Articles

The Nuclear-Encoded PsbW Protein Subunit of Photosystem II Undergoes Light-Induced Proteolysis†

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ABSTRACT: The repair of photoinhibitory damage to photosystem II involves the rapid degradation and turnover of the D1 reaction center subunit. Additional protein subunits which show a limited degradation at high light intensities are the complementary reaction center subunit, D2, and the two chlorophyll *a* binding proteins, CP 47 and CP 43. In this work, we provide the first evidence for light-induced degradation of a nuclear-encoded subunit of photosystem II, the recently discovered PsbW protein. This 6.1 kDa protein is predicted to have a single membrane span and was found to be closely associated with the photosystem II reaction center. The degradation of the PsbW protein was demonstrated by photoinhibitory experiments, both *in vitro*, using thylakoid membranes and photosystem II core particles, and *in vivo* using leaf discs. The PsbW protein showed almost the same rate and extent of degradation as the D1 protein, and its degradation was more pronounced compared to the D2 and CP 43 proteins. The degradation of the PsbW protein was shown to share many mechanistic similarities with the more well characterized D1 protein degradation, such as oxygen dependence, sensitivity to serine protease inhibitors, and high light triggering while the actual degradation could readily occur in total darkness. The degradation of the PsbW protein was impaired by protein phosphorylation, although this protein was not itself phosphorylated. This impairment was correlated to the phosphorylation of the D1 protein which has been shown to block its degradation during photoinhibitory conditions. It is concluded that the PsbW protein is not degraded as a direct consequence of primary photodamage but due to a general destabilization of the photosystem II complex under conditions were the D1 protein becomes degraded in the absence of a sufficient repair system. The results are discussed in terms of a requirement for coordination between degradation and protein synthesis/integration during the repair process of photodamaged photosystem II reaction centers.

Photosystem II (PS II)[1] is a multiprotein complex composed of nearly 30 different subunits encoded by both plastid and nuclear DNA (Andersson & Franzén, 1992; Vermaas et al., 1993; Andersson & Barber, 1994; Pakrasi, 1995). The reaction center is composed of the D1 and D2 proteins, which form a heterodimer ligating all the redox components required for primary photochemistry (Michel & Deisenhofer, 1986; Trebst, 1986; Barber et al., 1987; Nanba & Satoh, 1987). The heterodimer is possibly also involved in ligating the manganese cluster which catalyzes the process of water oxidation (Andersson & Styring, 1991; Nixon & Diner, 1992; Chu et al., 1995a,b). Other components associated with the heterodimer are the PsbI protein and the cyt b_{559} (Satoh, 1993).

The reaction center of PS II is the main target for photoinhibitory damage during high light illumination (Barber & Andersson, 1992; Prasil et al., 1992; Aro et al., 1993). Excess light leads to impaired electron transport, which in turn is thought to induce oxidative damage to the reaction center. This causes proteolysis of mainly the D1 protein and to some extent also the D2 protein and the chlorophyll *a* binding proteins, CP 43 and CP 47 (Schuster et al., 1988; Virgin et al., 1988; Trebst & Depka, 1990; Barbato et al., 1992; Andersson et al., 1994; Zer & Ohad, 1995). The majority of experimental observations demonstrated that the photodamaged subunits were proteolytically cleaved (Aro et al., 1993; Andersson et al., 1996) even though direct chemical cleavage of peptide bonds in the D1 protein by active oxygen species has also been proposed (Mishra & Ghanotakis, 1994; Miyao, 1994; Miyao et al., 1995).

The degradation of these proteins *in vivo* is considered to initiate the repair cycle of damaged PS II reaction centers (Adir et al., 1990; Melis, 1991; Aro et al., 1993). The phosphorylated forms of the D1 (denoted D1*) and D2 proteins are found to be poor substrates for proteolysis both *in vivo* and *in vitro* (Aro et al., 1992; Koivuniemi et al.,

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[1] Abbreviations: chl, chlorophyll; cyt b_{559}, cytochrome b_{559}; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CP, chlorophyll binding protein; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pefabloc SC, 4-(2-aminoethyl)benzenesulfonyl fluoride, hydrochloride; PpBQ, phenyl-*p*-benzoquinone; PS, photosystem; PVDF membrane, polyvinylidene difluoride membrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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1995), and the damaged D1* has been found to require light-dependent dephosphorylation prior to proteolysis (Koivuniemi et al., 1995; Rintamäki et al., 1996). Reversible phosphorylation of the D1 and D2 proteins in higher plants is therefore thought to be of significance for the regulation of the repair cycle (Kettunen et al., 1991; Aro et al., 1992; Elich et al., 1992) in coordinating the protein degradation and resynthesis events (Aro et al., 1993; Ebbert & Godde, 1994; Koivuniemi et al., 1995; Rintamäki et al., 1995).

A nuclear-encoded polypeptide of 6.1 kDa belonging to PS II (PsbW protein) was recently discovered and suggested to be closely associated with the reaction center proteins (Irrgang et al., 1995). This small subunit, which has a unique import and assembly route, was predicted to have one membrane span with its C-terminus exposed at the stromal side of the thylakoid membrane (Lorcovic et al., 1995). The function of the PsbW protein is not yet known, although a role in nucleation during biogenesis of the PS II complex has been suggested (Funk et al., 1995).

In this work, we demonstrate that the PsbW protein shows a pronounced degradation during high light treatment both *in vitro* and *in vivo* in a process which resembles the proteolysis of the D1 protein. The data will be discussed in terms of the mechanism of light-induced proteolysis and repair of the PS II complex the relationship between the newly discovered PsbW protein degradation and the D1 protein damage and degradation.

MATERIALS AND METHODS

Thylakoid membranes were isolated from spinach leaves (*Spinacia oleracea* L.) as described in Andersson et al. (1976). Oxygen evolving PS II core particles were prepared as in Ghanotakis et al. (1987).

Photoinhibitory illumination of thylakoid membranes and PS II core particles was carried out aerobically or anaerobically in a medium consisting of 20 mM MES, pH 7.4, 100 mM sucrose, 10 mM NaCl, and 5 mM $MgCl_2$ (incubation medium) at a concentration of 0.15 mg of chl mL^{-1}. The temperature during the experiments was kept at either 20 or 2 °C and illumination was performed using white light (4500 μmol of photons m^{-2} s^{-1}) for the indicated time periods. Under anaerobic conditions, the experiment was performed in sealed bottles in which the medium had been flushed with argon for 20 min prior to the addition of the sample.

Protein kinase activation was achieved by reduction of the plastoquinone pool in darkness by incubation of thylakoid membranes in the presence of 1 mg mL^{-1} NADPH and 10 mM ferredoxin. The protein phosphorylation was performed at 25 °C at 0.2 mg of chl mL^{-1} in 50 mM Hepes, pH 7.6, 100 mM sorbitol, 5 mM $MgCl_2$, and 5 mM NaCl, supplemented with 0.4 mM ATP and 100 μCi [γ-32P]ATP mL^{-1}. After incubation, excess reactants were removed by washing the thylakoids twice with incubation medium and 0.4 mM ATP was included after the last wash to prevent dephosphorylation. Quantification of autoradiograms was performed by laser scanning densitometry.

To study the proteolytic activity, PS II core particles were incubated with 4 mM Pefabloc SC (a serine protease inhibitor) at 0 °C for 2 h before illumination. Pefabloc SC was purchased from Boehringer Mannheim.

The degradation of PS II proteins *in vivo* was studied by illumination of leaf discs at 2200 μmol of photons m^{-2} s^{-1} for 2.5 h in the presence or absence of 2 mM lincomycin (Sigma Chemical Co., St Louis, MO), a chloroplast protein-synthesis inhibitor, as described in Rintamäki et al. (1996).

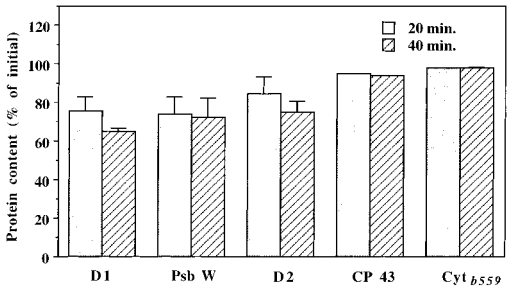

FIGURE 1: Degradation of PS II polypeptides during photoinhibitory illumination of thylakoid membranes. The membranes were subjected to photoillumination at 4500 μmol of photons m^{-2} s^{-1} at room temperature. Samples were withdrawn at 20 min and at 40 min. The relative amount of D1, PsbW, D2, CP 43, and cyt b_{559} proteins were followed by immunoblotting and quantified by laser scanning densitometry. The histograms give the average values of three independent experiments including error bars for the three former proteins.

SDS−PAGE was performed according to Laemmli (1970) with gels composed of a 12 to 22.5 % polyacrylamide gradient in the presence of 4 M urea (Ljungberg et al., 1986). Western blot analyses were performed according to Towbin et al. (1979). The antibodies used were polyclonal antisera raised against either the C-terminus or the N-terminus of the *psb*W gene product (Irrgang et al., 1995), the C-terminus of the D2 protein (kind gift from D. Sayre), an epitope of the A-B loop of the D1 protein, the CP 43 protein (kind gift from N.-H. Chua), and the α-subunit of cyt b_{559}. Immunodecorated proteins were detected with either ^{125}I-labeled protein A or alkaline phosphatase. Quantification of immunoblots were performed by laser scanning densitometry.

Oxygen evolution was measured in a Clark-type electrode at 20 °C and saturating light. PpBQ (0.4 mM) was used as electron acceptor.

RESULTS AND DISCUSSION

The D1 protein, to some extent, the D2 protein, and the two chlorophyll *a* binding proteins of PS II show light-induced degradation, which is associated with repair of photodamaged reaction centers (Prasil et al., 1992). In view of the possible tight association of the PsbW protein with the PS II reaction center (Irrgang et al., 1995), experiments were performed to investigate whether the PsbW protein showed any changes in response to high light exposure of thylakoid membranes. As shown in Figure 1, when high light stress was applied to thylakoid membranes, the typical degradation of the D1 and D2 protein was observed as judged by Western blots, in accordance with several previous studies (Schuster et al., 1988; Virgin et al., 1988; Trebst & Depka, 1990; Barbato et al., 1992).

Notably, under the same conditions, a pronounced degradation of the PsbW protein was found. After 20 min of illumination on the average of three different experiments, 26% of the PsbW protein was degraded (Figure 1) compared to 25% for the D1 and 15% for the D2 protein. After 40 min of illumination, the degradation of the three proteins was 28, 35, and 23%, respectively (Figure 1). The degradation rate of the PsbW protein was found to be almost as rapid as for the D1 protein and faster than that of the D2

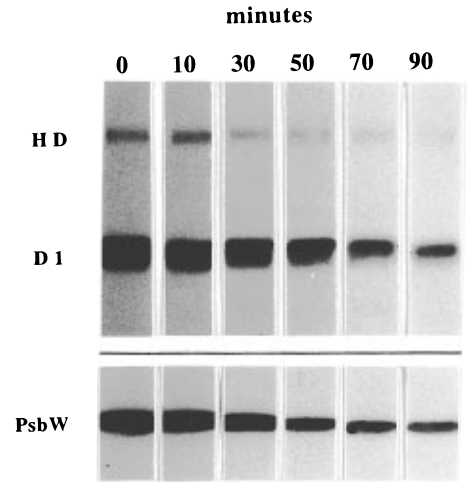

FIGURE 2: Degradation of the PsbW protein and the D1 protein in isolated PS II core particles during photoinhibitory illumination. The PS II core particles were subjected to strong illumination at 4500 μmol of photons m^{-2} s^{-1} for 0−90 min at room temperature. Samples were withdrawn at the indicated times and analyzed by immunoblotting. HD = heterodimer.

Table 1: Remaining Levels of D1 and PsbW Proteins in Thylakoid Membranes Subjected to Photoinhibitory Illumination[a]

	% of initial[b]	
protein	$-O_2$	$+O_2$
PsbW	91	72
D1	95	65

[a] Thylakoid membranes were kept under anaerobic conditions in sealed bottles flushed with argon prior adding samples. They were then subjected to 4500 μmol of photons m^{-2} s^{-1} at 20 °C for 40 min. [b] The remaining amounts of proteins were estimated by laser scanning densitometry of immunoblots.

protein (not shown). Under the same conditions, virtually no degradation of the CP 43 protein or the cyt b_{559} was observed (Figure 1). Antibodies raised against either the C-terminus or N-terminus of the PsbW protein revealed the same degradation kinetics. No degradation products could be detected by any of the antibodies for PsbW protein, suggesting a very efficient degradation process. Due to the somewhat higher sensitivity of the N-terminal antibody, this was used for further experiments.

The D1 protein has previously been shown to be degraded in isolated oxygen evolving PS II core particles (Virgin et al., 1990; De Las Rivas et al., 1992). The immunoblot shown in Figure 2 compares the degradation of both the D1 protein and the PsbW protein after subjecting the isolated PS II core particles to high light. After 30 min of illumination, 31% of the PsbW protein and 38% of the D1 protein were found to be lost with approximately the same kinetics.

In order to investigate whether the observed degradation of the PsbW protein was mechanistically related to the more well-characterized D1 protein degradation, several comparative experiments were performed. Initially, it was elucidated whether the degradation of the PsbW protein was dependent on the presence of oxygen as in the case for the D1 protein (Arntz & Trebst, 1986; Hundal et al., 1990; Kirilovsky & Etienne, 1991). Therefore, thylakoid membranes were illuminated under both aerobic and anaerobic conditions. Table 1 shows that the light-induced loss of the PsbW protein was highly dependent on the presence of oxygen. During

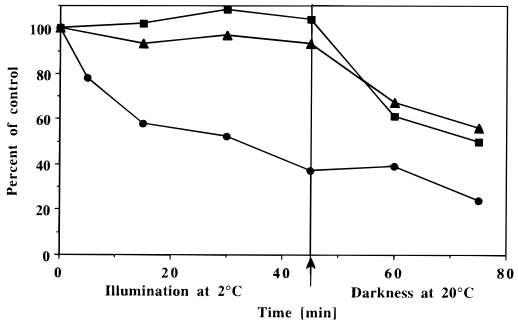

FIGURE 3: Degradation of PsbW and D1 proteins in darkness following photoinhibitory illumination of thylakoid membranes at low temperatures. Photoinhibitory illumination (4500 μmol of photons m^{-2} s^{-1}) was performed at 2 °C using thylakoid membranes. After 45 min in high light, the samples were transferred to 20 °C and total darkness (arrow). The loss of D1 protein (▲) and PsbW protein (■) were quantified by immunoblotting. PS II oxygen evolution (●) using PpBQ as electron acceptor was measured in all samples. The data are expressed as percentages of the values at a time point of 0 min.

the anaerobic conditions, as much as 91% of the PsbW protein remained after 40 min of high light treatment while only 72% remained in the presence of oxygen. The corresponding values for the D1 protein were 95 and 65%, respectively (Table 1).

To exclude the possibility that the PsbW protein degradation was not due to a light-induced chemical peptide cleavage as proposed for the D1 protein (Mishra & Ghanotakis, 1994; Miyao, 1994; Miyao et al., 1995), the photoinhibitory illumination was performed at a low temperature (2 °C), followed by transfer of the sample to 20 °C and total darkness, an approach that previously has been applied for the D1 protein degradation both *in vitro* and *in vivo* (Aro et al., 1990; Ottander et al., 1993; Andersson et al., 1994). Following strong illumination of the thylakoid membranes at 2 °C, the PS II electron transport was largely inactivated whereas no degradation of the PsbW protein or the D1 protein could be observed (Figure 3). However, when the illuminated thylakoid samples were transferred to 20 °C and total darkness, degradation of both the D1 protein and the PsbW protein commenced. These results are consistent with the concept that the degradation of both these proteins is of a proteolytic nature and not a chemical photocleavage considering the microsecond lifetime of singlet oxygen (Telfer et al., 1994). This is the active oxygen species during the applied acceptor-side photoinhibitory conditions (Vass et al., 1992).

To further investigate the direct or indirect involvement of proteolytic enzyme(s) in degradation of the PsbW protein, we performed photoinactivation experiments of the isolated PS II core particles after addition of a serine protease inhibitor, Pefabloc SC. Quantification of immunoblots (Table 2) from these experiments revealed that in the presence of the protease inhibitor only 12% of the PsbW protein was degraded compared to 31% in the absence of the inhibitor. For the D1 protein, the corresponding values were 20 and 37%, respectively (Table 2). This means that there is a partial inhibition of both the PsbW protein degradation (62%) and the D1 protein degradation (46%) in the presence of the protease inhibitor.

All experiments described above suggest that the degradation of the PsbW is mechanistically connected to the more well-characterized D1 protein degradation. It remains,

Table 2: Effect of a Serine Protease Inhibitor, Pefabloc SC, on the Degradation of the D1 and PsbW Proteins in PS II Core Complexes Subjected to Photoinhibitory Illumination[a]

protein	% of initial[b] −inhibitor	% of initial[b] +inhibitor
PsbW	69	88
D1	63	80

[a] PS II core complexes were illuminated in the presence or absence of a serine protease inhibitor at 4500 μmol of photons m^{-2} s^{-1} and 20 °C for 30 min. [b] The remaining amounts of proteins were estimated by laser scanning densitometry of immunoblots.

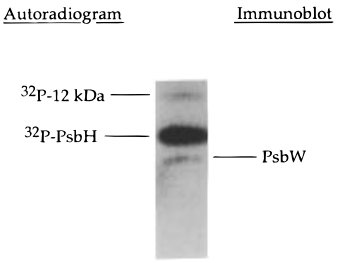

FIGURE 4: The PsbW protein is not a substrate for phosphorylation. An autoradiogram of [^{32}P]ATP phosphorylated thylakoid membranes was positioned on top of an immunoblot using an antibody against the PsbW protein. The latter was detected by alkaline phosphatase, allowing a distinct location of the PsbW protein with respect to the low molecular mass phosphoproteins, PsbH and phospho 12 kDa proteins.

however, to be established whether the PsbW protein is degraded as a consequence of direct photodamage or if its degradation is merely a consequence of a destabilized PS II complex due to the D1 protein degradation. In an attempt to discriminate between these two possibilities, we took advantage of earlier studies by Aro et al. (1992) and Koivuniemi et al. (1995) demonstrating that the phosphorylated D1 and D2 proteins are largely protected from degradation under photoinhibitory conditions. The phosphorylation of the two reaction center proteins occurs on threonine residues at the stromal exposed N-terminal portions of the proteins (Michel et al., 1988). According to amino acid sequence data of the PsbW protein (Lorcovic et al., 1995), only two threonine residues are found in this protein and they are both located at the N-terminus, exposed at the lumenal side of the membrane. However, on the stromal exposed C-terminal region, three serine residues are potentially available for phosphorylation. Experiments were, therefore, performed to elucidate if the PsbW protein can be phosphorylated and, if so, whether phosphorylation impairs its proteolysis or not. Isolated thylakoid membranes were phosphorylated with [^{32}P]ATP in the dark (Koivuniemi et al., 1995) and thereafter subjected to SDS−PAGE followed by Western blot and autoradiography on the same PVDF membrane (Figure 4). Autoradiography of the PVDF membrane visualized only the PsbH (9 kDa) protein (Farchaus & Dilley, 1986) and the phospho 12 kDa protein (Lindahl et al., 1995) in the lower molecular mass region. Immunoblot of the same PDVF membrane using an antibody against the PsbW protein and combined with alkaline phosphatase revealed a distinct polypeptide band clearly separated from the two phosphorylated low molecular mass polypeptides. This experiment demonstrates that the PsbW protein was not phosphorylated under conditions which lead

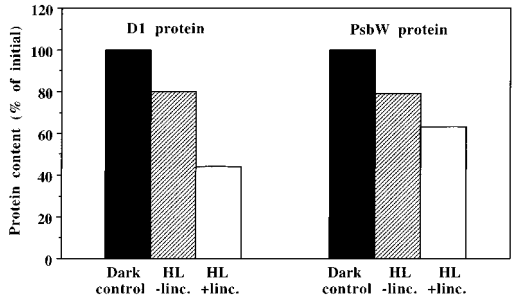

FIGURE 5: The PsbW protein can be degraded by photoinhibitory illumination *in vivo*. Leaf discs from intact leaves were illuminated *in vivo* at 2200 μmol of photons m^{-2} s^{-1}, 20 °C, for 2.5 h in the presence or absence of 2 mM lincomycin. Thylakoids were then isolated and separated by SDS−PAGE, subjected to immunoblotting and quantified by laser scanning densitometry. Degradation of the PsbW and D1 proteins were compared to the dark control leaves. HL = high light.

to phosphorylation of other PS II proteins including the D1 and D2 proteins.

In the next set of experiments, phosphorylated and unphosphorylated thylakoid membranes were subjected to the high light treatment and analyzed for protein degradation. In accordance with the previous studies *in vitro* (Koivuniemi et al., 1995), only residual D1 protein degradation was seen in the phosphorylated thylakoid membranes. Notably, despite not undergoing phosphorylation, the PsbW protein showed the same reduction in its degradation as observed for the phosphorylated D1 protein. Quantification of immunoblots revealed that, after 30 min of strong illumination of phosphorylated membranes, only 8% of both the PsbW and the D1 proteins was degraded compared to 30% for both proteins in the unphosphorylated membranes (not shown).

In an attempt to further characterize the significance of the PsbW protein degradation, experiments using whole leaf discs were performed. Earlier studies on D1 protein degradation *in vivo* (Ohad et al., 1984; Greer et al., 1986; Ottander et al., 1993; Rintamäki et al., 1996) have shown in the presence of lincomycin that the photodamaged D1 protein could not be replaced by a newly synthesized protein copy. Lincomycin inhibits the synthesis of chloroplast-encoded proteins and should therefore have no direct effect on the synthesis of the nuclear-encoded PsbW protein.

In these photoinhibitory experiments *in vivo*, there was a notable loss of the PsbW protein and its degradation appeared to be dependent on that of the D1 protein (Figure 5). As judged by the immunoblot, 66% of the D1 protein was degraded after 2.5 h of high light illumination in the presence of lincomycin compared to the dark control. Under the same conditions, 37% of the PsbW protein was degraded. Leaf discs were also illuminated with the same light intensity in the absence of lincomycin. As expected, the D1 protein was lost to a lesser degree (20%) due to reinsertion of newly synthesized protein copies (Figure 5). This relatively high net loss of the D1 protein in the absence of lincomycin could be explained by the repair system being slower than the rate of photodamage to the reaction center at the high light intensity. Also, under these experimental conditions, there was a loss of the PsbW protein. These *in vivo* observations, when combined with the *in vitro* experiments described above, give support to the view that PsbW degradation is dependent on the D1 degradation.

In conclusion, we have demonstrated that the newly discovered PsbW subunit of PS II can undergo light-induced

degradation. This is the first time such an event has been shown in association with a nuclear-encoded PS II subunit. During photoinhibitory conditions, the PsbW protein showed a higher rate of degradation compared to the D2 and CP 43 proteins. The loss of the PsbW protein was nearly as pronounced as that for the D1 protein. In this study, we did not analyze degradation of CP 47, but its degradation has previously been reported to be quite limited and in a similar range as for the CP 43 degradation (Zer & Ohad, 1995).

The fact that the PsbW protein can undergo degradation not only *in vitro* but also *in vivo* gives weight to the functional relevance of our current observations. Both the *in vitro* and *in vivo* results suggest that the degradation of PsbW protein was mechanistically connected to the light-induced degradation of the D1 protein. Considering that the PsbW protein is closely associated with the PS II reaction center (Irrgang et al., 1995) it could potentially be directly damaged by reactive radical species known to be created within the reaction center during conditions of excess excitation [for review, see Barber and Andersson (1992)]. However, in light of the data, particularly those involving protein phosphorylation, this is not the most likely explanation. Instead, a more indirect triggering for degradation is suggested to apply for the PsbW protein. Despite not being phosphorylated, the degradation of the PsbW protein is impaired to the same extent as the D1 protein, which undergoes a phosphorylation-dependant retardation of its light-induced degradation [for review, see Aro et al. (1993)]. We therefore conclude that the PsbW protein becomes susceptible for proteolysis as a consequence of PS II destabilization due to primary photodamage and proteolysis of the D1 reaction center subunit. This destabilization of the PS II complex will be particularly severe under conditions when a new copy of the D1 protein is not readily available for integration into the damaged PS II which, for example, is the case *in vitro*. Under *in vivo* conditions, as illustrated in Figure 5, such a destabilization will occur in the presence of chloroplast protein synthesis inhibitors or under severe light stress conditions when the regulated repair machinery lags behind the damaging events. This kind of reasoning may very well apply also for the degradation of the D2 protein and the two chlorophyll *a* binding proteins of PS II as suggested by Zer and Ohad (1995). A coordinated degradation and biosynthesis of the D1 protein seems to be very crucial (Aro et al., 1993, Komenda & Barber, 1995), if not, the repair of the photodamaged PS II complex is in danger. Not only could secondary proteolysis of additional plastid-encoded subunits, such as the D2 protein, occur but also, as shown in this work, degradation of certain nuclear-encoded subunits. This in turn would make the re-establishment of PS II function a very complicated process involving the coordinated expression of two genomes and import of new subunits into the chloroplast from the cytoplasm. When such a severe stress condition is reached, this could probably result in a chronic state of photoinhibition. The possibility that the PsbW protein may be involved in the assembly of the PS II complex as a nucleation subunit (Funk et al., 1995; Irrgang et al., 1995; Lorcovic et al., 1995) could add to this complication. However, this function is probably restricted to *de novo* assembly of PSII and does therefore not apply under the present turnover conditions.

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