The FtsH Protease slr0228 Is Important for Quality Control of Photosystem II in the Thylakoid Membrane of Synechocystis sp. PCC 6803*

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The cyanobacterium Synechocystis sp. PCC 6803 contains four members of the FtsH protease family. One of these, FtsH (slr0228), has been implicated recently in the repair of photodamaged photosystem II (PSII) complexes. We have demonstrated here, using a combination of blue native PAGE, radiolabeling, and immunoblotting, that FtsH (slr0228) is required for selective replacement of the D1 reaction center subunit in both wild type PSII complexes and in PSII subcomplexes lacking the PSII chlorophyll a-binding subunit CP43. To test whether FtsH (slr0228) has a more general role in protein quality control in vivo, we have studied the synthesis and degradation of PSII subunits in wild type and in defined insertion intermediates and unassembled PSII proteins markedly increased. Pulse-chase experiments showed that this was due to reduced rates of degradation in vivo. Importantly, analysis of epitope-tagged and green fluorescent protein-tagged strains revealed that slr0228 was present in the thylakoid and not the cytoplasmic membrane. Overall, our results show that FtsH (slr0228) plays an important role in controlling the removal of PSII subunits from the thylakoid membrane and is not restricted to selective D1 turnover.

All cellular organisms possess quality control mechanisms to prevent the accumulation of unwanted proteins (1). Of current interest are the processes by which damaged and unassembled proteins are removed from the thylakoid membrane, which is the location of the protein complexes involved in oxygenic photosynthetic electron transport in cyanobacteria and chloroplasts (2). Side reactions associated with the light reactions of photosynthesis lead to the production of a variety of potentially hazardous molecules (e.g. reactive oxygen species) that can oxidize components, such as amino acid side chains and pigment cofactors (3). Ultimately, unless the damage is repaired, there is a net reduction in photosynthetic performance known as photoinhibition (for review see Ref. 4). As yet, the repair pathways and, more generally, the quality control processes involved in the assembly of the thylakoid membrane protein complexes and removal of aberrant proteins remain poorly understood.

One component that is especially prone to photodamage is the photosystem II (PSII) complex, which is composed of over 25 membrane and peripheral proteins and is responsible for the light-driven oxidation of water and reduction of plastoquinone (5). The D1 protein, together with the homologous D2 protein, binds the cofactors involved in electron transfer through the complex (6). The D1 subunit is also the chief target for light-induced damage in PSII and undergoes rapid synthesis and degradation in the light to maintain PSII activity in what is termed the PSII repair cycle (4).

Although the precise mechanism of PSII repair is unknown, recent work has emphasized the importance of FtsH proteases for D1 turnover in vivo (7). This class of proteases is found throughout nature and is involved in the degradation of both soluble and membrane proteins (8). The prototypical Escherichia coli FtsH protease is a membrane-bound zinc metalloprotease and a member of the AAA (ATPases associated with a variety of cellular activities) class of proteins (9). The active form of the protease, most probably the hexamer (10), extracts the membrane-embedded parts of a substrate from the lipid bilayer for degradation in a hydrophilic environment (11).

The genome of the cyanobacterium Synechocystis sp. PCC 6803 encodes four putative FtsH homologues. Two of them, encoded by slr1390 and slr1604, are required for survival of the organism (12). In contrast, deletion of slr1463 does not result in any apparent phenotypic change (12), whereas ftsH (slr0228) insertion mutants showing light-sensitive growth are impaired in PSII repair and display slower rates of D1 degradation in vivo (13). These data and the observation that FtsH (slr0228) co-purified with His-tagged PSII (14) have led to a model in which FtsH (slr0228), possibly together with other FtsH homologues, is directly involved in the degradation of damaged D1 (7). A detailed analysis of the assembly and turnover of PSII subunits in ftsH (slr0228) insertion mutants has not yet been performed. Whether FtsH (slr0228) is located in the thylakoid or cytoplasmic membrane is also unknown and is an important point to resolve, because both sets of membranes are implicated in PSII assembly and repair (14, 15).

The Arabidopsis thaliana chloroplast also contains a number of FtsH homologues, all nucleus-encoded (16). Of these, FtsH2 (VAR2) has been shown to be involved in D1 degradation (17), but other FtsH members

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2 The abbreviations used are: PSII, photosystem II; BN, blue native; RCC, reaction center core; RC47, reaction center containing CP47; WT, wild type; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid; GFP, green fluorescent protein.
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are also likely to participate (18, 19). The possibility that FtsH proteases might have a more wide ranging role in quality control in the chloroplast has come from studies of a zinc-dependent proteolytic activity, assigned to FtsH, which is involved in the degradation of unassembled Rieske iron-sulfur protein in isolated chloroplasts (20). However, whether chloroplast FtsH has a role in removing unassembled proteins in planta has not yet been established.

In the present paper, we have taken a mutagenesis approach to testing whether FtsH (slr0228) has a general role in removing malfunctioning membrane proteins in *Synechocystis* sp. PCC 6803 in vivo. We have analyzed the effect of inactivating FtsH (slr0228) on the accumulation and degradation rates of PSII proteins in WT and in PSII mutants that are impaired to varying degrees in their ability to assemble the PSII holoenzyme. In such mutants, PSII subunits are typically inserted into the membrane in vivo, but they are usually rapidly degraded and fail to accumulate to appreciable levels (21). Overall, our results show that FtsH (slr0228) is needed both for selective D1 turnover during PSII repair and for the removal of unassembled PSII subunits and non-functional partially assembled PSII complexes. In addition, confocal microscopy and membrane fractionation indicate that the vast majority (if not all) of the enzyme is located in thylakoids and not in the cytoplasmic membrane.

**MATERIALS AND METHODS**

*Strains and Growth of Organisms*—The glucose-tolerant strain *Synechocystis* sp. PCC 6803 (22), referred to as wild type (WT), and its following previously constructed mutants were used in the study: (i) the CP43-less strain ΔCP43 with the psbC gene inactivated by a kanamycin resistance (kan<sup>R</sup>) cassette (21); (ii) the D1-less strain ΔD1 with the psba1, psba2, and psba3 genes inactivated by chloramphenicol (cm<sup>R</sup>), kan<sup>R</sup>, and spectinomycin (spec<sup>R</sup>) resistance cassettes, respectively (23); and (iii) the site-directed mutants D1-H198L and D1-H198V having the psba1 and psba2 genes inactivated by cm<sup>R</sup> and kan<sup>R</sup>, respectively, and the codon for residue His<sup>198</sup> in the psba3 gene replaced by codons for Leu or Val (24). The strains were grown in BG-11 containing 5 mM glucose. Solid medium contained, in addition, 10 mM Tes/NaOH, pH 8.2, 1.5% agar, and 0.3% sodium thiosulfate (25). 100–200-ml liquid cultures were grown at 29°C with shaking and as surface irradiance of 30 μmol m<sup>−2</sup> s<sup>−1</sup> at 29°C.

*Preparation of Membranes and Protein Analysis*—Total cyanobacterial membranes were prepared by breaking the cells with glass beads (150–200 μm in diameter) at 4°C followed by differential centrifugation (29). Thylakoid membranes were separated from cytoplasmic membranes by discontinuous sucrose density centrifugation followed by aqueous polymer two-phase partitioning (30, 31). Isolated membranes were analyzed by two-dimensional PAGE consisting of a blue native polyacrylamide gel electrophoresis (BN-PAGE, 5–14% linear gradient gel) in the first dimension and SDS-PAGE (12–20% linear gradient gel containing 7 M urea) in the second dimension (28). If not specified in the figure legend, standard protein analysis was performed by SDS-PAGE using the same gel as for two-dimensional analysis. The separated proteins were either stained by Coomassie Blue or transferred onto polyvinylidene difluoride membrane and immunodetected. The primary antibodies specific for D1, D2, CP43, CP47, and α-subunit of cytochrome b<sub>559</sub> were used as described in Ref. 28. Additionally, we used antibodies raised against *Chlamydomonas reinhardtii* CP43, against residues 184–203 of SbtA (the *Synechocystis* sodium-dependent bicarbonate transporter found in the cytoplasmic membrane) (32), and against *Strep* tag II (Qiagen). For autoradiography, the gel or the membrane with labeled proteins was exposed to x-ray film at laboratory temperature for 2–3 days.

*Fluorescence Microscopy*—Fluorescence micrographs were obtained with a Nikon PCM2000 laser-scanning confocal microscope equipped with a 100 milliwatt argon laser (488 nm) as described in Ref. 28. GFP fluorescence emission was selected with an interference band-pass filter transmitting from 500 to 527 nm, and chlorophyll fluorescence emission was selected with a 665-nm long pass filter. Images were scanned over a 47 × 47-μm window, with 512 × 512 pixels and a dwell time of 3 μs/pixel. Images were averaged from 10 scans. GFP and chlorophyll fluorescence images were smoothed and merged using EZ2000 viewer software (Nikon).

**RESULTS**

*FtsH (slr0228) Is Required for Selective D1 Replacement in WT in Vivo*—The PSII complex in *Synechocystis* assembles in a stepwise fashion through a number of distinct intermediates, including the PSII reaction center (comprising D1, D2, and cytochrome b<sub>559</sub>), a PSII core complex lacking CP43 (RC47), and the monomeric core complex reaction center core (RCC). The abundance of these and other assembly intermediates, plus free subunits, can be followed using two-dimensional blue native (BN)/SDS-PAGE and immuno-blotting (28). By analyzing cells pulse-labeled in the light with [35S]methionine/cysteine, the relative rates of synthesis of individual subunits can be assessed. In accordance with earlier work, we found that WT accumulated the monomeric PSII RCC together with smaller amounts of RC47 and unassembled CP47 and CP43 (Fig. 1A) (28). As reported previously (28), radiolabeling of the D1 protein in the RCC and RC47 complexes was higher than that of D2 and CP43 (Fig. 1B). This is consistent with the view that D1 is the main target for damage in PSII and shows the greatest rate of selective replacement or turnover. CP47 was, in contrast, labeled weakly, presumably

![Image](361x26 to 388x38)

![Image](389x26 to 416x38)

![Image](417x26 to 444x38)

![Image](445x26 to 472x38)

![Image](473x26 to 500x38)

![Image](501x26 to 528x38)

![Image](529x26 to 556x38)

![Image](557x26 to 584x38)

![Image](585x26 to 612x38)

![Image](613x26 to 640x38)

![Image](641x26 to 668x38)

![Image](669x26 to 696x38)

![Image](697x26 to 724x38)

![Image](725x26 to 752x38)

![Image](753x26 to 780x38)

![Image](781x26 to 808x38)
Because this subunit is recycled and damaged less frequently than the other PSII subunits.

PSII biogenesis was similarly analyzed in strain \( \Delta \text{ftsH} \) containing a disrupted \( \text{ftsH} \) (slr0228) gene (Fig. 1, C and D). A number of important differences compared with the WT were consistently observed (Fig. 1, A and B). First, \( \Delta \text{ftsH} \) showed similar extents of D1, D2, and CP43 radiolabeling in the RCC complex (Fig. 1D). This observation suggested that RCC in the mutant was assembled predominantly from newly synthesized D1, D2, and CP43 proteins and did not involve significant levels of selective D1 turnover. Second, despite the higher level of RC47 in the mutant, as deduced from the stained gel (Fig. 1, compare A and C) and D1 blot (Fig. 1E), the D1 and D2 proteins in this complex had incorporated less radiolabel in the \( \Delta \text{ftsH} \) mutant than in WT. This suggested that RC47 in \( \Delta \text{ftsH} \) originates from the disassembly of older RCC complexes, whereas in WT this complex represents either an assembly intermediate or results from the rapid disassembly of newly formed RCC. As judged from the autoradiograms (Fig. 1, B and D) and D1 blot (Fig. 1E), the mutant also contained increased levels of unassembled D1 protein in its three forms: precursor D1 (pD1), a C-terminally processed intermediate that still retained part of the C-terminal extension (iD1), and mature D1 (D1). These forms of D1 have already been assigned in WT in previous work using specific antibodies (28, 34).

**Influence of FtsH (slr0228) on D1 Turnover and the Accumulation of PSII Assembly Intermediates in a CP43-deficient Strain**—The results in Fig. 1 are consistent with the hypothesis that selective D1 turnover is impaired in the \( \Delta \text{ftsH} \) mutant. Although reproducible, the differences in D1 radiolabeling between \( \Delta \text{ftsH} \) and WT were rather small. To confirm more conclusively that selective D1 turnover was indeed impaired in the absence of FtsH (slr0228), we decided to analyze the effect of disrupting \( \text{ftsH} \) (slr0228) in a mutant that shows a much higher level of selective D1 turnover than WT. Such a mutant is \( \Delta \text{CP43} \), which lacks the \( \text{psbC} \) gene encoding the inner PSII antenna CP43 (35). Strain \( \Delta \text{CP43} \) accumulates reduced amounts of PSII (10–20% of WT level) in the form of RC47 and shows intense radiolabeling of D1 in pulse-labeling experiments, suggesting a high degree of selective D1 replacement (28). Fast turnover of D1 was confirmed by a pulse-chase experiment that showed the half-life of the D1 protein to be \( <20 \) min in \( \Delta \text{CP43} \) (Fig. 2A) compared with \( \sim 180 \) min for D1 in WT cells (not shown). When the \( \text{ftsH} \) (slr0228) gene was inactivated in the \( \Delta \text{CP43} \) strain, the growth rate of the resulting double mutant \( \Delta \text{CP43} \Delta \text{ftsH} \) markedly decreased (Fig. 2C). As revealed by \textit{in vivo} absorption spectra (not shown), the strain also accumulated 40% less chlorophyll (as estimated by the absorbance at 690 nm) and a significantly higher level of carotenoid (especially myxoxanthophyll) as revealed by high pressure liquid chromatography (not shown) on a per cell basis when compared with the original \( \Delta \text{CP43} \) strain. Estimation of the level of the D1, D2, and CP43 proteins by immunoblots showed a dramatically increased level of all three proteins as a consequence of disruption of the \( \text{ftsH} \) (slr0228) gene (Fig. 2B). Due to the high content of carotenoid, the two-dimensional pattern of PSII protein as determined by BN/SDS-PAGE was unavoidably smearable (Fig. 3, C–E). Nevertheless, this technique clearly showed that the vast majority of D1 was assembled into monomeric RC47 complexes plus a higher molecular mass complex that we attribute to dimeric RC47 (Fig. 3E). Together with the data in Fig. 2B, this means that the \( \Delta \text{CP43} \Delta \text{ftsH} \) strain accumulates substantially more RC47 complex than the WT. As in the \( \Delta \text{ftsH} \) strain, the \( \Delta \text{CP43} \Delta \text{ftsH} \) strain exhibited accumulation of radioactively labeled unassembled D1 protein. However, this accumulation was higher than in \( \Delta \text{ftsH} \), in agreement with higher synthesis of the D1 protein found in \( \Delta \text{CP43} \) compared with WT. Unassembled D1 protein could also be detected on the two-dimensional immunoblot in \( \Delta \text{CP43} \Delta \text{ftsH} \) but not in \( \Delta \text{CP43} \) (Fig. 3E). No decline in the intensity of the D1 radiolabeling in the pulse-chase experiment (Fig. 2A) and equivalent low levels of D1 and D2 radiolabeling in RC47 on the two-dimensional gel (Fig. 3D) confirmed the dramatic inhibition of selective D1 turnover in the \( \Delta \text{CP43} \Delta \text{ftsH} \) strain. Together these results showed that FtsH (slr0228) participates in fast D1 turnover \textit{in vivo} in the CP43-less strain and that its absence leads to higher accumulation of the RC47 complex.

**FtsH (slr0228) Is Needed to Degrade Unassembled PSII Subunits D2 and CP47**—To examine whether FtsH (slr0228) is involved in the removal of unassembled PSII proteins from the membrane, we investigated the effect of removing FtsH (slr0228) on the assembly status and quantity of PSII proteins in a strain lacking all three \( \text{psbA} \) genes encoding the D1 protein. Recent two-dimensional protein analysis of the D1-less strain \( \Delta \text{D1} \) revealed no accumulation of PSII complexes and only the presence of unassembled CP43 and smaller amounts of CP47 and D2 (28). Corresponding autoradiograms confirmed the absence of PSII complexes and the ongoing synthesis of CP43, CP47, and D2 (28). Analysis by SDS-PAGE of the D1 strain in which the slr0228 gene was also inactivated (\( \Delta \text{D1}/\Delta \text{ftsH} \)) suggested increased levels of CP47 (Fig. 4A). This was confirmed by more detailed immunochemical analysis that showed significantly increased levels of CP47 and D2 upon removal of FtsH (slr0228) (Fig. 4B). Although these proteins reached WT levels, they did not form PSII subcomplexes as judged by BN-PAGE (data not shown). In contrast to D2 and CP47, the levels of CP43 and the \( \alpha \)-subunit of cytochrome \( b_{599} \) were almost equal in all tested strains, suggesting that they were not a matter of control by FtsH (slr0228) (see next
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Whether the levels of the other low molecular mass PSII subunits (<10 kDa) were also affected by removal FtsH (slr0228) could not be assessed because of the lack of specific antibodies.

Pulse-chase experiments clearly showed that the increased level of D2 and CP47 was due to the inhibition of their degradation (Fig. 4A). In the case of CP47, a reduction in the rate of synthesis was also noticed. Surprisingly, degradation of CP43 was also largely inhibited in the absence of FtsH (slr0228), but in this case, synthesis of the protein was also markedly reduced, leading therefore to little overall effect on the accumulation of CP43, as detected by immunoblot. Importantly, removal of FtsH (slr0228) from the ΔD1 strain inhibited growth in a light-dependent manner (Fig. 4C).

Absence of FtsH (slr0228) Leads to Accumulation of Mutated D1 Protein with a Modified Binding Site for Reaction Center Chlorophyll—One physiological role for FtsH (slr0228) might be to remove aberrant proteins or complexes from the membrane that have incorporated an incorrect amino acid residue through, for example, mistranslation of mRNA. To test this possibility, we analyzed the effect of removing FtsH (slr0228) on the accumulation of D1 in site-directed mutants that do not normally accumulate D1. A key residue of D1 is D1-His198, which ligates chlorophyll P_D1, which in turn becomes oxidized following charge separation in PSII (24). In the case of mutants D1-H198L and D1-H198V, which are unable to provide an amino acid ligand to P_D1, there is a drastic decrease in the cellular content of D1 and consequently also of

FIGURE 2. Turnover (A) and steady-state levels of PSII proteins (B) in cells of the Synechocystis CP43-less mutant ΔCP43 and the double mutant ΔCP43/ΔFtsH and photoheterotrophic growth of their cells (C). A, pulse-radioabeled cells were exposed to white light (500 μmol of photons·m⁻²·s⁻¹) for 2 h in the presence of non-radioactive amino acids (chase). Thylakoids from cell aliquots taken at the times indicated were analyzed by SDS-PAGE (see “Materials and Methods”). The gel was stained (left panel), dried, and exposed to film (center panel). Identification of the stained and radioactive bands was performed by alignment with a blot containing immunodecorated bands of CP47, D2, and D1 (right panel). B, thylakoids of each strain containing 2, 1, and 0.5 μg of chlorophyll were immunoblotted using antibodies specific for D1, D2, and CP47. For comparison, the amount of proteins in WT corresponding to 2 μg of chlorophyll is also shown on the right edge of the membrane. Protein loading for all lanes is documented by Ponceau-stained subunits α and β of the ATP synthase (ATP synth stain). C, the cultures were grown at 29 °C in BG-11 containing 5 mM glucose with shaking and a surface irradiance of 30 μmol of photons·m⁻²·s⁻¹ of white light. For each time point, duplicate measurements of OD 750 were made and plotted as the average. The differences between the two values were less than the size of the symbols. rel.u., relative units.

FIGURE 3. Coomassie stain (A and C), autoradiogram (B and D), and D1 blot (E) of thylakoid membrane proteins from the CP43-less mutant ΔCP43 (A, B, and E) and the double mutant ΔCP43/ΔFtsH (C, D, and E) after separation by two-dimensional PAGE. Separation of proteins and designation of complexes was as described in the legend to Fig. 1. For E, the two-dimensional gel was electroblotted and immunodecorated using D1-specific antibody. ATP synth, ATP synthase.
PSII complexes (24). When the \textit{ftsH} (sr0228) gene was inactivated in these mutants, the amount of D1 detected in the membrane increased dramatically (Fig. 5), consistent with the proposed role for FtsH (sr0228) in quality control.

\textbf{FtsH (sr0228) Is Found in the Thylakoid Membrane—}Based on the detection of D1 in cytoplasmic membrane fractions, it has been suggested that PSII repair might occur in this membrane (36). Two experimental approaches were used to investigate the cellular location of FtsH (sr0228). In the first, the presence of \textit{Strep} II-tagged FtsH (sr0228) was tested in cytoplasmic and thylakoid membrane fractions isolated using the two-phase partition procedure developed by Norling and colleagues (30, 31). The effectiveness of the separation procedure was confirmed by the detection of CP43 and SbtA, which are found exclusively in the thylakoid (14, 30) and cytoplasmic (32) membranes, respectively (Fig. 6A). The epitope-tagged FtsH (sr0228) was only found in the thylakoid fraction (Fig. 6A).

As further confirmation, cells expressing FtsH (sr0228) tagged with GFP were imaged by confocal fluorescence microscopy. Control immunoblotting experiments confirmed that the FtsH-GFP fusion protein was membrane-bound (data not shown). Fluorescence spectra (data not shown) confirmed the presence of significant additional fluorescence in the green region, peaking at ~510 nm, which was seen in the FtsH-GFP cells but not in WT. Red chlorophyll fluorescence on images of FtsH-GFP cells (Fig. 6B, a) comes mainly from PSII and provides an excellent way of visualizing the thylakoid membranes in fluorescence micrographs (33). To check that the green fluorescence came from GFP and was not background autofluorescence from the cells, fluorescence micrographs were recorded with identical settings for WT cells (not shown). The background autofluorescence was, to some extent, variable from cell to cell but was always much lower than the green fluorescence from GFP-FtsH cells. Thus the green fluorescence images (Fig. 6B, b) report on the distribution of GFP and, hence FtsH, within the cells. The merged image (Fig. 6B, c) shows strong overlap between GFP and chlorophyll fluorescence, with the exception of a few cells in which chlorophyll fluorescence is very weak. This clearly indicates that FtsH is located in the thylakoid membranes. Optical resolution is quite adequate to distinguish between the location in the thylakoids and the periplasm/cytoplasmic membrane. This is documented by the images of the \textit{Synechocystis} strain expressing the TorA-GFP (33). TorA-GFP is a fusion protein comprising the Tat-specific targeting signal of \textit{E. coli} TorA linked to GFP. The fusion protein is localized in periplasm, and this compartment is seen as a halo outside the thylakoid membrane area (Fig. 6B, d). Similarly, the fluorescence originating solely from the cytoplasm is clearly distinguishable as documented by an image of an acridine orange-stained cell (Fig. 6B, e). Although the merged image (Fig. 6B, c) shows a rather similar distribution of FtsH and PSII, the brightest areas of GFP fluorescence did not always correspond to the brightest areas of PSII fluorescence. This suggests that there may be localized concentrations of FtsH and PSII within the thylakoid membrane system.

\textbf{DISCUSSION}

The analysis by two-dimensional BN-PAGE of radiolabeled membrane proteins in the Δ\textit{ftsH} strain and, more spectacularly, in the ΔCP43/Δ\textit{ftsH} double mutant provides clear data to support a role for FtsH (sr0228) in the selective replacement of D1 in the PSII repair cycle.
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| A | M     | S | TM | CM |
|---|-------|---|----|----|
| FtsH-Strep | 1 | 5 | 1 | 5 µg protein |
| CP43     |     |   |    |     |
| SbtA     |     |   |    |     |

B

![GFP fluorescence](image)

Merged images

![Acridine orange in cytoplasm](image)

Overall, our data, together with that of Silva *et al.* (13), suggest that the block in PSII repair in FtsH (slr0228) insertion mutants lies at the stage of removing full-length damaged D1 from the PSII complexes. The presence of unassembled radiolabeled D1 in the membrane argues against a limitation of newly synthesized D1 for PSII repair. There was also no evidence of significant fragmentation of PSII core complexes into individual subunits, although there were elevated levels of RC47, which might be an intermediate in PSII repair (28).

Our current working model therefore proposes that FtsH (slr0228) plays a direct role in the degradation of damaged D1 (7).

By analogy to what is known about the mechanism of *E. coli* FtsH (10), it is possible that FtsH (slr0228) removes D1 from the membrane in a highly processive reaction, without the accumulation of intermediates. Indeed we could obtain no evidence for the accumulation of D1 fragments in any of our experiments. However, we cannot yet dismiss an indirect role for FtsH in D1 turnover, such as in the recruitment or activation of other proteases or the trafficking of damaged D1 to the site of repair (19).

Importantly, we have obtained several lines of evidence to support the concept that the FtsH (slr0228) protease plays a more wide ranging role in the quality control of PSII than previously thought. First, D1 turnover was dramatically inhibited in a ΔCP43ΔFtsH double mutant compared with the parental ΔCP43 strain, and there was a substantial increase in the level of the PSII subcomplex RC47 (35). This result suggests that inactivating *ftsH* (slr0228) might be a useful way to obtain mutants that accumulate sufficient amounts of PSII complexes with modified structure and/or function for advanced biophysical and biochemical studies. These complexes are normally quickly recognized and degraded so their accumulation in the FtsH-containing strains is very limited.

Second, inactivation of *ftsH* (slr0228) in a D1-deficient strain led to increased levels of unassembled D2 and CP47 proteins due to inhibition of their degradation, as confirmed by radioactive pulse-chase experiments. In contrast, the steady-state level of unassembled CP43 was not dramatically affected by removal of FtsH (slr0228). However, pulse-chase radiolabeling revealed that CP43 was, in fact, degraded more slowly but that this was accompanied by a decreased rate of synthesis. Interestingly, the amount of CP43 in the ΔD1/ΔFtsH mutant remained close to the wild type level even when CP43 was unassembled and no longer part of a larger PSII complex. This suggests that synthesis and degradation are highly coordinated so that protein density in the thylakoid does not become too high.

Third, removal of FtsH (slr0228) caused a substantial increase in the levels of mutated D1 protein in site-directed mutants in which the chlorophyll ligand D1-His198 is replaced by Val and Leu. It is probable that the mutated D1 proteins are recognized as improperly assembled and, as in the ΔCP43 strain, are quickly removed. As spontaneous mutations occur rather frequently in cyanobacteria, FtsH (slr0228) may play a crucial role in preventing detrimental accumulation of such aberrant, non-functional proteins and their complexes.

Importantly, inactivation of *ftsH* (slr0228) has a drastic effect on the growth rate of *Synechocystis* sp. PCC 6803, both in a WT background (13) and in various PSII mutants, such as ΔCP43 (Fig. 2C) and ΔD1 (Fig. 4C). The growth defect displayed by the ΔD1/ΔFtsH strain provides compelling evidence for an important physiological role for FtsH (slr0228) beyond that of D1 degradation, such as in the biosynthesis of PSI (12) and possibly other thylakoid proteins.

The site of PSI repair in *Synechocystis* sp. PCC 6803 remains controversial. Our detection of FtsH (slr0228) in the thylakoid membrane, using two independent localization approaches, supports this compartment as the site of D1 degradation. If D1 is inserted into PSI co-translationally (37), this would mean that the thylakoid membrane is also the site of resynthesis of D1 during PSI repair, as suggested by Jansen *et al.* (15). If true, the D1 C-terminal processing protease CtpA, which is needed to reassemble the water-oxidizing complex (38), might not be restricted to the cytoplasmic membrane (14). Alternatively, if CtpA is absent from the thylakoid, D2 and cytochrome b559 (possibly as a complex) might shuttle to the cytoplasmic membrane to incorporate a newly synthesized D1 protein (39).
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Concluding Remarks—We have identified a member of the FtsH protease family, slr0228, which is important for both PSII repair and the removal of unassembled PSII subunits and complexes from the thylakoid membrane of *Synechocystis* sp. PCC 6803. Our work provides the first direct experimental evidence to support a wide ranging role for an FtsH protease in quality control in the thylakoid membrane *in vivo*. By analogy, FtsH proteases are also likely to fulfill a similar role in the chloroplast, as alluded to from earlier *in vitro* experiments (20).

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