



Quality control of Photosystem II: Cleavage and aggregation of heat-damaged D1 protein in spinach thylakoids

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Abstract

Moderate heat stress (40 °C, 30 min) on spinach thylakoids induced cleavage of the D1 protein, producing an N-terminal 23-kDa fragment, a C-terminal 9-kDa fragment, and aggregation of the D1 protein. A homologue of *Arabidopsis* FtsH2 protease, which is responsible for degradation of the damaged D1 protein, was abundant in the stroma thylakoids. Two processes occurred in the thylakoids in response to heat stress: dephosphorylation of the D1 protein in the stroma thylakoids, and aggregation of the phosphorylated D1 protein in the grana. Heat stress also induced the release of the extrinsic PsbO, P and Q proteins from Photosystem II, which affected D1 degradation and aggregation significantly. The cleavage and aggregation of the D1 protein appear to be two alternative processes influenced by protein phosphorylation/dephosphorylation, distribution of FtsH, and intactness of the thylakoids.

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1. Introduction

Various environmental stresses, such as strong visible light or moderate heat stress, have a significant impact on the structure and function of Photosystem II (PSII). The reaction center D1 protein is easily damaged, degraded, and removed from PSII for repair under

these conditions. The so-called damage and repair cycle of the D1 protein has been studied extensively during photoinhibition of PSII. Important aspects of the process are well documented, including generation and action of reactive oxygen species and cationic radicals, the function of proteases in the degradation and removal of the damaged D1 protein, insertion of a newly synthesized D1 protein in the PSII complex, and recovery from the damage [1–3].

Concerning the degradation of the damaged D1 protein in particular, significant efforts have been made to identify the proteases involved. Since chloroplasts have a prokaryotic origin, proteases homologous to those found in *Escherichia coli* degrade structurally unfavorable proteins, most notably the photodamaged D1 protein [4–6]. A homologue of the FtsH protease was identified and characterized in the chloroplasts of higher plants, and was suggested to be involved in the turnover of the thylakoid proteins [7]. Later works indicated that the photodamaged D1 protein was cleaved to a 23-kDa fragment in a GTP-dependent manner [8], which was subsequently degraded by FtsH [9]. Recently, however,

Abbreviations: PSII, Photosystem II; D1 and D2, reaction center binding proteins of PSII; FtsH, filamentation temperature sensitive H; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; Q_B, secondary plastoquinone electron acceptor; CP43, the antenna chlorophyll-binding protein of PSII with a relative molecular mass of 43 kDa; PsbO, P and Q, the extrinsic proteins of PSII involved in the regulation of oxygen evolution; HTG, *n*-heptyl-β-D-thiogluconate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; p-Thr, phosphothreonine; ECL, enhanced chemiluminescence; LHCII, the light-harvesting chlorophyll-protein complex associated with PSII; *M_r*, relative molecular mass

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growing evidence suggests that FtsH is involved directly in the degradation of the photodamaged D1 protein. In *Arabidopsis thaliana*, twelve genes encoding FtsH proteases were identified [10]. Nine were located in the chloroplasts (FtsH1, 2, 5–9, 11 and 12) and the other three in the mitochondria (FtsH3, 4 and 10) [11,12]. These homologues may have resulted from gene duplication events in *Arabidopsis*. Among them, FtsH2 and 8, and also FtsH1 and 5 are duplicated pairs, and the members of these pairs were shown to be interchangeable [13]. Loss of an FtsH2 and 5 was shown to be responsible for leaf variegation. The variegated mutants were sensitive to light stress [11,14–16], where photo-damaged D1 protein accumulated in PSII [16]. In the cyanobacterium *Synechocystis* PCC6803, four FtsH homologues have been identified [10], and a mutant lacking one of them (slr0228) was shown to be sensitive to light stress, and to accumulate photo-damaged D1 protein [17]. Indeed, FtsH (slr0228) was shown to be required for selective replacement of the damaged D1 protein [18].

More recently, we showed a possibility that FtsH is involved in degradation of the D1 protein under moderate heat stress in *Synechocystis* PCC6803, using mutant cells lacking FtsH (slr0228) [19]. The D1 protein was degraded by heat treatment at 40 °C for 30 min in wild-type cells, but not in mutants. The mutant cells did not grow under the moderate heat stress, whereas the wild-type cells grew normally. Thus, a repair mechanism worked in the wild-type cells to remove the heat-damaged D1 protein from PSII, and FtsH was suggested to be involved in this process. Moreover, we showed using *in vitro* experiments with spinach thylakoids that the D1 protein is cleaved and an N-terminal 23-kDa fragment is produced by heat treatment at 40 °C for 30 min. The D1 cleavage was stimulated by the addition of Zn and ATP. Solubilization and reconstitution of the protease activity in the thylakoids and identification of the proteases by MALDI-TOF mass spectrometry suggested that FtsH proteases (FtsH2 and 8) were responsible for the cleavage [20]. Since the addition of Q_B-binding herbicides effectively suppressed the D1 cleavage, the cleavage site of the D1 protein under moderate heat stress appeared to be on the DE-loop [20]. The cleavage site is probably the same as that proposed for the excess light-induced cleavage of the D1 protein on the acceptor side photoinhibition of PSII [21].

Although the details of proteolysis of the damaged D1 protein are becoming clear, the fate of the damaged D1 protein seems to be more complex than was previously thought. We have noted that not only cleavage but also aggregation of the D1 protein takes place under light stress conditions in spinach PSII [21–23]. The aggregation occurred between the D1 and D2 proteins (D1/D2), the D1 protein and the α -subunit of cytochrome *b*₅₅₉ (D1/*cytb*₅₅₉), and the D1 protein and CP43 (D1/CP43). The D1 aggregates were formed both by the acceptor-side and donor-side photoinhibition of PSII, which are differentiated based on the effects of oxygen on the aggregation: the protein aggregation induced by the acceptor-side photoinhibition of PSII is dependent on oxygen, while that by the donor-side photoinhibition is not [24]. Here we found that the damaged D1 protein formed aggregates with nearby polypeptides under moderate heat stress, in much the same way that the D1 aggregates were formed under light stress. Considering the two alternative pathways that the D1 protein takes after heat damage, i.e. cleavage or aggregation, it is important to understand the

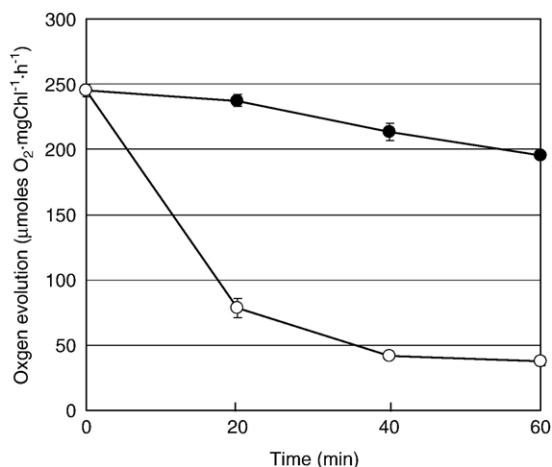


Fig. 1. Effects of moderate heat stress on the oxygen evolving activity of spinach thylakoids. Thylakoids were incubated at either 25 °C (●) or 40 °C (○) in the dark for the periods indicated. Data are averages \pm SD ($n=3$).

mechanism that determines the fate of the damaged protein. In this study, we examined localization of FtsH2 in spinach thylakoids. We subsequently characterized several steps responsible for the regulation of the fate of the heat-damaged D1 protein, i.e. dephosphorylation of the D1 protein and release of PsbO, P and Q proteins from PSII.

2. Materials and methods

2.1. Isolation of intact thylakoids, grana, stroma thylakoids, PSII-enriched membranes and PSII core complexes from spinach, and treatment of the thylakoids with Tris

Intact thylakoids and PSII-enriched membranes were prepared from spinach purchased from a local market as described previously [25]. The PSII core complexes were isolated by treatment of the PSII membranes with 2.0–2.6% (w/v) *n*-heptyl- β -D-thioglucoside (HTG) [26]. The grana and stroma thylakoids were separated by treating the thylakoids with 0.5% digitonin for 30 min at 4 °C followed by differential centrifugations. Digitonin (Sigma Chemicals Co.) was purified before use. Treatment of the thylakoids with alkaline Tris was carried out as previously described [22]. The samples were suspended in a solution containing 100 mM sorbitol, 50 mM Tricine-KOH (pH 7.6), 15 mM NaCl and 5 mM MgCl₂ (solution A). All preparation steps were carried out at 4 °C in darkness.

2.2. Heat treatment of the samples and measurement of the PSII activity

Membrane samples were suspended in solution A at chlorophyll concentrations of 0.4 mg ml⁻¹ in 1 ml plastic sample tubes. Samples were incubated at different temperatures (0–50 °C) in a thermo-regulated water bath for 30 min in darkness. Chlorophyll concentration was determined using 80% acetone extracts. Oxygen-evolving activity was measured with an oxygen electrode (Hansatech, UK) connected to a thermo-regulated water bath. The reaction mixture contained 50 mM MES (pH 6.5), 1 mM phenyl parabenzoquinone, 1 mM potassium ferricyanide, and thylakoids equivalent to 40 μ g of chlorophyll ml⁻¹. Saturating actinic light was provided by a tungsten lamp with a heat absorbing filter.

2.3. SDS-polyacrylamide gel electrophoresis and Western blot analysis

SDS/urea-PAGE and Western blot analysis were carried out as described [27]. The concentration of the acrylamide in the resolving gel was 12.5% (w/v). Each lane contained sample equivalent to 2.5 μ g chlorophyll. Antibodies prepared in our lab against the DE-loop of the D1 protein, and antibodies against the C-terminal of the D1 protein (Agrisera, Sweden) were used to detect the D1 protein and its cleavage

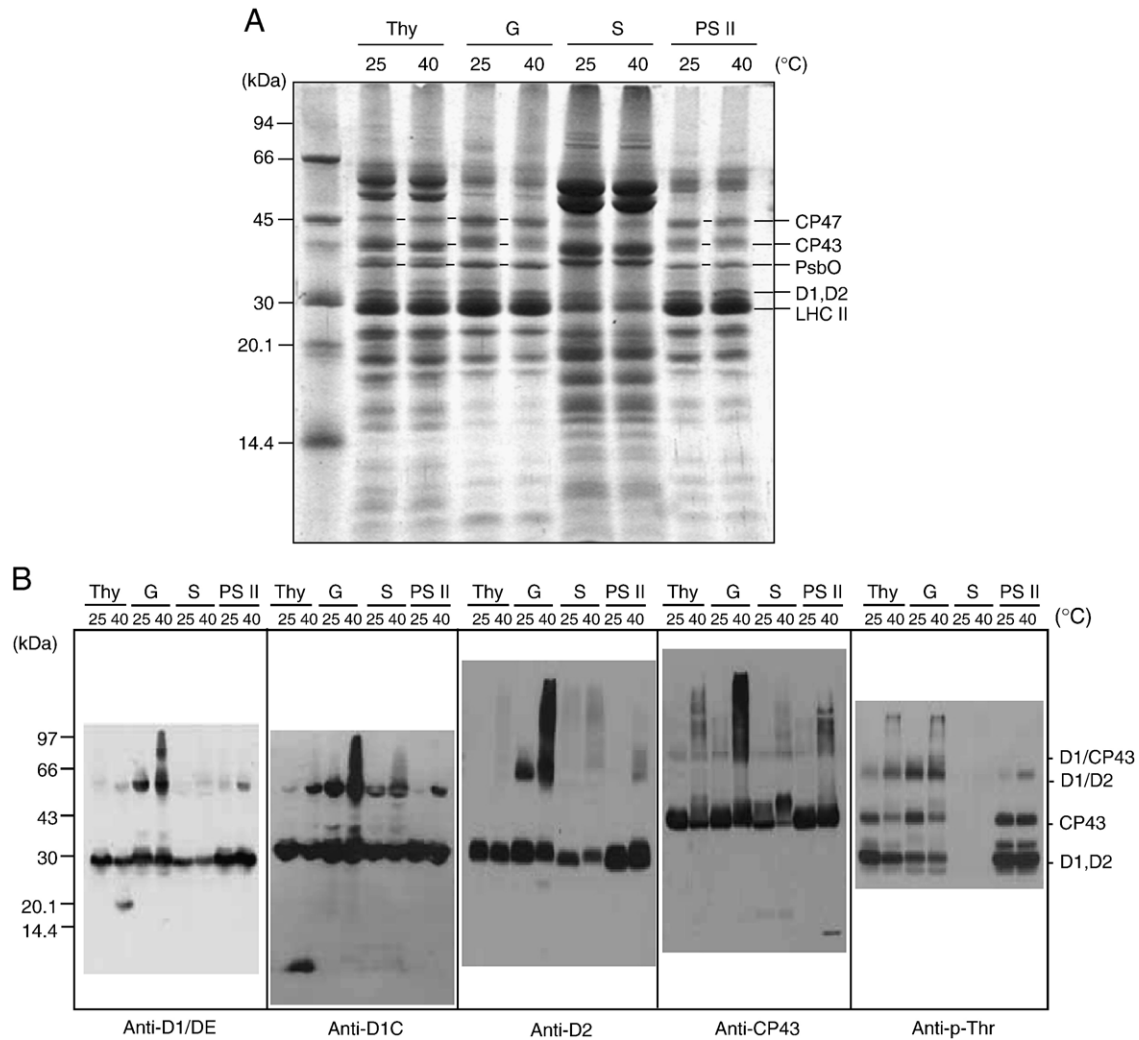


Fig. 2. Effects of moderate heat stress on the profiles of the proteins in various membrane samples. (A) Coomassie-blue staining of an SDS/urea-PAGE gel showing proteins in the thylakoids (Thy), grana (G), stroma thylakoids (S), and PSII-enriched membranes (PSII). Grana and stroma thylakoids were obtained by treating thylakoids with digitonin; PSII membranes were obtained by treating thylakoids with Triton X-100. Each membrane sample was incubated in darkness either at 25 or 40 °C for 30 min. Molecular markers are shown in the far-left lane. The positions of CP47, CP43, PsbO, D1, D2, and LHCII are indicated on the right. (B) Western blot analysis of the thylakoids and membrane samples before and after the moderate heat stress. Antibodies against the DE-loop of the D1 protein (D1–DE), the C-terminal part of the D1 protein (D1C), the D2 protein, CP43 and phosphothreonine (p-Thr) were used. The positions of CP43, D1, D2, the aggregates between the D1 and D2 proteins (D1/D2), and the aggregates between the D1 protein and CP43 (D1/CP43) are shown on the right hand side of the gel. Other conditions are the same as those in (A).

fragments. The anti-*Arabidopsis* FtsH2 (VAR2) antibody was kindly provided by Dr. W. Sakamoto of Okayama University, Japan. The Anti PsaA antibody was purchased from Agrisera, Sweden. The Anti-phosphothreonine (p-Thr) antibody (Zymed Laboratories) was used to monitor phosphorylation/dephosphorylation of the proteins in PSII. Anti-D2 and Anti-CP43 antibodies prepared in our lab were used for immunodetection of D2 and CP43. As an inhibitor of phosphatase, 1 mM NaF, was added where indicated. The bands that cross-reacted with the antibodies were detected by fluorography with enhanced chemiluminescence (ECL) reagents (Amersham, Japan). The density of the protein bands in the fluorogram was measured with Scion Image software.

3. Results

3.1. Inhibition of PSII activity in the thylakoids by moderate heat stress

Oxygen evolving activity of spinach thylakoids was measured under moderate heat stress (Fig. 1). At 40 °C, oxygen-evolving

activity decreased with a half decay time of 10–15 min, and the inhibition of the activity was almost saturated with 30 min incubation time.

3.2. Aggregation of the D1 protein under moderate heat stress

The oxygen-evolving activity in the thylakoids incubated at 40 °C showed saturation at 30 min. Therefore, profiles of the proteins in the thylakoids, grana, stroma thylakoids and PSII-enriched membranes were compared between 40 °C and 25 °C with 30 min incubation time (Fig. 2A). In Coomassie blue-stained gels of SDS/urea-PAGE, the loss of CP43 was most prominent in the grana. The lane loaded with the heat-treated grana showed diffused bands appearing in a higher M_r range (60–90 kDa). These changes are probably due to aggregation of CP43 with the D1 protein. Indeed, we identified the aggregates between the D1 protein and CP43 (D1/CP43) and between the D1 and D2 proteins (D1/D2) by

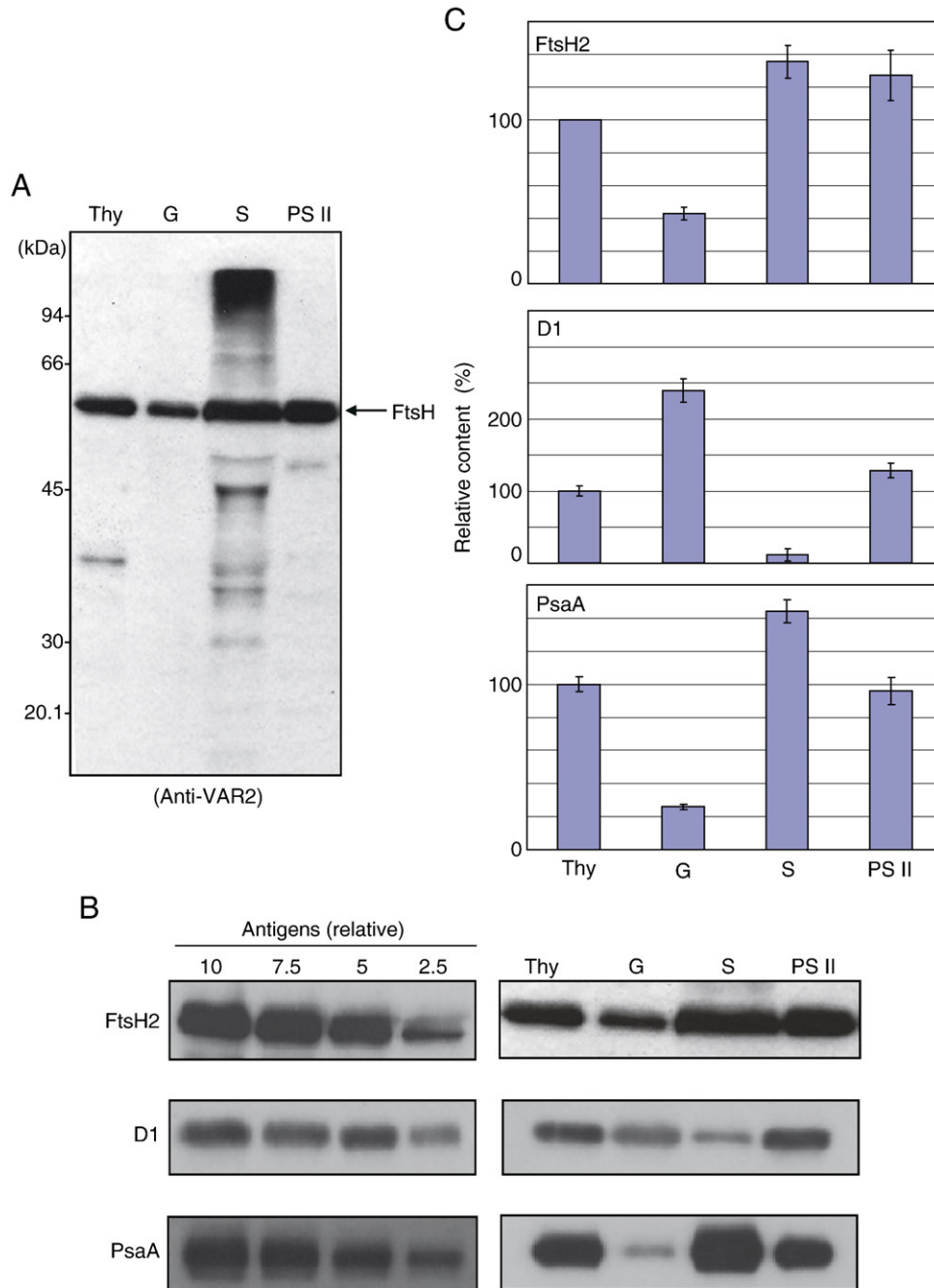


Fig. 3. Quantification of FtsH in various membrane samples kept at 25 °C. (A) Fluorogram of the Western blot analysis showing amounts of FtsH in the thylakoids (Thy), grana (G), stroma thylakoids (S), and PSII membranes (PSII). Grana and stroma thylakoids were obtained by treating thylakoids with digitonin; PSII membranes were obtained by treating thylakoids with Triton X-100. An antibody against VAR2 was used for immunodetection of FtsH (position of FtsH is shown by an arrow). (B) Fluorograms of the Western blot analysis carried out with specific antibodies against FtsH2 (VAR2), the DE-loop of the D1 protein and PsaA. Each lane contained sample equivalent to 2.5 μg chlorophyll. At the left of the gels, different amounts of the membranes (the stroma thylakoids were used for FtsH2 and PsaA, and the grana for the D1 protein) were separated, which were subsequently used for quantification of each band, as is shown in (C). In the immunoblotting of the D1 protein, the density of the band in the grana became significantly weak, because a part of the D1 protein was lost as the D1 aggregates that appeared at a higher M_r range of the gel, as was shown in the far-left fluorogram in Fig. 2B. Such D1 aggregation was not significant in the thylakoids, stroma thylakoids and the PS II-enriched membranes. (C) Diagram showing the relative amounts of FtsH2, the D1 protein and PsaA in each membrane sample. To show the total amount of the D1 protein in the grana, both the densities of original D1 band and of the aggregated D1 bands were combined. Values are averages \pm SD ($n=3$).

immunoblotting using the antibodies against D1, D2 and CP43 (Fig. 2B). The aggregation started at 30 °C, and was stimulated significantly at 40 °C (data not shown). Similar aggregates were previously reported in photoinhibition of PSII [21]. It should be noted that the D1 protein aggregated not only in the grana, but also

in the other membrane preparations (Fig. 2B), implying that the protein aggregation in each membrane under heat stress can be attributed to a common cause. The release of PsbO, P and Q from PSII under heat stress may contribute to this protein aggregation, as was demonstrated previously under light stress [21–23].

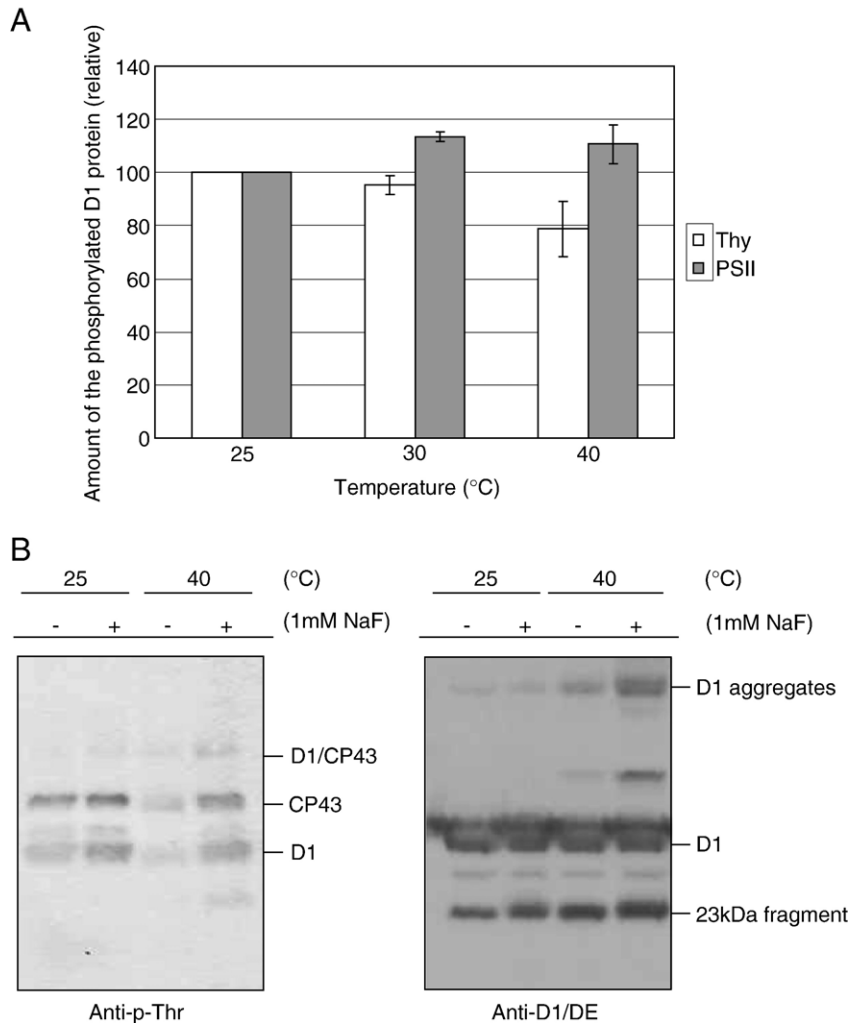


Fig. 4. Heat-induced dephosphorylation of the D1 protein in the thylakoids and the effects of a phosphatase inhibitor NaF. (A) Diagram showing the dephosphorylation of the D1 protein in the thylakoids (open bars) and in the PSII membranes (solid bars) at various temperatures. Data were obtained from the fluorogram of Western blot analysis with anti p-Thr. (B) Fluorogram showing the effects of NaF on the dephosphorylation of phosphoproteins (left) and on the formation of aggregates of the D1 protein (right) upon heat treatment (40 °C, 30 min) of the thylakoids. Western blot analysis with antibodies against D1–DE loop and p-Thr was carried out. Presence and absence of 1 mM NaF is denoted by “+” and “–” on the top of the gels, respectively. Positions of the D1 protein, CP43, aggregates and the 23-kDa fragment of the D1 protein are shown on the right hand side of each gel.

3.3. Cleavage of the D1 protein under moderate heat stress

The D1 protein was cleaved by the treatment of the thylakoids at 40 °C for 30 min, and thereby a 23-kDa fragment was detected by Western blot analysis with the antibody raised against the peptides of the DE-loop of the D1 protein (Fig. 2B). Previously, we showed the possibility that FtsH proteases are involved in the cleavage of the D1 protein in spinach thylakoids under moderate heat stress [20]. Here a C-terminal 9 kDa fragment of the D1 protein was also detected by immunoblotting with the antibodies raised against the peptide of the C-terminal part of the D1 protein (Fig. 2B). Judging from the sizes of the cleavage products and also from the inhibition of the cleavage by the addition of Q_B site-specific herbicides [20], it is likely that the cleavage takes place at the DE loop of the D1 protein. Interestingly, these degradation fragments were not observed in heat-treated grana or PSII-enriched membranes. Instead, significant aggregation of the D1 protein took place in these membranes, especially in the grana (Fig. 2B). The

lack of cleavage of the D1 protein in the grana and PSII membranes under heat stress has several possible explanations: first, absence of active FtsH proteases in these samples; second, absence of phosphatase activity, which is necessary for dephosphorylation of the N-terminal phosphothreonine residue (p-Thr) to initiate the cleavage of the D1 protein in these membranes. Finally, heat stress-induced release of PsbO, P and Q from PSII may facilitate the aggregation of the D1 protein.

3.4. Distribution of FtsH2 and 8 in the thylakoid membranes

The FtsH2 protease is most abundant in the FtsH family of *Arabidopsis* [28], and is probably involved in the degradation of photo- or heat-damaged D1 proteins. Previously, FtsH proteases were shown to be abundant in the stroma thylakoids using the antibody against FtsH from *E. coli* [7]. Here, we fractionated spinach thylakoids into grana and stroma thylakoids by digitonin treatment, and PSII membranes by Triton X-100 treatment. We

then examined the relative abundance of FtsH2 in each sample by Western blot analysis with a specific antibody against VAR2 (FtsH2 from *A. thaliana*) (Fig. 3A). It was shown previously that the antibody against FtsH2 reacts with FtsH8 as well [11]. When compared on an equal chlorophyll basis, FtsH2/8, which showed M_r of about 60 kDa in the fluorogram, are present in large quantities in the stroma thylakoids. The content of FtsH2/8 was small in the grana, and was 30% of that in the stroma thylakoids (Fig. 3B and C). The Triton-solubilized PSII membranes, which also represent the grana portion of the thylakoids, contained a larger amount of FtsH. The digitonin-fractionated grana and the Triton-fractionated PSII membranes cannot be compared with each other, because different solubilization conditions were employed. However, the data suggest that FtsH proteases are present not only in the stroma thylakoids but also in the grana. The grana region, where the PSII complexes are enriched, should be the major site where the D1 protein is damaged by excessive visible light or moderate heat stress. The presence of FtsH2/8 in the grana suggests a possibility that FtsH2/8 are involved in the recognition of the damaged D1 protein in the grana and/or the movement of the PSII complex containing the damaged D1 protein from the stacked to unstacked regions of the thylakoids.

3.5. Dephosphorylation of the PSII-related proteins under moderate heat stress

The D1, D2 proteins, CP43, PsbH and LHCII are reversibly phosphorylated [29–31], and dephosphorylation takes place under heat stress [31,32]. Dephosphorylation of the N-terminal Thr was shown to be necessary for the subsequent degradation of photodamaged D1 protein [33]. Thus the distribution and action of phosphatases in the thylakoids are probably crucial for the efficient degradation of the damaged D1 protein. We observed that the D1, D2 proteins and CP43 were phosphorylated in the thylakoids and the phosphorylation was more prominent in the grana and the PSII-enriched membranes (Fig. 2B). By contrast, these proteins in the stroma thylakoids were not phosphorylated. This observation is consistent with the earlier report that the phosphorylated D1 protein is located predominantly in the grana [34].

Upon heat treatment at 40 °C for 30 min, the D1, D2 proteins and CP43 in the thylakoids were dephosphorylated, while no significant dephosphorylation occurred in the grana and the PSII-enriched thylakoids (Fig. 2B). We subsequently examined the heat-induced protein dephosphorylation with the thylakoids and the PSII membranes in detail (Fig. 4A). Approximately 15% of the phosphorylated D1 protein was dephosphorylated at 40 °C in the thylakoids, while no dephosphorylation occurred in the PSII membranes. To further investigate the relationship between the dephosphorylation and aggregation of the D1 protein, we examined the effects of NaF, a phosphatase-inhibitor. When 1 mM NaF was added to the thylakoids during the moderate heat treatment, the dephosphorylation of the phosphoproteins was prevented, while the aggregation of the D1 protein was stimulated slightly (Fig. 4B). The stimulation of the D1 aggregation by the addition of NaF, however, was not clearly seen in the grana, stroma thylakoids and PSII membranes (data not shown). Thus, phosphorylation and dephosphorylation states of the D1 protein

may be critical for the determination of the fate of heat-damaged D1 protein in the thylakoids, but that is not necessarily the case with other membranes.

3.6. The relationship between the heat-induced release of PsbO, P and Q from PSII and the cleavage and aggregation of the heat damaged D1 protein

Heat stress induces the release of the extrinsic PsbO, P and Q proteins from PSII [35]. These structural changes in the donor side of PSII may have a considerable impact on the cleavage and aggregation of the D1 protein, since significant aggregation of the D1 protein was demonstrated previously in the PSII

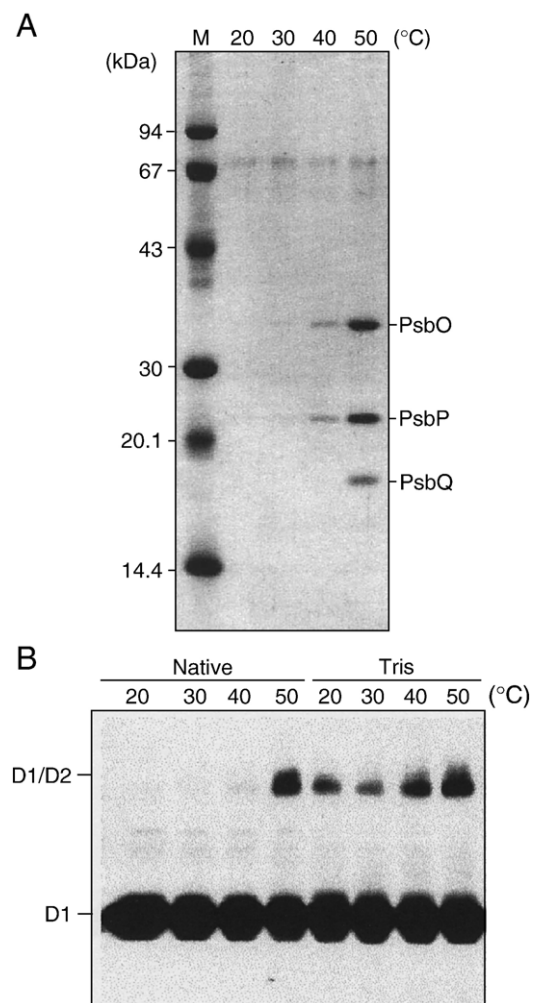


Fig. 5. Release of the PsbO, P and Q proteins and aggregation of the D1 protein in the PSII membranes under moderate heat stress. (A) The PSII membranes were treated at 20–50 °C for 30 min, and the supernatants after centrifugation were subjected to SDS/urea-PAGE. Coomassie staining showed the release of PsbO, P and Q from the PSII membranes at higher temperatures. The positions of PsbO, P and Q are indicated at the right hand side of the gel. Marker proteins are shown in the lane M. (B) Aggregation of the D1 protein in the PSII membranes under the heat stress (temperature, 20–50 °C) was estimated by Western blot analysis with the antibody against the DE-loop of the D1 protein. “Native” and “Tris” at the top of the gel indicate the non-treated and alkaline Tris-washed thylakoids, respectively. Each membrane sample was incubated at the indicated temperature for 10 min. The position of the D1 protein and D1/D2 aggregates is shown at the left hand side of the gel.

membranes lacking PsbO, P and Q under photoinhibitory conditions [22]. It is notable that the D1 protein was not cleaved in the stroma thylakoids under moderate heat stress (Fig. 2B), even though FtsH proteases were abundant in the stroma thylakoids (Fig. 3) and the phosphatase activity was high in this membrane region (Fig. 4). These results may be explained by the disturbance of the donor-side of PSII by heat stress, which presumably has a more significant effect on the D1 protein than the action of the FtsH proteases or phosphatases. Indeed, we observed release of PsbO, P and Q from PSII by heat treatment of the PSII membranes at 40 °C. (Fig. 5A), and also marked aggregation of the D1 protein (Fig. 5B). The release of protein was confined to PsbO, P and Q, as judged from Western blot analysis (data not shown). The increase in the D1 aggregation in stroma thylakoids upon heat treatment may have shielded the cleavage of the D1 protein. In the thylakoids, the effect of heat in the generation of the D1 aggregates was more significant at alkaline pH (pH 8) where the extrinsic PsbO, P and Q are released from PSII. This is in comparison with neutral pH (pH 7) or slightly acidic pH (pH 6) where the extrinsic proteins were associated with PSII (data not shown). In the Tris-washed PSII membranes where all the extrinsic proteins of PSII are completely removed, heat treatment induced more significant aggregation of the D1 protein (Fig. 5B). These data suggest that the stability of the donor-side of PSII is essential for the efficient degradation of the damaged D1 protein.

4. Discussion

Moderate heat stress has a significant impact on the structure and function of PSII. The reaction center-binding D1 protein is one of the main targets of heat stress. Recently the possibility was shown that the D1 protein denatured by heat stress in spinach thylakoids is cleaved by FtsH proteases [20]. Chloroplast FtsHs are membrane-bound zinc-dependent metalloproteases with one or two trans-membrane α -helices on the N-terminal side, and a large hydrophilic domain containing an ATP binding site and a catalytic site on the C-terminal side exposed to the stroma [7,12]. In this study, we examined the distribution of the FtsH proteases in spinach thylakoid membranes. We fractionated the thylakoids into grana and stroma thylakoids by treatment with 0.5% digitonin, and then carried out SDS/urea-PAGE and Western blot analyses with antibodies against VAR2, which corresponds to FtsH2 of *Arabidopsis* (Figs. 2 and 3). Apparently, the homologue of FtsH2 was much more abundant in the stroma thylakoids than in the grana. According to the phylogenetic tree of FtsH proteases, FtsH2 and 8 are closely related to each other [12], and therefore, the antibody against FtsH2 cross-reacted with FtsH8 as well [11]. The homologues of FtsH2 and 8 were detected in spinach thylakoids by MALDI-TOF mass spectrometry [20], so that at least these two protease species seem to be abundant in the stroma thylakoids. The Western blot analysis with the antibody against FtsH2 indicated that considerable amounts of the homologues of FtsH2 and/or 8 are present in the PSII-enriched membranes (Fig. 3). The FtsH protease was detected even in the PSII core complexes prepared by the treatment of the PSII

membranes with HTG (data not shown). In *Synechocystis* PCC6803, FtsH was copurified with PSII complexes [17,36]. Thus, it is likely that some FtsH proteases are closely associated with PS II. In *E. coli*, FtsH is present in the inner membrane forming a membrane-bound hexameric ring [37]. The size of the hexamer is 6–7 nm in diameter [38], which is smaller than, but almost comparable to ATP synthase which has a diameter of 10 nm and projects on the stroma side of the thylakoid membranes. This implies that the large hexameric FtsH complexes are excluded from the stacked region of the thylakoids in much the same way as the ATP synthases are. The FtsH that is localized in the grana core is possibly not a large hexameric complex but a monomeric FtsH. At present, the exact form of the proteases located in each membrane domain is not known, and identification of these FtsH species is being undertaken.

It was shown earlier that heat treatment induces unstacking of the thylakoids. Stacking and unstacking are controlled by the electrostatic interaction of the membrane surface in general [39]. We measured the time course of thylakoid unstacking during moderate heat stress (data not shown). Judging from the effective unstacking of thylakoids induced by heat stress, it is likely that hydrogen bonding, in addition to the electrostatic interaction, participates in the membrane stacking. Raising the temperature may weaken the hydrogen bonds that contribute to the membrane–membrane interaction, and thereby cause unstacking of the thylakoids. The stacking and unstacking of the thylakoids should result in a significant change in the distribution of FtsH proteases as well as PSII complexes in the thylakoids. The FtsH proteases enriched in the stroma thylakoids might diffuse to the newly unstacked regions of the thylakoids and bind to the damaged D1 protein in the PSII complex more easily. The PSII complexes in the stacked regions of the thylakoids may also migrate to the stroma thylakoids.

We observed a significant loss of CP43 from the PSII complexes in the grana upon heat treatment, which was detected in the Coomassie-stained gel after SDS/urea-PAGE (Fig. 2A). Protein aggregates may be formed by CP43 crosslinking to the damaged D1 protein. Indeed, it was demonstrated by Western blot analysis that the D1 protein forms aggregates with the D2 protein and CP43 under the moderate heat stress (Fig. 2B). The D1 protein, as well as the other two proteins, might form potentially interactive surfaces of the proteins when exposed to heat stress. Interestingly these proteins were also shown to form aggregates under photoinhibitory conditions, especially in the donor side photoinhibition [21]. Thus, we assume that a common mechanism functions to determine the fate of the damaged D1 protein in photoinhibition and “heat inhibition” of PSII.

In earlier studies and in recent studies also, it was shown that the D1 protein, the D2 protein, CP43, the PsbH protein and LHCII are phosphorylated [40,41]. The phosphorylated D1 protein is exclusively located in the grana [34], and is a poor substrate for proteolytic enzymes [42]. Subsequently, it was demonstrated in photoinhibition experiments with higher plants that reversible phosphorylation of the D1 protein is crucial for controlling the turnover of the D1 protein, and that the N-terminal p-Thr must be dephosphorylated before the photodamaged D1 protein is degraded [43]. On the contrary, there is an argument that reversible

phosphorylation is not necessarily related to the turnover of the D1 protein under light stress [43]. Dephosphorylation of the D1 protein, D2 protein, CP43 was observed when spinach and *Arabidopsis* thylakoids were subjected to heat stress [32,33]. Thylakoid-bound phosphatases were shown to function in the dephosphorylation of the phosphoproteins [40,41]. One of them was purified from spinach thylakoids and was shown to have characteristics of Ser/Thr phosphatases of the PP2A family [41]. It is possible that aggregation of the D1 protein observed here reflects failure in degrading the damaged protein. The D1 aggregation was shown to be most prominent in the heat-treated grana where the D1 protein remained phosphorylated. As was observed in the degradation of the photodamaged D1 protein, phosphatases probably play a key role in the initiation of the heat-induced proteolysis of the D1 protein. In the grana isolated by digitonin treatment of the thylakoids, little phosphatase activity may be present, and thereby D1 proteolysis should not be initiated. This, in turn, increases the possibility that the damaged D1 protein form aggregates. By contrast, all the phosphoproteins were dephosphorylated in the heat-treated stroma thylakoids, suggesting the presence of the phosphatase activity in this membrane region (Fig. 2B). It was suggested that phosphorylation of the D1 protein retards the degradation of the damaged D1 protein during photoinhibition in conditions where rapid replacement of the damaged D1 protein is not possible [44]. Our observation, however, suggests that the damaged and phosphorylated D1 protein tends to form aggregates with the nearby polypeptides, which probably leads to malfunction of PSII. Thus the accumulation of these inactive PSII complexes might cause serious damage to the chloroplasts and eventually lead to plant cell death [21]. The dephosphorylation of the N-terminal Thr seems to be a prerequisite condition for the cleavage of the D1 protein during heat stress, and the D1 cleavage probably takes place at the DE-loop of the D1 protein [20]. Therefore, we speculate that the dephosphorylation at the N-terminal p-Thr of the D1 protein affects the conformation of the DE-loop of the protein, although the exact mechanism underlying this effect is not yet known. It should be noted here that the stroma thylakoids, which are rich in FtsH proteases and phosphatases, did not show clear cleavage of the D1 protein upon heat stress. To explain this, we showed the release of PsbO, P and Q as the most influential element in protein aggregation (Fig. 5). At 40 °C, the PsbO, P and Q proteins were released from the PSII membranes. Previously, the release of the PsbO protein from PSII was shown to facilitate aggregation of the D1 protein in photoinhibition [21,23]. Similar stimulation of D1 aggregation was observed upon heat treatment of all the membrane samples examined here (Fig. 2B). These results suggest that the disturbance of the donor-side of PSII has an intense effect on the conformation of the damaged D1 protein. Efficient degradation of the heat-damaged D1 protein may be possible only when the D1 protein is dephosphorylated and recognized by FtsH without much release of PsbO, P and Q proteins from PSII. Indeed, we experienced that the intactness of the thylakoids is essential for clear detection of heat-induced cleavage of the D1 protein.

Photoinhibition and “heat inhibition” of PSII share certain similarities in the cleavage of the damaged D1 protein. However, photoinhibition differs greatly from “heat inhibition” in that the

extrinsic PsbO, P and Q proteins are scarcely released from PSII under illumination [45]. These extrinsic proteins were easily removed from PSII in moderate “heat inhibition”, and as a result, the damaged D1 protein tends to form aggregates that resist proteolysis by specific proteases. Under natural heat stress conditions, the effects of light stress and those of heat stress would be superimposed on each other. The results of our experiments suggest that photoinhibition becomes more prominent under heat stress conditions as was shown previously [46], since the donor side of PSII is disturbed by heat stress, which may enable photoinhibition to occur more easily.

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