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The role of the FtsH and Deg proteases in the repair of UV-B radiation-damaged Photosystem II in the cyanobacterium *Synechocystis* PCC 6803

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Abstract

The photosystem two (PSII) complex found in oxygenic photosynthetic organisms is susceptible to damage by UV-B irradiation and undergoes repair *in vivo* to maintain activity. Until now there has been little information on the identity of the enzymes involved in repair. In the present study we have investigated the involvement of the FtsH and Deg protease families in the degradation of UV-B-damaged PSII reaction center subunits, D1 and D2, in the cyanobacterium *Synechocystis* 6803. PSII activity in a ΔFtsH (slr0228) strain, with an inactivated slr0228 gene, showed increased sensitivity to UV-B radiation and impaired recovery of activity in visible light after UV-B exposure. In contrast, in ΔDeg-G cells, in which all the three *deg* genes were inactivated, the damage and recovery kinetics were the same as in the WT. Immunoblotting showed that the loss of both the D1 and D2 proteins was retarded in ΔFtsH (slr0228) during UV-B exposure, and the extent of their restoration during the recovery period was decreased relative to the WT. However, in the ΔDeg-G cells the damage and recovery kinetics of D1 and D2 were the same as in the WT. These data demonstrate a key role of FtsH (slr0228), but not the Deg proteases, for the repair of PS II during and following UV-B radiation at the step of degrading both of the UV-B damaged D1 and D2 reaction center subunits.

Keywords: Photosystem II; D1-protein; D2-protein; FtsH protease; UV-B damage

1. Introduction

Ultraviolet-B (UV-B, 280–320 nm) radiation is a harmful component of sunlight that damages all forms of life including photosynthetic microbial organisms [1,2]. In plants and cyanobacteria a well-documented effect is the inhibition of photosynthesis leading to decreased oxygen evolution and CO_2 fixation [3–5]. Within the photosynthetic apparatus the most UV-B sensitive component is the light-energy converting Photosystem II (PSII) complex, whose electron transport capacity is inhibited and protein structure is damaged (for reviews see [6,7]). Inside PSII, the primary target of UV-B radiation is the Mn cluster of the water-oxidizing complex [8–10], with additional effects at the Tyr-Z and Tyr-D electron donors [11], and the Q_A and Q_B quinone electron acceptors [7,9,12]. UV-B has negative effects not only on electron transport of PSII but also on the key reaction center proteins, D1 and D2, which are degraded under UV-B irradiation [12–15].

To avoid permanent inhibition of PSII function due to the loss of the D1/D2 heterodimer, a tightly regulated repair process occurs in the thylakoid membranes of cyanobacteria and plants to replace the damaged proteins with new, fully functional copies [16–19]. The main steps of the PSII repair cycle are thought to involve: (i) a structural change to signal the need for damaged subunits to be removed after photodamage; (ii) monomerization and partial disassembly of the dimeric PSII complex to allow access to the damaged protein subunits; (iii) degradation of the damaged D1 and D2 subunits and synchronized replacement by newly synthesized copies, and (iv) reassembly of the extrinsic proteins and the Ca–Mn cluster

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PSII, photosystem II; WOC, water oxidation complex

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to the complex, which can occur only after C-terminal processing of the D1 subunit by the CtpA protease. In contrast to photodamage by visible light, where mainly the D1 subunit is damaged and repaired, UV-B radiation damages the D1 and D2 proteins to almost the same extent and the repair process includes *de novo* synthesis of both subunits [15].

As yet the proteases involved in PSII repair following UV-B damage have not been identified and it still remains unclear the extent to which non-enzymatic reactions are involved in protein degradation. *In vitro* studies using isolated PSII membrane fragments or detergent-solubilized PSII complexes indicated the formation of a 20-kDa C-terminal D1 fragment via a non-enzymatic mechanism, which would place the primary cleavage site of D1 to the middle of the second transmembrane helix [14]. However, this fragment did not accumulate to a significant amount, and the D1 protein was degraded also under conditions when the 20-kDa C-terminal fragment could not be observed. The degradation of D2 also appeared to be independent of protease activity in isolated PSII reaction center complexes [20]. Small amount of the 20 kDa D1 fragment was also observed when intact leaves were illuminated by UV-B. However, this fragment was unstable in visible light pointing to the possibility that protease activity is involved in the final step of the degradation process [21].

In the case of visible-light damage the FtsH and Deg proteases have both been implicated in PSII repair *in vivo*. There are four predicted FtsH proteases in the cyanobacterium *Synechocystis* 6803, designated slr0228, slr1604, slr1390 and sll1463. Insertional mutagenesis experiments revealed that two genes were absolutely required for cell viability (slr1390 and slr1604), one had no obvious phenotype (sll1463), and the fourth (slr0228) caused an altered pigmentation due to a 60% decrease in the content of PSI [22]. FtsH (slr0228) mutants were subsequently found to show visible-light sensitive growth, impaired PSII repair and retardation of D1 degradation *in vivo* [23]. The persistence of full-length D1 protein in the FtsH (slr0228) mutant, the co-purification of slr0228 with His-tagged PSII [23], and the exclusion of the functional role of other cyanobacterial proteases in the cleavage of damaged D1 protein has led to a general model for PSII repair in which FtsH complexes alone are able to degrade visible-light damaged D1 [19]. FtsH protease activity has also been associated with the degradation of oxidatively damaged D1 protein *in vivo* in higher plants [24,25].

In contrast an alternative view emphasizes the involvement of the DegP/HtrA or Deg proteases in PSII repair and D1 degradation following visible light stress, both in chloroplasts [26] and cyanobacteria [27]. In *Synechocystis* 6803 there are 3 members of the Deg family of proteases: HtrA (slr1204), HhoA (sll1679) and HhoB (sll1427) [28]. In the model of Huesgen et al. [27], which is partially supported by *in vitro* data [29], D1 is proposed to be cleaved in periplasmic-exposed loops by the HhoA protease. However, it has been recently reported that although the Deg proteases are required for photo-tolerance, they are not involved in D1 turnover following visible-light stress [19,30]. Whether FtsH and Deg proteases have a role in the response to UV-B damage is unclear.

Recent microarray data have indicated that UV-B radiation strongly induces the transcript levels of the *ftsH* (*slr0228)* gene in *Synechocystis* 6803 [31,32]. This observation points to the possibility that the FtsH (slr0228) protease could be involved in the repair of UV-damaged PSII complex similarly to its previously documented role in visible light stress [22].

Here we have studied the effect of inactivating the *slr0228* gene and the 3 *deg* genes of *Synechocystis* 6803 on PSII repair during and after UV-B damage. Our results show that in the Deg triple mutant, D1 and D2 degradation proceeds at the same rate as in the WT during UV-B radiation, and the efficiency of PSII repair is unaffected. However, in the ΔFtsH (slr0228) mutant PSII repair is largely abolished, and D1 and D2 protein degradation is retarded. Our work demonstrates the participation of the FtsH (slr0228), but not of the Deg proteases, in the repair of UV-damaged PSII, and importantly, that removal of damaged PSII subunits is mainly enzymatic *in vivo*.

2. Materials and methods

2.1. Synechocystis strains and growth conditions

The glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was used to construct the mutants [33]. Cells were routinely grown in BG-11 medium in a rotary shaker at 30 °C under a 5% CO_2-enriched atmosphere and 40 μE/m^{-2} s^{-1} light intensity. The ΔFtsH (slr0228) mutant was constructed by interrupting the *slr0228* gene with a chloramphenicol-resistance cassette [23]. The three DegP/HtrA genes were inactivated stepwise using the plasmids described earlier [34]: first *hhoA*, then *hhoB* to generate the *hhoAhhoB* double mutant and finally the *htrA* to give the ΔDeg-G triple mutant [30]. The genes were interrupted by chloramphenicol, erythromycin and kanamycin-resistance cassettes, respectively.

2.2. UV-B treatment

UV-B radiation was performed in open, square glasses in which 100 ml cell culture of 6.5 μg Chla/ml formed 1 cm layer height, maintained in suspension by magnetic agitation. UV-B light was provided by a Vilbert–Lourmat lamp, with maximum emission at 312 nm, in combination with 0.1 mm cellulose acetate filter (Clarfoil, Courtalouds Chemicals, UK) yielding an intensity of 12 μE m^{-2} s^{-1} at sample surface. In some cases, a protein–synthesis inhibitor, either lincomycin (at 300 μg/ml) or spectinomycin (at 200 μg/ml), was added to the cell culture. For the recovery experiments, visible light was produced by an array of halogen spot lamps in the 40–50 μE m^{-2} s^{-1} intensity ranges.

2.3. Oxygen evolution measurements

PSII activity was assessed by measuring the light-saturated rate of oxygen evolution from whole cells, in the presence of 0.5 mM 2,5-dimethyl-p-benzoquinone as electron acceptor, using a Hansatech DW2 O_2 electrode. Usually, 1 ml of cells at 6.5 μg Chl a/ml was used in each measurement.

2.4. Fluorescence measurements

Flash-induced increase and subsequent decay of chlorophyll fluorescence yield was measured by a double-modulation fluorometer (P.S.I. Instruments, Brno, Czech Republic) [35], in the 150 μs to 100 s time range, in samples which were dark adapted for 3 min prior to measurements, as described by [9].

2.5. Thylakoid preparation and protein analysis

Thylakoid membranes were prepared by breakage of the cells with glass beads (150–200 μm in diameter) at 4 °C followed by differential centrifugation

according to [18]. Protein composition was assessed by electrophoresis in a denaturating gel containing 6 M urea. After solubilization thylakoid extracts adjusted at 0.7–1 μg Chl *a* per lane were loaded and the gel was run overnight at 18 °C. Proteins were transferred onto a nitrocellulose membrane (0.45 μm, Schleicher and Schuell, Germany) by wet blotting. The membrane was incubated with an antibody raised against the C-terminus of pea D1 protein, and then with secondary antibody–alkaline phosphatase conjugate. The antigen–antibody complexes were visualized by colorimetric reaction using the BCIP–NBT system. The linearity of the immuno response was checked by loading dilution series of samples. The bands from the scanned blots were quantified using a NIH program, ImageJ.

2.6. RT-PCR detection of gene expression

Quantitative PCR (Q-PCR) was carried out on an ABI 7000 Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA) using SYBR green PCR Master mix of the same manufacturer. rnpB gene expression level was used as internal control for quantification of the gene expression data. Primer pairs for the individual sequences were designed using Primer Express 2.0 program (ABI). Clustalw alignments of the homologous sequences of the ftsH and degP families, respectively, were carried out for aiding the selection of primers mapping to unique sequence regions of the respective genes. Primers with the following sequences were used: slr0228–329F: CTGTCCGCAACAATGGCAT, slr0228–426R: GCTGGAACGGCGGAAGA; slr1604–210F: CAGCGGTGGTCCTCCCTAC, slr1604–286R: CCACGTTGTGTTGGGTGAGA; slr1390–69F: GATGGGTTTACTGGTAGCTGGC, slr1390–203R: GGTGTGGCTTCTCCATTGCT; sll1436–208F: CTCAAACCGGAAGCAGAGGA, sll1436–304R: AGCGCTTGGGTAACTCCAGA; rnpB–72F: GCGGCCTATGGCTCTAATCA, rnpB–197R: GGCGTTACCCAGCAAGTTTG; sll1427–703F:GGCCACATCGGTCATCGTAT, sll1427–826R: TGACGGCGAAGTTTTAACCAA; sll1679–663F: GAGCCACTGGTAGGGCAGAA, sll1679–792R: GGTGCCAGTAAAGTGGTGGTG; slr1204–289F: CCTGCCAATGAGAGCTTAGCA, slr1204–366R: CACGTCCACGACAAAATTGC.

Ten mL of samples were harvested by centrifugation and total RNA was isolated by hot phenol method [36] with minor modifications. The crude RNA was further purified and freed from DNA contamination using NusleoSpinRNA kit (Macherei Nagel, Düren, Germany) as per the manufacturer's instruction. 2 μg of the RNA was reverse transcribed using H-MuLV (Fermentas). Aliquots of the resulted cDNA was used in the Q-PCR reaction as template.

3. Results

3.1. Effects of inactivating the deg and ftsH genes on PSII activity in UV-irradiated cells

To investigate the physiological role of the Deg and FtsH proteases in the repair of UV-damaged PSII, we studied the UV-B induced loss of oxygen-evolving activity in the ΔDeg-G (with inactivated *slr1204*, *sll1679* and *sll1427* genes) and ΔFtsH (slr0228) (with an inactivated *slr0228* gene) mutants. In the absence of an inhibitor of protein synthesis, the WT and ΔDeg-G cells lost about 50% of their initial oxygen-evolving activity during 120-min UV-B irradiation, which was fully regained during a subsequent 120-min recovery period under normal growth conditions (Fig. 1A, B). In the ΔFtsH (slr0228) strain the activity loss during UV-B exposure was significantly higher (~80%), and the recovery under visible light was only partial (Fig. 1C). In the presence of a protein synthesis inhibitor, the WT and ΔDeg-G cells now showed an accelerated loss of oxygen evolution under UV-B exposure resulting in about ~70% activity decrease after 120 min, and almost complete loss of activity after 240 min exposure (Fig. 1A and B). However, in the ΔFtsH (slr0228) strain inhibition of protein synthesis did not accelerate further the loss of oxygen evolution (Fig. 1C), so that the kinetics were similar to that seen in the WT and ΔDeg-G cells in the presence of a protein synthesis inhibitor. Overall these data indicated that PSII repair was functioning with almost equally well in the Deg mutant as in WT, but was severely inhibited in the FtsH (slr0228) mutant.

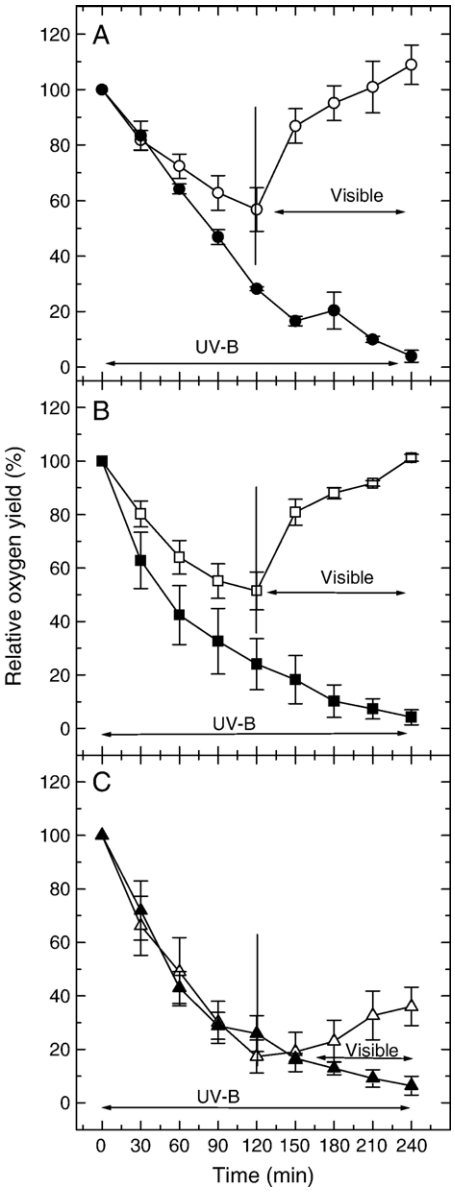

Fig. 1. The effect of UV-B illumination on PSII activity in the *deg* and *ftsH* (slr0228) mutants. WT (A), ΔDeg-G (B) and ΔFtsH (slr0228) (C) cells were exposed to UV-B light. The experiments were performed either in the presence (full symbols) of a protein synthesis inhibitor (lincomycin for WT and ΔFtsH (slr0228), and spectinomycin for ΔDeg), or in the absence (empty symbols) of protein synthesis inhibitors. In the presence of protein synthesis inhibitors cells were exposed only to UV-B light, whereas in the absence of protein synthesis inhibitors 120 min UV-B exposure was followed by a recovery period under visible light of 40 μE m^{-2} s^{-1} as indicated on the horizontal arrows. PSII activity was followed by oxygen evolution measurements in the presence of 1 mM DMBQ as an artificial electron acceptor. The data represent the average of three independent experiments, and shown after normalization to the oxygen evolution rates measured in the non-irradiated control cells.

The effect of UV radiation on the function of PSII can also be followed by measuring the kinetics of flash-induced chlorophyll fluorescence relaxation [37]. In the presence of DCMU, which occupies the Q_B-binding site and inhibits Q_A-to-Q_B electron transfer, the fluorescence relaxation reflects the recombination of Q_A^- with positively charged donor components of PSII. In non-irradiated cells the relaxation follows hyperbolic kinetics with about 1 s time constant, which arises from the recombination of Q_A^- with the S_2 state of the water-oxidizing complex [9] (Fig. 2A–C, squares). As a consequence of UV-B irradiation a faster component (with a 5–10 ms time constant) appears (Fig. 2A–C, circles), reflecting the recombination of Q_A^- with Tyr-Z^{ox} in PSII centers in which the electron transport between the Mn cluster and Tyr-Z has been inactivated [9]. The fraction of PSII centers showing the fast decaying component was about the same in the WT and ΔDeg-G cells, and substantially higher in the ΔFtsH (slr0228) cells. In the WT and ΔDeg-G cells the fast phase completely disappeared during recovery under visible light (Fig. 2A and B, triangles) demonstrating the restoration of normal electron transfer in the PSII complex. However, in the ΔFtsH (slr0228) cells there was only a small extent of restoration and the relaxation kinetics were dominated by the fast component even after 120 min recovery. The initial amplitude of the fluorescence signal reflects the amount of PSII centers that are capable of reducing Q_A. These include fully active centers with functional Mn cluster of the water-oxidizing complex, as well as centers with a non-functional Mn cluster and inactive in oxygen evolution, but which are nevertheless still able to transfer an electron from Tyr-Z to Q_A.

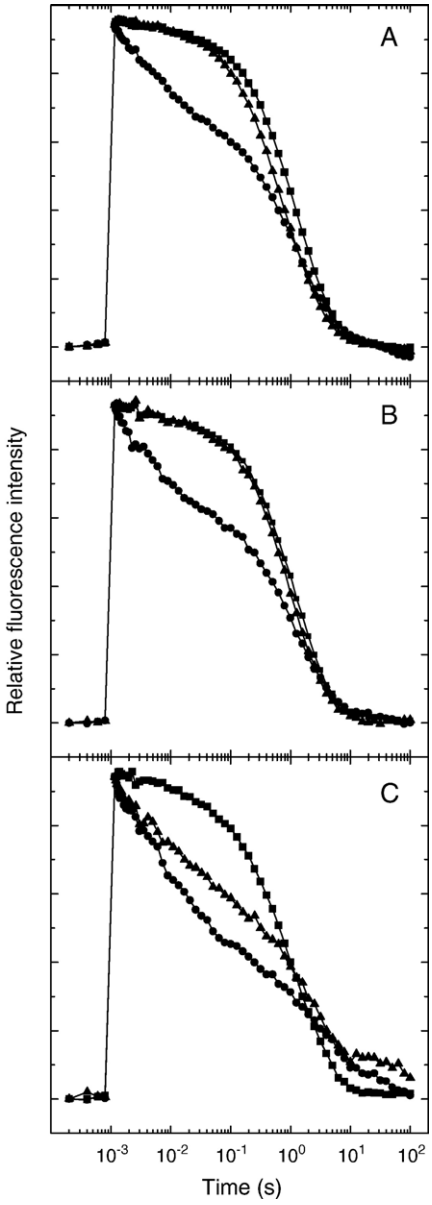

Fig. 2. Damage and recovery of flash-induced chlorophyll fluorescence in UV-B illuminated cells of ΔDeg-G and ΔFtsH. Cells were exposed to UV-B light followed by recovery under visible light of 40 $\mu Em^{-2}\ s^{-1}$. PSII function was followed by measuring flash-induced chlorophyll fluorescence in the presence of DCMU. The kinetics of fluorescence relaxation are shown for WT (A), ΔDeg-G (B) and ΔFtsH (slr0228) (C) cells before (squares) and after 120 min UV-B treatment (circles), as well as after 60 min recovery (triangles) after normalization to the same initial value.

3.2. UV-induced degradation of the D1 and D2 proteins in the Deg and FtsH (slr0228) mutants

In order to clarify the role of the Deg and FtsH proteases in D1 protein degradation we followed the time course of D1 protein levels during UV-B light treatment and subsequent recovery in visible light. In WT and ΔDeg-G cells, the amount of D1 decreased during the UV-B illumination to about 65% of the initial value, but its amount was restored to the original level in visible light. In contrast, in the ΔFtsH (slr0228) cells the amount of D1 was practically unchanged (Fig. 3) despite the severe inhibition of PSII activity shown in Fig. 1. To study the rate of D1 degradation without the compensating effect of *de novo* protein synthesis the experiments were also performed in the presence of a protein synthesis inhibitor. Under these conditions the UV-induced loss of the D1 protein was strongly accelerated in the WT and ΔDeg-G cells, but their kinetics were practically identical (Fig. 4). However, in the ΔFtsH (slr0228) cells the rate of D1 loss was significantly slower than in the WT or ΔDeg-G (Fig. 4). It is of note that UV-B radiation induced a specific 20 kDa C-terminal fragment in spinach BBY and core particles [14], as well as in higher plant leaves [21]. However, such fragment was not observed in whole cells of the WT, ΔFTSH (slr0228), or ΔDeg-G strains (Fig. 4A, C). We used the same C-terminal directed D1 antibody as was used in the earlier work [14], thus, the lack of the fragment should reflect the different behavior of isolated plant PSII and whole *Synechocystis* cells.

The involvement of specific proteases in the degradation of the reaction center proteins during PSII repair has so far been studied in detail only for D1, although the D2 subunit can also be degraded under extreme conditions [7,15]. UV-B irradiation provides a convenient tool to study the role of proteases in D2 degradation since UV-B light induces D2 loss to an extent comparable with that of D1. To this end we followed the kinetics of D2 abundance under the same conditions as was done for D1. The data in Fig. 5 show that in the absence of protein synthesis inhibitors the D2 protein was lost to the same

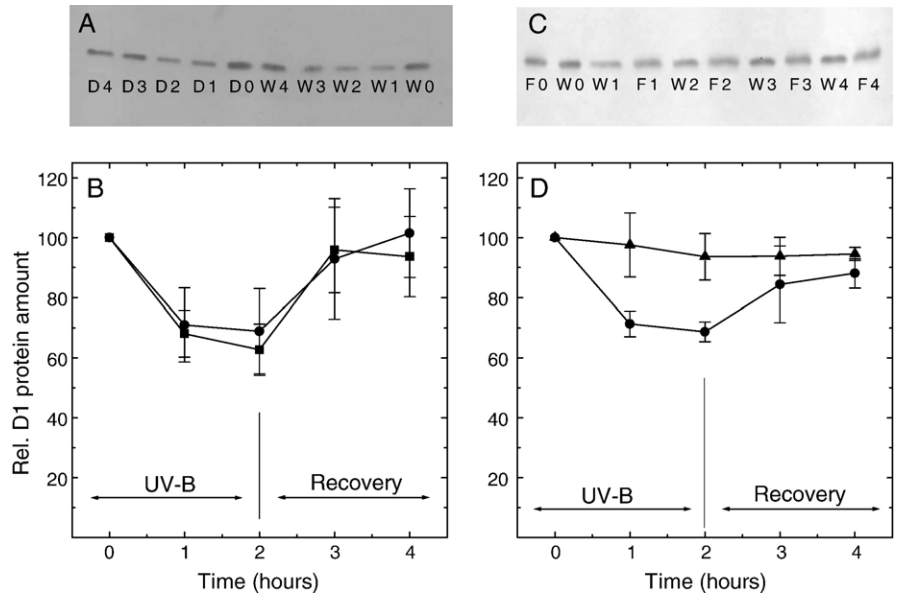

Fig. 3. D1 protein content in the absence of protein synthesis inhibitor in UV-B illuminated WT and mutant *Synechocystis* 6803 cells. Cells were exposed to UV-B radiation followed by recovery under visible light of 40 $\mu Em^{-2} s^{-1}$. Thylakoids were isolated at the indicated time points and D1 protein amount was determined by immunoblotting. (A and C) Immunoblots of D1 obtained in WT (W0,..,W4), ΔDeg-G (D0, …, D4) and ΔFtsH (slr0228) (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1) and 2 h (W2, D2, F2) UV-B exposure followed by 1 h (W3, D3, F3) and 2 h (W4, D4, F4) of recovery. (B and D) Changes in the D1 protein amount obtained from densitograms of blots of WT (circles), ΔDeg-G (squares) and ΔFtsH (slr0228) (triangles) thylakoids. The data are shown after normalization to the value at the 0 time point.

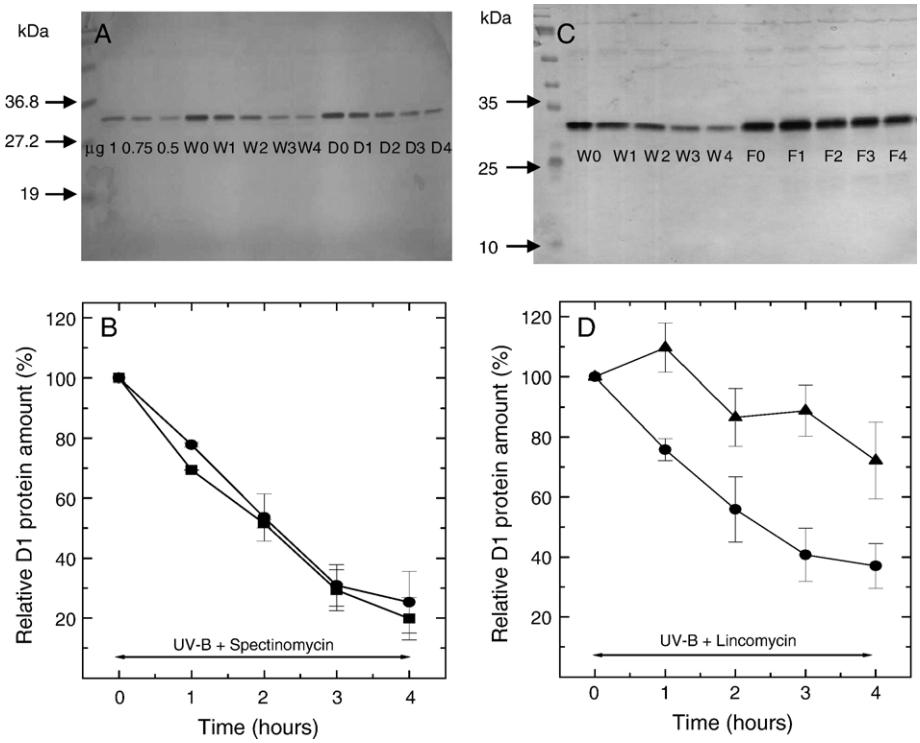

Fig. 4. D1 protein content in the presence of a protein synthesis inhibitor in UV-B illuminated WT and mutant *Synechocystis* 6803 cells. Cells were exposed to UV-B irradiation in the presence of protein synthesis inhibitors (spectinomycin for ΔDeg-G and its WT control, or lincomycin for ΔFtsH (slr0228) and its WT control). Thylakoids were isolated at the indicated time points and D1 protein amount was determined by immunoblotting. (A and C) Immunoblots obtained in WT (W0,..,W4), ΔDeg-G (D0, …, D4) and ΔFtsH (slr0228) (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1),…,4 h (W4, D4, F4). On the left side of panel A, a dilution series is shown with 0.5, 0.75 and 1.0 µg Chl/lane loading, whereas, the other samples contained 1,5 µg Chl. (B and D) Changes in the D1 protein amount obtained from densitograms of blots of WT (circles), ΔDeg-G (squares) and ΔFtsH (slr0228) (triangles) thylakoids. The data are shown after normalization to the value at the 0 time point.

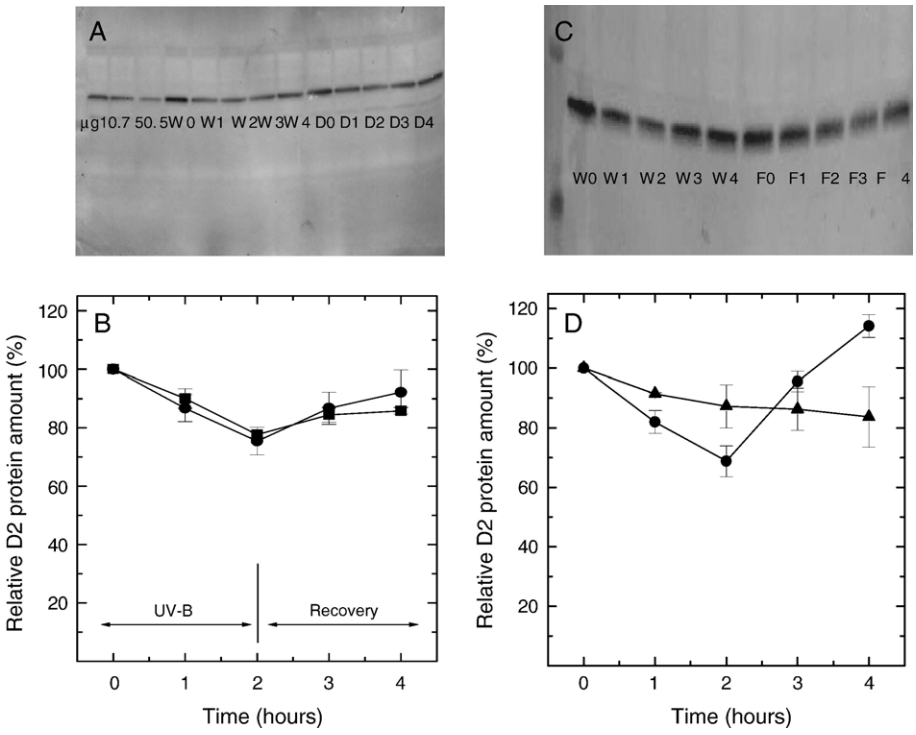

Fig. 5. D2 protein content in the absence of protein synthesis inhibitor in UV-B illuminated WT and mutant *Synechocystis* 6803 cells. Cells were exposed to UV-B irradiation followed by recovery under visible light of 40 $\mu Em^{-2}\ s^{-1}$. Thylakoids were isolated at the indicated time points and levels of D2 were determined by immunoblotting. (A and C) Immunoblots of D2 obtained in WT (W0,..,W4), ΔDeg-G (D0, …, D4) and ΔFtsH (slr0228) (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1) and 2 h (W2, D2, F2) UV-B exposure followed by 1 h(W3, D3, F3) and 2 h (W4, D4, F4) of recovery. On the left side of panel A, a dilution series is shown with 0.5, 0.75 and 1.0 µg Chl/lane loading, whereas, the other samples contained 1,5 µg Chl. (B and D) Changes in D2 levels obtained from densitograms of blots of WT (circles), ΔDeg-G (squares) and ΔFtsH (slr0228) (triangles) thylakoids. The data are shown after normalization to the value at the 0 time point.

extent as seen for D1 and was restored during the recovery period. The kinetics of D2 loss and recovery were almost identical for the WT and ΔDeg-G cells (Fig. 5B). However, loss of D2 during UV-B irradiation was retarded in ΔFtsH (slr0228) as compared to the WT, and its recovery in visible light was severely inhibited (Fig. 5D). Experiments performed in the presence of protein synthesis inhibitors confirmed the differential effect of inactivating the Deg proteases and FtsH (slr0228) on D2 protein degradation, since the kinetics of D2 loss were similar for the WT and ΔDeg-G cells in the presence of spectinomycin (Fig. 6A, B), whereas, the loss of D2 was much slower in ΔFtsH (slr0228) than in WT in the presence of lincomycin (Fig. 6C, D).

4. Discussion

4.1. FtsH (slr0228), but not the Deg proteases, is required for the recovery of UV-B inhibited PSII activity

Comparison of PSII activity in the presence of inhibitors of protein synthesis revealed that WT, ΔFstH (slr0228) and ΔDeg-G were equally susceptible to UV-B-induced damage. However, in comparison to the WT, the UV-B induced loss of PSII activity is accelerated in ΔFstH (slr0228), but remains unaffected in the ΔDeg-G cells when assessed in the absence of protein synthesis inhibitors. Restoration of PSII activity following UV-B exposure is also affected differentially in the two mutants: In the absence of all three Deg proteases, recovery proceeds like in the WT; however, the lack of the FtsH (slr0228) protease suppresses the recovery although does not block it completely (Fig. 1). The parallel oxygen evolution and chlorophyll fluorescence relaxation measurements demonstrate that FtsH (slr0228) is required for restoring electron transfer between the Mn cluster and the acceptor side of PSII via Tyr-Z (Figs. 1 and 2). The similar extent of effects caused by FtsH (slr0228) inactivation and by the presence of a protein synthesis inhibitor demonstrates that the absence of FtsH (slr0228) interrupts the PSII repair cycle and prevents the replacement of UV-B damaged PSII subunits with newly synthesized copies. The lack of significant effect of deleting the three Deg homologues shows that in contrast to FtsH (slr0228) the Deg protease family is not required for repair of UV-damaged PSII.

4.2. FtsH(slr0228), but not the Deg protease family, is required for degradation of UV-B damaged D1 and D2

In the PSII repair cycle the damaged D1 protein has to be removed and degraded before it can be replaced by a newly synthesized copy in the reaction center complex [18]. In contrast to visible light, which induces preferential damage and repair of D1, UV-B light leads to a similar extent of damage of both D1 and D2 [15]. Acceleration of D1 and D2

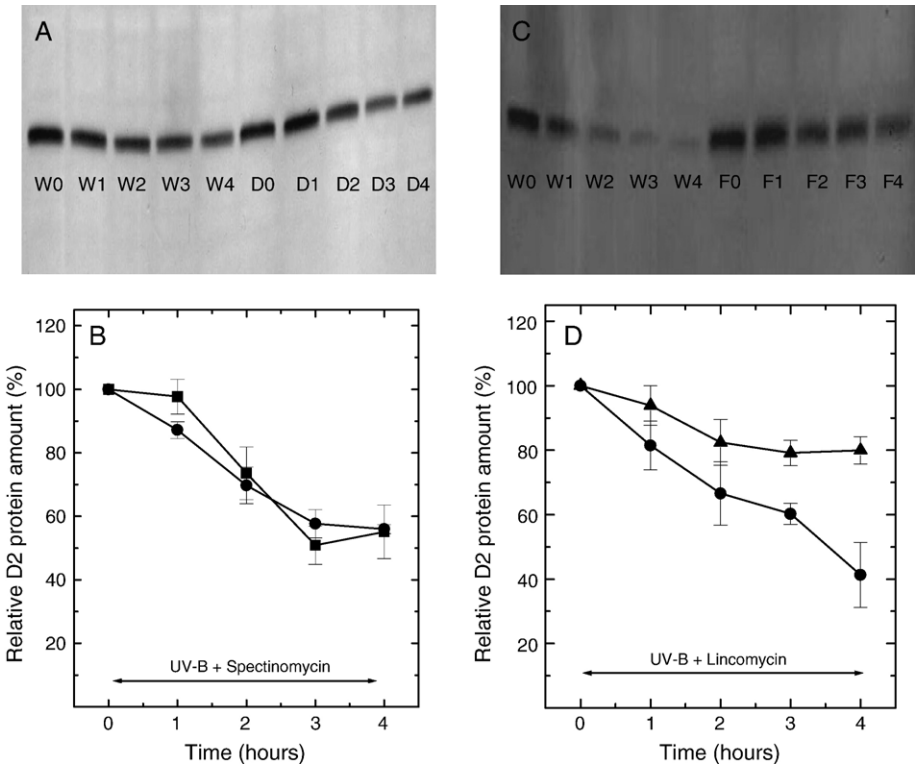

Fig. 6. D2 protein content in the presence of a protein synthesis inhibitor in UV-B illuminated WT and mutant *Synechocystis* 6803 cells. Cells were exposed to UV-B irradiation in the presence of protein synthesis inhibitors (spectinomycin for ΔDeg-G and its WT control, or lincomycin for ΔFtsH (slr0228) and its WT control). Thylakoids were isolated at the indicated time points and D2 levels were determined by immunoblotting. (A and C) Immunoblots obtained in WT (W0,..,W4), ΔDeg-G (D0, …, D4) and ΔFtsH(slr0228) (F0,…,F4) cells after 0 h (W0, D0, F0), 1 h (W1, D1, F1),…,4 h (W4, D4, F4). (B and D) Changes in D2 levels were obtained from densitograms of blots of WT (circles), ΔDeg-G (squares) and ΔFtsH (slr0228) (triangles) thylakoids. The data are shown after normalization to the value at the zero time point.

degradation under UV-B exposure and retardation of their resynthesis both under UV-B and visible light in the ΔFtsH (slr0228) mutant, as compared to the WT, show that FtsH (slr0228) is involved in the degradation of not only the D1 but also of the D2 protein. Our present results demonstrate that FtsH (slr0228) is involved in the removal of light damaged D2 subunit embedded in the PSII complex providing example for the more general role of this protease besides the selective degradation of D1. In contrast, the lack of a significant effect upon inactivating the three Deg homologues shows that the Deg proteases are not absolutely required for degradation of UV-B damaged D1 and D2.

Previous *in vitro* studies showed the formation of a 20-kDa C-terminal D1 fragment via a non-enzymatic mechanism when isolated thylakoid membrane particles were treated with UV-B light suggesting that this particular cleavage site of D1 is located in the middle of the 2nd transmembrane helix of D1 [14]. In case of the D2 protein a 22-kDa N-terminal D2 fragment was observed when isolated D1/D2 reaction center complexes were exposed to UV-B light in the presence of the quinone analogue DBMIB [20]. The 20 kDa fragment of D1 was also observed in UV-B irradiated leaves, although it was unstable in the presence of visible light [21]. However, these D1 and D2 fragments were formed only in a minor amount in the isolated membrane particles, and were not observed in the present study at all when whole *Synechocystis* cells were irradiated with UV-B light. In addition, fragments did not accumulate in the absence of FtsH of Deg proteases (Figs. 4 and 6) either. Therefore, we must conclude that the degradation pathways involving non-proteolytic D1 and D2 fragment formation are not significant in intact *Synechocystis* 6803 cells.

Housekeeping proteases, like the FtsH family, recognize misfolded or otherwise structurally modified proteins. Accord-

Table 1
The effect of UV-B irradiation on the relative levels of *deg* and *ftsH* transcripts in *Synechocystis* 6803

Synechocystis 6803 gene	mRNA induction(relative transcript level)
Deg proteases	
htrA/slr1204	1.03±0.13
hhoA/sll1679	2.31±0.33
hhoB/sll1427	0.89±0.33
FtsH proteases	
slr0228	11.5±2.8
slr1604	4.84±1.44
slr1390	2.75±0.63
sll1463	1.82±0.34

Cells were exposed to UV-B light for 90 min and the mRNA levels of the indicated genes were determined by quantitative RT PCR. The data represent the ratio of transcript levels in the UV-B treated and untreated control cells after normalization to the *rnpB* reference gene. The mean vales and standard errors are calculated from three independent experiments.

ing to a recent model of Nixon et al. [19], FtsH in cyanobacteria and chloroplasts is proposed to form a hexameric ring in the membrane, and the damaged protein is translocated through a central pore in an ATP-driven process and subsequently degraded at a Zn^{2+}-activated center in a highly processive reaction. Based on what is know about the orientation of FtsH in chloroplasts [38], the protease domain of FtsH (slr0228) is likely to be located on the cytoplasmic side of the thylakoid membrane rather than the luminal side [39]. This would mean that removal and degradation of UV-B damaged D1 and D2 also proceeds from the cytoplasmic side of the PSII complex. Proteolysis could therefore be initiated from the N-terminus of the full size protein or a fragment produced by a cleavage in a cytoplasmically exposed loop region. The N-termini of both D1 and D2 are located at the periphery of the PSII complex [40,41] and so ideally placed for engaging with FtsH.

Although our results demonstrate that the Deg homologues of *Synechocystis* are not required for the degradation of UV-damaged D1 and D2, the approximately 2-fold UV-B induction of the *sll1679* gene encoding HhoA (Table 1) indicates some role for this protein as well in the UV-B stress response other than the involvement in D1 and D2 turnover.

Whether the other FtsH homologues besides slr0228 are involved in PSII repair in *Synechocystis* 6803 is unclear. Recent work has demonstrated that two different types of FtsH subunit participate in PSII repair in *Arabidopsis thaliana* [42]. These so-called type-A and type-B subunits are suggested to form a hetero-hexameric complex [43]. Both types of subunit are made of a pair of redundant copies (type-A: FtsH1 and FtsH5, type-B: FtsH2 and FtsH8), and the complete absence of either the A- or B-types is lethal. Based on a recent phylogenetic analysis, FtsH (slr0228) and FtsH (slr1390) in *Synechocystis* 6803 would correspond to type-B subunits, and FtsH (slr1604) to a type-A subunit [44]. Thus, by analogy to chloroplasts, FtsH complexes in *Synechocystis* 6803 might also be composed of various heterocomplexes including an FtsH (slr1604)/FtsH (slr0228) complex, which would fit well with the observed UV-B induction of these two genes (Table 1). Our present data also show that some PSII repair (Fig. 1C), and D1 and D2 degradation persists in the ΔFtsH (slr0228) mutant (Figs. 4 and 6). This residual repair activity might be because FtsH (slr1390) can operate as a type-B subunit or that other types of proteases are able to catalyze PSII subunit degradation albeit with reduced effectiveness. Clarification of the interplay of the different FtsH copies in the turnover of the D1 and D2 subunits of PSII will be an important challenge of research in the near future.

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