitro systems (see Fig. 6).

The finding that such a large percentage of D2, CP43 and CP47 could accumulate in the membrane as "free," while little free D1 protein was found, has important implications for the model of PSII (re)assembly. It suggests that the synthesis of the D1 protein is well regulated in intact chloroplasts and probably also in vivo, while the D2 protein, CP43, and CP47 are synthesized in their relative abundance. The amount of synthesized D1 protein seems to depend on the need and possibilities for incorporation into a PSII complex. This suggests that other PSII core proteins (e.g. the D2 protein) play a regulatory role in the translation of the D1 protein (see Refs. 11 and 24), possibly through a cotranslational incorporation of the D1 protein into existing PSII complexes. In this respect it is worth noting that the D2 protein seems to be relatively abundant in pseudopolysome preparations containing ribosomes, psbA mRNA, and small membrane fragments.5

In a future study5 we will apply pulse-chase experiments to follow the process of elongation and termination of translation and to resolve the steps of incorporation of the D1 protein into PSII in more detail.

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4 R. Kettunen, K. J. van Wijk, B. Andersson, and E.-M. Aro, unpublished results.

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