Involvement of the HtrA family of proteases in the protection of the cyanobacterium *Synechocystis* PCC 6803 from light stress and in the repair of photosystem II

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Photosystem II (PSII) is prone to irreversible light-induced damage, with the D1 polypeptide a major target. Repair processes operate in the cell to replace a damaged D1 subunit within the complex with a newly synthesized copy. As yet, the molecular details of PSII repair are relatively obscure despite the critical importance of this process for maintaining PSII activity and cell viability. We are using the cyanobacterium *Synechocystis* sp. PCC 6803 to identify the various proteases and chaperones involved in D1 turnover *in vivo*. Two families of proteases are being studied: the FtsH family (four members) of Zn²⁺-activated nucleotide-dependent proteases; and the HtrA (or DegP) family (three members) of serine-type proteases. In this paper, we report the results of our studies on a triple mutant in which all three copies of the *htrA* gene family have been inactivated. Growth of the mutant on agar plates was inhibited at high light intensities, especially in the presence of glucose. Oxygen evolution measurements indicated that, under conditions of high light, the rate of synthesis of functional PSII was less in the mutant than in the wild-type. Immunoblotting experiments conducted on cells blocked in protein synthesis further indicated that degradation of D1 was slowed in the mutant. Overall, our observations indicate that the HtrA family of proteases are involved in the resistance of *Synechocystis* 6803 to light stress and play a part, either directly or indirectly, in the repair of PSII *in vivo*.

Keywords: photoinhibition; photosystem II; HtrA; DegP; D1 protein repair cycle

1. INTRODUCTION

The D1 polypeptide of the PSII reaction centre complex was originally identified in the chloroplast because of its high rate of turnover in the light (Bottomley *et al.* 1974; Eaglesham & Ellis 1974). This property is now thought to be a reflection of a repair process to replace damaged D1 within a defective PSII complex with a newly synthesized copy (Kyle *et al.* 1984; Ohad *et al.* 1984). Damage to D1 in the light occurs through a variety of mechanisms (Barber & Andersson 1992) and manifests itself through the irreversible oxidation of amino acid sidechains (Sharma *et al.* 1997) and changes in protein structure (He *et al.* 1991; Ruan *et al.* 2001). When the rate of damage exceeds the rate of repair, there is a net loss of PSII activity that leads to a decline in photosynthetic activity and ultimately cell death (reviewed by Melis 1999).

The abundance and relatively high rate of turnover of D1 compared with other thylakoid membrane proteins means that it is an attractive system for studying the repair of membrane protein complexes. The key aspects of studying D1 turnover and PSII repair are to understand

how the PSII complex is maintained in a stable state whilst the damaged D1 subunit, lying at the heart of this large multi-subunit complex, is degraded and replaced and how the various co-factors are inserted. As yet, the identity of the proteases and assembly factors involved in PSII repair in the intact organism are, to our knowledge, unknown, although important clues are emerging both from studies *in vitro* and from analysis of D1 turnover in defined mutants.

The recent publication of the entire genome sequence from the cyanobacterium *Synechocystis* sp. PCC 6803 (Kaneko *et al.* 1996) has meant that the role of potential proteases in D1 turnover can be examined *in vivo* through the analysis of defined knockout mutants. We have chosen the FtsH and HtrA (the latter is also known as DegP) families of proteases for study because of their conserved role in nature in the degradation of damaged or unassembled proteins (reviewed by Pallen & Wren 1997) and also because these types of protease are found in the chloroplast (Lindahl *et al.* 1996; Itzhaki *et al.* 1998) and, more recently, have been linked to D1 degradation from studies *in vitro* (Lindahl *et al.* 2000; Haußühl *et al.* 2001). Of the FtsH family of proteases in *Synechocystis* 6803, we have so far identified one member (Cyanobase designation slr0228) that is important for optimizing the D1 repair cycle, probably at an early step in D1 degradation (Bailey *et al.* 2001).

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Figure 1. (a) Disruption of the *htrA* (ORF slr1204), *hhoA* (ORF sll1679) and *hhoB* (ORF sll1427) genes in the chromosome of *Synechocystis* 6803. Position of the kanamycin- (Km^R), chloramphenicol- (Cm^R) and erythromycin- (Em^R) resistance cassettes in each of the genes is indicated together with relevant restriction sites and restriction fragment sizes (see § 2). Arrows within the resistance cassettes indicate the direction of transcription of the enzymes conferring antibiotic-resistance. (b) Confirmation of the disruption of the *htrA*, *hhoA* and *hhoB* genes in Δ by PCR using the forward (F) and reverse (R) gene-specific primers indicated in (a). In the control experiments, DNA from the glucose-tolerant strain (WT) that was used to generate the mutant was amplified.

Here, we describe experiments aimed at testing the importance of the HtrA protease family for D1 turnover in *Synechocystis* 6803. Like *Escherichia coli*, there are three potential members of this family in *Synechocystis* 6803, termed HtrA (or DegP) (Cyanobase designation slr1204), HhoA (or DegQ) (Cyanobase designation sll1679) and HhoB (or DegS) (Cyanobase designation sll1427) (Pallen & Wren 1997). *E. coli* HtrA, an extrinsic membrane protein attached to the periplasmic side of the cytoplasmic membrane (Skorko-Glonek *et al.* 1997), is involved in the degradation of unfolded proteins, particularly after heat stress (Lipinska *et al.* 1989) and acts as a chaperone at lower temperatures (Spiess *et al.* 1999). Little is known about the *Synechocystis* homologues. Due to the possibility that the three potential members of this family might show some overlap in function, our studies have initially focused on the construction and characterization of a triple mutant of *Synechocystis* 6803 in which all three members have been knocked out.

2. MATERIAL AND METHODS

(a) Disruption of the *htrA* family of genes

The *htrA*, *hhoA* and *hhoB* genes were amplified from *Synechocystis* 6803 genomic DNA using the PCR and cloned into pBluescript. The *htrA* gene (size 1359 bp) was interrupted by inserting a kanamycin-resistance cassette into the *Pst*I site located at nucleotide position 725. For the *hhoA* gene (size 1185 bp), a chloramphenicol-resistance cassette was inserted between nucleotide positions 310 and 733 after removal of the 423 bp *Sma*I fragment. For *hhoB* (size 1251 bp), an erythromycin-resistance cassette was inserted between positions 135 and 816 after removal of the 681 bp *Hinc*II fragment. The orientations of the resistance cassettes with respect to the direction of transcription of the marker enzyme are indicated in figure 1. The triple mutant was constructed in the glucose-tolerant strain of *Synechocystis* 6803 using standard transformation procedures

(Williams 1988). The stepwise inactivation of the *htrA* gene family was performed: first *hhoA*, then *hhoB* to generate an *hhoA*, *hhoB* double mutant and finally *htrA* to give the triple mutant, designated in this paper as Δ . The genotypes of the single and double mutants were confirmed through Southern analysis and PCR (data not shown).

(b) Growth conditions

All strains were grown in the light at 30–33 °C in BG-11 mineral medium as described by Williams (1988), supplemented where necessary with 5 mM glucose and antibiotics. Liquid cultures (4 l) were bubbled with 5% (v/v) CO_2 in air and grown at an incident light intensity of $40 \mu E m^{-2} s^{-1}$.

(c) Photosystem II activity measurements and photoinhibition experiments

PSII activity was assessed by measuring the light-saturated rate of oxygen evolution from whole cells using 1 mM 2, 6-dichloro-*p*-benzoquinone and 2 mM potassium ferricyanide as artificial electron acceptors (Nixon *et al.* 1995). Chlorophyll concentrations were determined by extracting into methanol and measuring the absorbance at 666 nm (Komenda & Barber 1995). For photoinhibition experiments, the cells were concentrated to $20 \mu g ml^{-1}$ chlorophyll and exposed at 31 °C to heat-filtered white light at an incident light intensity of $600 \mu E m^{-2} s^{-1}$ either in the absence or presence of spectinomycin at $200 \mu g ml^{-1}$. Thylakoid membranes were isolated after the breakage of the cells with glass beads (Komenda & Barber 1995).

(d) Other biochemical techniques

The SDS-PAGE and immunoblotting of thylakoid membranes were performed as described by Nixon *et al.* (1995). Antibodies specific for FtsH were kindly made available by Professor T. Ogura (University of Kumamoto, Japan).

Figure 2. The growth of mutants lacking the HtrA family of proteases is more sensitive to high light. Cells were re-streaked onto BG-11 agar plates either containing (+glucose) or lacking (–glucose) 5 mM glucose and grown at the light intensities indicated. The WT control strain is shown below the dashed lines, whereas the mutants with the disrupted *htrA* family of genes are above the lines.

3. RESULTS

(a) Disruption of the *htrA* gene family in *Synechocystis*

One of the advantages of using *Synechocystis* 6803 as a model photosynthetic organism is the ease of generating chromosomal knockout mutants. This process involves the cell taking up DNA, such as a plasmid carrying a target gene disrupted by a selectable marker, which then inserts into the chromosome through homologous recombination to inactivate the WT gene (Williams 1988). Each of the three members of the *htrA* gene family was amplified using the PCR and cloned into a plasmid vector. The coding regions were then disrupted either by insertion of an antibiotic-resistance cassette or by partial deletion and insertion of a cassette (see § 2 and figure 1). Stepwise transformation led to the generation of a chloramphenicol-, kanamycin- and erythromycin-resistant mutant in which all the members of the *htrA* gene family had potentially been disrupted. PCR analyses confirmed that this mutant had indeed segregated fully so that all of the genome copies were inactivated (figure 1).

(b) Growth properties of the mutant

To test whether growth of Δ was susceptible to light stress, cells were re-streaked onto agar plates and incubated at three different light intensities. The extent and rate of growth of Δ was similar to the WT at low light, but at higher intensities there was a noticeable inhibition (figure 2). This effect was most profound when glucose was included in the growth medium. These observations indicated that disruption of the *htrA* gene family had compromised the ability of *Synechocystis* to withstand light stress.

Figure 3. Effect of high light on PSII activity in Δ (triangles) and WT (squares). Oxygen evolution was measured from cells taken at various time-points during exposure to $600 \mu\text{E m}^{-2} \text{s}^{-1}$ of white light, either in the absence (black symbols) or presence (white symbols) of spectinomycin at $200 \mu\text{g ml}^{-1}$.

(c) Effect of high light on photosystem II activity in the mutant

The PSII repair cycle was assessed in Δ by measuring the rate of light-saturated oxygen evolution, a measure of the number of active PSII complexes, from cells as a function of time of exposure to high light, either in the presence or absence of spectinomycin, an inhibitor of protein synthesis that blocks the repair cycle (figure 3). The cells examined in this part of the study were grown photoautotrophically in the absence of added glucose. In the presence of spectinomycin, the WT and Δ showed similar

kinetics of PSII inactivation, indicating that the rate of damage to PSII was similar in Δ and the WT. In the absence of spectinomycin, however, the level of PSII activity displayed by Δ was markedly reduced compared with that of the WT, especially in the first 2 h of illumination, but still greater than that observed in the presence of spectinomycin. In a separate experiment, Δ and WT samples were returned to the growth-light conditions after a high-light period that reduced PSII activity to less than 50% of original levels. In the presence of spectinomycin there was no restoration of PSII activity, whereas in its absence, PSII activity in both the WT and Δ recovered to their original levels within 90 min (data not shown). Together, these data indicated that under the experimental conditions used, Δ was able to synthesize active PSII, albeit less effectively than the WT

(d) Rate of D1 degradation in the mutant

A widely used method to study the rate of D1 degradation in *Synechocystis* 6803 is to perform immunoblotting experiments using D1-specific antibodies under conditions in which D1 synthesis is blocked through the addition of a protein synthesis inhibitor, such as lincomycin (Komenda *et al.* 2000). The loss of bulk D1 is then a reflection of its rate of degradation, although there are limitations in the interpretations of these experiments (see § 4). In the experiments described here, spectinomycin was added rather than the usual lincomycin, as the Δ mutant contains an erythromycin-resistance cassette, which also confers resistance to lincomycin. For the WT control, the level of D1 decreased to *ca.* 50% of original levels during the 6 h period of high-light treatment (figure 4a), whereas in the absence of spectinomycin, there was much less loss of D1 in the WT (figure 4b), consistent with active D1 synthesis. For the Δ mutant, there was no significant loss of D1 in the presence of spectinomycin (figure 4b). Interestingly, in the absence of spectinomycin, there appeared to be a transient loss of D1 in the Δ mutant in the first 2 h of exposure to high light, which was restored at later time-points (figure 4b). On comparison with a dilution series, the amount of D1 in Δ at the 2 h time-point was estimated to be *ca.* 60% of the original level (data not shown).

(e) Levels of FtsH in the *htrA* triple mutant

Previous work has implicated a role for FtsH (slr0228) in the early steps of D1 degradation (Bailey *et al.* 2001). To assess whether inactivation of the HtrA family of proteases had affected the expression of the various FtsH proteases, immunoblotting was performed on the samples obtained from the Δ mutant and WT during exposure to high light, either in the presence or absence of protein synthesis. The results presented in figure 4 indicate that, under both conditions, there were similar amounts of immunodetectable FtsH in the Δ and WT mutants.

4. DISCUSSION

As a first step towards addressing the roles of the HtrA family of proteases in the PSII repair cycle and in resistance to photoinhibition, we have constructed a novel mutant of *Synechocystis* 6803 in which all three members of the family have been inactivated. PCR analyses con-

Figure 4. Levels of D1 in Δ and WT following exposure to high light. Thylakoid membranes were isolated at various time-points from cells exposed to high light in a similar experiment to that shown in figure 3 either in (a) the presence of spectinomycin or (b) the absence of spectinomycin. Thylakoid samples containing 1 μ g of chlorophyll were separated by SDS-PAGE and specific antibodies were used to detect D1 (α -D1) and FtsH (α -FtsH) in a single immunoblotting experiment. In (a), Coomassie-blue staining of a duplicate gel was used to check the amount of protein loaded. Approximate sizes of pre-stained markers are indicated. In (a) the 6 h time-point for the WT appears slightly underloaded. Light-saturated oxygen evolution rates at the start of the high-light treatment were *ca.* 320 μ mol O₂ mg chl⁻¹ h⁻¹ and 130 μ mol O₂ mg chl⁻¹ h⁻¹ for the WT and Δ mutant, respectively. After 6 hours, the oxygen evolution rates in the presence of spectinomycin had declined by *ca.* 60% in both strains.

firmed that Δ had fully segregated and that all loci were disrupted (figure 1). The ability to generate such a mutant indicates that the *htrA* gene family is not crucial for the viability of *Synechocystis* 6803 under the low-light growth conditions that we use, although at this stage we cannot discount totally the presence of second-site suppressor mutations in Δ . Interestingly, growth of Δ is inhibited at higher light intensities, especially in the presence of glucose (figure 2). Possibly this phenotype reflects an enhanced susceptibility to photo-oxidative stress, which

Figure 5. Simplified model for D1 turnover. D1 degradation is catalysed by a large FtsH complex. The replacement D1 polypeptide may be inserted into PSII co-translationally via the Sec translocon or post-translationally via a large prohibitin complex.

would be in line with current ideas on the role for HtrA in the removal of oxidized proteins from cellular membranes (Skorko-Glonek *et al.* 1999).

PSII activity assays revealed that the synthesis of active PSII could still occur in the Δ mutant during the 6 h of high-light treatment. At the moment, it is not clear whether this process reflects the specific replacement of damaged D1 in a repair process, or whether it represents the synthesis of the entire PSII complex. Importantly, the rate of damage to PSII in Δ , as assessed by the loss of oxygen evolution in the presence of spectinomycin, is similar to that observed in the WT (figure 3). Thus, the loss of PSII activity in Δ in the absence of spectinomycin (figure 3) is not due to an enhanced rate of damage to PSII in Δ but rather an effect on the ability to replace the damaged D1 subunit or synthesize a new PSII complex. From the data shown in figure 4, it appears that the rate of D1 degradation is slowed in the mutant. Effects on the other steps of a repair pathway, particularly the synthesis of D1, which is extremely sensitive to oxidative stress (Nishiyama *et al.* 2001), will be assessed in the future in pulse-labelling experiments.

Although our data implicate the HtrA family of proteases in D1 degradation, it is important to realize that this effect may be direct or indirect. For instance, the loss of the HtrA family of proteases might affect the levels of other proteases within the cell or the trafficking of damaged complexes to the site or sites of repair. It should also be noted that the impairment in D1 degradation in Δ in the presence of spectinomycin (figure 4a) gives information on the capacity of the cell to degrade D1 at the time of addition of the protein synthesis inhibitor, and may not reflect D1 degradation in the absence of inhibitor during the high-light treatment. It is possible that additional proteases are synthesized during the high-light stress conditions to allow the cell to repair damaged PSII. This may explain the reduction in D1 levels observed in Δ within the first 2 h of exposure in the absence of spectinomycin (figure 4b). From the data presented in figure 4, it also did not appear that the level of immunodetectable

FtsH was substantially affected in Δ , although the particular antibody that we used did not allow us to discriminate between the various members of the FtsH family.

The question arises as to the importance of each of the members of the HtrA family of proteases. Recent work on an *htrA* mutant of *Synechocystis*, still containing intact copies of *hhoA* and *hhoB*, indicates that D1 can still be degraded in this strain when cells are illuminated by high light in the absence of a protein synthesis inhibitor (Funk *et al.* 2001). This mutant is also able to grow photoautotrophically at WT rates at a light intensity of $50 \mu\text{E m}^{-2} \text{s}^{-1}$ (Funk *et al.* 2001). However, an *htrA* mutant constructed by our group shows a very similar phenotype to Δ described here with regard to an inhibition in growth at high light on agar plates supplemented with glucose. By contrast, the *hhoA/hhoB* double mutant behaves like WT (data not shown). Consequently, of the three proteases, HtrA appears to be the most important for the protection of *Synechocystis* to high light, but at light intensities and under growth conditions that might not have been examined previously.

At the moment, little is known about the biogenesis and repair of PSII within *Synechocystis* 6803. Immunochemical studies support the idea that the cytoplasmic membrane, rather than the thylakoid membrane, might actually be a site of synthesis for some of the PSII subunits, including the D1 and D2 polypeptides, the extrinsic PsbO subunit and cytochrome *b559* (Smith *et al.* 1992; Smith & Howe 1993; Zak *et al.* 2001). As discussed by Smith & Howe (1993), the cytoplasmic membrane might also be a site for D1 degradation and PSII repair. Consequently, proteases found in the periplasm may have a role in D1 degradation. Further work must therefore be directed at probing the site of degradation of damaged PSII and the location of the HtrA and FtsH families of proteases in *Synechocystis*. For *E. coli*, HtrA (Skorko-Glonek *et al.* 1997), HhoA (Waller & Sauer 1996) and HhoB (Alba *et al.* 2001) are present in the periplasm, whilst in *Arabidopsis thaliana* chloroplasts, three HtrA/DegP homologues (DegP1, DegP5 and DegP8) are in the lumen (Itzhaki *et al.* 1998;

Schubert *et al.* 2002) and a fourth (DegP2) is found on the stromal surface of the thylakoid (Haußühl *et al.* 2001).

Previous studies of an engineered *ftsH* null mutant of *Synechocystis* have implicated FtsH (slr0228) in an early step of D1 degradation (Bailey *et al.* 2001). A direct role for FtsH in binding to PSII has come from the detection of FtsH in His-tagged PSII preparations of *Synechocystis* 6803 (Silva & Nixon 2001). Together, these data support a direct involvement of FtsH (slr0228) in the biogenesis and repair of PSII. Analysis of a null mutant of its closest chloroplast homologue, termed VAR2 or FtsH2, also indicates an important role for FtsH in the early steps of D1 degradation in *A. thaliana* (Bailey *et al.* 2002). Together, these studies indicate a conserved role for FtsH in the turnover of damaged D1 in oxygenic photosynthetic organisms. One possible mechanism involves the ATP-driven pulling, and subsequent proteolysis, of damaged full-length D1 (or break-down products) through a large FtsH pore in the thylakoid membrane (Langer 2000; Silva & Nixon 2001). Recent work has identified the Sec translocon as a potential component of the co-translational pathway for D1 insertion into the thylakoid membrane (Zhang *et al.* 2001). By analogy to recent results from the study of the assembly of membrane complexes in mitochondria (Nijtmans *et al.* 2000), a prohibitin complex, which has also been detected in His-tagged PSII preparations isolated from *Synechocystis* 6803 (Silva & Nixon 2001), might play a part in stabilizing newly synthesized full-length D1 prior to incorporation into *Synechocystis* PSII (Silva & Nixon 2001). A model for D1 turnover taking account of these new findings is presented in figure 5.

By contrast, other biochemical studies have suggested an important role for the HtrA homologue, DegP2, in the first step of D1 degradation in chloroplasts (Haußühl *et al.* 2001). *E. coli*-expressed DegP2 was shown to cleave the D1 polypeptide between transmembrane helices 4 and 5 (Haußühl *et al.* 2001), in a region that is assumed to be the site of the primary cleavage of damaged D1 *in vivo* (Greenberg *et al.* 1987). In addition, a chloroplast FtsH protease (FtsH1) has been shown to degrade a photoinduced 23 kDa N-terminal D1 fragment *in vitro* (Lindahl *et al.* 2000). Together, these two proteases have been suggested to cooperate in the removal of damaged D1 from the membrane (Haußühl *et al.* 2001).

Questions remain concerning the physiological importance of DegP2 and FtsH1 in D1 turnover and their contribution to total D1 turnover, especially if D1 degradation should proceed via a number of different pathways. In chloroplasts and cyanobacteria, this might be complicated by the presence of several members of the FtsH and HtrA protease family (Adam *et al.* 2001) with potential for overlap of function. However, the distinctive phenotype for the *var2* mutant indicates that the activity of VAR2 cannot be replaced totally by the other members of the chloroplast FtsH family nor by other proteases such as DegP2 (Chen *et al.* 2000; Bailey *et al.* 2002).

Future work must also be directed towards identifying the environmental factors regulating the expression of the various chloroplast and cyanobacterial proteases. For instance, DegP2 is induced by salt stress and desiccation (Haußühl *et al.* 2001). There is also the question of how does FtsH2 (VAR2) fit into the DegP2/FtsH1 model for

D1 turnover? Clearly, a combination of biochemical studies using purified components and analysis of D1 turnover in defined mutants is required to answer these questions.

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Discussion

C. Howe (Department of Biochemistry, University of Cambridge, Cambridge, UK). We first proposed several years ago (see Smith & Howe 1993) that the cytoplasmic membrane of cyanobacteria was involved in the assembly of PSII, and possibly also in repair. So I wondered if you had looked at the intracellular distribution of proteins such as FtsH?

P. J. Nixon. As far as I am aware, studies on the location of PSI and PSII have relied on the isolation of pure cytoplasmic membranes, free from thylakoid contamination. While these non-thylakoid membranes are assumed to be cytoplasmic membranes, there is still a possibility that they contain other types of so-far uncharacterized membrane, including 'intermediate' membranes connecting the thylakoid to the cytoplasmic membrane and possibly vesicular membrane. Nevertheless, your early work and the more recent studies from Pakrasi and co-workers (Zak *et al.* 2001) provide strong support for the presence of some, but not all, subunits of PSII in the cytoplasmic membrane. Unfortunately, our studies of the FtsH and HtrA proteases are relatively recent and we have not yet had the opportunity to investigate their location in the cell. I do agree though that their distribution is a most important question to answer and it is an area that we are actively pursuing.

Our work does, however, indicate that FtsH subunits are present in His-tagged CP47 preparations. Since the consensus is that cytoplasmic membranes do not contain CP47, our data suggest that these particular FtsH subunits, assigned recently by Kashino *et al.* (2001) to FtsH (slr0228) and FtsH (slr1604), can be found in the thylakoid membrane.

T. Hundal (*Department of Biochemistry, Linköping University, Linköping, Sweden*). I wish to ask you the same question that I asked Professor Aro. Do you have any evidence that suggests that fragments of the degrading D1 protein trigger the synthesis of new D1 protein?

P. J. Nixon. Our data on the FtsH (slr0228) null mutant indicate that the rates of D1 synthesis and D1 degradation are slowed compared with the wild-type control. This is consistent with the synchronized replacement of damaged D1 with a newly synthesized copy. Whether D1 fragments trigger the synthesis of new D1, we cannot say.

B. Andersson (*Rector, Linköping University, Linköping, Sweden*). I am pleased to see that the biochemical and mutant approaches seem to give a rather consistent picture of the proteases that potentially are involved in D1 protein degradation. However, in the mutant studies one should try to see accumulation of degradation fragments. The pulling model by FtsH is interesting indeed and should be developed in the future, including the ATP-dependent closing and opening of the pore.

P. J. Nixon. Yes, both the biochemical and mutant approaches support the involvement of the FtsH and DegP/HtrA proteases in D1 degradation. However, there are still insufficient data to make definitive conclusions as to their precise roles. According to a current model for D1 degradation in the chloroplast (Haußühl *et al.* 2001), damaged D1 is cleaved initially by the DegP2 protease and the N-terminal break-down product is degraded by the FtsH1 homologue. Accordingly, it would be predicted that *ftsH* mutants might show the accumulation of D1 breakdown products. Yet our work on the *ftsH* (slr0228) *Synechocystis* mutant as well as the *var2* mutant of *Arabidopsis thaliana* (Bailey *et al.* 2002) implicates a role for FtsH at an early stage of D1 degradation, either at or before the primary cleavage step. In these mutants, damaged D1 is relatively stable in the membrane and there is no accumulation of breakdown fragments. This could be because these particular FtsH proteins have direct or indirect effects on the recruitment of additional proteases, such as DegP/HtrA homologues, to the damaged PSII complex. Alternatively, FtsH might play a more direct role in the primary cleavage reaction than had previously been thought.

The possibility that damaged thylakoid proteins are pulled through an ATP-gated FtsH pore within the membrane is an extremely attractive idea and one that will guide future experimentation.

P. Fromme (*Max-Volmer-Laboratorium für Biophysikal-*

ische Chemie und Biochemie, Technische Universität Berlin, Berlin, Germany). I have two questions. Firstly, is the level of the two proteases increased with increased light intensity?

Secondly, is either of the two proteases also attached to photosystem I?

P. J. Nixon. I am not aware of experiments that have looked at the effect of light intensity on the protein levels of individual members of the FtsH and DegP/HtrA protease families in *Synechocystis* 6803. However, recent work has indicated that the mRNA levels for FtsH subunits slr0228 and slr1604 increase significantly following high light treatment (Hihara *et al.* 2001).

Whether FtsH or HtrA bind to PSI is unclear at the moment.

J. Komenda (*Department of Autotrophic Microorganisms, Institute of Microbiology, Czech Academy of Sciences, Trebon, Czech Republic*). In the FtsH mutant, do you see the D1 precursor or not?

P. J. Nixon. In pulse-chase experiments on the slr0228 mutant we observed two labelled bands in the D1 region—the upper one appears to be chased into the lower band with time. This observation is consistent with slower processing of precursor D1 in the mutant but as yet we have no direct immunochemical evidence to support this.

Additional references

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GLOSSARY

- Δ: *Synechocystis* 6803 mutant in which all three members of the *htrA* gene family (*htrA*, *hhoA* and *hhoB*) have been activated
- ORF: open reading frame
- PSII: photosystem II
- SDS–PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis
- WT: wild-type