Degradation Pattern of Photosystem II Reaction Center Protein D1 in Intact Leaves

The Major Photoinhibition-Induced Cleavage Site in D1 Polypeptide Is Located Amino Terminally of the DE Loop

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Photoinhibition-induced degradation of the D1 protein of the photosystem II reaction center was studied in intact pumpkin (Cucurbita pepo L.) leaves. Photoinhibition was observed to cause the cleavage of the D1 protein at two distinct sites. The main cleavage generated an 18-kD N-terminal and a 20-kD C-terminal degradation fragment of the D1 protein. This cleavage site was mapped to be located clearly N terminally of the DE loop. The other, less-frequent cleavage occurred at the DE loop and produced the well-documented 23-kD, N-terminal D1 degradation product. Furthermore, the 23-kD, N-terminal D1 fragment appears to be phosphorylated and can be detected only under severe photoinhibition in vivo. Comparison of the D1 degradation pattern after in vivo photoinhibition to that after in vitro acceptor-side and donor-side photoinhibition, performed with isolated photosystem II core particles, gives indirect evidence in support of donor-side photoinhibition in intact leaves.

PSII is a key component in capturing light energy in the form of chemical energy in photosynthesis. PSII is a multiprotein complex embedded in the thylakoid membrane that mediates electrons derived from water at the lumenal (donor) side of the thylakoid membrane to primary and secondary quinone acceptors on the stromal (acceptor) side of the thylakoid membrane. PSII reaction center proteins D1 and D2 are intrinsic membrane-spanning proteins, each consisting of five transmembrane α helices connected by lumenal and stromal loops (Trebst, 1986). Together, the D1 and D2 proteins bind all the redox components participating in PSII electron transfer and create oxidative power strong enough to break water molecules. This makes the protein framework vulnerable because mismatches in electron transfer create potentially dangerous oxidants that can (photo)damage the PSII reaction center proteins D1 and (to a lesser extent) D2 in a phenomenon known as photoinhibition. The photodamaged proteins are subsequently degraded (for reviews, see Prasil et al., 1992; Aro et al., 1993b). D1 protein is known to have a rapid turnover even in moderate light (Mattoo et al., 1981), and the turnover rate increases with increasing irradiance (Kyle et al., 1984; Aro et al., 1993a; Tyystjärvi and Aro, 1996). The light-induced degradation of the D1 protein occurs in intact leaves, isolated thylakoids, and various PSII preparations. Most experimental data suggest that the D1 protein is initially cleaved by a proteinase closely associated with the reaction center complex of PSII (Virgin et al., 1990; Shipton and Barber, 1991).

Although under intense research, the in vivo mechanism(s) of the light-induced irreversible damage of the electron transport in PSII and the actual proteolytic degradation of the D1 protein are still poorly understood. In vitro studies with various PSII preparations have given evidence for two different mechanisms of photoinhibition, the acceptor-side (Setlik et al., 1990; Styring et al., 1990; Vass et al., 1992) and the donor-side mechanisms (Theg et al., 1986; Jegerschöld et al., 1990). Acceptor-side photoinhibition is induced when the forward electron flow from PSII is blocked because of complete reduction of the plastoquinone acceptors. Donor-side photoinhibition occurs when the reduction of P680+ and TyrZ+ is hampered because of impaired electron donation from the oxygen-evolving complex. Both types of photoinhibition lead to the formation of highly reactive oxidants that induce photoinhibitory damage in the PSII reaction center and subsequent degradation of the D1 protein.

The elucidation of the photoinhibition-induced degradation pattern of the D1 protein is important for several reasons. First, the degradation pattern may give indirect evidence of the photoinhibition mechanism. Furthermore, the search for the D1-specific protease is greatly facilitated by localization of the primary cleavage site(s) in the D1 polypeptide. There is a consensus in the literature that the two different mechanisms of photoinhibition yield different degradation patterns of the D1 protein in vitro (De Las Rivas et al., 1992). After acceptor-side photoinhibition, the main D1 degradation fragments are a 23-kD N-terminal and a 10-kD C-terminal fragment. This pattern of fragments suggests cleavage in the stromal DE loop of the D1 protein. Donor-side photoinhibition results in the formation of an N-terminal and a C-terminal D1 degradation

Abbreviations: Chl, chlorophyll; F v/F m, ratio of variable fluorescence to maximal fluorescence.

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fragment with apparent molecular masses of 10 and 24 kD, respectively. The initial cleavage would therefore be expected to occur in the luminal loop connecting helices A and B of the D1 protein (De Las Rivas et al., 1992). In addition, degradation products of the D1 protein in the 16- to 18-kD range have been observed in vitro (Aro et al., 1990; Virgin et al., 1990; Shipton and Barber, 1991, 1992; Barbato et al., 1992b; De Las Rivas et al., 1992; Salter et al., 1992; Friso et al., 1993) and also in PSII reaction centers isolated from illuminated pea leaves (Shipton and Barber, 1994). These D1 degradation fragments have been proposed to originate from cleavage in the luminal loop connecting helices C and D (Barbato et al., 1992b; Friso et al., 1993).

In vivo, the study of the degradation pattern of the D1 protein is hampered by rapid proteolysis of the primary degradation fragments. In the present study we have further elucidated the degradation pattern of the D1 protein during photoinhibition in vivo. We report degradation fragments that originate from two different cleavage sites in the D1 protein: the main cleavage site is located N terminally of the DE loop, and the other site was mapped to the DE loop.

MATERIALS AND METHODS

Pumpkin (Cucurbita pepo L.) plants were grown at the PPFD of 300 μmol m⁻² s⁻¹ (moderate light) or 1000 μmol m⁻² s⁻¹ (high light) at 23°C. Fully expanded leaves of 3- to 5-week-old plants were used in the experiments. For the photoinhibition experiments pumpkin leaves were detached and the petioles were immersed in water or in either aqueous chloramphenicol solution (3 mM) or lincomycin (2 mM) and incubated in darkness for 3 h. After the preincubation, the leaves were illuminated at either the PPFD of 1100 or 2500 μmol photons m⁻² s⁻¹ in saturated humidity. The temperature of illuminated leaves was kept at 20°C. A 1200-W HMI arc lamp (Quasar, Strand Lightning, Rome, Italy) was used as a light source.

To study the time course of photoinhibition, leaf discs were taken periodically during illumination for the measurement of \( F_\text{r} / F_\text{max} \). The \( F_\text{r} / F_\text{max} \) was measured with a pulse amplitude modulation fluorometer after a 30-min dark adaptation of the leaf discs. The leaf discs taken for the quantification of the D1 protein were immediately frozen in liquid nitrogen and stored at -70°C until isolation of the thylakoid membranes. Thylakoid membranes were rapidly isolated at 0 to 4°C according to Leto et al. (1985) and frozen in liquid nitrogen. When the light-induced D1 protein phosphorylation was under study, 10 mM NaF was added to the thylakoid isolation buffers.

The isolation of PSII core particles was done according to either van Leeuwen et al. (1991) or Ghnoutakis et al. (1987). Illumination of PSII core particles was done at the Chl concentration of 300 μg/mL in either 50 mM HEPES-KOH (pH 7.6), 10 mM NaCl, and 400 mM Suc, or in 50 mM Tris-HCl (pH 8.0), 0.1 M sorbitol, and 5 mM MgCl₂ with 1 mM 2,6-dichloro-p-benzoquinone as an electron acceptor. The PPFD was 4500 μmol m⁻² s⁻¹ white light, and the temperature was kept at 20°C. Trypsin digestion of the D1 protein in isolated thylakoid membranes was carried out at 18°C and at the trypsin (Sigma) concentration of 50 μg/mL in 10 mM sodium-phosphate buffer (pH 7.4), 20 mM NaCl, and 100 mM Suc. The Chl concentration was 300 μg/mL. After digestion, SDS-PAGE solubilizing buffer was added to the samples and they were immediately frozen in liquid nitrogen.

The thylakoids were solubilized and the polypeptides were separated by SDS-PAGE (essentially according to Laemmli, 1970) in 14% polyacrylamide gels, including 4 μL urea. The separation of the unphosphorylated and phosphorylated D1* (Koivuniemi et al., 1995) forms of the D1 protein was performed in 15% SDS-PAGE gels, including 6 μL urea, and with a long electrophoretic run. In some experiments, as indicated in the text, SDS-PAGE was performed by omitting urea from the gels and from the solubilizing buffer. After the electrophoresis, polypeptides were electroblotted to Immobilon P membrane (Millipore) and the D1 protein was immunodetected with a Bio-Rad chemiluminescence kit. Four different rabbit polyclonal anti-D1α antisera were used to identify the D1 protein and fragments derived from it. The antisera are anti-D1α (Herrman et al., 1985), anti-D1C (a kind gift of Dr. P. Nixon, Imperial College, London), anti-D1N (Barbato et al., 1991), and anti-D1DE (Research Genetics, Huntsville, AL). The anti-D1C and anti-D1N recognize epitopes located at the C-terminal and N-terminal ends of the D1 protein, respectively. The anti-D1C is raised against amino acids 333 to 353 from pea D1 polypeptide, and the anti-D1N is raised against the N terminus of wheat D1 protein, produced by cleavage in the specific Lys²⁵⁰ of the wheat D1 protein. The anti-D1α was raised against the D1 protein purified from the alga Bumilleriopsis filiformis and the anti-D1DE was raised against amino acids 234 to 242 from Synecocystis PCC 6803 D1 protein. Bio-Rad prestained SDS-PAGE standards were used to estimate the apparent molecular masses of the polypeptides. Chl was determined according to Arnon (1949).

RESULTS

Primary Products of Photoinhibition-Induced Degradation of the D1 Protein in Intact Leaves

Exposure of high-light-grown pumpkin leaves to the PPFD of 2500 μmol m⁻² s⁻¹ resulted in about a 50% loss of PSII photochemical activity within 3 h (measured as a decrease in \( F_\text{r} / F_\text{max} \)), with only a minor decrease in the steady-state D1 protein content of the thylakoids (Fig. 1). Continued illumination did not induce any additional loss of PSII activity, but the D1 protein content slowly decreased. Photoinhibition was accompanied by the formation of degradation products of the D1 protein with apparent molecular masses of 18, 20, and 23 kD (Fig. 2). The 18- and 20-kD D1 degradation fragments (Fig. 2A) are N terminal and C terminal in origin, since they were recognized by anti-D1N and anti-D1C, respectively (Fig. 2, B and C). The epitopes recognized by anti-D1N or anti-D1C are located at the very N terminus (see below) or very C terminus (see "Materials and Methods") of the D1 polypeptide, respectively, which sup-
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Figure 1. Time course of PSII photoinhibition ($F_{v}/F_{\text{max}}$) and the loss of the D1 protein (•) from the thylakoid membranes during illumination of high-light-grown pumpkin leaves at PPFD 2500 ±mol m$^{-2}$ s$^{-1}$, 20°C. $F_{v}/F_{\text{max}}$ was measured from leaf discs after 30 min of dark incubation, and the D1 protein content was measured immunologically (anti-D1$_{\text{DE}}$) from thylakoids isolated from the illuminated leaves. Chl (0.75 µg) was loaded in each well.

Figure 2. Identification of the degradation products of the D1 protein in thylakoid membranes isolated from high-light-treated pumpkin leaves. Polypeptides were separated by urea-SDS-PAGE and electroblotted to a PVDF membrane, and the immunodetection of the D1 protein was done using anti-D1$_{\alpha}$ (A), anti-D1$_{\text{DE}}$ (B), anti-D1$_{\text{N}}$ (C), and anti-D1$_{\text{C}}$ (D). Lanes 1, Control thylakoids isolated from nonphotoinhibited leaves; lanes 2 and 4, thylakoids isolated from leaves illuminated at a PPFD of 2500 ±mol m$^{-2}$ s$^{-1}$, 20°C, for 1.5 and 3 h, respectively; lanes 3 and 5, same as lanes 2 and 4, respectively, but the illumination was performed in the presence of chloramphenicol. Ten micrograms of Chl was loaded in each well. The estimated apparent molecular masses of the D1 degradation fragments (18, 20, and 23 kD) and a putative translation intermediate (25 kD) are indicated.

Comparison of the in Vivo D1 Fragments with the Photoinhibition-Induced Degradation Pattern of the D1 Protein in Isolated PSII Core Particles

To determine whether the two putative in vivo cleavage sites were related to the acceptor-side and donor-side photoinhibition mechanisms deduced from in vitro experiments, we first compared the in vivo degradation pattern...
Figure 3. A, Immunoblot demonstrating the time course of the appearance of in vivo D1 protein degradation products, and comparison of the D1 degradation pattern between in vivo photoinhibition and in vitro donor-side photoinhibition. Moderate-light-grown pumpkin leaves were illuminated at a PPFD of 1100 μmol m⁻² s⁻¹, 20°C, in the presence of lincomycin for 0 to 240 min as indicated above each lane. Donor-side photoinhibition was performed by illumination of PSII core particles (300 μg Chl/mL in 50 mM Tris-HCl [pH 8.0], 0.1 M sorbitol, 5 mM MgCl₂, and 1 mM 2,6-dichloro-p-benzoquinone) at a PPFD of 4500 μmol m⁻² s⁻¹, 20°C, for 30 min. After illumination, thylakoid polypeptides were separated by urea-SDS-PAGE and electroblotted to a PVDF membrane, and immunodecoration was performed with anti-D1ₑ or anti-D1α as indicated at the bottom of the immunoblots. Eight micrograms (intact leaves) or 5 μg (PSII core particles) of Chl was applied in each well. CTL, Nonilluminated control sample. The estimated apparent molecular masses of the putative D1 synthesis fragment (25 kDa, on the left side) and D1 degradation fragments (18, 20, 23, and 25 kDa, on the right side) are indicated. The D1/D2 heterodimer is indicated. B, Degradation pattern of the D1 protein after acceptor-side photoinhibition of isolated PSII cores (300 μg Chl/mL in 50 mM Hepes-KOH [pH 7.6], 10 mM NaCl, and 400 mM Suc) at 4500 μmol m⁻² s⁻¹, 20°C, for 30 min. Immunodecoration was with anti-D1α. Five micrograms of Chl was loaded in both wells. The estimated molecular masses of the D1 degradation fragments are given and shown by arrows, and the positions of the 41-kD adduct and the D1/D2 heterodimer are indicated.

of the D1 protein to that obtained by illuminating PSII core particles in the presence of an electron acceptor (donor-side photoinhibition; De Las Rivas et al., 1992, and refs. therein). The most intense D1 degradation fragments obtained from donor-side photoinhibition in vitro were the same 20-kD, C-terminal and 18-kD, N-terminal D1 degradation fragments also produced during in vivo photoinhibition (compare Figs. 2 and 3A, specifically the immunodecoration with anti-D1α). Also, a D1 fragment of 25 kDa was detected during the illumination (Fig. 3A).

Illumination of PSII core particles without added electron donors or acceptors (acceptor-side photoinhibition; Shipton and Barber, 1994) resulted in a poor yield of D1 degradation fragments and led to the appearance of the 41-kD adduct (Fig. 3B; see Barbato et al., 1992a), which never accumulated during in vivo photoinhibition (Figs. 2, 3A, and 4). In this acceptor-side photoinhibition experiment, the most intense D1 fragment recognized by anti-D1C was observed in the 8-kD range (Fig. 3B). This band, however, was also present in the control sample (Fig. 3B). The 8-kD D1 fragment (Fig. 3B) was overestimated by the electrophoretic system used.
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20-kD, C-terminal fragment (Figs. 2 and 3A) could be produced by a cleavage in the DE loop, we decided to compare recently been reported by Kim et al. (1994).

Urea Hampers the Estimation of the Molecular Size of the D1 Protein Degradation Products

Urea is known to affect the mobility of the reaction center polypeptides D1 and D2 in SDS-PAGE in relation to standard polypeptides. Because both polypeptides have an apparent molecular mass of 32 kD, the difference in mobility must be related to different conformations rather than to a difference in molecular mass. Minor changes such as phosphorylation (Kettunen et al., 1991; Elich et al., 1992) or the change of only one amino acid (Tyystjärvi et al., 1994) affect the mobility of the D1 protein in SDS-PAGE even if the polypeptides are solubilized. To determine whether the size of our 20-kD, C-terminal degradation product of D1 protein was only an overestimation of a considerably smaller fragment, the electrophoretic separations were repeated by omitting urea both from the gels and from the solubilizing buffer.

As shown in Figure 4, the C-terminal degradation fragment of the D1 protein runs, with respect to the standard polypeptides, much faster in SDS-PAGE gels without urea than in the urea gels. The estimated molecular masses were 13 and 20 kD and from the solubilizing buffer. The open arrows indicate the migration of the C-terminal D1 degradation fragment in the presence (A) and absence (B) of urea in the gel. The immunolabeled band at approximately 28 kD (B) is the D1 conformer.

The three small trypsin digestion fragments of the D1 protein, recognized by anti-D1α, had apparent molecular masses of 16, 12, and 8 kD in urea-SDS-PAGE (Fig. 5A). The 12- and 8-kD D1 protein fragments are C terminal in origin (Fig. 5C). If urea was omitted from SDS-PAGE, the 12-kD, C-terminal D1 fragment induced by trypsin digestion migrated together with the 9-kD D1 fragment (data not shown). Although urea affected the mobility of trypsin-digested, C-terminal D1 fragments, the clear difference in the mobility of trypsin-digested and photoinhibition-induced D1 fragments further confirmed that our photoinhibition-induced, 20-kD, C-terminal D1 fragment did not originate from the cleavage of the D1 protein in the DE loop but clearly originated N terminally from it. Trypsin digested D1 protein also at the N terminus. This is evident from the appearance of a large fragment that was recognized by anti-D1α (Fig. 5A) and anti-D1C (Fig. 5C) but not by anti-D1N (Fig. 5B). The epitopes, therefore, recognized by anti-D1N were located at the very N terminus of the D1 polypeptide and were lost because of trypsin digestion. Moreover, we want to emphasize that phosphorylation of the D1 protein (D1*) somehow affected the N-terminal epitopes so that they were no longer recognized by anti-D1N (Fig. 6), as also reported by Rintamäki et al. (1995).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Immunoblot demonstrating that the conformation greatly affects the mobility of the C-terminal degradation fragment of the D1 protein in SDS-PAGE. Intact pumpkin leaves were illuminated at a PPFD of 2500 μmol m⁻² s⁻¹, 20°C, for 1.5 h, thylakoids were isolated, and the D1 protein fragments were analyzed by immunoblotting using anti-D1α. Ten micrograms of Chl was applied in each well. A, Immunoblot from SDS-PAGE with 4 M urea. B, Immunoblot from SDS-PAGE without urea. The molecular masses and the migration of the prestained standard polypeptides (soybean trypsin inhibitor, 27.5 kD, and lysozyme, 18.5 kD) are indicated with bars for both gels. The open arrows indicate the migration of the C-terminal D1 degradation fragment in the presence (A) and absence (B) of urea in the gel. The immunolabeled band at approximately 28 kD (B) is the D1 conformer.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Immunoblot from trypsin digestion of D1 protein in intact pumpkin thylakoids. Trypsin (50 μg/mL) digestion was performed at 18°C. Polypeptides were separated by urea-SDS-PAGE, electroblotted to a PVDF membrane, and analyzed by immunoblotting with anti-D1α (A), anti-D1N (B), and anti-D1C (C). Lane 1, Thylakoids before trypsin digestion; lane 2, trypsin digestion for 15 min; lane 3, trypsin digestion for 30 min. Chl (4.5 μg) was loaded in each well. The D1 fragments are indicated by arrows, and the estimated molecular masses of the three small D1 fragments are given. The immunolabeled band at approximately 31 kD in C is the D1 conformer.
Figure 6. Immunoblot demonstrating that anti-D1\textsubscript{N} does not recognize (A) but that anti-D1\textsubscript{DE} does recognize (B) the phosphorylated form of the D1 protein, the D1* in thylakoid membranes isolated from illuminated pumpkin leaves. Thylakoid polypeptides were separated by urea-SDS-PAGE, electroblotted to an Immobilon P membrane, and immunodecorated first with anti-D1\textsubscript{N} (A) and subsequently with anti-D1\textsubscript{DE} (B). Lane 1, Dark-incubated leaves; lane 2, moderate-light-grown leaves illuminated at 1000 \(\mu\text{mol photons m}^{-2}\ \text{s}^{-1}\), \(20^\circ\text{C}\), for 10 min; and lane 3, illuminated for 1 h. Chl (0.75 \(\mu\text{g}\)) was loaded in each well.

**DISCUSSION**

The survival strategy of high-light-grown plants is the high capacity to repair photodamaged PSII by a fast turnover of the D1 protein during exposure to high light (Kettunen et al., 1992; Tyystjärvi et al., 1992). The finding that increasing amounts of D1 degradation products are observed only with photoinhibitory light (Fig. 3A) indicates that the rate of initial cleavage of the D1 protein under these conditions exceeds the rate of the further proteolysis of the primary cleavage products.

In vivo photoinhibition-induced D1 degradation fragments in pumpkin leaves are summarized in Figure 7. Our model of D1 degradation is based on site-specific antibodies in recognition of D1 fragments; sequencing of the D1 fragments, to our knowledge, has not yet been successful. We show two distinct cleavage sites in the D1 protein during illumination of intact pumpkin leaves. The main cleavage occurs somewhere in the middle of the D1 protein, thus creating the 20-kD, C-terminal and the corresponding 18-kD, N-terminal degradation fragments (Figs. 2 and 7). These fragments are likely to appear in concert in the course of illumination. Although it is difficult to predict the exact cleavage site only on the basis of apparent molecular masses of the fragments in urea-SDS-PAGE (Figs. 2 and 4; Kim et al., 1994), it is evident that the cleavage that yields these 18- and 20-kD fragments does not occur in the stromal loop connecting \(a\) helices D and E. By comparing the size and migration of trypsin-induced, C-terminal fragments and photoinhibition-induced fragments in SDS-PAGE with and without urea, we can conclude that this primary cleavage occurs clearly N terminally of the stromal DE loop of the D1 protein (Figs. 4 and 5), possibly at the luminal side of the thylakoid membrane.

The other, although less frequent, cleavage occurs C terminally from Glu\textsuperscript{242} and produces the minor 23-kD fragment (recognized by anti-D1\textsubscript{DE}; Figs. 2D, 3A, and 7), which apparently is the same N-terminal D1 degradation product first reported by Greenberg et al. (1987). Unexpectedly, however, the 23-kD degradation fragment of the D1 protein was not recognized by anti-D1\textsubscript{N}. To solve this discrepancy, we further tested the specificity of anti-D1\textsubscript{N} and discovered that it recognizes the N terminus of the D1 protein only in its unphosphorylated form (Fig. 6). The phosphorylated form of D1 protein, D1*, has a slower mobility in SDS-PAGE and can be separated from the unphosphorylated D1 protein with a long electrophoretic run (Callahan et al., 1990; Kettunen et al., 1991; Elich et al., 1992; Koivuniemi et al., 1995). D1* did not cross-react with anti-D1\textsubscript{N} (Fig. 6). Therefore, it seems likely that the minor, 23-kD degradation fragment of the D1 protein (Figs. 2D and 3A) induced by in vivo photoinhibition of intact leaves is phosphorylated in its N terminus and for that reason is not recognized by anti-D1\textsubscript{N}. The same reasoning is valid for anti-D1\textsubscript{DE}, even though it cross-reacts with both intact proteins, D1 and D1*, by recognizing the more C-terminal epitopes. Anti-D1\textsubscript{DE} was raised against the D1 protein iso-
lated from algae, and lower plants generally do not phosphor-
ylate the D1 protein (De Vitry et al., 1991; Rintamäki et
al., 1995). However, the possibility that the 23-kD, N-ter-
minal D1 fragment is too faint to be seen in immunoblots
decorated with anti-D1N and anti-D1α cannot be excluded.

Under photoinhibitory light conditions in vivo, the D1 protein
is mostly phosphorylated (Fig. 6B) in all higher plants studied so far (wheat, pea, pumpkin; Rintamäki et
al., 1996b). However, the phosphorylated D1* is not as
good a substrate for the D1-specific proteinase as the unphos-
phorylated the D1 protein (Aro et al., 1992), which partly
explains why photoinhibitory illumination of PSII core particles isolated from thylakoids phosphorylated
with [32P]ATP results in the formation of only a small
amount of the 23-kD, phosphorylated D1 degradation
product (Salter et al., 1992). Phosphorylated D1 protein is probably first dephosphorylated (Koivuniemi et al., 1995; Rintamäki et al., 1996a) and only subsequently cleaved to
produce the prominent 18- and 20-kD in vivo D1 degrada-
tion fragments. The finding that, after high-light illumina-
tion, anti-D1, recognized both the intact D1 protein and
the N-terminal 18-kD D1 fragment (Fig. 3B) suggests that
D1 dephosphorylation had occurred before the proteolytic
degradation of the D1 protein.

Considerable amounts of the 23-kD N-terminal degrada-
tion product of the D1 protein can be detected by
radioactive-labeling experiments after only short pulse pe-
riods (Greenberg et al., 1987; Kim et al., 1994). This may
suggest that the cleavage of the D1 protein in the stromal
DE loop plays a role in elimination of the newly synthe-
sized D1 protein copies with aberrant folding and incorrect
conformation for proper assembly of the PSII complex. It is
questionable, however, whether this degradation mecha-
nism is directly related to that induced by PSII photoinhi-
bitory and photodamage of the D1 protein in vivo. The
photoinhibition-induced in vivo degradation fragments of
the D1 protein, reminiscent of acceptor-side photoinhi-
bition, appear only during severe photoinhibition (Fig. 3;
Cánovas and Barber, 1993) in conditions that might induce
drastic conformational modification of the D1 protein and
thereby trigger its degradation even in phosphorylated
form. The possibility that alterations in the conformation
(near or at the DE loop) lead to acceptor-side-type D1
degradation is further supported by the finding that the 23-
and 9-kD D1 fragments can be produced even in darkness
if a phenol-type inhibitor, PN08, binds to the secondary
quinone acceptor site in the D1 polypeptide (Nakajima et
al., 1995). During in vivo photoinhibition, this kind of
photoinhibition-induced D1 degradation might reflect a
situation in which the acceptor side of PSII is destroyed,
which leads to conformational modification and exposure
of the cleavage site in the DE loop to proteolysis.

We have shown that the cleavage of the D1 protein
during in vivo photoinhibition seldom occurs in the DE
loop, and that this cleavage probably involves phosphory-
lated D1 protein and releases a phosphorylated 23-kD,
N-terminal fragment. More frequently, however, the pho-
todamaged D1 protein is cleaved N terminally from the DE
loop and produces a C-terminal D1 fragment and the cor-
responding unphosphorylated N-terminal fragment with
apparent molecular masses of 20 and 18 kD, respectively
(Figs. 2 and 7). In many respects, this degradation pattern
resembles the donor-side-photoinhibition-induced D1 degrada-
tion pattern deduced from in vitro experiments (Friso et
al., 1993; Fig. 3B). It is questionable, however, whether
the in vivo photoinhibition mechanism can be deduced on
the basis of the D1 fragmentation pattern. If so, the main in
vivo photoinhibition mechanism would more likely be the
donor-side mechanism than the acceptor-side mechanism.
This is in accordance with the recent results by Russell et al.
(1995) and Tyystjärvi and Aro (1996), who studied in vivo
photoinhibition using methods other than immunodetect-
ing the D1 fragmentation pattern.

Knowledge about the pattern and regulation of D1 protein
cleavage in vivo is just emerging. For the understanding of
the balanced turnover of the PSII reaction center protein D1,
it is important to know how the D1 protein is degraded once
it has been damaged. Moreover, D1 protein kinase(s) and
phosphatase(s) seem to have an additional role in controlling
the rate of D1 protein degradation. Although in vitro systems
have provided an excellent model system for studies of D1
protein degradation, in vivo studies are now urgently needed
to discover the D1-specific proteinase and to show the regu-
lar aspects of D1 turnover.

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