Quality Control of Photosystem II

CLEAVAGE OF REACTION CENTER D1 PROTEIN IN SPINACH THYLAKOIDS BY FtsH PROTEASE UNDER MODERATE HEAT STRESS

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When spinach thylakoids were subjected to moderate heat stress (40 °C for 30 min), oxygen evolution was inhibited, and cleavage of the reaction center-binding protein D1 of photosystem II took place, producing 23-kDa N-terminal fragments. The D1 cleavage was greatly facilitated by the addition of 0.15 mM ZnCl₂ and 1 mM ATP and was completely inhibited by 1 mM EDTA, indicating the participation of an ATP-dependent metalloprotease(s) in the D1 cleavage. Herbicides 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, bromoxynil, and ioxynil, all of which bind to the Q₈ site, inhibited the D1 cleavage, suggesting that the DE-loop of the D1 protein is the heat-sensitive cleavage site. We solubilized the protease by treating the thylakoids with 2 M KSCN and detected a protease activity in the supernatant by gelatin activity gel electrophoresis in the 70–80-kDa region. The antibodies against tobacco FtsH and Arabidopsis FtsH2 reacted with a 70–80-kDa band of the KSCN-solubilized fraction, which suggests the presence of FtsH in the fraction. In accordance with this finding, we identified the homolog to Arabidopsis FtsH8 in the 70–80-kDa region by matrix-assisted laser desorption ionization time-of-flight mass analysis of the thylakoids. The KSCN-solubilized fraction was successively reconstituted with thylakoids to show heat-induced cleavage of the D1 protein and production of the D1 fragment. These results strongly suggest that an FtsH protease(s) is involved in the primary cleavage of the D1 protein under moderate heat stress.

Photosystem II (PS II)² is prone to various environmental stresses, the most prominent being strong visible light. Under light stress conditions, the reaction center-binding D1 protein of PS II is damaged and is promptly replaced by a newly synthesized D1 protein (1–4). This process is referred to as photoinhibition and repair of PS II, and a lot of effort has been made to elucidate the details of the process. Two basic mechanisms have been shown to operate in the photoinhibitory steps (3, 4). In the acceptor side mechanism of photoinhibition, over-reduction of the acceptor side of PS II by excessive light induces charge recombination between the oxidized primary electron donor P680⁺ and the reduced primary electron acceptor Pheo⁻, leading to the formation of triplet state P680. The triplet state P680 subsequently reacts with molecular oxygen to form a highly reactive singlet oxygen, which is very destructive to the D1 protein. The site of damage in the D1 protein is the stroma-exposed DE-loop of the D1 protein. In the donor side mechanism of photoinhibition, the D1 protein is damaged by endogenous cat-ionic radicals such as P680⁺ and chlorophyll⁺ generated by illumination of PS II that has an impaired oxygen-evolving system. In this case, electron donation from water to the reaction center is inefficient, and photodamage to the D1 protein takes place at the lumen-exposed AB-loop.

The photodamaged D1 protein is degraded by proteolytic enzymes and removed from PS II (5). In the acceptor side photoinhibition, 23-kDa N-terminal and 9-kDa C-terminal fragments are formed after cleavage of the D1 protein at the DE-loop (6). In the donor side photoinhibition, the D1 protein is damaged at the lumen-exposed AB-loop, whereby 10-kDa N-terminal and 24-kDa C-terminal fragments are produced (6). Identification of the proteases responsible for primary cleavage and secondary degradation of the D1 protein is currently a central topic of research on photoinhibition and repair of PS II. FtsH (filamentation temperature-sensitive) proteases are ATP- and zinc-dependent metallo-type proteases. The proteases mediate degradation of membrane proteins in bacteria, mitochondria, and chloroplasts (7). In chloroplasts, the FtsH proteases are anchored to thylakoid membranes, with two transmembrane segments at the N terminus and a large C terminus part exposed to the stroma (8). The large soluble part has an AAA (ATPase associated with various cellular activities) ATPase domain with chaperone functions and a proteolytic center where the zinc-binding motif HEXXH exists. FtsH forms a hexameric ring, and substrate proteins are translocated through a central cavity in an ATP-dependent manner. The substrates are subsequently digested at the zinc-binding site. From a series of work done with Escherichia coli, the degrada-
Heat-induced Cleavage of the D1 Protein

In this work, we investigate the effects of moderate heat stress on the D1 protein of spinach thylakoids in detail, and we show a specific degradation of the D1 protein under moderate heat stress among the PS II polypeptides. Several experiments, including MALDI-TOF mass analysis of the thylakoids and solubilization and reconstitution of proteases, demonstrated that an FtsH protease(s) is responsible for the primary cleavage of the D1 protein under moderate heat stress conditions.

**EXPERIMENTAL PROCEDURES**

**Isolation of Thylakoids**—Fresh spinach leaves were purchased from a local market, and intact chloroplasts were isolated as reported previously (29). Thylakoid membranes were obtained by osmosis of the intact chloroplasts with a hypotonic solution containing 5 mM MgCl₂ and 10 mM Hepes-KOH (pH 7.5). The mixture was subsequently centrifuged at 15,000 × g for 10 min after which the pellets were suspended in a solution containing 0.1 M sorbitol, 15 mM NaCl, 5 mM MgCl₂, and 50 mM Tricine-KOH (pH 7.6) (TC solution). After washing...
Heat-induced Cleavage of the D1 Protein

Heat Treatment—Heat treatment of the thylakoids was conducted by placing the samples in black plastic sample tubes (1 ml volume) and incubating them in a circulating water bath for a given time at 40 °C. Where indicated, either metal salts, ATP, EDTA, or other protease inhibitors were added to the thylakoids before heat treatment at 40 °C for 30 min.

Treatment of Thylakoids with Salts and Detergents—To remove the extrinsic proteins, thylakoids adjusted to 0.4 mg of chlorophyll/ml were washed with TC solution containing either 1 M NaCl, 1 M CaCl$_2$, 0.2 M Na$_2$CO$_3$, or 2 M KSCN. After incubation at 4 °C for 30 min, each suspension was centrifuged at 15,000 × g for 5 min. The pellets were washed once by the same centrifugation and finally suspended in a TC solution at 0.4 mg of chlorophyll/ml. For detergent treatment, the thylakoids were incubated with a TC solution containing either 0.1% Triton X-100, 0.1% SDS, or 0.1% Nonidet P-40 at 4 °C for 30 min. Then the samples were washed and suspended in the TC solution as described above. The salt- or detergent-treated thylakoids were used for the following heat stress experiments.

Solubilization and Reconstitution of the Protease Obtained by KSCN Treatment of Thylakoids—Thylakoids adjusted to 1 mg of chlorophyll/ml were incubated with solution A containing 2 M KSCN at 4 °C for 30 min. After centrifugation at 35,000 × g for 10 min, the supernatant was centrifuged twice more at 35,000 × g for 10 min to remove small fragments of the thylakoid membrane. The supernatant was dialyzed with a Spectra/Por 7 membrane for 3 h in a cold room with three changes of buffer solution containing 10 mM Tricine-KOH and 5 mM MgCl$_2$ (pH 7.5) and subsequently centrifuged twice at 35,000 × g for 10 min to obtain a clean supernatant. The supernatant was concentrated with an Amicon ultrafree-MC centrifugal filter (10,000 Nominal Molecular Weight Limit (NMWL) filter unit). The concentrated supernatant (protein concentration, 2.0 mg/ml) was added to either KSCN-treated or nontreated thylakoids adjusted at 0.4 mg of chlorophyll/ml, and the samples were incubated at 4 °C for 30 min. Protein concentration was measured with a BCA protein assay reagent (Pierce).

SDS/Urea-PAGE, Western Blot Analysis, and Gelatin Activity Gel Electrophoresis—SDS/urea-PAGE and Western blot analysis were carried out as described previously (31–33). The antibodies used for Western blot analysis were as follows: antibodies against the D1 protein (AB-loop and DE-loop), the D2 protein, CP43, CP47, the α-subunit of cytochrome b$_{559}$ LHCII, DS9 protein (FtsH from tobacco) and VAR2 (FtsH2 from *Arabidopsis*). Horseradish peroxidase-conjugated anti-rabbit antibody (Bio-Rad) was used as secondary antibody. Immuno-decorated bands were detected by fluorography with ECL (Amersham Biosciences). Protease activity was assayed by gelatin activity gel electrophoresis as described previously (33). The density of the proteins in the fluorogram was measured by the use of Scion Image software.

Measurements of Oxygen Evolution and Chlorophyll Fluorescence—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 μg of chlorophyll/ml. Saturating actinic light was given by a slide projector.

twice by centrifugation at 15,000 × g for 5 min with TC solution. The thylakoids were suspended in the same buffer solution at 0.4 mg chlorophyll/ml. We carried out all the preparation steps at 4 °C in a dark room under a green safe light. Chlorophylls were determined using the absorption coefficient of 0.0016 × ε$_{554}$ (Mackinney (30)).

FIGURE 2. Effects of metal salts (A), EDTA (B), and ATP (C) on moderate heat-induced cleavage of the D1 protein in thylakoids. A, various concentrations of ZnCl$_2$, MnCl$_2$, CuCl$_2$, FeCl$_3$, and MgCl$_2$ were added to the thylakoids suspended in a TC buffer (without MgCl$_2$) that had been adjusted at 0.5 mg of chlorophyll/ml, and the suspensions were subjected to heat stress at 40 °C for 30 min. Production of the 23-kDa D1 fragment was assayed by SDS/urea-PAGE and Western blot analysis with the antibody against the DE-loop of the D1 protein. For quantitative determination of the D1 fragment, a standard curve was obtained by treatment of different amounts of thylakoids at 40 °C for 30 min. The density of the D1 fragment was determined using Scion Image software. Data are the average of five measurements, and deviations are within 10%. B, effects of 1 mM EDTA on production of the 23-kDa fragment of the D1 protein of thylakoids in a TC buffer under moderate heat stress were determined. The other conditions were the same as those in A. C, effects of 1 mM EDTA on production of the D1 fragment generated by moderate heat stress were determined. The other conditions were the same as those in A. Data are the average of five measurements ± S.D.
Heat-induced Cleavage of the D1 Protein

Mass Spectrometry—Protein bands from SDS-gel stained with silver nitrate (34) were manually excised. In-gel trypsin digestion was performed with modified trypsin (Promega, WI), according to Shevchenko et al. (35). Tryptic peptides were extracted twice from the gel bands by shaking in 50% acetonitrile, 5% trifluoroacetic acid and concentrated with a vacuum centrifuge. The resulting peptides were desalted with a Perfect-Pure C-18 Tip (Eppendorf), eluted with 0.1% trifluoroacetic acid, 50% acetonitrile solution containing saturated α-cyano-4-hydroxycinnamic acid, and finally spotted on the MALDI target plate. MALDI-TOF mass analysis of the peptides was carried out with an Autoflex MALDI-TOF mass spectrometry (Bruker Daltonics). Peptide masses were searched against the NCBI nonredundant protein data base using Mascot (Matrix Science) and ProFound search engines. Alignment of Arabidopsis protein sequences was performed with a ClustalW 1.82 program.

RESULTS

Effect of Moderate Heat Stress on Oxygen Evolution and the D1 Protein—We examined the effects of moderate heat stress on oxygen evolution and degradation of the D1 protein using spinach thylakoids. Oxygen evolution activity was not much affected by the incubation of thylakoids at 20–25 °C for 30 min but was inhibited at temperatures above 30 °C (Fig. 1A). In parallel with that, production of a 23-kDa fragment of the D1 protein was detected at temperatures higher than 30 °C by SDS/urea-PAGE and Western blot analysis of the thylakoids with an antibody against the DE-loop of the D1 protein. The cleavage of the D1 protein reached its maximum at 40 °C (Fig. 1B). The degradation of the D1 protein proceeded with incubation time at 40 °C (Fig. 1C). We detected the same 23-kDa fragment by immunoblotting with an antibody against the AB-loop of the

![Figure 3: Effects of herbicides on the production of the 23-kDa fragment of the D1 protein under moderate heat stress.](Image)

![Figure 4: Solubilization of the protease responsible for the cleavage of the D1 protein from thylakoids (A), and detection of protease activity in the supernatant of KSCN-treated thylakoids by gelatin activity gel electrophoresis (B) and by Western blot analysis with the antibody against Arabidopsis FtsH2 (C).](Image)
FIGURE 5. Silver staining of thylakoids and the supernatant of KSCN-treated thylakoids (A) and mass spectrometric analysis of thylakoids (B). A, thylakoids and the supernatant of the KSCN treatment were also silver-stained, and the stained bands in the range of 60–80 kDa of both the thylakoids and the supernatant of the KSCN treatment were subjected to an Autoflex MALDI-TOF mass spectrometry to identify the proteins in the two samples. The identified proteins are shown on the right-hand side of the gel. The relative molecular masses are shown on the left-hand side of each gel. B, MALDI-TOF mass spectrum of tryptic peptides of a putative spinach FtsH band. Peptide masses were searched using Mascot and ProFound programs, both of which assigned the band to the same protein, Arabidopsis FtsH. Peptide mass peaks, the mass/charge ratios (m/z values) of that were assigned to the computed m/z values of the tryptic peptides of Arabidopsis FtsH8, were shown with the measured m/z values, and the residues of Arabidopsis FtsH8 are depicted in B. The sequence coverage of tryptic digestion was 33.1%. C, alignment of Arabidopsis FtsH2, FtsH8, and tobacco FtsH-like protein. Alignment of Arabidopsis FtsH2 (RefSeq accession number NP_850156), FtsH8 (RefSeq accession number NP_563766), and tobacco FtsH-like protein Puf (GenBank™ accession number AAD17230) sequences was performed with a ClustalW 1.82 program. Peptide sequences assigned to peptide ions obtained from MALDI-TOF mass spectrometry are underlined. Some peptide ions were assigned to peptide sequences that have a nonconserved amino acid.
Heat-induced Cleavage of the D1 Protein

AtPtuH2
MAASACLVGLGSLTVTTTKQLSKFGSRTQPSSVIRTSKVD---VYKASLGDKQKQSG 58
AtPtuH8
MAASACLVGLGSLTVTTTKQLSKFGSRTQPSSVSITASVQL---LKSLDGKQKQSG 47
NtPtfC
MATTSSVCIGSNLSSTHIQVFTQDYYQKIFSNNPSSSKTSRDYAVSALQ-OPDSE 59
**:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** **:** """"FIGURE 5---continued"""
Heat-induced Cleavage of the D1 Protein

D1 protein, suggesting the fragment to be an N-terminal one (data not shown). On the other hand, a C-terminal 9-kDa fragment was detected using a D1 antibody raised against the C-terminal sequence of the protein (data not shown). Among the various polypeptides in PS II, the D1 protein was the only protein that clearly showed a cleavage product upon heat treatment of the thylakoids; the D2 protein, the α-subunit of cytochrome b559, CP43 and CP47, and LHCIi did not cleave under the same heat stress (Fig. 1D).

Involvement of an ATP-dependent Metalloprotease in Moderate Heat-induced Cleavage of the D1 Protein—Cleavage of the D1 protein under moderate heat stress (40 °C for 30 min) was significantly stimulated by the addition of 0.05 mM ZnCl2, and the effect of ZnCl2 was maximum at 0.15 mM (Fig. 2B). The cleavage was also enhanced by the addition of 0.05 mM ATP, and the effect of ATP was saturated at 1 mM (Fig. 2C). The serine protease inhibitors 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride and aprotinin, as well as cysteine protease inhibitors E64 and leupeptin, had no effects on the heat-induced cleavage of the D1 protein (data not shown).

Effects of Herbicides on the Moderate Heat-induced Cleavage of the D1 Protein—The size of the cleavage product of the D1 protein observed under moderate heat stress (23 kDa) was similar to that detected with the acceptor side photoinhibition of PS II, suggesting the sites of cleavage of the D1 protein under light and heat stress are close to each other or probably the same. The addition of herbicides such as 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU), bromoxynil, and ioxynil, which all bind to the Qa site on the DE-loop of the D1 protein and inhibit oxygen evolution, also inhibited heat-induced cleavage of the D1 protein in the thylakoids (Fig. 3). These features are quite similar to those observed with the cleavage of the D1 protein by excessive illumination. A specific protease(s) probably recognized the heat stress-induced conformational changes in the DE-loop of the D1 protein, and the cleavage site of the D1 protein under heat stress may be the same as that under the acceptor side photoinhibition of PS II.

Solubilization of the Metalloprotease from Thylakoid Membranes—To solubilize the protease, thylakoid membranes were first treated with various salts to release the peripheral proteins. Washing thylakoids with either 1 mM NaCl, 1 mM CaCl2, or 0.2 mM Na2CO3 did not eliminate the protease activity, because the D1 protein was cleaved upon heat treatment after these salt treatments (Fig. 4A). By contrast, heat-induced cleavage of the D1 protein was not observed after treatment of thylakoids with 2 mM KSCN. Washing of thylakoids with 0.1% Triton X-100, 0.1% SDS, or 0.1% Nonidet P-40 also prevented the cleavage of the D1 protein (data not shown). Therefore, these detergents and chaotropic anions were effective in removing the protease from the thylakoids.

Detection of Protease Activity by Gelatin Activity Gel—We assayed the protease activity in the supernatants obtained by treatment of thylakoids with Triton X-100, SDS, Nonidet P-40, or KSCN using gelatin activity gel electrophoresis. Of the samples examined, only the supernatant of KSCN-treated thylakoids showed protease activity, which was seen as a transparent band on the blue background of the substrate gelatin in the gel (Fig. 4B). The relative molecular mass of the protease was 70–80 kDa. FtsH1 was suggested to be included in the supernatant by Western blot analysis of the KSCN-treated supernatant with a specific antibody against VAR2, which represents FtsH2 of Arabidopsis (Fig. 4C). The antibody reacted with a single protein band of about 77 kDa. Western blot analysis with an antibody against DS9, which corresponds to FtsH of tobacco, also showed the same profile (data not shown).

Mass Spectrometry—We tried to identify the protease in the supernatant of the KSCN-washed thylakoids by silver staining and the following mass spectrometry. However, the amounts of the protein in the 70–80-kDa region in the supernatant are too small to be detected by silver staining. We subsequently determined the proteins in the thylakoids by silver staining (Fig. 5A) and mass spectrometry (Fig. 5, B and C). Peptide mass fingerprinting with MALDI-TOF mass spectrometry assigned the protein band of 70–80 kDa to Arabidopsis FtsH8 protein with significant possibility. Arabidopsis FtsH2 and tobacco FtsH-like protein Pftf were also shown to be related to the specific band (Fig. 5C). Because the amino acid sequences of spinach FtsH proteases are unknown, this is a tentative assignment. These results suggest that the 70–80-kDa protein band contains both spinach FtsH2/8 orthologs.

Reconstitution of Protease Activity—We used the supernatant of the thylakoids washed with 2 mM KSCN for reconstitution with the thylakoids. The supernatant was added to either KSCN-treated or nontreated thylakoids, after which the effects of heat treatment on the D1 protein were examined (Fig. 6). In the reconstitution with KSCN-treated thylakoids, a slight enhancement of D1 protein degradation was observed by addition of the supernatant, whereas D1 degradation was markedly increased resulting in the reconstitution of the supernatant with native thylakoids. Degradation of the D1 protein in these reconstitution experiments was completely inhibited by EDTA, whereas degradation was not stimulated by addition of ZnCl2. Aprotinin, an inhibitor of serine-type proteases, had no effect on the reconstituted protease activity.

DISCUSSION

In this study, we showed the possibility that FtsH protease is responsible for the degradation of the D1 protein damaged under moderate heat stress. The N-terminal 23-kDa fragment of the D1 protein, detected by Western blot analysis with the antibody against the DE-loop of the D1 protein, increased by raising the temperature from 20 to 40 °C, parallel to the inhibition of oxygen evolution (Fig. 1). The degradation of the D1 protein was stimulated by the addition of ATP and zinc and inhibited by EDTA (Fig. 2). The protease was solubilized by the treatment of the thylakoids with 2 mM KSCN (Fig. 4A) and was shown to have a relative molecular mass of 70–80 kDa by gelatin zymography (Fig. 4B). The supernatant of the KSCN-treated thylakoids cross-reacted with the antibody against Arabidopsis VAR2 (FtsH2) (Fig. 4C). In the thylakoids, we detected the homolog of Arabidopsis FtsH8 by mass spectrometry as the
Heat-induced Cleavage of the D1 Protein

During moderate heat stress, only the D1 protein was cleaved in the thylakoids, and no degradation was detected with other PS II subunit proteins such as D2, CP43, and CP47 (Fig. 1D). At the present stage, the reason why the D1 protein is particularly prone to the moderate heat stress among all the PS II subunits is unknown. Although there is no supporting evidence yet, it is possible that only the D1 protein shows a large conformational change in the stroma-exposed loop structure of the protein during the limited heat stress. The structural change may be caused either by direct perturbation with heat or by indirect effects of heat stress. It was shown previously that heat induces dephosphorylation of the threonine residue at the N-terminal region of the D1 protein in spinach thylakoids (26, 27). As has been indicated with photoinhibition of PS II (37), dephosphorylation of the D1 protein should be a prerequisite condition for the recognition of the heat-damaged D1 protein by a specific protease. Indeed, the addition of a phosphatase inhibitor NaF blocked the heat-induced degradation of the D1 protein (data not shown), suggesting that protein dephosphorylation is crucial for the proteolysis of heat-damaged D1 protein. The particular susceptibility of the D1 protein to the moderate heat stress may be partly due to more efficient dephosphorylation of the D1 protein, which should result in faster alteration in its conformation, compared with the other phosphorylated proteins such as D2, CP43, and LHCII. However, it was shown previously that CP43 is first dephosphorylated and is detached from the PS II complex to ensure removal of the photodamaged D1 protein (38). Phosphorylation and dephosphorylation of the PS II core proteins appear to be catalyzed by kinases and phosphatases that are different from those working in phosphorylation and dephosphorylation of LHCII (39, 40). Distribution of phosphatase around PS II and regulation of the phosphatase activity should affect dephosphorylation rates of the D1 protein.

Another possibility responsible for the specific degradation of the D1 protein is limited accessibility of the FtsH protease to the PS II proteins. According to the turnover model of PS II under light stress, CP43 is released first from the photodamaged PS II complex, which is followed by removal of the damaged D1 protein. The spatial arrangement of polypeptides in PS II (36) indicates that there is no FtsH or no extra space for the presence of a hexameric FtsH complex in the PS II complex. Thus, access of FtsH to the damaged D1 protein becomes possible only after CP43 is removed from PS II. There may be a tight regulation mechanism for the proteases in approaching the place of proteolysis to avoid random degradation of polypeptides. Small polypeptides around the D1 protein and CP47, such as PsbI, -J, -K, and -Z, may play a positive role in heat-induced damage to the D1 protein. That was shown to be true in the present study (Fig. 3). A conformational change in the DE-loop of the D1 protein, such as unfolding of its short α-helical structure, may occur easily following oxidative damage to the constituent amino acids by heat stress or thermal perturbation of the local structure by heat stress. Such an unstructured region might act as the initiation site for degradation, because degradation signals that are buried are transiently exposed. It is suggested that AAA proteases can sense the folding state of the hydrophilic domains of membrane proteins (7).
Heat-induced Cleavage of the D1 Protein

regulation of the removal of CP43 and disintegration of the damaged D1 protein, which is the initial step of the D1 repair process. As to the location of the FtsH protease, we found that they are present in the PS II-enriched membranes and also in the core complex of PS II prepared from spinach thylakoids (data not shown). These data suggest that the proteases exist in close proximity to PS II, probably in between the PS II core and the surrounding LHC complexes.

This highly specific damage to the D1 protein might be confined to a narrow temperature range above the physiological temperature, i.e. 30 – 40 °C. Temperatures higher than that should cause irreversible damage to all the protein components in spinach PS II. Indeed, we detected rather significant aggregation of proteins at 45 °C (data not shown). The amounts of the 23-kDa degradation product of the D1 protein were apparently decreased at 45 °C compared with 40 °C, because aggregation of the D1 protein may have competed with the degradation of the D1 protein. There is no evidence so far suggesting that FtsH proteases are involved in the degradation of the protein aggregates. When EDTA was added to inhibit FtsH, the amount of the aggregation of the D1 protein induced by the moderate heat stress did not change (data not shown).

The membrane-bound protease(s) of the thylakoids was solubilized with detergents (Triton X-100, SDS, and Nonidet P-40) and the chaotropic reagent KSCN. All these reagents removed the proteolytic activity effectively from the thylakoids, because the subsequent heat treatment did not produce the 23-kDa fragment of the D1 protein. From the Western blot analysis, Triton X-100, SDS, and Nonidet P-40 were shown to solubilize a larger amount of FtsH proteases compared with the treatment with 2 M KSCN, but the protease activity in the supernatants of the former treatments was nil (data not shown). This is presumably due to inactivation of the protease by the detergent treatment or inefficient removal of the detergents by dialysis before application of the samples to the protease activity assay. Successful reconstitution of protease activity with the supernatants of KSCN-washed and untreated thylakoids supports the view that FtsH is involved in the primary cleavage of the D1 protein under heat stress (Fig. 6). The reconstitution was more complete with untreated thylakoids compared with the KSCN-treated thylakoids, probably because KSCN treatment distorted the structure of PS II, making it difficult to reconstitute proteolysis of the heat-damaged D1 protein. Alternatively, the protease activity might have been affected by the remaining KSCN upon reconstitution.

Excessive light stress and mild heat stress probably share the same feature in many aspects. The sites of damage by the acceptor side photoinhibition of PS II and the moderate heat-induced inhibition of PS II are probably the same DE-loop of the D1 protein as was described above, and production of 23-kDa N-terminal and 9-kDa C-terminal fragments of D1 protein was observed under both stress conditions. The present study strongly suggests that FtsH plays a crucial role in the primary cleavage of the D1 protein damaged by heat stress as well as by light stress. Recently we showed that FtsH is indispensable for turnover of the D1 protein under heat stress using an FtsH (slr0228)-deleted mutant of Synechocystis sp. PCC6803 (41). According to the phylogenic tree of FtsH proteins from various organisms, slr0228 of Synechocystis (Synechocystis FtsH2) has homology to slr1309 (Synechocystis FtsH1) and also to Arabidopsis FtsH2 and FtsH8 (16). The essential role of Synechocystis FtsH2 was also demonstrated in the same cyanobacterial mutant subjected to light stress (13). In this study, we showed with mass analysis that the FtsH protease homologous to Arabidopsis FtsH8 is present in spinach thylakoids. A serine protease DegP2 was shown previously to be responsible for the primary cleavage of the D1 protein under light stress (14). DegP2 is loosely bound to the stroma-exposed surface of the thylakoids and is therefore removed by washing the thylakoids with salts. After treating the thylakoid membranes with various salts and subjecting them to heat stress, cleavage of the D1 protein still took place (Fig. 4). These results suggest that DegP2 is not the protease involved in the primary cleavage of the D1 protein under heat stress.

The results presented here strongly suggest that FtsH is a key protease in degradation of the D1 protein in moderate heat-stressed thylakoids. The turnover process of the photodamaged PS II under light stress has been studied extensively (1-4, 42), and according to these results, monomerization of the PS II dimer and regulation of turnover of the D1 protein by protein phosphorylation/dephosphorylation are crucial for recognition and degradation of the damaged D1 protein by a specific protease(s). The same condition may apply to heat-induced damage of PS II. Probably the quality control mechanisms of PS II under light and heat stresses are fundamentally similar in many aspects.

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Heat-induced Cleavage of the D1 Protein

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