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Structural Changes of the Spinach Photosystem II Reaction Center After Inactivation by Heat Treatment

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Abstract: The structural changes in the electron donor side of the PSII reaction center have been monitored since heat treatment (45℃ for 5 min) of thylakoids is known to decrease the oxygen evolving activity. In heat-treated spinach chloroplast thylakoids, the inhibitory effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the electron transport activity of the PSII reaction center from diphenyl carbazide to dichlorophenolindophenol became reduced approximately 3.8 times and [^{14}C]-labeled DCMU binding on the D1 polypeptide decreased to 25~30% that of intact thylakoid membranes, implying that the conformational changes of the DCMU binding pocket, residing on the D1 polypeptide, occur by heat treatment. The accessibility of trypsin to the NH_2-terminus of the cytochrome *b-559* α-subunit, assayed with Western blot using an antibody generated against the synthetic peptide (Arg-68 to Arg-80) of the COOH-terminal domain, was also increased, indicating that heat-treatment caused changes in the structural environments near the stromal side of the cytochrome *b-559* α-subunit, allowing trypsin more easily to cleave the NH_2-terminal domain. Therefore, the structural changes in the electron donor side of the PSII reaction center complexes could be one of the reasons why the oxygen evolving activity of the heat-treated thylakoid membranes decreased.
Key words: cytochrome *b-559*, D1 polypeptide, heat treatment, photosystem II reaction center.

In oxygenic photosynthesis of plants, the reactions take place in a specialized subcellular organelle called the chloroplast. The chloroplast has two membrane-embedded photochemical reaction centers: Photosystem II (PSII) and Photosystem I (PSI). They are highly structured complexes of geometrically arranged pigments and proteins embedded in thylakoid membranes. Each functions to move electrons against a redox potential gradient utilizing the energy captured by an excited chlorophyll. PSII is an integral membrane protein complex of the chloroplast thylakoid membrane, which contains a number of prosthetic groups that are necessary to extract electrons from water (yielding molecular oxygen and hydrogen ions) and to pass them through an electron transport chain to PSI.

The PSII reaction center, the isolated minimal functional preparation of PSII, where the initial conversion of light into biochemical energy takes place, consists of four major polypeptides D1 (32 kDa), D2 (34 kDa), and the α- (9 kDa) and β- (4 kDa) subunits of cytochrome *b-559* (Nanba and Satoh, 1987; Yocum, 1991). The D1 polypeptide had been implicated in PSII function at the reducing side (Mattoo *et al.*, 1981) as well as the oxidizing side (Metz *et al.*, 1986) of the membrane. The Q_B site, residing in the D1 polypeptide, is the target of many structurally different herbicides such as atrazine (Pfister *et al.*, 1981) and diuron (Mattoo *et al.*, 1981). The herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is a highly specific inhibitor of plant and algal photosynthetic electron transport. One molecule of DCMU per PSII complex has been reported to inhibit the light reaction at the site close to the Q_B by competing with quinone for a binding site (Horton *et al.*, 1976). Cytochrome *b-559* consists of a heme group and two subunits, α- and β-polypeptides, encoded by psbE and psbF genes on the spinach plastid genome, respectively (Widger *et al.*, 1984; Herrmann *et al.*, 1984; Westhoff *et al.*, 1985), and each subunit spans the thylakoid membrane once as an α-helix (Tae *et al.*, 1988; Tae and Cramer, 1994). The orientation of cytochrome *b-559* α- and β-polypeptides in thylakoids determines the position of the heme in the thylakoid membrane,but their function is still unknown (Tae *et al.*, 1988; Tae and Cramer, 1994).

The structural and functional aspects of PSII are interrelated. In order to maintain the stable photosynthetic activity of the reaction center, the structual integrity

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of the D1, D2, and cytochrome *b-559* complex is essential. The fact that an environmental stress such as drought, flood, high intensity light, or high temperature can cause a decrease in photosynthetic yield is primarily ascribed to damages in the PSII reaction center. It has been shown that heat-treatment (45℃, 3 min) causes a decrease in the redox potential of the hydroquinone-reducible cytochrome *b-559* and in the oxygen evolution rate of PSII (Cramer *et al.*, 1981). In this paper, the degenerative effects of high temperature (45 ℃ for 5 min) on PSII activity have been examined in terms of the structural intactness of the PSII reaction center.

Materials and Methods

Preparation of intact and heat-treated thylakoids

Spinach thylakoid membranes were prepared by osmotic shock from intact chloroplasts (Tae and Cramer, 1994). The latter were made from approximately 35 g of leaves by (i) briefly homogenizing in a medium consisting of 0.33 M sorbitol, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2% BSA, and 50 mM HEPES, pH 7.5; (ii) coarse filtration of the homogenate and centrifugation at 1,500×*g* for 3 min (4℃); and (iii) further purification on a Percoll gradient. The osmotic shock medium was as in Tae and Cramer (1994). The thylakoid membranes were resuspended in 50 mM sucrose, 10 mM NaCl, and 20 mM HEPES, pH 7.5 after centrifugation at 7,000×*g* for 10 min. The chlorophyll concentration was adjusted to 1 mg chl/ml according to Lichtenthaler (1987). The heat-treated thylakoid membranes were prepared by incubating the resuspended thylakoid membranes at 45℃ for 5 min.

Trypsin treatment of thylakoid membranes

Two different kinds of thylakoid membranes, structurally and functionally intact and heat-treated membranes, were incubated with trypsin (Sigma, T-1005) (Chl : trypsin=50 : 1) in 20 μg of chl/reaction for the indicated times (0, 15, 30, and 60 min) at room temperature (ca. 20℃). The digestion reactions were terminated by adding three different kinds of protease inhibitors (2 mM PMSF, 4 mM benzamidine, and 4 mM ε-aminocaproic acid). The chlorophyll molecules were extracted with 80% (w/v) acetone.

Measurement of DCMU effects on DCPIP reduction in the PSII complex

The electron transfer activity from Q_A to Q_B of the PSII reaction center was measured with 0.4 mM diphenyl carbazide (DPC) and 40 μM dichlorophenolindophenol (DCPIP) at room temperature on an Aminco dual-wavelength monochromator with a computer-controlled stepper motor (High-Sync AC synchronous/DC stepper motor; Bodine Electric Co.). The measuring beam (half bandwidth, 2 nm) and the actinic light, arranged to pass vertically through the cuvette, was blocked by a Balzer's DT-Gruen filter to prevent any interference from fluorescence and detected by an S11 EMI 9524 photomultiplier. Various concentrations of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (0.1 to 10 μM) were added to intact or heat-treated thylakoid membranes and the electron transport activity from the artificial electron donor (DPC) to the acceptor (DCPIP) through the PSII reaction center was measured.

[^{14}C]-labeled DCMU binding assay

In order to quantitate the stoichiometry of functional PSII complexes of intact and heat-treated thylakoid membranes, [^{14}C]DCMU binding to PSII complexes was measured (Tae and Cramer, 1992). Various (4.5~91.3 nM) concentrations of radiolabeled DCMU (243 μCi/mg; Amersham, kindly donated by W.F.J. Vermaas) were added to 1 mL of thylakoid membranes (20 μg chl/ml) in 50 mM sucrose, 10 mM NaCl, and 20 mM HEPES, pH 7.5. To correct for the amount of [^{14}C] DCMU bound to membranes other than the PSII complex, control experiments were performed in which [^{14}C]DCMU binding was measured in the presence of 40 μM unlabeled atrazine, which is a specific inhibitor of the quinone or DCMU binding site in the PSII reaction center. After incubation for 15 min at room temperature in dim light, the membranes were sedimented in a microcentrifuge, and 0.8 ml of the supernatant was mixed with scintillation cocktail and assayed.

Western blot analysis of trypsin-treated thylakoid membranes

Trypsin-digested thylakoid membrane proteins were separated on a 15~20% (w/w) gradient SDS-polyacrylamide gel containing 6 M urea. The gel was incubated in 30 mM Tris-HCl, pH 8.0, including 17 mM boric acid, 2 mM SDS, and 20% methanol for 10 min at room temperature. Polypeptides were transferred to nitrocellulose paper (pore size: 0.45 μm, Hybond-C, Amersham) with a semi-dry transfer blotter (130-mA constant current, 30 min) (Model TE 70, Hoefer Scientific Instruments). The paper was washed in TBS buffer (10 mM Tris-HCl, pH 7.5, and 0.15 M NaCl), incubated in a "Seal-a-Meal™" bag with 10% milk casein on a nutator (2 h, room temperature), removed from the bag, and washed in TBS buffer. After nutation with antibody against the cytochrome *b-559* α-subunit (Tae *et al.*, 1988; Vallon *et al.*, 1989) in TBS buffer containing 0.5% (w/v) BSA for 2 h, and washing in TBS buffer

containing 0.1% (w/v) Nonidet-40, the paper was again incubated with a second antibody [goat anti-rabbit IgG conjugated with horseradish peroxidase (type VI, Accurate Chemical and Scientific Corp.)] in TBS buffer containing 0.5% BSA. The paper was nutated for 1 h, washed in TBS buffer containing 0.1% (w/v) Nonidet-40, and stained for 10 min with 0.017% 4-chloro-1-naphthol and hydrogen peroxide in TBS buffer.

Results and Discussion

Reduced inhibitory effect of DCMU on DCPIP reduction in heat-treated thylakoids

X-ray crystallographic analysis of the reaction center from T4 mutant (Tyr-222 of L-subunit changed to Phe) of *Rps. viridis* with bound DCMU, whose structure is very similar to that of the higher plant PSII, provided structural details of DCMU-D1 polypeptide interactions. In *Rps. viridis*, Glu-212 interacts with DCMU. In the D1 polypeptide of PSII, Ala-251 is suggested to be at the equivalent position of Glu-212, which is in close contact with DCMU and gives DCMU more space when binding in PSII (Sinning, 1992). It is, however, surprising that the mutational change of a single residue that is involved in neither quinone nor herbicide binding can block the herbicide binding of the bacterial reaction center, implying that the structural integrity of the DCMU binding niche is important for its binding.

Since heat-treated thylakoids have decreased the oxygen evolving activity (from water to 2,6-dichloro-p-benzoquinone) to 30% of the intact thylakoids (Cramer *et al.*, 1981), the damaged sites along the electron transport pathway of the PSII complex have been monitored after heat-treatment by measuring the DCMU inhibitory effect on the electron transport from the special chlorophyll of PSII (P680) to the quinone binding site (Q_B) by use of DPC and DCPIP. In intact thylakoids, the concentration of DCMU that inhibits 50% of the electron transfer from DPC to DCPIP was 170 nM. On the other hand, 650 nM of DCMU was needed to block 50% of the electron transport activity in heat-treated thylakoids (Fig. 1). The decreased sensitivity (approximately 3.8 times) to the DCMU inhibitory effect reflects the impaired structures of the DCMU binding niche of the D1 polypeptide in the heat-treated PSII reaction center, implying that the D2 polypeptide and cytochrome *b-559* also undergo structural changes by heat-treatment since D1 is tightly bound to D2-cytochrome *b-559* to form the PSII reaction center. The decreased sensitivity might be ascribed to the impaired structure of all reaction centers or completely competent structure of a small fraction of the reaction center. In other words, there are two alternative possibilities

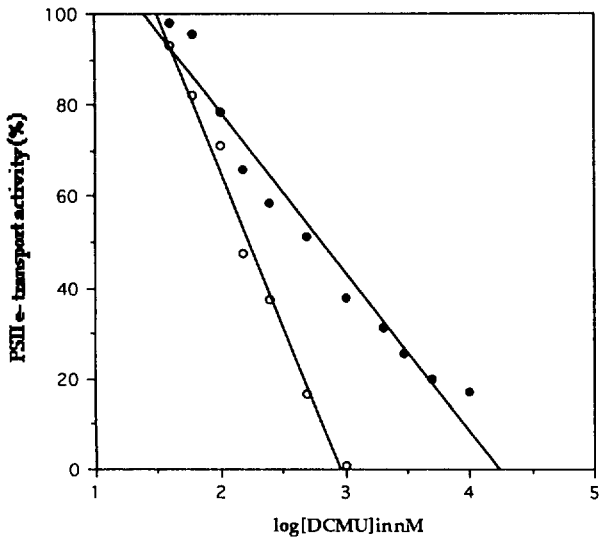

Fig. 1. Inhibitory effect of DCMU on DCPIP reduction in heat-treated thylakoids. Assays were performed at 15 μg chl/ml, with 40 μM DCPIP, 0.4 mM DPC and various concentration (10 nM to 10 μM) of DCMU. The reduction of DCPIP was measured at 550~500 nm. Samples were mixed before each assays with a magnetic stirrer that was turned off before measurement. The open and closed circles represent intact and heat-treated thylakoid membranes, respectively. The relative PSII electron transport activity of 100% means that no DCMU is added to block the electron transport.

for the decreased inhibition of DCMU on heat-treated PSII: (i) all PSII reaction centers are altered in their structures with a consequent decreased sensitivity towards DCMU; or (ii) most of the PSII complexes are damaged, where DCMU cannot bind, but a small fraction is intact and has an electron transport rate between DPC and DCPIP similar to that of the intact reaction center. In order to assay the concentration and functional state of the PSII reaction centers, binding of [^{14}C]DCMU to the PSII complexes in thylakoids can be used.

[^{14}C]-labeled DCMU binding assay

The stoichiometry of binding of [^{14}C]DCMU can be used to assay the concentration and functional state of the PSII reaction center (Tae and Cramer, 1992). The binding of [^{14}C]DCMU to the thylakoid membranes as a function of DCMU concentration was assayed to quantitate the number of PSII reaction centers *in vivo* in intact and heat-treated thylakoids (Fig. 2). Scatchard plot analysis showed that (i) heat-treated and intact thylakoid membranes have a similar number of functional PSII complexes per unit chlorophyll (the intercept of X-axis, 875 chlorophyll/DCMU, mol/mol), but (ii) the binding affinity (the negative value of the slope) of DCMU of heat-treated thylakoids was approximately 1/4 that of intact thylakoids (binding affinities

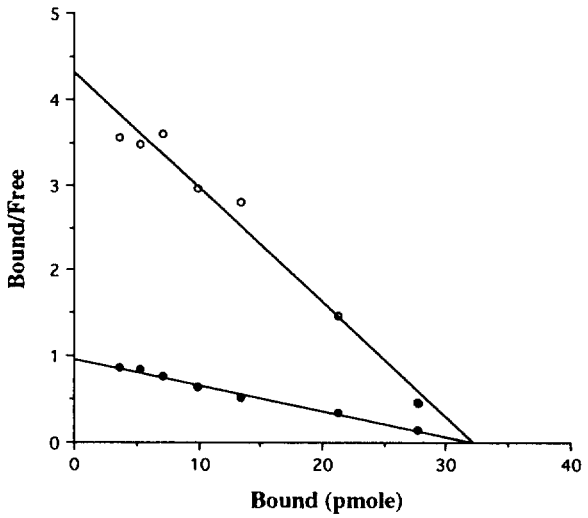

Fig. 2. [^{14}C]DCMU binding assays of intact and heat-treated thylakoid membranes. 4.5~91.3 nM of radiolabeled diuron was used to determine a total number of PSII reaction centers in thylakoids. The X-axis intercept and a negative value of the slope indicate the number of the PSII reaction center and the binding constant, respectively. The open and closed circles represent intact the heat-treated thylakoid membranes, respectively.

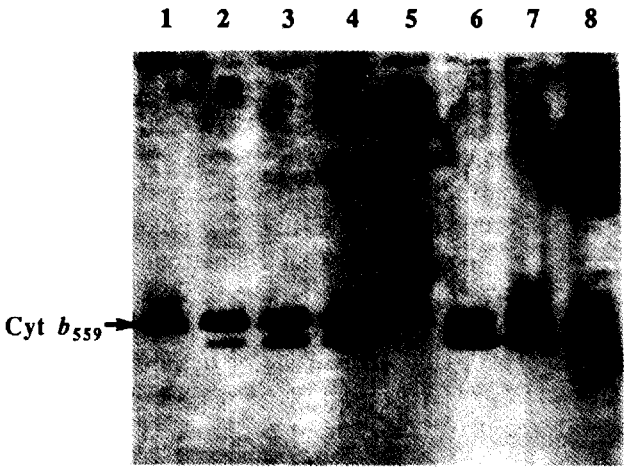

Fig. 3. Effect of heat treatment on accessibility of the NH_2-terminal domain of the cytochrome *b-559* α-subunit to trypsin. Intact (lanes 1~4) and heat-treated (lanes 5~8) thylakoid membranes were proteolyzed with trypsin (trypsin : chlorophyll, 1 : 50) for 0 (lanes 1 and 5), 5 (lanes 2 and 6), 10 (lanes 3 and 7), and 15 min (lanes 4 and 8), and the proteins were separated on the 15~20% gradient SDS-polyacrylamide gel including 4 M urea. Each lane was loaded with the membrane equivalent of 10 μg of chlorophyll.

of intact and heat-treated thylakoids: 1.34×10^{-1} μM and 3.03×10^{-2} μM, respectively). The number of binding sites of DCMU located on both intact and heat-treated thylakoid membranes turned out to be similar to each other, approximately 1 reaction center per 875 chlorophylls, but in heat-treated thylakoids the affinity for DCMU binding decreases approximately to 25~30 % that of intact thylakoids. Therefore, inactivation of approximately 30% of the oxygen evolving activity of PSII by heat-treatment (45℃, 5 min) can be ascribed to all PSII reaction centers altered in their structures, resulting in the decreased sensitivity towards DCMU. The structural intactness of the PSII reaction center would be an important factor to maintain the electrochemical properties of the PSII complexes. The heat damage to the PSII complex is likely to be a permanent change since the oxygen evolving activity of heat-treated thylakoid membranes has never been restored after incubation on ice for more than 30 min in the dark (data not shown).

Increased trypsin accessibility to the NH_2-terminal domain of the cytochrome *b-559* α-subunit in heat-treated thylakoids

Trypsinolysis of intact thylakoid membranes caused a trace amount of the original cytochrome *b-559* α-subunit (apparent molecular weight: 10 kDa) to shift to a band of slightly smaller size (Fig. 3, lanes 2~4) that also reacted with the antibody generated against the synthetic tridecapeptide of the cytochrome *b-559* α-subunit, spanning Arg-68 to Arg-80 (Tae *et al.*, 1988). As the incubation time with trypsin increases, the cleaved band intensities increase (Fig. 3, lanes 2~4). The magnitude of the band shift has been determined to be 762± 227 Da (n=7). Topography of the cytochrome *b-559* α- and β-subunit of spinach has been determined (Tae *et al.*, 1988; Tae and Cramer, 1994). Cytochrome *b-559* is the essential component of the PSII reaction center for its electrophotochemical activity. It spans the thylakoid membrane once and the NH_2- and COOH-termini are exposed to the stromal and lumenal side of the thylakoid membranes. Therefore, trypsin is likely to be accessible to the NH_2-terminus of the α-subunit and cleave the carboxylic side of Arg-7, based on the size of the trypsin cleaved fragment. Since the COOH-terminus, containing the antigenic site (Arg 68 to Arg 80), of the cytochrome *b-559* α-subunit is exposed to the lumen of thylakoids and the total amount of intact and trypsin-cleaved cytochrome *b-559* does not change during the course of the trypsin digestion, based on the densitometric scanning of the bands (data not shown), trypsin is not likely to be accessible to the COOH-terminal domain of cytochrome *b-559*, implying that the thylakoid membrane maintains its intactness during trypsinolysis.

Trypsin digestion of heat-treated thylakoids (45℃, 5 min), even for 5 min at room temperature, however, caused approximately 75% of the parental band to shift to a position on the gel of a digested band (Fig. 3, lane 6), indicating that trypsin is more easily accessible

to the Arg-7 site of heat-treated thylakoids compared to that of intact thylakoids (Tae *et al.*, 1988). This result implies that heat-treatment causes structural changes near the NH_2-terminal domain of the cytochrome *b-559* α-subunit, allowing trypsin more easily to cleave the Arg residue at position 7. Since cytochrome *b-559* is tightly bound to the D1 and D2 polypeptides, the essential components of the PSII reaction center, and the position of the cytochrome *b-559* heme is likely to be located near the quinone binding niche of the D1 polypeptide, the accessibility of trypsin to the NH_2-terminus of the cytochrome *b-559* α-subunit could be an indicator of the structural perturbation of the PSII reaction center by heat-treatment. It was concluded that heat-treatment of thylakoid membranes causes a structural change of the DCMU binding sites near the D1 polypeptide that increased the accessibility of trypsin to the NH_2-terminal domain of the cytochrome *b-559* α-subunit. The increase in trypsin accessibility implies a corresponding increase in solvent accessibility that could explain that the lowered E_m of the cytochrome associated with the low potential form is due to the changes in the membrane electrostatic environment (Krishtalik *et al.*, 1993). In conclusion, the structural changes in the electron donor side of the PSII reaction center complexes could be one of the reasons why the oxygen evolving activity of the heat-treated thylakoid membranes decreased.

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