Novel approach reveals localisation and assembly pathway of the PsbS and PsbW proteins into the photosystem II dimer

Ellinor Thidholm[a,b,c,1], Viktoria Lindström[a,1], Christophe Tissier[d], Colin Robinson[d], Wolfgang P. Schröder[b,c], Christiane Funk[a,*]

[a] *Arrhenius Laboratories for Natural Sciences, Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden*
[b] *Södertörns Högskola (University College), Bipontus, P.O. Box 4101, SE-141 04 Huddinge, Sweden*
[c] *Karolinska Institute, Department of Medical Nutrition, Novum, SE-141 86 Huddinge, Sweden*
[d] *Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK*

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Abstract A blue-native gel electrophoresis system was combined with an in organello import assay to specifically analyse the location and assembly of two nuclear-encoded photosystem II (PSII) subunits. With this method we were able to show that initially the low molecular mass PsbW protein is not associated with the monomeric form of PSII. Instead a proportion of newly imported PsbW is directly assembled in dimeric PSII supercomplexes with very fast kinetics; its negatively charged N-terminal domain is essential for this process. The chlorophyll-binding PsbS protein, which is involved in energy dissipation, is first detected in the monomeric PSII subcomplexes, and only at later time points in the dimeric form of PSII. It seems to be bound tighter to the PSII core complex than to light harvesting complex II. These data point to radically different assembly pathways for different PSII subunits.

Key words: Assembly; PsbS; PsbW; Photosystem II dimer; Import; Blue-native polyacrylamide gel electrophoresis

1. Introduction

The chloroplast proteome of higher plants has been estimated to comprise between 2000 and 3500 proteins [1]. As the chloroplast genome only encodes roughly 90 proteins, the majority of the proteins are nuclear-encoded and must be imported into the chloroplast from the cytosol. Most of the imported proteins appear to use a common chloroplast import mechanism [2,3], whereas at least four distinct pathways have been identified for the subsequent targeting of proteins to the thylakoid network. Depending on the translocation mechanism, they are often referred to as the Sec-dependent, signal recognition particle-dependent, ΔpH-dependent and spontaneous pathways (for reviews see [4–6]).

Some imported proteins are functionally active in the chloroplast as single proteins, but the majority form elements of various multi-subunit complexes. In the thylakoid membrane, where photosynthetic electron transport and ATP synthesis occur, four major multi-subunit complexes are embedded: photosystem I (PSI; 540 kDa), photosystem II (PSII; 250 kDa), the cytochrome b_6f complex (260 kDa) and the ATP synthase (520 kDa). PSII in higher plants plays a key role in the overall photosynthetic process as it catalyses the light driven oxidation of water to molecular oxygen, and the reduction of plastoquinone to plastoquinol. At present more than 30 proteins have been identified or suggested to be part of this complex. The supramolecular organisation of PSII has for a long time been a matter of debate. Various biochemical analyses of the PSII complex have shown a dimeric organisation [7–9]. Furthermore, dimeric PSII/light harvesting complex II (LHCII) supercomplexes can be isolated directly from the thylakoid membranes, supporting a dimeric organisation of the complex in vivo [10]. More evidence was provided by 2D and 3D crystallisation studies, which revealed two regions of high density with an apparent two-fold rotational symmetry axis in plants and the thermophilic cyanobacterium *Synechococcus elongatus*, respectively [11–14].

The assembly and regeneration of multi-subunit thylakoid membrane protein complexes is very poorly understood. Recent investigations on plastid-encoded subunits suggest that assembly of the monomeric PSII complex is strongly dependent on newly synthesised D1 protein, which seems to be co-translationally inserted into the complex [15,16]. The assembly pathways undertaken by newly imported nuclear-encoded photosynthetic subunits, on the other hand, have not been analysed. Here, we devised a method to study the assembly of nuclear-encoded PSII proteins by combining an in organello import assay with a modified blue-native polyacrylamide gel electrophoresis (BN-PAGE) system [17,18]. The PsbW subunit was earlier shown to be important for the stabilisation of the PSII dimer in transgenic *Arabidopsis thaliana* plants [19]. This 6.1 kDa nuclear-encoded peptide consists of a single transmembrane helix and is located close to the PSII reaction centre [20]. In contrast to other thylakoid membrane proteins, its C-terminus is exposed to the stroma while the N-terminus is situated in the thylakoid lumen [21].

*Corresponding author. Present address: Department of Biochemistry, Umeå University, SE-90187 Umeå, Sweden.
Fax: (46)-90-786-7661.
E-mail address: christiane.funk@chem.umu.se (C. Funk).

[1] These authors contributed equally to this work.

Abbreviations: BN-PAGE, blue-native polyacrylamide gel electrophoresis; β-DM, *n*-dodecyl-β-D-maltoside; LHCII, light harvesting complex II; pPsbW, pPsbS, precursors of PsbW and PsbS, respectively; PSI, PSII, photosystem I and photosystem II, respectively; PVDF, polyvinylidene difluoride; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS, sodium dodecyl sulphate

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The localisation and assembly of the PsbS protein into PSII monomers and dimers were also analysed. The PsbS protein is involved in photoprotection of the LHC of PSII. Its location within the complex is a matter of debate, since it is absent in the LHCII/PSII supercomplex for which a 3D structure has been obtained [22]. While PsbW was found to be localised mainly in the PSII dimer, PsbS could be detected in both the monomeric and dimeric forms of PSII. It seems to be bound tighter to the PSII core complex than to the LHCII.

2. Materials and methods

2.1. Chloroplast isolation

Intact chloroplasts from pea (*Pisum sativum*) were prepared from seedlings grown for 8 days under a 12 h light/dark period at a light intensity of 50–100 μE m^{-2} s^{-1} [23]. High-light-treated plants were exposed to 2000 μE m^{-2} s^{-1} for 3 h before the chloroplast preparation. The chlorophyll content was determined in 80% acetone, calculated as in [24].

2.2. Generation and import of precursor proteins

The precursor proteins were prepared in vitro by transcription of the cDNAs using appropriate RNA polymerase followed by translation in a wheat germ lysate in the presence of [^{35}S]methionine. Precursor proteins were then incubated with intact pea chloroplasts essentially as in [25–27]. The import was terminated at different time points by washing the intact chloroplasts in 1 ml of ice-cold buffer (50 mM HEPES-KOH, pH 8.0, 330 mM sorbitol) per 25 μg chlorophyll. The sample was divided in fractions for BN-PAGE (15 μg chlorophyll) and sodium dodecyl sulphate (SDS)–PAGE (10 μg chlorophyll) and sedimented at 1200×*g* for 1.5 min in a microfuge at 4°C. The chloroplasts were resuspended and lysed in buffer (10 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$), at 1 ml/15 μg chlorophyll, then pelleted at 10 000×*g* for 3 min at 4°C. The thylakoid membrane pellet was prepared for SDS–PAGE or BN-PAGE as described below.

Mutagenesis of *Arabidopsis* pre-PsbW was carried out using the Quikchange™ method (Stratagene) according to the manufacturer's instructions.

2.3. Sample preparation for BN-PAGE

Thylakoid membrane pellets corresponding to 15 μg of chlorophyll were resuspended in 40 μl of BN-PAGE solubilisation buffer (50 mM Bis-Tris–HCl, pH 7.0 (4°C), 750 mM ε-amino-*n*-caproic acid and 20% glycerol) according to [17] and dodecyl-β-D-maltoside was added to a final concentration of 0.8%. After vortexing briefly, solubilisation was carried out on ice, in the dark, for 50 min, followed by 10 min centrifugation at 18 000×*g* in a microfuge at 4°C. The supernatant was supplemented with Serva blue G-250 from 5% stock in 500 mM ε-amino-*n*-caproic acid to a detergent/Serva blue G-250 ratio of 4:1 and directly loaded onto the gel.

2.4. BN-PAGE

BN-PAGE was carried out using the Hoefer SE 600 system (18×16×0.15 cm) from Amersham Pharmacia Biotech, according to [17,28] with modifications. The concentration of ε-amino-*n*-caproic acid was reduced by a factor of 13 (3×gel buffer: 150 mM Bis-Tris–HCl, pH 7.0 (4°C) and 10 mM ε-amino-*n*-caproic acid) and the acrylamide gradient in the separation gel was 4–13%. The upper running buffer contained 0.012% Serva G-250. The gel was run at 0°C at 5 mA, 90 V for 16 h. After the run the gel was washed for up to 6 h in a solution containing 30% ethanol and 10% acetic acid, then dried and exposed to a PhosphorImager plate or Biomax MS Kodak film. The plate was developed in the PhosphorImager (Fuji, type FLA 3000) after 2 days. The film was developed after 3 weeks. To analyse the protein content of the different bands the BN gel was electroblotted directly on polyvinylidene difluoride (PVDF) membrane, which was then immunostained according to [29].

2.5. 2D analysis of the BN-PAGE

Protein bands were cut out of the BN gel, washed in H_2O and homogenised in Laemmli solubilising buffer (0.125 M Tris–HCl pH 6.8, 20% glycerol, 4% SDS and 10% β-mercaptoethanol). Solubilisation was carried out for at least 3 h at 40°C and 30 min at 75°C. The bands were then loaded onto a 17.5% SDS gel containing 4 M urea and run at 10 mA overnight at 4°C [30]. Thylakoids, PSII membrane particles, prepared from pea according to [31], and PSI particles prepared from spinach (*Spinacea oleracea*) according to [32] were loaded as markers. The proteins were then silver-stained using the method of Wray et al. [33].

2.6. Isolation of PSII dimers using sucrose density gradient centrifugation

PSII dimers were isolated essentially as in [10]. Pea thylakoid membranes corresponding to 130 μg of chlorophyll were loaded on a continuous sucrose gradient. The supercomplex fraction containing the dimer was collected after centrifugation and spun down at 540 000×*g* for 30 min. The pellet was resuspended in BN-PAGE solubilising buffer and loaded onto a BN-PAGE, without the addition of further dodecyl-β-D-maltoside.

3. Results and discussion

3.1. BN-PAGE separates PSII monomers and dimers

BN-PAGE has been found to be an extremely useful tool to study intact protein complexes as it separates highly active, native membrane protein complexes [17,34] according to their molecular mass. The dye Coomassie G-250 introduces negative charges into the protein complexes and the presence of ε-amino-*n*-caproic acid supports the solubilising properties of neutral detergents [28]. Running the gel at pH 7.0 avoids protein denaturation and the use of Tricine as a trailing ion optimises the resolution in acrylamide gradient gels. Our aim was to adapt the system originally described by Schägger and von Jagow [28] to enable the separation of monomeric and dimeric forms of the PSII complex in order to study its assembly. Various factors in the sample preparation procedure were therefore optimised, including a reduction in the *n*-dodecyl-β-D-maltoside (β-DM) detergent concentration (to 0.8%) and an increase in the solubilisation time to 50 min on ice. To avoid heating of the sample during electrophoresis, the gel was run at 0°C and 5 mA for 16 h. Finally, the concentration

Table 1
Western blot analysis of the BN-PAGE complexes

BN band	Immune response with antibodies raised against:							
	D1	PsbS	PsbW	CP43	LHCII	PsaH	Rubisco	$F_1\beta$
1, 2 and 3	X	X	X	X	x			
4						X		X
5							X	
6						X		x
7	X	X		X				
8	X	x						
9					X			x

Distinct immune response is marked X and weaker immune response is marked x.

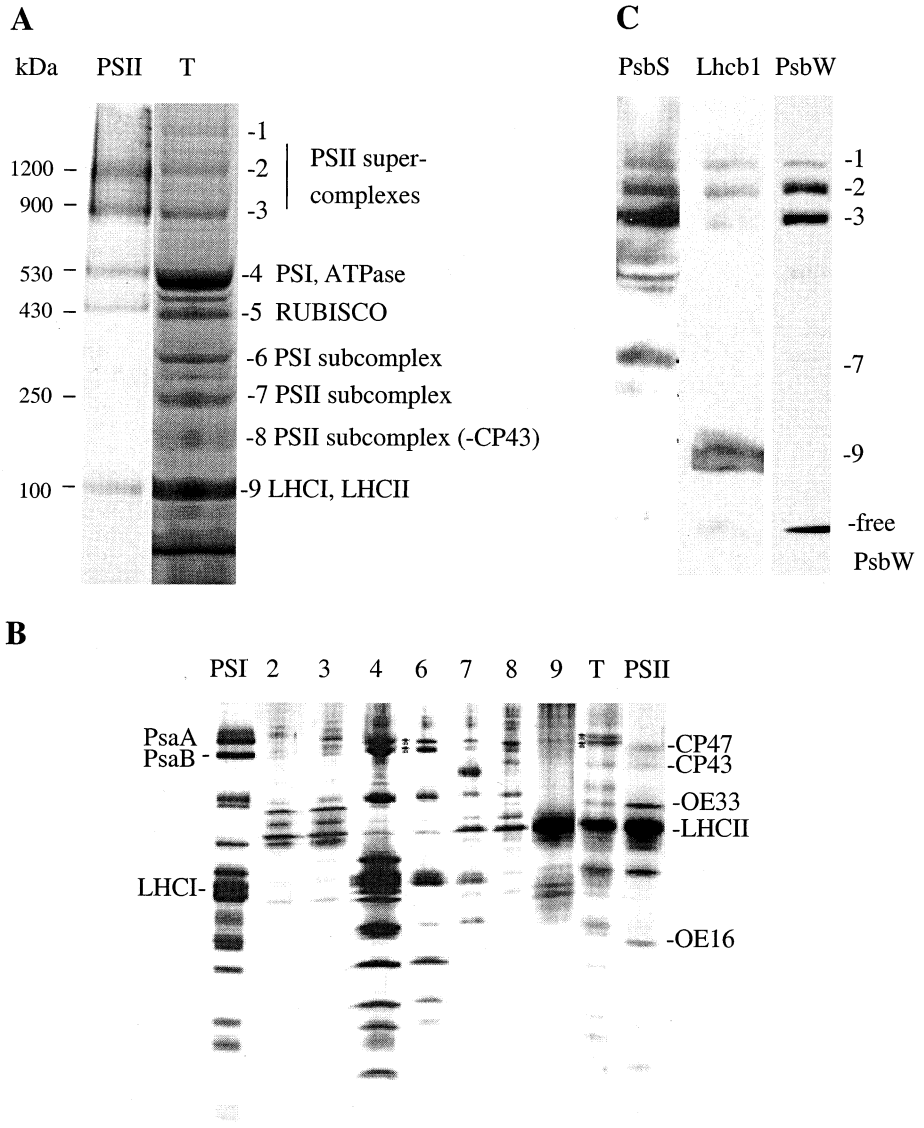

Fig. 1. A: BN polyacrylamide gel (4–13%), which resolves dimeric PSII supercomplexes and monomeric PSII subcomplexes. The PSII supercomplexes were isolated from pea by low resolution sucrose gradient centrifugation according to [10] (PSII). Lane 2 (T) shows pea thylakoid membranes corresponding to 15 μg of chlorophyll solubilised in 0.8% β-DM for 50 min on ice. C: Immunoblots of thylakoid membranes resolved on the BN gel. After BN-PAGE the bands were electroblotted onto PVDF membrane and immunostained with antisera raised against PsbS, Lhcb1 and PsbW. B: 2D analysis of the BN-PAGE bands. The major bands found after BN-PAGE were cut out, solubilised and run on denaturing SDS–PAGE with 17.5% acrylamide and 4 M urea. Thylakoids (T), PSII membrane fragments (PSII) and PSI preparation (PSI) corresponding to 1.5 and 1 μg chlorophyll, respectively, were used as markers. Silver stain was used for visualisation of the individual protein subunits.

of glycerol in the solubilisation buffer and the reduced concentration of ε-amino-*n*-caproic acid in the gel proved to be critical to obtain distinct dimeric PSII bands.

Fig. 1A shows a BN-PAGE of thylakoid membranes run with the above-described modifications. At least nine (numbered) distinct protein complex bands could be separated from solubilised thylakoid membranes (lane marked T). The content of the individual BN bands was determined by denaturing SDS–PAGE (Fig. 1B) and further characterised by performing direct immunoblotting against some selected marker proteins (Fig. 1C, Table 1). The silver-stained polypeptide profiles are shown in Fig. 1B together with a lane of total thylakoids (T), and separately isolated PSI and PSII preparations. The three uppermost bands (1–3) in Fig. 1A, denoted PSII supercomplexes, varied in relative intensities between different preparations and in many cases only two bands were observed (bands 2 and 3). They contain chlorophyll and are clearly green (see also Fig. 2A, lane T for colour print). The polypeptides in bands 2 and 3 co-migrated exactly with the proteins of a PSII preparation in an SDS–PAGE (Fig. 1B). Immunoblotting analysis supported the protein identification of the SDS–PAGE, and furthermore showed that the BN-PAGE separation was very good. Some of the immunoblotting results are shown in Fig. 1C and clearly show the majority of PsbW, PsbS, and a minor fraction of Lhcb1 being present in bands 1–3 (Fig. 1C, Table 1).

To obtain support for dimeric organisation of PSII bands 1–3, dimeric LHCII/PSII supercomplexes were isolated directly from pea thylakoids by sucrose gradient centrifugation according to [10] and loaded onto the BN-PAGE (Fig. 1A,

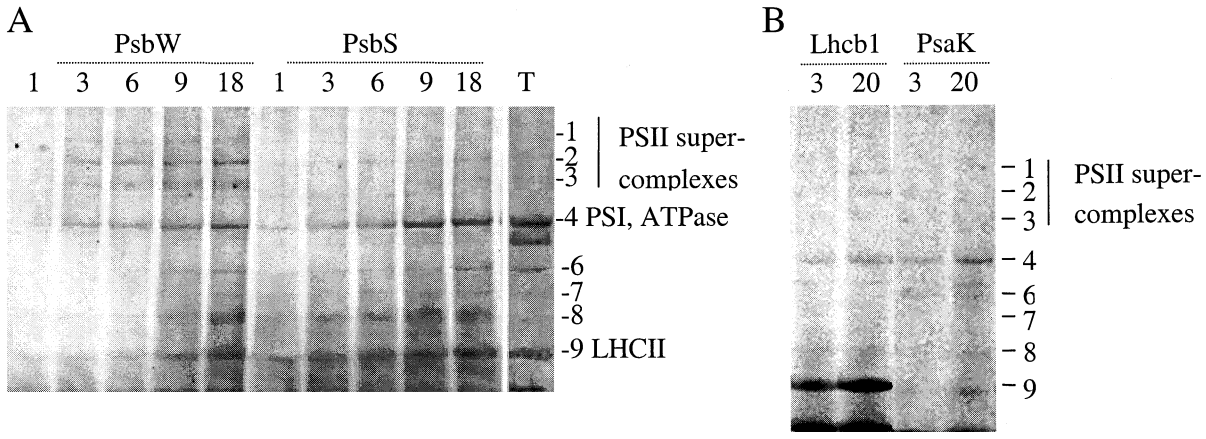

Fig. 2. Time course of the assembly of imported PsbW and PsbS on BN-PAGE detected by autoradiography. In vitro translated PsbW and PsbS precursors were incubated with intact chloroplasts and the import was terminated after 1, 3, 6, 9 and 18 min. Import mix corresponding to 15 μg chlorophyll was analysed on the BN gel and the radiolabelled proteins were visualised by autoradiography. Lane 'T' shows pea thylakoid membranes resolved by BN-PAGE. B: Assembly studies of Lhcb1 and PsaK using BN-PAGE and autoradiography. Import into intact chloroplasts was performed for 3 and 20 min. Import mix corresponding to 15 μg chlorophyll was analysed on the BN gel and the radiolabelled proteins were visualised by autoradiography.

PSII lane). As seen in the figure the isolated supercomplexes resolved after BN-PAGE in two strong bands of around 1200 kDa and 900 kDa, which co-migrate precisely with the upper bands from thylakoid membrane BN bands. Thus, BN band 2 (1200 kDa) probably corresponds to a complex of a PSII dimer (2×250 kDa), plus six LHCII trimers (110 kDa), while BN band 3 (900 kDa) consists of a PSII dimer plus four LHCII trimers. These findings are in good agreement to the supercomplex organisation suggested by Dekker and co-workers [35]. The weak band in the PSII lane with a molecular mass of around 500 kDa could thus be a PSII dimer without LHCII. Based on SDS–PAGE, immunoblotting and the co-migration analysis of isolated PSII supercomplexes we conclude that the BN gel is able to resolve dimeric PSII supercomplexes.

The other BN gel bands were characterised by similar means. Bands 7 and 8 were identified as two different PSII subcomplexes; their protein content was similar to PSII membrane fragments and Western blotting showed that these complexes contain the D1 and the PsbS protein together with some proteins of the minor antenna complex. Interestingly, the anti-CP43 antibody recognised this protein only in band 7, but not in band 8. These two bands clearly represent PSII in its monomeric form, the only difference being the presence or absence of CP43. Barber and co-workers isolated CP47 reaction centre complexes that were depleted of CP43, using sucrose gradient centrifugation and the detergent β-DM [36]. Therefore it seems that CP43 is more readily removed from the PSII core than CP47, despite the fact that both proteins are likely to be structurally similar.

The composition of bands 4 and 6 is dominated by the PSI and ATP synthase complexes. Immunoblotting of the PSI subunit PsaH showed this protein to be present only in bands 4 and 6 and a similar result was obtained for the ATP synthase (Table 1, PsaH and $F_1\beta$). Indeed, the abundant α- and β-subunits of the ATP synthase, apparent in the thylakoid protein sample in Fig. 1B (lane T), are also clearly apparent in lanes 4 and 6 of Fig. 1B (denoted by asterisks). It appears from these data that the two abundant complexes of the stromal, non-appressed lamellae (those thylakoids in contact with the stroma) are not resolved. One possibility is that they co-migrate in this system, but we believe that band 4 (and to a lesser extent band 6) actually represents total, partially solubilised stromal lamella contents, which run as a single band in this gel system.

Band 9 contains the main fraction of LHCII. The molecular mass of 110 kDa corresponds to trimeric complexes. Immunoblotting (Fig. 1C) as well as the silver-stained SDS gel (Fig. 1B) revealed that most of the Lhcb1 is found in this fraction (Fig. 1C). However, the presence of some LHCI could also be detected in the SDS gel of band 9. This fraction might therefore represent partially solubilised stromal lamella and/or those proteins that were separated from the photosystems after solubilisation.

3.2. Localisation of PsbW and PsbS in the PSII dimer

One of the advantages of our BN gel system is that it can be directly electroblotted onto PVDF membrane and used for immunostaining (Fig. 1C). We used this advantage to study the localisation of PsbW and PsbS in native PSII complexes. The exact location of PsbS within the PSII complex is still unclear and disputed. Biochemical analyses suggested the pro-

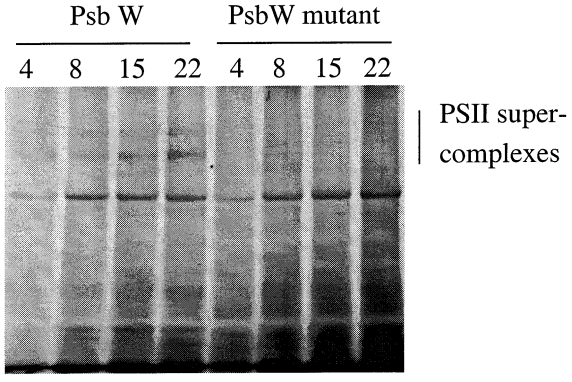

Fig. 3. Time course of the assembly of imported PsbW protein and a mutated PsbW precursor lacking the negatively charged N-terminal domain. In vitro translated precursor of PsbW was incubated with intact chloroplasts and the import was terminated after 4, 8, 15, and 22 min. Exactly the same procedure was performed on a mutated PsbW protein that has its N-terminal domain modified resulting in a positive net charge (D82N, E83Q, L92H, P93H, F94H).

tein to be connected to the PSII core complex [37,38], while its recently observed involvement in the photoprotective non-photochemical quenching of PSII implies close connection to LHCII [39]. PsbS appears not to be present in the 2D PSII crystals investigated by Barber and co-workers [22]. However, in this study we were able to immunodetect PsbS in dimeric PSII supercomplexes as well as in monomeric PSII, but not in the LHCII fraction (see Fig. 1C) after BN-PAGE. It therefore seems to be more tightly bound to the PSII core than to the fractions of LHCII. PsbW, on the other hand, is localised exclusively in the dimeric form of PSII. No immunoreaction could be seen in the PSII monomers (Fig. 1C). Previous studies have shown PsbW to be important for dimerisation of PSII [19] and its exclusive location in bands 1–3 is a further indication that these bands do represent PSII dimers.

3.3. Time course of assembly of newly imported PsbW and PsbS

After localising PsbW and PsbS in the various PSII bands, we used the BN gel system to analyse the assembly of newly imported, in vitro synthesised PSII subunits. The imported proteins are radiolabelled and can thus be specifically analysed in terms of assembly characteristics and kinetics. Besides the precursors of PsbW and PsbS we examined as a control Lhcb1, the well-characterised major element of the LHCII complex, and a low molecular weight protein of PSI, PsaK. All precursors were rapidly imported into the chloroplasts and processed to mature size as investigated on SDS–PAGE (not shown).

The assembly kinetics of PsbW and PsbS are shown in Fig. 2A. Lane T displays the electrophoretic separation of thylakoid complexes in the BN system for easier appointment of the different bands. In this experiment, pPsbW and pPsbS were imported into chloroplasts over a time course of up to 18 min, samples were removed, the chloroplasts lysed and the thylakoid membranes rapidly analysed by BN-PAGE. Immediately after import the PsbW protein was detected in band 4, to some extent in band 9 (stroma thylakoid fractions) and bands 1–3 (the PSII supercomplexes) of the autoradiogram. The kinetics of appearance of bands 1–3 are essentially identical to the import kinetics (not shown), indicating that this protein is assembled into the PSII dimer very soon after import and insertion into the stroma thylakoid membranes. At later time points the imported PsbW was also found in the PSII subcomplex (band 8) on the BN gel.

The appearance of PsbW in band 4 is consistent with this band representing the contents of the stroma lamellae. After import, PsbW is initially inserted into the stroma lamellae and it is therefore expected that a proportion of the imported protein should be found in this fraction. As new precursor is imported continually during the time course, the intensity of this band does not peak and subsequently decline, but instead gradually increases during the import reaction. However, after longer import times PsbW could also be detected in the PSII subcomplexes, which reflects an inability of this PsbW fraction to assemble into dimers. The appearance of the imported, labelled PsbW in multiple BN bands contrasts markedly with the immunoblot of PsbW in total thylakoid samples, where PsbW is found exclusively in bands 1–3 (see Fig. 1C), indicating that the presence of labelled PsbW in the other BN bands is not due to disassembly of these complexes in the BN gel system, but must reflect an inability of these PsbW molecules to assemble into PSII. This could be explained by the fact that in our experiments the newly imported nuclear-encoded protein is present in the stroma thylakoid at a large surplus compared to the normal in vivo situation. Hence, the assembly of the PSII complex can only proceed as long as other proteins needed for the assembly are available. Thus, the lack of 'normal' proteins/complexes to interact with is likely to induce new, probably hydrophobic protein interactions in the thylakoid membrane. However, the fact that a proportion of the imported PsbW is not assembled into dimeric PSII complexes indicates that only a limited amount of PSII assembly is occurring in these chloroplasts. The limiting factor for the process of PSII assembly will be the amount of other unassembled protein subunits present in the thylakoid membrane. In these experiments we used young, highly active leaf tissue and as the assembly of the plastid-encoded proteins continues in isolated chloroplasts, the assembly will not stop directly. Our data show that a certain fraction of the PSII particles are under assembly at the time of the protein import, and some of the newly imported PsbW becomes incorporated.

Fig. 2A also shows the assembly kinetics of PsbS. Here, the autoradiogram of the BN gel profile differs from that of PsbW. Even though also PsbS is observed first in bands 4 and 9, the stroma lamellae fractions, it assembles immediately and strongly with the monomeric PSII subcomplexes (bands 7 and 8). Labelling in bands 1–3 appears after longer import times and the intensities of the bands are somewhat weaker, possibly indicating a less efficient assembly into PSII supercomplexes. In the immunoblot of total thylakoid samples PsbS is detected in the PSII dimer as well as in the monomeric fraction (Fig. 1C). These results strongly suggest that the PsbW and PsbS proteins associate with the PSII complex at different stages of assembly. Assembly or exchange of PsbW into the PSII dimer is more efficient than that of PsbS and we propose that this reflects the unique position of PsbW in the dimeric complex. PsbW stabilises the dimeric PSII structure [19] and may be the last protein to be incorporated into the dimer. It appears to assemble into PSII at the same moment as the dimer is created. An additional explanation for the fast incorporation of the PsbW protein into PSII dimers could be the high turnover rate of the PsbW protein that has been reported earlier [40].

To verify the rapid assembly of newly imported PSII subunits shown by BN-PAGE and to exclude non-specific binding of the precursors to the thylakoid complexes in the BN-PAGE, we carried out control tests on Lhcb1 and PsaK, a low molecular weight PSI protein, which is not expected to appear in the PSII supercomplexes (Fig. 2B). After insertion into the stroma lamellae fraction, Lhcb1 could be detected very weakly in bands 1–3, instead accumulating primarily in band 9. This is expected since much of the LHCII complex tends to separate from the PSII core complex and to run separately in this system (as band 9, see Fig. 1B,C). Newly incorporated Lhcb1 seems to complex with the 'free' trimers, only a small amount is assembled or exchanged in the PSII dimers. Meanwhile the PsaK protein appeared exclusively in the stroma thylakoid fraction (band 4) and no migration with any PSII fraction could be detected (Fig. 2B).

3.4. Analysis of PSII dimerisation assembly using a PsbW protein with modified N-terminus

The rapid assembly of the newly imported PsbW into the PSII dimer, together with the finding that this protein was not detected in native PSII monomers, suggests that this particular subunit is incorporated towards the end of the PSII assembly process. This is also consistent with the result that the PsbW protein stabilises the dimerised structure of PSII [19]. To investigate which part of the PsbW protein is involved in the dimer stabilisation, we replaced two of the three negative charged N-terminal amino acids with neutral amino acids, and three positively charged histidines were added (D82N, E83Q, L92H, P93H, F94H). The net charge of the lumenal exposed N-terminus was thereby changed from minus three to plus two in the mutated PsbW precursor. This change had no influence on the import characteristics of the protein as monitored on SDS–PAGE (not shown). However, the autoradiogram (Fig. 3) clearly shows that the removal of the negative charges modifies the assembly of PsbW into the PSII dimer. The radiolabelled mutated PsbW protein is exclusively detected in the stroma lamellae fraction, it does not assemble into the dimeric PSII supercomplex after import into chloroplasts. Meanwhile, native PsbW protein imported under identical conditions assembled into the dimeric PSII bands as shown earlier (Fig. 3). Therefore, the N-terminal domain seems to be critical for the role of PsbW in stabilising the PSII dimer. The exact mechanism for this process is not clear at the moment, but other modifications of the PsbW protein are currently under investigation to address this question.

4. Conclusion

By adapting the previously described BN-PAGE technique we were able to resolve monomeric and dimeric PSII complexes, providing a useful technique for localisation studies. Using this technique we could show that in the native system the PsbW protein is localised exclusively in the PSII dimer, whereas PsbS could be immunodetected in the PSII monomer and dimer, but not in the LHCII fraction.

The additional combination of in organello assay and BN-PAGE represents a powerful new method for the analysis of how nuclear-encoded thylakoid proteins assemble into complexes. Due to the in vitro assay, the importance of specific amino acids can be investigated by the use of site-directed mutated precursor proteins. In this report we have shown that the PsbW protein apparently inserts directly into the dimeric PSII supercomplex with rapid kinetics. The negatively charged N-terminal domain of the PsbW protein seems to be of special importance. Furthermore, we could show that PsbW and PsbS associate with the PSII complex at different stages of assembly.

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