Synthesis and assembly of thylakoid protein complexes: multiple assembly steps of photosystem II

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To study the synthesis and assembly of multisubunit thylakoid protein complexes, we performed [^{35}S]Met pulse and chase experiments with isolated chloroplasts and intact leaves of spinach (*Spinacia oleracea* L.), followed by Blue Native gel separation of the (sub)complexes and subsequent identification of the newly synthesized and assembled protein subunits. PSII (photosystem II) core subunits were the most intensively synthesized proteins, particularly *in vitro* and at high light intensities *in vivo*, and could be sequestered in several distinct PSII subassemblies. Newly synthesized D1 was first found in the reaction centre complex that also contained labelled D2 and two labelled low-molecular-mass proteins. The next biggest PSII subassembly contained CP47 also. Then PsbH was assembled together with at least two other labelled chloroplast-encoded low-molecular-mass subunits, PsbM and PsbT$_c$, and a nuclear-encoded PsbR. Subsequently, CP43 was inserted into the PSII complex concomitantly with PsbK. These assembly steps seemed to be essential for the dimerization of PSII core monomers. Intact PSII core monomer was the smallest subcomplex harbouring the newly synthesized 33 kDa oxygen-evolving complex protein PsbO. Nuclear-encoded PsbW was synthesized only at low light intensities concomitantly with Lhcb polypeptides and was distinctively present in PSII–LHCII (where LHC stands for light-harvesting complex) supercomplexes. The PsbH protein, on the contrary, was vigorously synthesized and incorporated into PSII core monomers together with the D1 protein, suggesting an intrinsic role for PsbH in the photoinhibition-repair cycle of PSII.

Key words: Blue Native gel electrophoresis, D1 protein, photosystem II assembly, PsbH, PsbW, thylakoid.

INTRODUCTION

The biogenesis and assembly steps of photosynthetic multiprotein complexes in the thylakoid membrane are poorly known, although the structures of these complexes have been resolved with high resolution [1–3]. Biogenesis and turnover of functional thylakoid protein complexes, the products of both the chloroplast and nuclear genomes, requires a stoichiometric synthesis and sequential assembly of the protein subunits and a ligation of a number of various cofactors.

Most emphasis, concerning both the structure and protein assembly, has so far been put on PSII (photosystem II), composed of more than 20 polypeptides [1,4]. The PSII–RC (reaction centre) complex, which consists of the D1 and D2 proteins, the α- and β-subunits of the cytochrome b_{559} (Cyt b_{559}) and the 4 kDa PsbI protein, forms the heart of PSII and is capable of charge separation [5]. Two light-harvesting chlorophyll *a* binding proteins, CP43 and CP47, are located on the opposite sides of the RC complex [1,6]. The proteins of the OEC (oxygen-evolving complex), which in higher plants and green algae consist of the PsbO, PsbP and PsbQ proteins, are bound to the luminal surface of the PSII core complex. Additionally, more than 10 LMM (low molecular mass) proteins have been resolved in the structure of PSII [1,4]. The functional PSII core complex of higher plants is located in grana membranes as a dimer, and is associated with the light-harvesting antenna complex. In PSII–LHCII (where LHC stands for light-harvesting complex) supercomplexes [7], the CP29 (Lhcb4) and CP26 (Lhcb5) proteins anchor the LHCII trimers consisting of Lhcb1 and Lhcb2 to the PSII core dimers. CP24 (Lhcb6), together with CP29 and CP26, apparently facilitates the binding of additional LHCII trimers, also containing Lhcb3, to the edge of the supercomplex [8].

Regarding the assembly of PSII proteins, a number of studies have revealed a co-ordinated accumulation in the thylakoid membrane of D1, D2 and CP47 [9–11], which suggests the function of these proteins as early assembly partners after or during the co-translational membrane insertion of the PSII subunits [12]. So far, however, the assembly of only the major chloroplast-encoded PSII subunits (D1, D2, CP43 and CP47) has been extensively addressed [11–14]. Less attention has been paid to nuclear-encoded subunits, and the assembly sequence of most of the LMM subunits has also remained obscure during PSII biogenesis and repair. On the other hand, studies with knock-out mutants of various LMM proteins have given essential information on the requirement of LMM subunits for stable assembly of PSII. For example, both the α- and β-subunits of Cyt b_{559} (PsbE and PsbF) have been shown essential for any stable assembly of PSII in higher plants [15,16], whereas many other LMM proteins are dispensable for autotrophic growth, particularly in cyanobacteria [17,18], but also for some LMM subunits in higher plants [19,20]. Distinct differences in the requirement of several LMM subunits for successful assembly and/or stability of PSII indeed exist between different organisms (for a review see [21]).

In the present study, we performed *in vitro* and *in vivo* [^{35}S]Met pulse–chase experiments with developing spinach (*Spinacia oleracea* L.) leaves and intact chloroplasts to understand which thylakoid proteins (chloroplast- and nuclear-encoded ones) and protein complexes are synthesized and assembled *in vitro* in intact chloroplasts and *in vivo* under low- and high-light conditions. Under all labelling conditions, the PSII core monomer was the

Abbreviations used: BN, Blue Native; Cyt, cytochrome; LHC, light-harvesting complex; LMM, low molecular mass; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; OEC, oxygen-evolving complex; PFD, photon flux density; PSII, photosystem II; RC, reaction centre.

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most vigorously synthesized thylakoid protein complex. Its assembly could be dissected into five distinct intermediate stages with the association of 13 labelled PSII core subunits, followed by PSII dimerization and finally the attachment of Lhcb polypeptides to form PSII–LHCII supercomplexes.

EXPERIMENTAL

Plant material and isolation of intact chloroplasts

Spinach (*S. oleracea* L.) was grown in a controlled growth chamber under a PFD (photon flux density) of 400 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} with a 10 h light/14 h dark rhythm at 23 °C. For *in vitro* translations, spinach leaves were harvested 1 h after the lights were turned on. Intact chloroplasts were isolated by Percoll™ step-gradient centrifugation [22]. Chlorophyll content was determined by the method described in [23].

In vitro translation

In vitro translations in isolated chloroplasts were performed essentially as described in [24]. After a 5 min preincubation of chloroplasts (0.5 μg $\cdot$ μl^{-1}) at 20 °C under low light intensities, [^{35}S]Met was added to a final concentration of 0.5 μCi $\cdot$ μl^{-1}, and chloroplasts were pulse-labelled for 2.5, 5 or 10 min under PFD of 50 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} followed by 2.5–30 min chase in the presence of 10 mM unlabelled methionine. The translation was stopped by adding a 10-fold volume of ice-cold lysis buffer (7 mM magnesium acetate, 118 mM potassium acetate and 46 mM Hepes/KOH, pH 7.6). Subsequently, the thylakoids were washed twice with 5 mM $MgCl_2$, 10 mM NaCl and 25 mM Mes/NaOH (pH 6.0). A mixture of protease inhibitors (2 μg $\cdot$ ml^{-1} antipain, 2 μg $\cdot$ ml^{-1} leupeptin and 0.1 mg $\cdot$ ml^{-1} Pefabloc; Roche, Indianapolis, IN, U.S.A.) was included in the lysis and wash solutions.

In vivo pulse labelling

Discs were cut from leaves harvested 2 h after the lights were turned on or from plants that were pre-illuminated at 22 °C under PFD of 1500 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} for 2 h. The leaf discs were pressed against coarse sandpaper to facilitate the incorporation of [^{35}S]Met while floating on a solution containing 6.7 μCi $\cdot$ ml^{-1} [^{35}S]Met in 0.4 % (v/v) Tween 20. Labelling was performed under PFDs of 50 or 600 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} at 22 °C, as indicated. After a 2 h pulse, leaf discs were rapidly washed with 0.4 % Tween 20 and the thylakoid membranes were isolated by the method described in [25].

BN (Blue Native)/PAGE, SDS/PAGE and immunoblotting

BN/PAGE was performed principally as described in [26] with minor modifications. Thylakoids were resuspended in medium A (25 mM Bistris/HCl, pH 7.0, 20 %, w/v, glycerol and 0.25 mg $\cdot$ ml^{-1} Pefabloc) to a final concentration of 0.5 mg $\cdot$ ml^{-1} chlorophyll, and an equal volume of 2 % (w/v) dodecyl β-D-maltoside (Sigma), freshly prepared in medium A, was added. Thylakoids were then solubilized on ice for 1 min and centrifuged at 18 000 ***g*** at 4 °C for 12 min. The supernatant was supplemented with 1/10 volume of a buffer (100 mM Bistris/HCl, pH 7.0, 0.5 M ε-amino-*n*-caproic acid, 30 % (w/v) sucrose and 50 mg $\cdot$ ml^{-1} Serva Blue G) and loaded on to a gel with a 5–12 % gradient of acrylamide in the separation gel. Electrophoresis was performed (using the Hoefer Mighty Small system from Amersham Biosciences) at 0 °C for 3.5 h by gradually increasing the voltage from 75 to 200 V. After BN/PAGE, the lanes were cut out and incubated in sample buffer [27] containing 5 % (v/v) 2-mercaptoethanol for 30 min at room temperature (21 °C), followed by separation of the protein subunits of the complexes with SDS/PAGE (15 % polyacrylamide) and 6 M urea.

After electrophoresis, the proteins were visualized by silver staining and autoradiography, or they were electroblotted on to a PVDF (Millipore, Watford, Herts., U.K.) membrane. Autoradiograms were quantified with Fluorchem image analyser (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). Western blotting with chemiluminescence detection was performed with standard techniques using protein-specific antibodies. The PsbR antibody was raised against oligopeptides corresponding to the amino acids 1–15 and 20–34 in the spinach PsbR protein (Eurogentec, Seraing, Belgium). Antibodies raised against LHCI and LHCII subunits were purchased from AgriSera (Vännäs, Sweden). D1, D2 and PsbZ antibodies were also produced against oligopeptides as described earlier [12,28]. Antibodies raised against other PSII subunits mentioned here were kindly provided by Dr R. G. Herrmann (Ludwig-Maximilians-Universitat Munchen, Munich, Germany; Cyt b_{559}), Dr R. Barbato (Università del Piemonte Orientale, Alessandria, Italy; CP43, CP47 and PsbO) and Dr W. Schröder (Umeå University, Umeå Plant Science Center, Umeå, Sweden; PsbW). It is noteworthy that, after a two-dimensional electrophoresis, immunodetection with various antibodies is often severely hampered.

MS and N-terminal amino acid sequencing

In-gel trypsin digestion and sample preparation for MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS analysis were performed manually as described in [29]. Samples were loaded on to the target plate by the dried-droplet method using α-cyano-4-hydroxycinnamic acid as a matrix. MALDI–TOF analysis was performed in reflector mode on a Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.). Internal mass calibration of the spectra was based on trypsin autodigestion products (*m*/*z* 842.5094 and 2211.1046). Proteins were identified as the highest-ranking result by searching in the NCBI database, using Mascot (www.matrixscience.com). The search parameters allowed carbamidomethylation of cysteine, one miscleavage of trypsin and 50 p.p.m. mass accuracy. For positive identification, the score of the result should be over the significance threshold level.

For N-terminal amino acid sequencing, thylakoid membrane proteins were transferred on to a PVDF membrane after SDS/PAGE and analysis was performed with an Applied Biosystems model 477A protein sequencer equipped with an on-line Applied Biosystems model 120A phenylthiohydantoin amino acid analyser.

RESULTS

Assembly of thylakoid protein complexes in isolated intact chloroplasts

Chloroplasts isolated from young rapidly expanding spinach leaves were used for *in vitro* translations to incorporate [^{35}S]Met into all the protein subunits of the thylakoid membrane complexes as much as possible, and not only to the D1 protein with high turnover rate [30]. It became clear, however, that the PSII repair cycle is always taking place in parallel with the *de novo* synthesis of PSII complexes, even though the chloroplasts were isolated from young leaves. Very short (2.5 min) pulse labelling was necessary to allow the chase of protein assemblies to gradually bigger-sized complexes, as shown in the autoradiogram of the BN/PAGE gel (Figure 1A, for identification of complexes see the

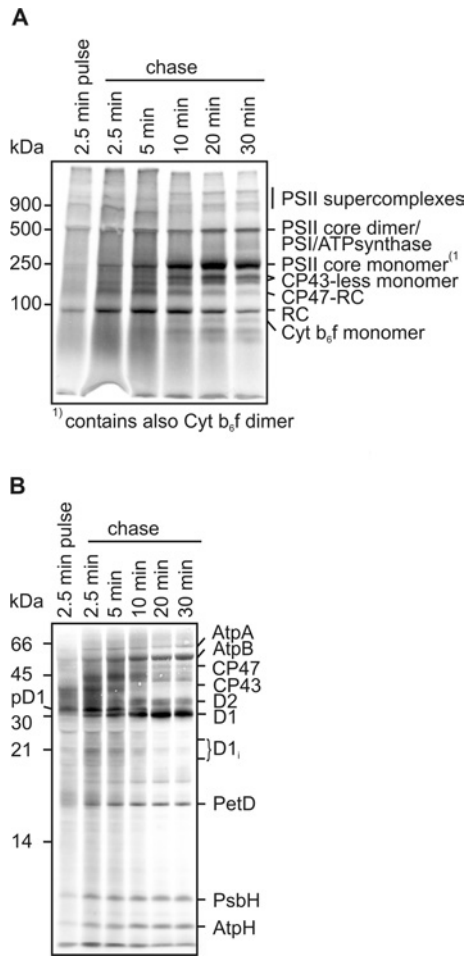

Figure 1 Autoradiograms demonstrating the synthesis and assembly of thylakoid membrane protein complexes in intact chloroplasts

Chloroplasts isolated from young spinach leaves were pulse-labelled for 2.5 min in the presence of [^{35}S]Met followed by a chase from 2.5 to 30 min. (**A**) BN/PAGE separation of newly assembled protein complexes and subcomplexes. PSII core dimer co-migrates with the PSI and ATP synthase, and the PSII core monomer with the Cyt b_6f dimer. (**B**) SDS/PAGE separation of labelled thylakoid proteins.

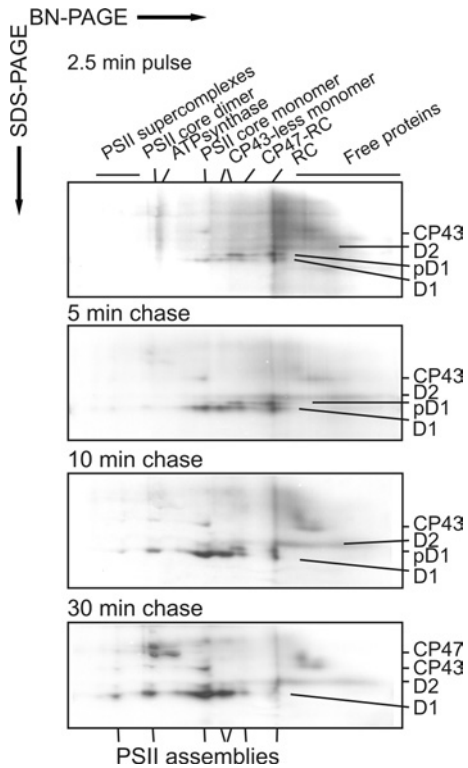

Figure 2 Time course of incorporation of the major labelled PSII core proteins into PSII assemblies of increasing size

The lanes of the BN/PAGE gel in Figure 1(**A**) were subjected to SDS/PAGE in the second dimension to separate the radioactively labelled proteins in distinct PSII subassemblies.

following sections). Prolonging the pulse to 5 min hampered the chase of protein complexes. After a 2.5 min pulse, a protein complex of approx. 100 kDa had accumulated some radioactivity. For 2.5, 5 and 10 min chases, bigger complexes also became clearly visible in the autoradiograms (~150, 180, 220, 250 and 500 kDa) and, on gradually increasing the chase times to 30 min, the ~250 and 500 kDa protein complexes were the most dominating ones, whereas the label clearly decreased in the smaller complexes (Figure 1A). Accumulation of the label in PSII monomer complexes is probably due to the ongoing PSII repair cycle and the relatively inefficient migration of PSII from stroma to grana thylakoids, a prerequisite for PSII dimerization, under the *in vitro* labelling conditions [31].

The pulse–chase samples were also analysed in denaturing one-dimensional SDS/PAGE to reveal whether any individual newly synthesized polypeptide starts degrading during the chase. This was not the case, at least for the PSII proteins, as shown in Figure 1(B). The label decreased with increasing chase time only in the precursor D1 (pD1) and the D1 protein translation intermediates ($D1_i$) of approx. 25 and 17 kDa [12], with concomitant increase in label accumulation in mature D1 of 32 kDa.

Identification of thylakoid proteins synthesized and assembled *in vitro* in intact chloroplasts

To identify the labelled newly synthesized and assembled polypeptides in each PSII (sub)complex, the lanes of the BN/PAGE in Figure 1(A) were subjected to SDS/PAGE in the second dimension (Figure 2) and the major PSII proteins, D1, D2, CP43 and CP47, were identified by immunoblotting (results not shown). The smallest assembly intermediate, seen already in the pulse sample, contained labelled D1 (or pD1, precursor D1) together with labelled D2 protein (Figure 2). During chase, the assembly of labelled CP43 protein could be detected only in the PSII core monomer. A short pulse (2.5 min) that was required for the chase of radioactivity from smaller to bigger PSII subassemblies (Figure 2) did not allow us to identify the assembly of other [^{35}S]Met-labelled PSII proteins. Indeed, both CP47 and most of the LMM polypeptides escaped the detection limits. To accumulate more radioactivity into CP47 (11 methionine residues) and the six chloroplast-encoded LMM subunits (PsbF, PsbH, PsbI, PsbK, PsbM and $PsbT_c$), which in spinach contain one to three methionine residues, we next increased the pulse time to 10 min followed by a 10 min chase. This resulted in a steady-state condition with all different radiolabelled PSII subassemblies shown in Figure 2 being present in the same gel, and all ten chloroplast-encoded proteins, known to incorporate methionine residues in spinach, could be visualized in different PSII subassemblies (Figures 3B and 3C). It was also tested that a further increase in labelling time (up to 60 min) did not reveal any new polypeptides in the autoradiograms (results not shown).

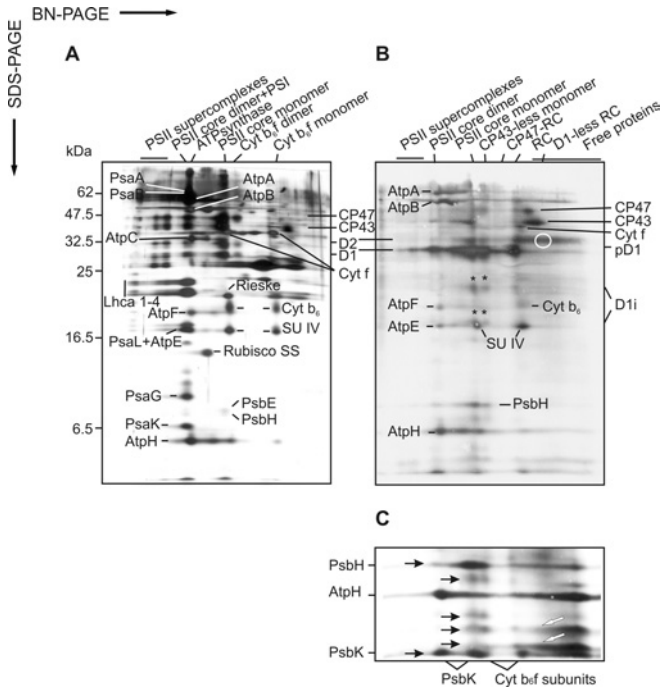

Figure 3 Assembly of the newly synthesized thylakoid membrane proteins into complexes in intact chloroplasts

(**A**) Silver-stained gel showing the distribution of thylakoid membrane proteins and protein complexes after two-dimensional BN/PAGE/SDS/PAGE separation. Molecular-mass standards for the second dimension SDS/PAGE are given on the left of the gel. (**B**) Autoradiogram showing the [^{35}S]Met-labelled translation products in different thylakoid protein assemblies after a 10 min pulse followed by a 10 min chase with excess of unlabelled methionine in intact chloroplasts. D2 protein of the apparent D1 receptor complex is denoted by a white circle. (**C**) Longer exposure of the lower part of the autoradiogram in (**B**), showing the assembly of translated LMM proteins into PSII complexes. The labelled LMM subunits, which are already present in the D1 receptor complex, are indicated by open arrows and subunits of bigger PSII assemblies are indicated by black arrows. $D1_i$ (*), D1 intermediate; SU, subunit; SS, small subunit.

A two-dimensional BN/SDS/PAGE gel stained with silver is shown as a reference to indicate the location and protein composition of the major thylakoid complexes (Figure 3A). An autoradiogram in Figure 3(B) depicts the newly synthesized [^{35}S]Met-labelled translation products and also reveals the presence of early PSII subassemblies not visible in silver-stained gels (Figure 3A) due to a low amount of such intermediate complexes. The identification of the newly synthesized [^{35}S]Met containing thylakoid proteins is presented in Table 1. In addition to the mature D1 protein, residual amounts of a precursor form of D1 (pD1) were seen in the smallest PSII subassemblies (for assigning the subcomplexes, see the next section). Some labelled translation products of approx. 17 and 25 kDa (marked with * in Figure 3B) in PSII subassemblies were identified as D1 synthesis intermediates by immunodetection with an antibody produced against the N-terminus of the D1 protein (results not shown, see also Figure 1B). These intermediates diminished during prolonged chase times (see Figure 1B). Altogether, six labelled proteins below 10 kDa (LMM proteins; Figure 3C, black arrows) were found in PSII subassemblies. PsbH and PsbK were definitively identified by N-terminal sequencing (Table 1). Four other labelled LMM PSII subunits represent PsbF, PsbI, PsbM and $PsbT_c$, which are the only additional chloroplast-encoded LMM subunits in spinach that contain a methionine residue. The N-terminal formylmethionine of PsbI, PsbM and $PsbT_c$, which is usually cleaved off, does not undergo processing in the case of spinach proteins [32,33]. It is also worth noting that spinach PsbE does not contain methionine residues.

Besides PSII proteins, several chloroplast-encoded subunits of ATP synthase ($CF_1\alpha$, $CF_1\beta$, CF_oI, $CF_1\varepsilon$ and CF_oIII encoded by the *atpA*, *atpB*, *atpF*, *atpE* and *atpH* genes respectively) were assembled into the complex and the Cyt *f*, Cyt b_6 and subunit (SU) IV proteins were found to be assembled into both the monomer and dimer forms of the Cyt b_6f complex (Figure 3B).

Subunit composition of the PSII assembly intermediates formed in intact chloroplasts

The newly synthesized D2 protein formed a long horizontal band in the second dimension gel (see Figure 3B), demonstrating a multiplicity of assembly intermediates of the newly synthesized D2 protein. Two labelled LMM proteins (Figure 3C, open arrows) were apparently assembled with the D2 protein before the assembly of the newly synthesized D1 (pD1) and were assigned as PsbF and PsbI, previously observed in the PSII–RC complex [34]. The PsbE protein was also detected in the PSII–RC complex by immunoblotting (Figure 4A).

The next PSII subcomplex with increasing size was present in autoradiograms only in small amounts (Figures 2 and 3B). Immunoblotting experiments, however, revealed the presence of CP47 in this subcomplex (Figure 4B), being in agreement with the size increment of the complex. However, a concomitant assembly of some unlabelled LMM subunits cannot be excluded. The complex was denoted as CP47–RC. Successful immunodetection of CP47 required 20 μg of chlorophyll to be loaded on to BN/PAGE gels (normally 10 μg), but in autoradiograms only the D1 protein was detectable (Figures 3B and 4B). Therefore it is conceivable that mostly unlabelled, *a priori* synthesized, CP47 is present in PSII subassemblies in *in vitro* experiments. Two subsequent assembly steps shown in Figures 1(A) and 2 were not separated from each other in Figure 3(B), but they collectively contained a newly synthesized PsbH protein and two other labelled chloroplast-encoded LMM subunits. Assuming that the PsbF and PsbI are present already in the PSII–RC complex, these labelled proteins must represent the PsbM and $PsbT_c$ subunits, both spinach proteins harbouring only one methionine residue. The fifth complex with increasing size showed an association of the CP43 protein together with yet another chloroplast-encoded newly synthesized LMM protein (Figures 3B and 3C), forming the PSII core monomer. Edman sequencing revealed this chloroplast-encoded LMM protein as PsbK (Table 1). Labelling of PsbK became even more distinguished in the PSII core dimer complex (Figures 3C and 5).

To obtain further information about the subunit composition of various PSII subassemblies (Figure 2), the chloroplast-encoded PsbZ protein that does not incorporate any radioactive methionine, and two nuclear-encoded subunits PsbW and PsbO were localized in similar BN/SDS/PAGE gels by immunodetection (Figure 4A). The PSII core monomer was revealed as the smallest PSII subassembly containing any PsbZ in spinach (Figure 4A). Surprisingly, a large portion of the protein was found free and not associated with any of the PSII subassemblies. Similarly, the PSII core monomer was the smallest subassembly where PsbO and PsbW could be found (Figure 4A). It should be noted, however, that PsbW was most distinctively associated with PSII supercomplexes, which also harbour the LCHII antenna polypeptides.

Synthesis and assembly of thylakoid proteins and protein complexes in leaves

We next performed [^{35}S]Met-labelling experiments with leaf discs cut from young spinach plants to detect the synthesis and assembly

Table 1 Identification of [^{35}S]Met-labelled thylakoid proteins

After *in vitro* or *in vivo* pulse labelling, the protein complexes were separated on BN/PAGE followed by SDS/PAGE. Identification was based on MALDI–TOF-MS, immunodetection (ID) and Edman sequencing (ES). For MALDI–TOF-MS analysis, the protein spots were cut from silver-stained gels, and for N-terminal sequencing, proteins were first transferred on to a PVDF membrane and then stained with Coomassie Blue.

Protein complex	Protein name, gene	Method of identification	Molecular mass (kDa)	Matched peptides/total	NCBI accession no.	N-terminal sequence
PSII	D1, *psbA*	ID	30*			
	D2, *psbD*	ID	32*			
	CP47, *psbB*	ID	46*			
	CP43, *psbC*	ID	43*			
	Cyt b_{559} α, *psbE*	MS, ID	9.4	3/4	11497544	
	PsbH, *psbH*	ES, ID	7.6		P05146	ATQTVESSSRSR
	PsbK, *psbK*	ES	4.3		P12163	XLPXAYAFLS
	PsbO, *psbO*	ID, MS	33*			
	PsbR, *psbR*	ID	10*			
	PsbW, *psbW*	ES, ID	5.93		Q41387	LVDERMST
LHCII	Lhcb1	ID	27*			
	Lhcb2	ID	26*			
	Lhcb3	ID	23*			
PSI	PsaG, *psaG*	MS	11*	3/3	131194	
	PsaK, *psaK*	ES	6.5*		P14627	XXFIXSXTNLIMV
LHCI	Lhca1	ID	22			
	Lhca2	ID	25			
	Lhca3	ID	26			
	Lhca4	ID	22			
Cyt b_6f	Cyt *f*, *petA*	MS	35	14/17	11497541	
	Cyt b_6, *petB*	MS	24	4/4	11497556	
	Rieske, *petC*	MS	27	6/6	2914267	
	Subunit IV, *petD*	MS	18	2/2	11497557	
ATP synthase	$CF_1\alpha$, *atpA*	MS	56	12/12	114527	
	$CF_1\beta$, *atpB*	MS	51.7	9/10	14718062	
	$CF_1\gamma$, *atpC*	MS	36.7	9/11	755801	
	$CF_1\varepsilon$, *atpE*	MS	14.8	3/4	11497534	
	CF_0I, *atpF*	MS	21.0	5/9	11497510	
	CF_0III, *atpH*	ES	5.5*		7636092	MNPLIAA
Rubisco	Rubisco large subunit, *rbcL*	MS	53.2	9/10	11497536	
	Rubisco small subunit, *rbcS*	MS	14.8	5/6	20150190	

* Based on migration on SDS/PAGE.

of thylakoid proteins under conditions where both the chloroplast- and nuclear-encoded proteins can be synthesized. Incorporation of label into intact leaves and subsequently into the thylakoid proteins required much longer pulse times compared with that in isolated chloroplasts, making the chase of the assembly intermediates impossible. *In vivo* translations were performed, however, to search for labelled newly synthesized proteins in PSII assembly intermediates, which were not present in *in vitro* translations. A 2 h pulse labelling of intact leaves under a PFD of 50 μmol $\cdot$ $m^{-2} \cdot s^{-1}$ allowed the synthesis of several nuclear-encoded PSII proteins including the various LHCII polypeptides and PsbO, the 33 kDa OEC protein (Figure 5, Table 1). The PSII core monomer was the smallest subcomplex harbouring some radioactively labelled PsbO, although most of the newly synthesized PsbO was detected unassembled. It was confirmed also by immunoblotting (Figure 4A) and MS analyses (results not shown) that no PsbO was present in any smaller subcomplexes. Interestingly, yet another PSII protein was found in CP43-less and intact PSII core monomers (Figure 5). No corresponding protein was detected in *in vitro* autoradiograms even after prolonged translations in intact chloroplasts (results not shown), strongly suggesting the nuclear origin of this protein. The size of 10 kDa, together with nuclear origin, hinted towards the PsbR protein, which prompted us to prepare an antibody against PsbR. The specificity of the antibody was tested with *Arabidopsis psbR* T-DNA insertion lines (M. Suorsa and E.-M. Aro, unpublished work). Indeed, the labelled 10 kDa protein was identified as PsbR by immunoblotting (Figure 5, immunoblot on the left side of the autoradiogram). Additionally, the synthesis and assembly at low light intensities of several LHCI proteins, the PsaG and PsaK subunits of PSI and nuclear-encoded subunits of the ATP synthase ($CF_1\gamma$, encoded by the *atpC* gene) and Cyt b_6f (Rieske iron-sulphur protein, encoded by the *petC* gene) were identified.

PsbW had so far escaped detection in autoradiograms, although it is another nuclear-encoded LMM subunit of PSII, which contains a methionine residue. To enhance the synthesis and assembly of the PsbW protein, which was demonstrated previously to be degraded to almost the same extent as the D1 protein during photoinhibitory light treatment [35], we next applied different light conditions for pulse experiments (Figures 6A–6C). Both immunoblotting (Figure 6B, lanes 1 and 2) and Edman sequencing (Table 1) revealed the migration of PsbW in SDS/PAGE just above the AtpH protein. The nuclear origin of this protein was further confirmed by labelling experiments in the presence of lincomycin and cycloheximide (Figure 6B, lanes 3 and 4). Interestingly, the newly synthesized labelled PsbW protein was present in low light translations (Figure 6B, lane 2; Figure 6C, left panel), but completely absent from high light translations (Figure 6B, lane 5; Figure 6C, right panel). A 2 h illumination of leaves under a PFD of 1500 μmol $\cdot$ $m^{-2} \cdot s^{-1}$, before pulse labelling under a PFD of 50 μmol $\cdot$ $m^{-2} \cdot s^{-1}$, reduced the synthesis (or membrane insertion) of most of the nuclear-encoded thylakoid proteins, whereas the synthesis of the chloroplast-encoded PSII subunits was not affected significantly (Figure 6A, compare lanes 1 and 2). When

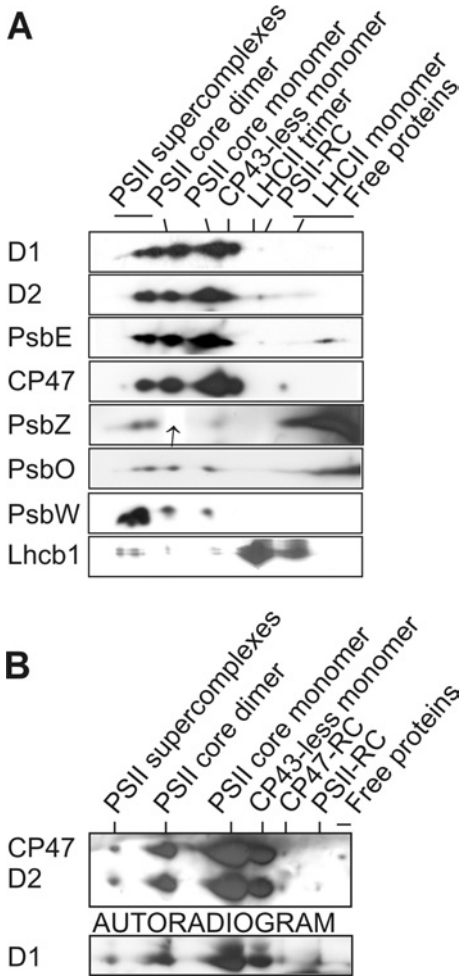

Figure 4 Immunodetection of PSII proteins in different PSII subassemblies separated by two-dimensional BN/SDS/PAGE

(**A**) After *in vitro* or *in vivo* translation, the thylakoid membranes (corresponding to 10 μg of chlorophyll) were separated first on BN/PAGE followed by SDS/PAGE. Proteins were transferred on to a PVDF membrane and immunodetected with different PSII protein antibodies. Immunoblots were obtained with D1, D2, PsbE and CP47 antibodies after *in vitro* translations and with others after *in vivo* translations. An arrow in the PsbZ immunoblot points to an area where an abundant amount of chlorophyll has prevented the binding of the antibody to the PsbZ protein. Note that the antibody raised against the Lhcb1 protein cross-reacts with the Lhcb2 protein. (**B**) Thylakoid membranes corresponding to 20 μg of chlorophyll were subjected to BN/SDS/PAGE as described above and proteins were detected with antibodies against D2 and CP47. Below is an autoradiogram of the same PVDF membrane showing the location of the [^{35}S]Met-labelled D1 protein in different PSII subassemblies.

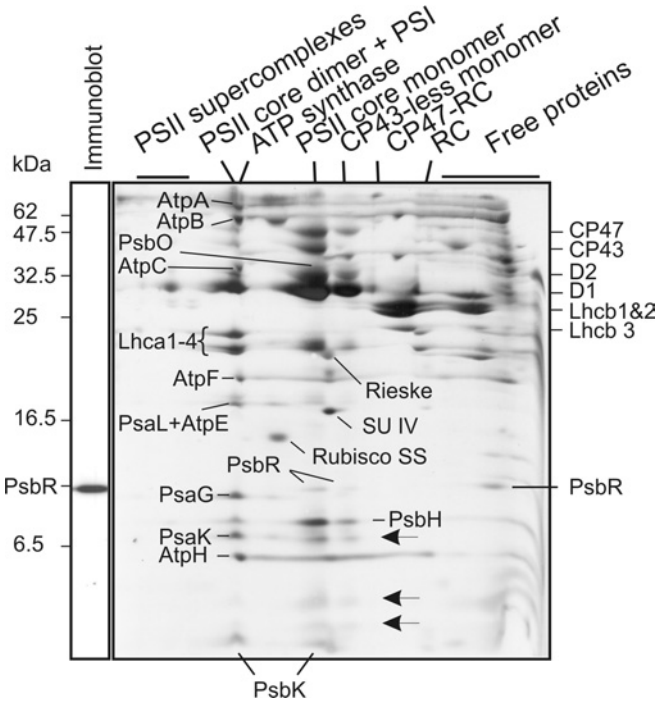

Figure 5 An autoradiogram showing the *in vivo* [^{35}S]Met-labelled thylakoid membrane proteins

Detached leaves were pulse-labelled for 2 h under PFD of 50 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} and the thylakoid membrane proteins (corresponding to 20 μg of chlorophyll) were separated first on BN/PAGE gel followed by SDS/PAGE. For identification of the newly synthesized proteins see Table 1. The labelled LMM subunits, which are present in the CP43-less PSII core monomer, are indicated by black arrows. On the left-hand side of the autoradiogram is an immunoblot showing the migration of the PsbR protein.

the pulse labelling was performed under a PFD of 600 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} (Figure 6A, lane 3; Figure 6C, right panel) and compared with a parallel experiment performed under 50 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} (Figure 6A, lane 1; Figure 6C, left panel), the accumulation and assembly of newly synthesized D1, D2 and PsbH proteins prominently increased, whereas the accumulation of most nuclear-encoded thylakoid proteins was decreased under these conditions. It is noteworthy that the synthesis of PsbW was decreased concurrently with that of the other nuclear-encoded proteins (e.g. the LHCII proteins), when the leaves were exposed to high light illumination (Figure 6B, lane 5). In this connection, it is also interesting to note that the *in vitro* labelling pattern of thylakoid proteins (Figure 6A, lane 4) very closely resembled the *in vivo* translations at high light intensity (Figure 6A, lane 3).

Relative turnover rates of the major protein subunits in PSII

When assessing the translation and assembly efficiencies of various [^{35}S]Met-labelled PSII core proteins, the number of methionine residues for each protein must be taken into account. Mature D1 and CP47 incorporate 11 methionine residues, whereas D2 and CP43 have eight methionine residues. Of the chloroplast-encoded LMM PSII subunits, PsbH has three and the PsbF, PsbI, PsbK, PsbM and PsbT$_c$ polypeptides have only one methionine in the mature spinach protein. Quantitative analysis of the label in each polypeptide, incorporated into the PSII core monomer complex during *in vitro* translations, expectedly revealed the most efficient incorporation of newly synthesized D1 protein into PSII (Table 2). Newly synthesized PsbH and D2 proteins were incorporated into PSII core monomer complexes two to three times less frequently when compared with the newly synthesized D1. Newly synthesized CP43 and CP47 proteins, on the other hand, were only scarcely present in PSII core monomer complexes, approximately in 10 % of the centres incorporating newly synthesized D1 protein. From these results, it can be concluded that, besides the D1 proteins, the PsbH and D2 proteins are also frequently replaced by newly synthesized protein copies in the PSII complex.

Since the high synthesis rate of the PsbH protein was a somewhat unexpected result, we next tested whether the PsbH protein also shows a high rate of degradation when spinach leaves were exposed to high light intensity. To this end, spinach leaves were first illuminated at low light intensities (PFD of 100 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) and subsequently exposed to growth light and photoinhibitory illumination (PFD of 1500 μmol $\cdot$ m^{-2} $\cdot$ s^{-1}) for 3 h. Analyses of the PsbH, D1, CP43, PsbW and Lhcb1 protein contents after

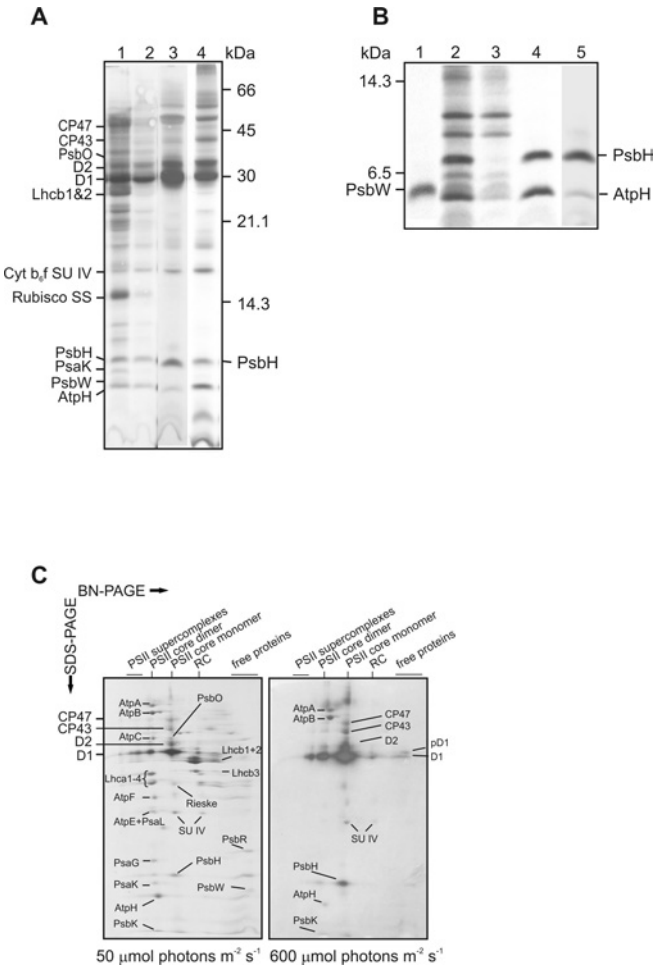

Figure 6 Autoradiograms demonstrating the translation efficiency of the nuclear- and chloroplast-encoded thylakoid membrane proteins at low and high light intensities

(**A**) Before thylakoid isolation and protein separation in SDS/PAGE, the leaves were pulse-labelled for 2 h under PFD of 50 μmol · m^{-2} · s^{-1} (lane 1), the leaves were first treated with a high-intensity light under PFD of 1500 μmol · m^{-2} · s^{-1} for 2 h and then pulse-labelled for 2 h under a low-intensity light (lane 2) or the leaves were pulse-labelled under PFD of 600 μmol · m^{-2} · s^{-1} for 2 h (lane 3). Thylakoids from *in vitro* translation for 10 min showing the chloroplast-encoded translation products are in lane 4. Thylakoids corresponding to 10 μg of chlorophyll were loaded on to each lane. (**B**) Demonstration of the synthesis of nuclear-encoded PsbW only at low-intensity light. PsbW immunoblot (lane 1) and an autoradiogram (lanes 2–5) showing labelled thylakoid proteins after *in vivo* 2 h pulse labelling under PFD of 50 μmol · m^{-2} · s^{-1} (lane 2), after a similar pulse labelling in the presence of lincomycin (lane 3) and in the presence of cycloheximide (lane 4), and after *in vivo* pulse labelling under high-intensity light (600 μmol · m^{-2} · s^{-1}) for 2 h (lane 5). (**C**) BN/PAGE/SDS/PAGE separation of thylakoid membrane complexes and protein subunits after pulse labelling of intact leaves for 2 h under a PFD of 50 μmol · m^{-2} · s^{-1} (left panel) and under a PFD of 600 μmol · m^{-2} · s^{-1} (right panel) to emphasize the differences in translation efficiency and assembly of the chloroplast- and nuclear-encoded proteins under low- and high-light conditions.

such treatments by immunoblotting revealed a degradation of both the D1 and the PsbH proteins during photoinhibitory illumination, whereas no degradation of the CP43, PsbW and Lhcb1 proteins was recorded (Figure 7). In contrast with growth light conditions, the repair of PSII complexes under photoinhibitory light apparently could not keep pace with degradation of damaged proteins, resulting in a partial loss of both the D1 and PsbH proteins. When lincomycin was present during illumination, the degradation of D1 and PsbH occurred already at growth light intensity and at high light intensity all PSII proteins were degraded possibly because of a complete collapse of the PSII complexes

Table 2 Comparison of the relative assembly rates of newly synthesized PSII core proteins into the PSII core monomer complex during *in vitro* pulse-labelling experiments at low-light conditions

To allow direct comparison of the assembly of the newly synthesized proteins, the intensity of the protein bands of the PSII core monomers on autoradiograms was corrected for the number of methionine residues in each protein (for details, see the text). Incorporation of [^{35}S]Met into the D1 protein was regarded as a reference and was given a value of 1.00. Measurements were made from three independent translation experiments.

PSII subunit	Normalized distribution of [^{35}S]Met in proteins of the PSII core monomer complex
D1	1.00
D2	0.33 ± 0.02
CP47	0.08 ± 0.04
CP43	0.11 ± 0.05
PsbH	0.39 ± 0.04

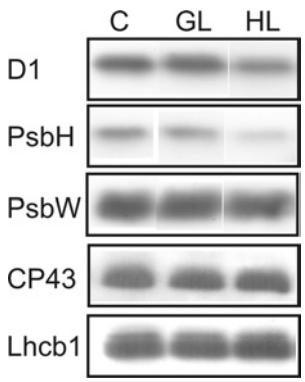

Figure 7 Degradation of the PSII core proteins

Spinach leaves were incubated under low light intensities (PFD of 100 μmol · m^{-2} · s^{-1}) (C, control sample) and then transferred for 3 h to PFD of 400 μmol · m^{-2} · s^{-1} (GL, growth light) and to PFD of 1500 μmol · m^{-2} · s^{-1} (HL, high light). Thylakoids were then isolated, proteins separated by SDS/PAGE and the D1, PsbH, PsbW and Lhcb1 proteins were immunodetected with protein-specific antibodies.

(results not shown). These results are in agreement with our pulse experiments both *in vivo* (Figure 6) and *in vitro* (Figure 3), showing that the newly synthesized PsbH protein is incorporated into the PSII monomer complex more frequently when compared with other PSII subunits, except for the D1 protein.

DISCUSSION

Five assembly steps of the PSII core monomer

Under all pulse labelling conditions, the PSII core proteins accumulated most radioactivity. Assembly of the D1 protein into PSII has been previously studied by *in vitro* translations in intact chloroplasts followed by fractionation of the PSII subassemblies in sucrose gradients [11–13]. Those experiments revealed only the assembly of major chloroplast-encoded PSII proteins D1, D2, CP43 and CP47, whereas the synthesis and assembly of PSII LMM subunits and the nuclear-encoded subunits, many of which also accumulate [^{35}S]Met, have remained elusive. In the present study, we used the two-dimensional BN/SDS/PAGE system for separation of both the *in vivo* and *in vitro* labelled and assembled thylakoid proteins and protein complexes, which allowed us to separate five distinct assembly intermediates of the PSII core monomer and to assign the assembly of 13 PSII core subunits. The summary of these PSII assembly steps is presented in Figure 8.

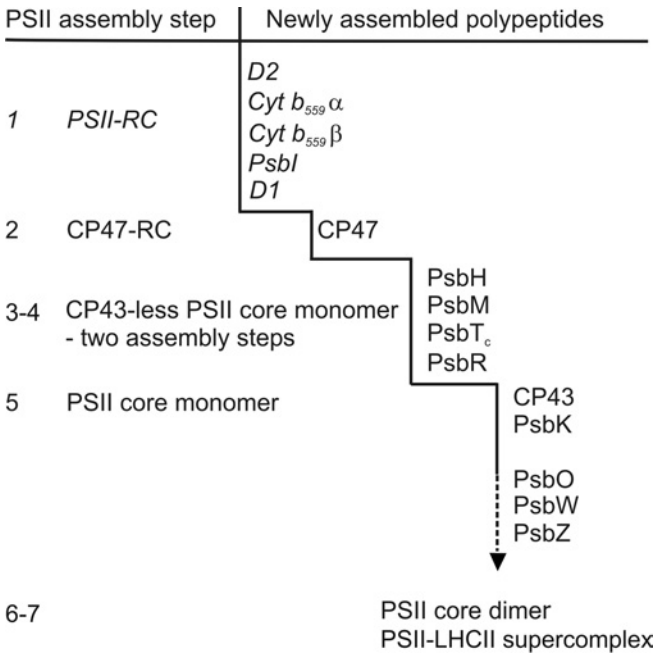

Figure 8 Presumed assembly steps of 15 PSII subunits

The order of the assembly of PSII subunits is based on the incorporation of each radioactively labelled protein subunit into a distinct PSII assembly intermediate. Only the assembly of the PSII–RC complex, shown in italics, has been extensively addressed in previous studies [11–13].

The first assembly step of PSII, leading to the formation of the PSII–RC complex (Figure 8, step 1 in *italics*), has been described previously [11,12]. It involves an assembly of the D1 protein into a previously formed 'receptor' complex composed of the D2, the α- and β-subunits of Cyt b_{559} and the PsbI proteins [34,36,37]. In the present study, we show that CP47 is directly associated with the RC complex, hence denoted as CP47–RC, before any labelled LMM subunit (Figure 8, step 2). The next two (Figure 8, steps 3 and 4) labelled PSII subassemblies with increasing size, collectively denoted here as a CP43-less monomer (see Figures 1 and 2), showed an association of PsbH and two other labelled chloroplast-encoded LMM proteins, the PsbM and $PsbT_c$ subunits, as well as one nuclear-encoded subunit, PsbR of approx. 10 kDa. PsbH, PsbM and $PsbT_c$ apparently stabilize the assembly of CP47. All of them are predicted to contain one transmembrane helix and have a molecular mass of 9, 3.7 and 3.8 kDa respectively [38,39].

The radioactively labelled nuclear-encoded PsbR protein, which is found only in higher plants, is also associated with the CP47–RC subcomplex. The physiological role of PsbR still remains unknown [40]. It has been suggested to be an extrinsic protein bound relatively tightly to the luminal surface of the thylakoid membrane in the vicinity of the water-splitting site [38] and was cross-linked with CP47 [41]. A prediction of transmembrane protein regions by the TMHMM program (http://www.cbs.dtu.dk/biolinks/), however, suggested a 22-amino-acid-long transmembrane helix located close to the C-terminus of PsbR. Thus it is possible that PsbR is an integral membrane protein with most of the N-terminus located on the luminal side of the thylakoid membrane. Based on the location and on the order of assembly before the OEC proteins, PsbR might have a role in anchoring some of the OEC proteins to PSII.

The next (Figure 8, step 5) biggest radiolabelled subassembly of PSII represented the PSII core monomer complex with assembled CP43. Intriguingly, an association of yet another labelled LMM subunit, identified as the PsbK protein, accompanied the assembly of the CP43 protein. In accordance with this result, a recent study [42] with *Chlamydomonas* revealed that PsbK is tightly bound to CP43, and moreover the assembly of PsbK was shown to occur only in the presence of CP43. Late association of PsbK with the PSII core monomer agrees with a peripheral location for PsbK in PSII [4].

The PSII core monomer was the smallest PSII assembly harbouring the newly synthesized (^{35}S-labelled) PsbO protein of OEC, and the assembly was confirmed by MS identification. In structural analysis, PsbO has been localized towards the CP47/D2 side of the PSII core [4,7], yet the assembly of CP43 protein was also a prerequisite for stable association of this OEC protein.

PsbW and PsbZ are probably located in the periphery of the PSII complex

PsbW is one of the few nuclear-encoded LMM subunits in PSII, and studies on its location in PSII have produced controversial results. Our immunoblotting approach, however, located PsbW predominantly to PSII–LHCII supercomplexes, and only minor amounts were found in PSII core dimers or core monomers (Figure 4A). In variance with these results, earlier studies have shown the presence of PsbW even in PSII–RC preparations [37,43,44]. Co-purification of PsbW with these preparations suggests that, although PsbW might be among the final proteins to be assembled into the PSII core monomer, it binds tightly to some of the PSII–RC proteins. More recent studies indeed seem to address the role of PsbW in a higher organization of PSII. *In organello*, imported PsbW was shown to assemble into PSII at the same moment with PSII core dimerization [45] and the *psbW*-deficient mutant was shown to lack the PSII supercomplexes [20]. Our observation that PsbW is synthesized only at low light intensities concomitantly with Lhcb proteins and is abundantly associated with PSII–LHCII supercomplexes corroborates these more recent results ([20,45]) and suggests the role for PsbW as a linker for LCHII binding to the PSII complex. This view is further strengthened by the fact that PsbW is present only in the grana-localized PSII complexes and is missing from PSII complexes located in the stroma-exposed thylakoid regions (preliminary results from our laboratory).

The most recently characterized chloroplast-encoded LMM subunit of PSII is PsbZ [19]. This 6.5 kDa protein is predicted to contain two membrane-spanning segments. The smallest PSII assembly found to harbour the PsbZ protein in spinach was the intact PSII core monomer containing CP43 (immunoblot in Figure 4). Notably, however, most of the PsbZ was found to be released from PSII complexes after a two-dimensional BN/SDS/PAGE of thylakoid membranes, together with the LHCII proteins (Figure 4). The PsbZ-deficient tobacco plants cannot assemble PSII–LHCII supercomplexes and their CP26 content is also decreased [19]. Because PsbZ apparently affects the binding of LHCII trimers, probably through stabilizing the association of CP26 with CP43, it is probably located towards the periphery of the PSII core, close to CP43.

Synthesis and assembly of thylakoid proteins at low and high light intensities

Studies on *in vivo* synthesis and assembly of thylakoid proteins at low and high light intensities revealed a distinct discrimination between the chloroplast- and nuclear-encoded proteins. Several nuclear-encoded thylakoid proteins were synthesized and incorporated into the membrane and/or the protein complexes only at low light intensities. These proteins include the Lhca, PsaG and PsaK proteins of PSI and the Lhcb and PsbW proteins of PSII. High light intensities, on the other hand, strongly favoured the

synthesis and assembly of chloroplast-encoded proteins, particularly the D1, D2 and PsbH proteins, whereas the synthesis and assembly of nuclear-encoded proteins was hardly recognized. *In vivo* high light translations and the assembly patterns of thylakoid proteins thus strongly resembled the *in vitro* translation patterns obtained in intact chloroplasts. Incorporation of labelled subunits into the Cyt b_6f complex and ATP synthase was not significantly affected by the light intensity during pulse labelling, although a lesser synthesis and assembly of nuclear-encoded subunits was observed in these complexes at high light intensity.

PsbH protein is frequently replaced in PSII core monomers

The accumulation of labelled PsbH into the PSII core monomer complexes turned out to be surprisingly high compared with that of CP43 and CP47, and even higher than the replacement of D2, which is known to become occasionally degraded in PSII [46,47]. This result, together with a specific disappearance of the PsbH protein from thylakoid membranes concomitantly with D1 when the leaves were illuminated with photoinhibitory light (Figure 7) or even at growth light when lincomycin was present, strongly suggests a high turnover rate for the PsbH protein. It is thus conceivable that the PsbH protein has a critical role in the PSII repair cycle. Degradation of the damaged D1 protein probably occasionally destabilizes PsbH and thereby exposes it to proteolysis.

The physiological role and function of this 9 kDa phosphoprotein is poorly understood. Mutant studies with *Chlamydomonas* have demonstrated the need of PsbH for stable accumulation of PSII cores [10,48]. Recently it was shown in cyanobacteria that PsbH is required both for a correct structure of the Q_B binding site and for initiation and completion of the PSII repair cycle [49]. Studies with *Arabidopsis* have revealed the phosphorylation of PsbH at two distinct sites, at the N-terminal Thr-2 and Thr-4 residues, of which the latter site is highly sensitive to the ambient light condition, being rapidly dephosphorylated when plants are placed in darkness [50]. However, the significance of the phosphorylation or the apparently high turnover rate of PsbH in PSII complexes is currently not known and calls for further investigation.

Conclusion

Taken together, the *in vitro* and *in vivo* pulse labelling of thylakoid membrane proteins, the latter being performed both under low- and high-light conditions, allowed us to dissect the assembly of PSII complexes into seven phases (five for PSII monomer followed by the formation of dimers and PSII–LHCII supercomplexes). This was accompanied by the identification of a stepwise association of 15 subunits (13 labelled and two identified by immunoblotting) and the late attachment of the Lhcb polypeptides (Figure 8). Synthesis of all essential PSII core proteins by the chloroplast translation machinery apparently facilitates the hierarchical and concerted assembly of PSII subunits on turnover of the D1 protein, by ensuring a rapid replacement of all those protein subunits that occasionally become targeted to degradation together with the D1 protein. Opposite to chloroplast-encoded PSII subunits, the translation and membrane insertion of nuclear-encoded thylakoid proteins were in general strongly down-regulated at light intensities exceeding the growth light conditions.

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