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Maintenance of functional Photosystem II by D1 protein turnover

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Introduction

Water splitting in PS II involves a series of dangerous reactions potentially harmful for both the functional and structural properties of the complex. Despite a number of protection mechanisms there is, however, always an inherent inhibition occurring in PS II with a very low quantum yield (Tyystjärvi and Aro, 1996; Anderson et al., 1997). Thus, even at low light irradiances oxidative damages are occurring in PS II and lead to an irreversible damage of the PS II complex (Keren et al., 1997). With increasing irradiances such damages occur more and more frequently. Visible light induced oxidative damage is directed mainly towards only one specific target protein, the D1 protein in the reaction center of PS II (Mattoo et al., 1989), whereas the other protein components of PS II remain largely unaffected. As long as plants can cope with the light-induced damage to the D1 protein by constant D1 synthesis and repair of PS II, no visible or measurable symptoms of photoinhibition of photosynthesis can be recorded *in vivo*. Indeed, such constant turnover of the D1 protein in PS II cannot be detected by monitoring chlorophyll fluorescence parameters or by measurements of steady state oxygen evolution. However, as soon as the capacity for repair of damaged PS II centers is exceeded at increasing irradiances by the more frequently occurring damaging reactions, an irreversible inhibition of PS II can be recorded *in vivo* (Aro et al., 1993). A mere light-induced irreversible inhibition of PS II is not common *in vivo* but occurs more often when high irradiances prevail in combination with other stress factors like drought, low or high temperatures, CO_2 limitation or nutrient deprivation, which generally hamper the repair of damaged PSII. Here we provide some insights into the mechanisms involved in the maintenance of functional PSII via rapid D1 protein turnover.

Materials and methods

Plants were grown at 25C at a photosynthetic photon flux density of 300 (spinach) or 600 (pea) µmol photons m^{-2} s^{-1}. Pea leaves were pulse-labeled with ^{35}S-methionine and the synthesis and degradation (chase experiments) of the D1 protein at various light intensities was studied as described earlier (Aro et al. 1993). Thylakoid isolation, SDS-PAGE, autoradiography and immunodetections were performed according to standard methods, and the Blue-native (BN) gel analysis of thylakoid protein complexes according to Kugler et al. (1997) with minor modifications. *In vitro* translations in intact spinach chloroplasts, thylakoid subfractionations, sucrose density fractionation of the thylakoid membrane, isolation of ribosome nascent chain complexes (RNCs) and immunoprecipitations were done essentially as described in Zhang et al. (1999; 2000; 2001).

Results

Labeling of intact leaves with ^{35}S-Methionine

Labeling of intact fully expanded pea leaves with ^{35}S-methionine revealed strong accumulation of radioactivity in the D1 reaction center protein of PS II (Fig. 1A) with only minor labeling of other thylakoid proteins. Presence of lincomycin completely inhibited the synthesis of D1. As shown in Fig. 1B, the accumulation of label in the D1 protein increased first nearly linearly with increase in the light intensity and then gradually reached saturation. Similar curve was obtained when analysing the degradation of the damaged D1 protein by chase experiments. Monitoring the photochemical efficiency of leaves in these various light intensities revealed no decrease in Fv/Fm at light intensities up to 1400 µmol photons m^{-2} s^{-1} (not shown). Exposure of leaves to higher light intensities induced a decrease in Fv/Fm (measured after 30 min dark-incubation of leaves to allow restoration of non-photochemical quenching), and these conditions also induced an accumulation of irreversibly damaged PSII centers in the thylakoid membrane (Fig. 1B).

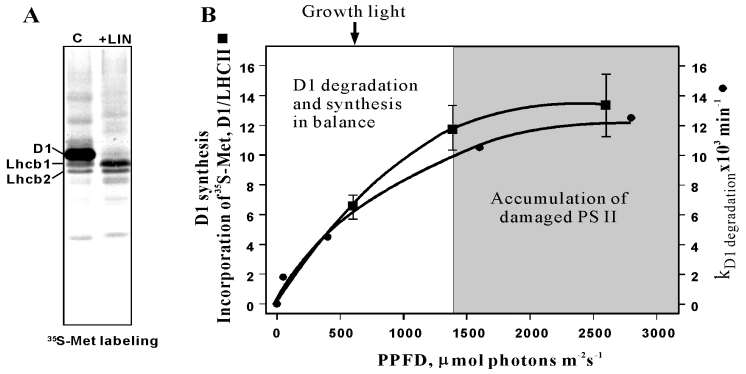

Fig. 1. A. Pulse labeling of intact pea leaves with ^{35}S-methionine for 1h in the absence (C) and presence (+LIN) of lincomycin. B. Rate of synthesis and degradation of the D1 protein at various light intensities. Pulse-chase experiments with intact leaves were applied. Plants were grown at a PPFD of 600 µmol $m^{-2}s^{-1}$.

Analysis of PSII complexes in thylakoid subfractions of high-light treated leaves

We next isolated thylakoid membranes from mature leaves illuminated at 2000 µmol photons m^{-2} s^{-1} for 2 h. Thylakoids were fractionated by digitonin and subjected to BN-gel separation of thylakoid protein complexes. Polypeptide composition of separated complexes was then resolved in second dimension SDS-PAGE followed by silver staining (Fig. 2) and MALDI-TOF analysis of protein components.

PSII supercomplexes, dimers and monomers were the dominating PSII complexes in appressed thylakoid regions (Fig. 2A). No disassembled PSII was present in appressed membranes even at high illumination. In non-appressed stroma-exposed thylakoid regions the PSII monomers were predominant but also a smaller complex lacking CP43 but having the other major PSII core proteins D1, D2 and CP47 assembled, was clearly distinguished (Fig. 2B). This complex was present, however, only as a minor pool of PSII complexes.

Another set of leaves was fed with lincomycin prior to the high light illumination in order to prevent D1 synthesis and thereby the repair of PSII. This treatment induced strong accumulation of damaged PSII centers (60 % inactivation of PSII with only 20 % loss of D1, not shown). Fractionation of the thylakoid membrane from such leaves revealed intact PSII complexes in grana appressions (Fig. 2C) whereas the non-appressed membranes were almost

devoid of PSII complexes (Fig. 2D). Thus, a portion of PSII complexes in grana was photodamaged but remained structurally intact (with core proteins phosphorylated, data not shown). With time, however, these complexes become degraded upon entering the stroma-exposed thylakoid domain (not shown).

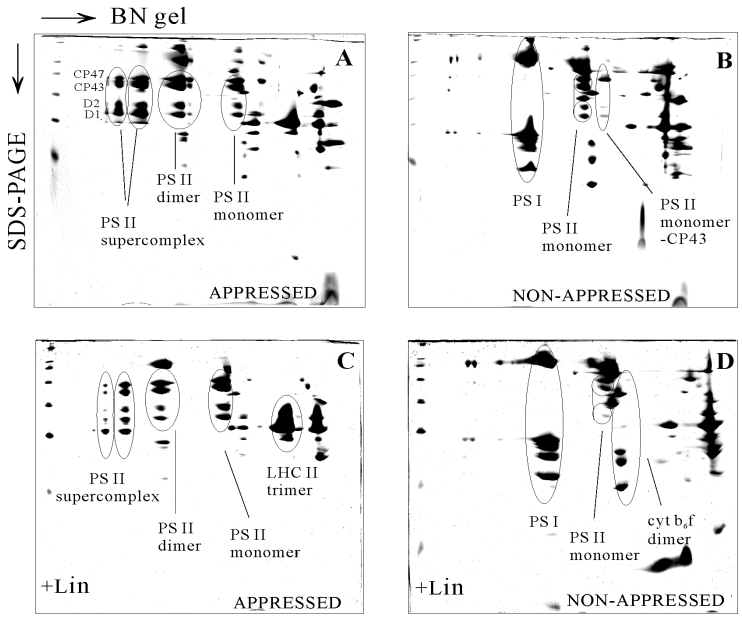

Fig. 2. Blue-native gel/SDS-PAGE analysis of thylakoid protein complexes in appressed grana (A and C) and non-appressed stroma-exposed (B and D) membranes. Intact leaves were illuminated at 2000 μmol photons $m^{-2}s^{-1}$ for 2 h in the absence (A and B) and in the presence of lincomycin (+LIN) (C and D) prior to thylakoid isolation and subfractionation. The gels were silver stained and protein spots of various complexes were identified by MALDI-TOF. PSII supercomplexes, dimers, monomers and monomers lacking CP43 are indicated in the figure. In panel D also the location of PSI and the dimer of $Cytb_6f$ complex are indicated.

Co-translational insertion of nascent D1 chains into the thylakoid membrane occurs via a cpSecY translocation channel

High turnover rate of the D1 protein, together with distinct pausing of ribosomes during *in vitro* translation (Kim et al., 1991; Zhang et al., 1999), offers unique opportunities to examine specific protein-protein interactions while the D1 protein is translated on thylakoid membrane-bound ribosomes, translocated across the membrane and integrated into the lipid bilayer. By analogy with bacterial export system, a chloroplast homologue of SecY (cpSecY) is a conceivable candidate for a translocon component mediating the translocation and insertion of chloroplast-encoded proteins into the thylakoid membrane. To elucidate a possible interaction of cpSecY with D1 elongation intermediates, the isolated RNCs were first purified in BN-gel to release possible contaminating protein complexes. The BN-gel of separated protein complexes was further subjected to denaturing SDS-PAGE and silver staining (Fig. 3A). To identify various thylakoid membrane complexes, similar gels were subjected to Western blot analysis with distinct antibodies (Fig. 3B). Contaminating thylakoid protein complexes were efficiently released from RNCs by BN-gel separation, as indicated by immunoblots with anti-D1 and anti-cyt f (Fig. 3B)

Most importantly, anti-cpSecY recognized two distinct complexes in the BN-gel (Fig. 3A and B): one migrating at about 100 kDa and the major one at about 900 kDa, both clearly resolved in the separating gel. The major cpSecY complex in RNCs was distinct from other thylakoid protein complexes, and co-migrated in the BN-gel with ribosomes, as evidenced by immunodetection with anti-ribosome L21 (not shown).

The protein complex in the BN-gel containing most of cpSecY together with ribosomes was extracted from the gel and subjected to immunoprecipitation with anti-D1. The D1 intermediates of different lengths were efficiently precipitated from this cpSecY-ribosome complex (Fig. 3C, indicated by filled stars). This result clearly demonstrates that both the D1 nascent polypeptides and the cpSecY complex are tightly associated with ribosomes. Further co-immunoprecipitation and crosslinking experiments indicated that cpSecY resides in the vicinity of D1 elongation intermediates and provided evidence for a transient interaction of cpSecY with D1 elongation intermediates during the biogenesis of D1 (data not shown).

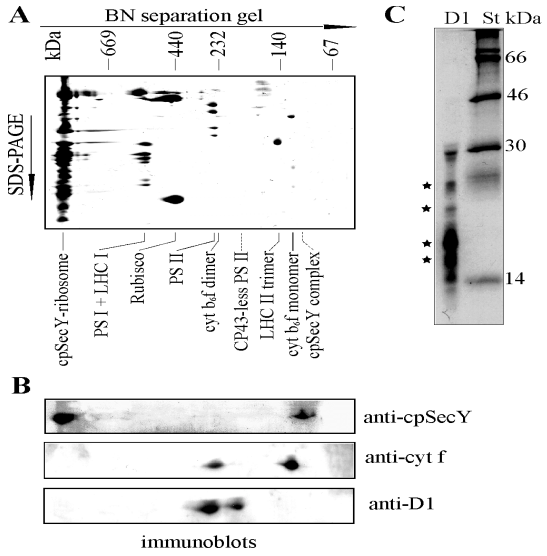

Fig. 3 Analysis of RNC preparation by a BN-gel. (A) Two-dimensional resolution of protein complexes in RNC preparation. RNCs were solubilized with 1 % DM and separated by a BN-gel (acrylamide gradient of 5-12 %). A lane of a BN-gel was run in a second dimension on a 15 % SDS-urea-PAGE and silver stained. Designations for the identities of protein complexes, as resolved by the first dimension BN-gel, are given at the bottom of the silver-stained gel and the molecular weight of standards on the top of the gel. (B) Representative immunoblots from second dimensional SDS-PAGEs of RNCs. Horizontal strips of immunoblots with anti-cyt f, anti-D1 and anti-cpSecY are shown. (C) Association of cpSecY and nascent D1 chains with ribosomes. After a short pulse-labeling of 2.5 min in intact chloroplasts, RNCs were isolated and purified in a BN-gel. The proteins, extracted from the high molecular mass complex containing cpSecY and ribosomes, were subjected to immunoprecipitation with anti-D1. Filled stars indicate D1 nascent chains of 14, 17, 22 and 25 kDa. Molecular mass markers (kDa) are indicated to the right of the autoradiogram.

D1 nascent chains interact with the D2 protein

After a short pulse labeling in intact chloroplasts, most of the labeled thylakoid membrane proteins are incorporated into PS II complexes, pointing to a cotranslational assembly manner (Zhang et al., 1999). To gain more direct evidence of the interaction of D1 with other PS II core proteins during cotranslational translocation/insertion process, we isolated RNCs from pulse-labeled chloroplasts and performed immunoprecipitations with specific antibodies against D1, D2 and CP47. Typically, the D1 elongation intermediates of 17, 22 and 25 kDa were detected by immunoprecipitation with anti-D1 and subsequent autoradiography (Fig.

4A, lane D1). Interestingly, not only the anti-D1 but also anti-D2 was competent in precipitating D1 elongation intermediates, particularly the 25 kDa D1 intermediate (Fig. 4A, lane D2). None of D1 elongation intermediates could be immunoprecipitated with anti-CP47 under denaturating conditions.

Under non-denaturation conditions (co-immunoprecipitation), however, both anti-D2 and also anti-CP47 faithfully precipitated the D1 elongation intermediates of 17, 22 and 25 kDa (Fig. 4B).

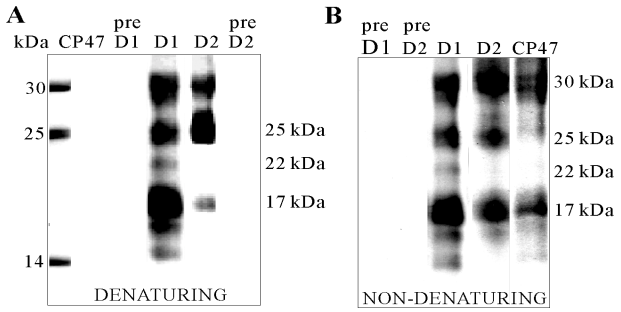

Fig. 4. Interaction of D1 elongation intermediates with other PS II core proteins. After 2.5 min pulse-labeling, RNCs were isolated and subjected to immunoprecipitation with anti-D1, anti-D2, anti-CP47, and D1 and D2 presera as controls, under denaturating (A) and non-denaturating (B) conditions. Antisera used for immunoprecipitation is shown on the top of each lane. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by autoradiography.

Appearance of newly synthesized precursor/mature D1 protein in PSII subcomplexes

After *in vitro* pulse labeling in intact chloroplasts for 2.5 min and subsequent chase of 5 min to allow termination of D1 translation elongation (Zhang et al., 2000), the thylakoid membranes were isolated, solubilized with dodecyl maltoside and subsequently applied to sucrose density fractionation. After 26 h centrifugation, 20 fractions were collected from the gradients and subjected to SDS-PAGE and autoradiography.

After 5 min chase, most of labeled D1 protein was present in the reaction center or PSII monomer (+/- CP43) complexes (Fig. 5). Only 8 % of radiolabeled D1 was free in the membrane. About half of the D1 protein was present as a precursor form (pD1) and the other half as a processed mature D1 protein. pD1 form was found as a free protein, in the reaction center complexes and also in PSII monomers lacking CP43 (fraction 9). Notably, only the processed D1 protein was present in PSII monomers with CP43 attached. No radiolabeled proteins were found in appressed membranes after 5 min chase (not shown).

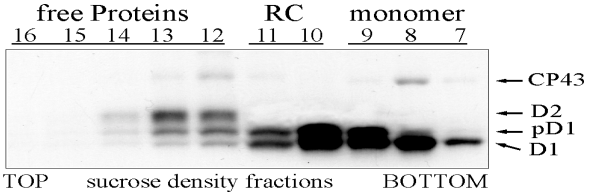

Fig. 5. Newly synthesised pD1/D1 protein can be trapped directly (5 min chase) in the reaction center and monomer complexes in the non-appressed thylakoid domains. Only minor amounts of free pD1/D1 were present, as shown by autoradiogram. Newly synthesised labeled D2 protein is never incorporated into PSII complexes using this experimental approach. Newly synthesised CP43, however, can be found as a component of PSII monomers.

Distribution of newly assembled PSII complexes between appressed and non-appressed membrane regions

After 5 min chase the labeled pD1/D1 proteins were found only in non-appressed thylakoid domains (not shown). Prolongation of the chase to 20 min still revealed most of labeled D1 in non-appressed stroma thylakoids where it was all processed and mainly present in PSII monomers. Labeled D1 protein was, however, now also found in appressed grana membranes, mostly in PSII dimers and to a much lesser extent in PSII monomers (Fig. 6).

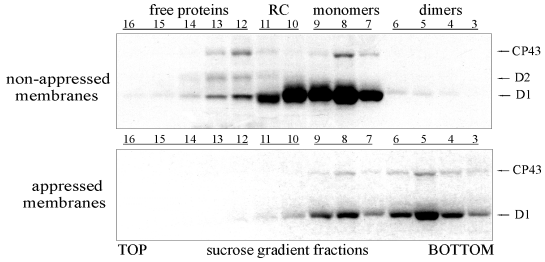

Fig. 6. Autoradiogram of newly synthesised proteins in PSII complexes of appressed and non-appressed thylakoid domains after 20 min chase.

Thiol-disulfide regulation of post-translational assembly steps of PSII

Application of thiol alkylating agent N-ethylmaleimide (NEM) during the 20 min chase revealed strong thiol regulation of PSII assembly. This concerns both the elongation of D1 (Zhang et al. 2000) and post-translational processing and assembly of D1 into PSII (Fig. 7). Alkylation of thiol groups by NEM nearly completely prevented the processing of pD1 and the assembly of PSII monomers.

Discussion

Replacement of the photodamaged D1 protein in PSII complexes is a continuous and complicated process occurring in thylakoid membranes of all organisms pursuing oxygenic photosynthesis. The rate constant for PSII photodamage increases linearly with increase in light intensity (Tyystjärvi and Aro, 1996) but the repair of PSII via D1 protein turnover reaches a saturation level generally at light intensities two to three fold that experienced during growth (Fig. 1B). Constant D1 protein turnover makes it possible to study the mechanisms of D1 replacement in isolated intact chloroplasts. The site for D1 degradation and synthesis of the new D1 copy is restricted to non- appressed thylakoid domains where ribosomes have an access. Thus in cyanobacteria

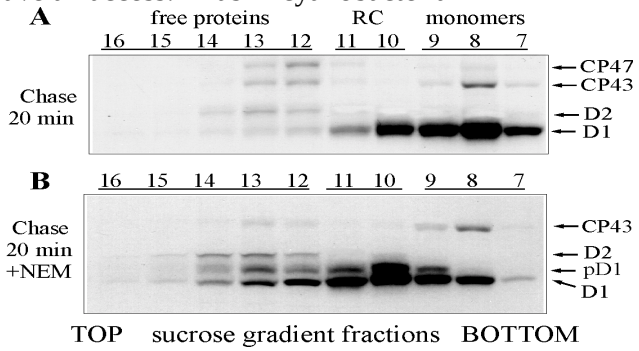

Fig. 7. Posttranslational assembly of pD1/D1 into PSII subcomplexes is strongly under thiol-disulfide regulation. 5 mM NEM was applied during the 20 min chase period. The labeled proteins were visualized by autoradiography.

and some algae the damage and repair of PSII are not spatially segregated; the damaged D1 protein can be more or less directly degraded and replaced with a new D1 copy. In higher plants, on the other hand, the damaging reactions occur mainly in appressed thylakoid domains whereas repair sites are strictly limited to stroma-exposed thylakoid domains (Fig. 2). Moreover, the degradation of damaged D1 and subsequent insertion of a new D1 copy into PSII are tightly synchronized. Phosphorylation of PSII core polypeptides D1, D2 and CP43 seems to play a crucial role in this respect (Baena-Gonzalez et al., 1999). Phosphorylated, albeit damaged, PSII centers do not fall apart in the grana (Fig. 2A, C) but migrate to stroma exposed regions in a highly coordinated way, yet to be mechanistically elucidated. Upon arrival on stroma thylakoids, the PSII complex undergoes dephosphorylation of CP43, D2 and D1 proteins (Baena-Gonzalez et al., 1999). This apparently allows the opening of the center and accessibility of a stromal protease to degrade the damaged D1 protein. Recently, the bacterial homologues DegP2 and FtsH were demonstrated to be involved in the degradation of D1 protein (Lindahl et al., 2000; Haussühl et al., 2001)

A new D1 protein copy, encoded by *psb*A mRNA, first cotranslationally inserts into a cpSecY translocation channel in the thylakoid membrane and the D1 elongation intermediates transiently interact with cpSecY. After termination of translation, such interaction no more exists. Thus, besides a well-characterized role in posttranslational translocation of nuclear encoded proteins, cpSecY also functions in cotranslational insertion of chloroplast encoded proteins (Zhang et al., 2001).

It has recently become clear that translation elongation is the most critical phase determining the success of D1 protein synthesis when damaged PSII centers are repaired (Zhang et al., 2000), though translation initiation is strictly controlled in chloroplasts (Somanchi and Mayfield, 2001). Elongation of *psbA* mRNA translation is arrested if nascent D1 chains do not start interacting with D2 protein, thereby allowing the termination of translation (Zhang et al., 2000; Tyystjärvi et al. 2001). The 25 kDa D1 elongation intermediate is tightly complexed with the D2 protein and can be immunoprecipitated from RNCs with anti-D2, whereas only loose interaction with D2 was observed for the 17 kDa D1 nascent chain (Fig. 4). It is, thus, conceivable that the transmembrane domains of the D1 nascent chains laterally exit from the cpSecY translocation channel and interact with other PS II core proteins. Structural and functional flexibility of the translocon is probably required for the efficient protein folding and assembly during the biogenesis of multi-protein PS II complex. CP47 is possibly also present in the complex when D1 elongation intermediates are interacting with D2. CP43, however, is clearly associated with the complex only after termination of *psbA* mRNA translation (Fig. 5). This assembly step is closely coordinated with processing of the precursor D1 protein. Indeed, unprocessed D1 is never met in PSII complexes with CP43 associated. These coordinated assembly steps then probably allow a safe ligation of the Mn cluster and attachment of oxygen evolving proteins with other PSII proteins. Both the cotranslational (Zhang et al. 2000) and posttranslational (Fig. 7) assembly steps of PSII are under thiol redox regulation.

Immediately after termination of translation the pD1/D1 protein is found only in non-appressed thylakoids as a component of reaction center and monomer complexes of PSII. Migration of monomer complexes to appressed grana membranes is clearly a slow process, but when entering the grana appressions, efficient dimerization of PSII takes place (Fig. 6).

Acknowledgement

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