

# Degradation Pattern of Photosystem II Reaction Center Protein D1 in Intact Leaves<sup>1</sup>

## 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.

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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 luminal (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  $\alpha$  helices connected by luminal 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<sup>+</sup> and TyrZ<sup>+</sup> 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 proteinase 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

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Abbreviations: Chl, chlorophyll;  $F_v/F_{max}$ , ratio of variable fluorescence to maximal fluorescence.

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 PPF of 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (moderate light) or 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (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 PPF of 1100 or 2500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  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  $F_v/F_{\text{max}}$ . The  $F_v/F_{\text{max}}$  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 Ghanotakis et al. (1987). Illumination of PSII core particles was done at the Chl concentration of 300  $\mu\text{g/mL}$  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  $\text{MgCl}_2$  with 1 mM 2,6-dichloro-*p*-benzoquinone as an electron acceptor. The PPF was 4500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white light, and the temperature was kept at 20°C.

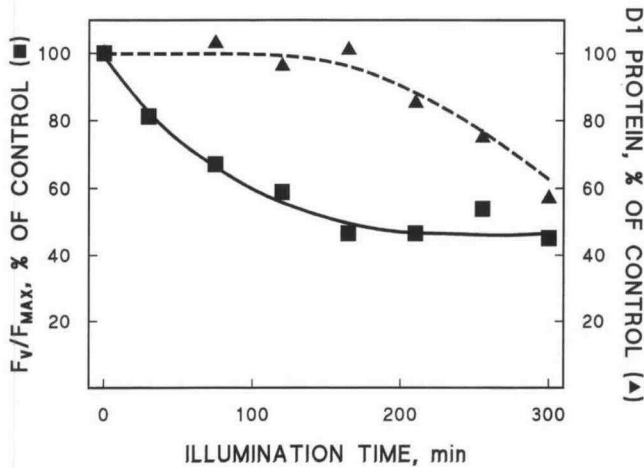
Trypsin digestion of the D1 protein in isolated thylakoid membranes was carried out at 18°C and at the trypsin (Sigma) concentration of 50  $\mu\text{g/mL}$  in 10 mM sodium-phosphate buffer (pH 7.4), 20 mM NaCl, and 100 mM Suc. The Chl concentration was 300  $\mu\text{g/mL}$ . After digestion, SDS-PAGE 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 M 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 M 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-D1 antisera were used to identify the D1 protein and fragments derived from it. The antisera are anti-D1 $\alpha$  (Herrman et al., 1985), anti-D1<sub>C</sub> (a kind gift of Dr. P. Nixon, Imperial College, London), anti-D1<sub>N</sub> (Barbato et al., 1991), and anti-D1<sub>DE</sub> (Research Genetics, Huntsville, AL). The anti-D1<sub>C</sub> and anti-D1<sub>N</sub> recognize epitopes located at the C-terminal and N-terminal ends of the D1 protein, respectively. The anti-D1<sub>C</sub> is raised against amino acids 333 to 353 from pea D1 polypeptide, and the anti-D1<sub>N</sub> is raised against the N terminus of wheat D1 protein, produced by cleavage in the specific Lys<sup>238</sup> of the wheat D1 protein. The anti-D1 $\alpha$  was raised against the D1 protein purified from the alga *Bumilleriopsis filiformis* and the anti-D1<sub>DE</sub> was raised against amino acids 234 to 242 from *Synechocystis* 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 PPF of 2500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  resulted in about a 50% loss of PSII photochemical activity within 3 h (measured as a decrease in  $F_v/F_{\text{max}}$ ), 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-D1<sub>N</sub> and anti-D1<sub>C</sub>, respectively (Fig. 2, B and C). The epitopes recognized by anti-D1<sub>N</sub> or anti-D1<sub>C</sub> are located at the very N terminus (see below) or very C terminus (see "Materials and Methods") of the D1 polypeptide, respectively, which sup-



**Figure 1.** Time course of PSII photoinhibition ( $F_v/F_{max}$ , ■) and the loss of the D1 protein (▲) from the thylakoid membranes during illumination of high-light-grown pumpkin leaves at PPFD  $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $20^\circ\text{C}$ .  $F_v/F_{max}$  was measured from leaf discs after 30 min of dark incubation, and the D1 protein content was measured immunologically (anti-D1<sub>DE</sub>) from thylakoids isolated from the illuminated leaves. Chl ( $0.75 \mu\text{g}$ ) was loaded in each well.

ports the notion that these 18- and 20-kD D1 fragments are derived from two different domains in the D1 polypeptide. Importantly, the 23-kD D1 (Fig. 2D) fragment originates from the N terminus of the D1 polypeptide, since it was recognized by DE-loop-specific antibody (anti-D1<sub>DE</sub>, raised against amino acids 234–242 in D1 polypeptide) but not by anti-D1<sub>C</sub>.

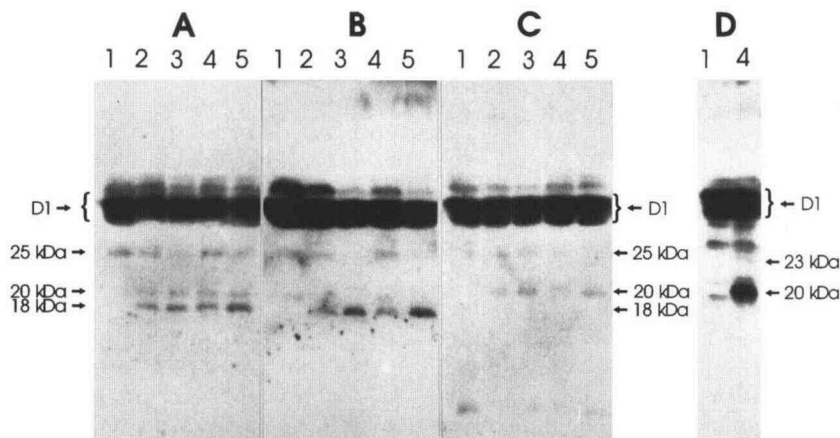
A putative translation intermediate of the D1 protein, with an apparent molecular mass of 25 kD, is seen in Figure 2, A

and B. This band was present at the beginning of high-light illumination and disappeared upon illumination if D1 synthesis was inhibited (Fig. 2, A and B, lanes 3 and 5; Fig. 3A). The origin of this 25-kD D1 fragment as a translation intermediate is also supported by the inability of anti-D1<sub>C</sub> to recognize it (Fig. 2C). A minor, low-molecular-mass band was also detected by anti-D1<sub>C</sub> (Fig. 2C). Since this band was also seen in the control lane, we do not consider it to be a light-induced D1 degradation product.

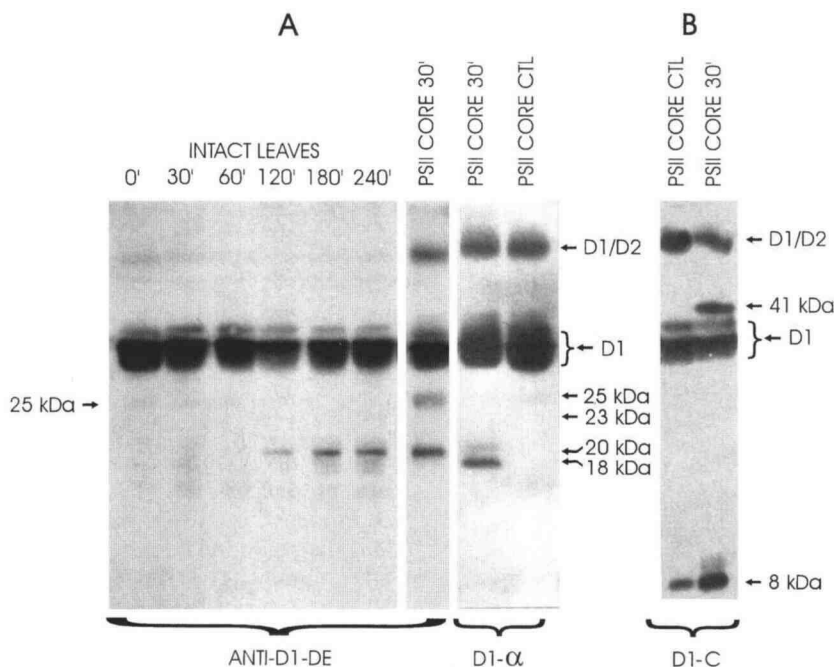
The 20-kD C-terminal degradation fragment of the D1 protein was the first to appear during illumination (Fig. 3A). The minor, N-terminal, 23-kD degradation fragment (Figs. 2D and 3A), recognized by anti-D1<sub>DE</sub>, appeared later in the course of photoinhibition (Fig. 3A). This kind of D1 degradation pattern suggests that two cleavage mechanisms function during photoinhibition of intact leaves: the first producing the 20-kD, C-terminal D1 fragment and its counterpart, the 18-kD, N-terminal degradation fragment, and the second cleavage producing the 23-kD, N-terminal D1 fragment. The appearance of a small, approximately 10-kD, C-terminal degradation product (Cánovas and Barber, 1993), which would complement the 23-kD, N-terminal fragment, would have been expected. However, we did not observe such a degradation fragment during illumination of intact leaves (Fig. 2C).

#### 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 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<sub>α</sub> (A), anti-D1<sub>N</sub> (B), anti-D1<sub>C</sub> (C), and anti-D1<sub>DE</sub> (D). Lanes 1, Control thylakoids isolated from nonphotoinhibited leaves; lanes 2 and 4, thylakoids isolated from leaves illuminated at a PPFD of  $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $20^\circ\text{C}$ , 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.



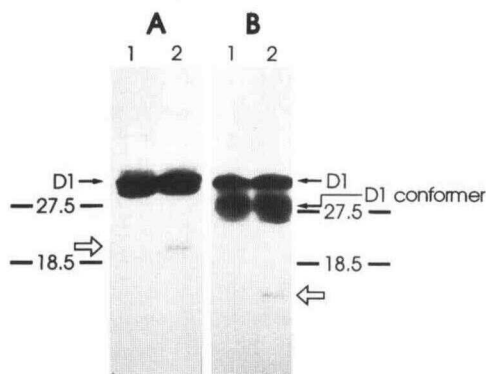
**Figure 3.** A, Immunoblot demonstrating the time course of the appearance of *in vivo* D1 protein degradation products, and comparison of the D1 degradation pattern between *in vivo* photoinhibition and *in vitro* donor-side photoinhibition. Moderate-light-grown pumpkin leaves were illuminated at a PPFD of  $1100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $20^\circ\text{C}$ , 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 ( $300 \mu\text{g Chl/mL}$  in  $50 \text{ mM Tris-HCl}$  [pH 8.0],  $0.1 \text{ M sorbitol}$ ,  $5 \text{ mM MgCl}_2$ , and  $1 \text{ mM 2,6-dichloro-}p\text{-benzoquinone}$ ) at a PPFD of  $4500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $20^\circ\text{C}$ , 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-D1<sub>DE</sub> or anti-D1 $\alpha$  as indicated at the bottom of the immunoblots. Eight micrograms (intact leaves) or  $5 \mu\text{g}$  (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 kD, on the left side) and D1 degradation fragments (18, 20, 23, and 25 kD, 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 ( $300 \mu\text{g Chl/mL}$  in  $50 \text{ mM Hepes-KOH}$  [pH 7.6],  $10 \text{ mM NaCl}$ , and  $400 \text{ mM Suc}$ ) at  $4500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $20^\circ\text{C}$ , for 30 min. Immunodecoration was with anti-D1<sub>C</sub>. 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-D1 $\alpha$ ). Also, a D1 fragment of 25 kD 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-D1<sub>C</sub> 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) appeared when grana

particles were further purified with detergents to make PSII core preparations (data not shown). This finding suggests that the procedure used to isolate PSII preparations may alter the conformation of the D1 protein so that cleavage sites in the D1 polypeptide become exposed. The D1 protein will subsequently be cleaved, possibly by the same proteinase that functions during photoinhibition, which is also supported by the finding that the yield of these D1 fragments increased during illumination *in vitro* (Fig. 3B).

We conclude that the main photoinhibition mechanism *in vivo* resembles a donor-side mechanism as far as the D1 protein degradation pattern is concerned. In addition to this direct comparison of *in vivo* and *in vitro* D1 degradation fragments, we also considered the possibility that the photodamaged D1 protein *in vivo* is always primarily cleaved in the DE loop, producing the 23-kD, N-terminal fragment. This fragment could then be rapidly degraded further to a more stable, 18-kD fragment observed in our experiments (Fig. 2). Although the size of the 20-kD, C-terminal fragment (Figs. 2 and 3A) did not fit this model, the possibility that our electrophoretic system overesti-



**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  $2500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $20^\circ\text{C}$ , for 1.5 h, thylakoids were isolated, and the D1 protein fragments were analyzed by immunoblotting using anti-D1<sub>DE</sub>. 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 pre-stained standard polypeptides (soybean trypsin inhibitor, 27.5 kDa, and lysozyme, 18.5 kDa) 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 kDa (B) is the D1 conformer.

mated the size of this C-terminal D1 degradation fragment needed to be excluded by the experiments described below.

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

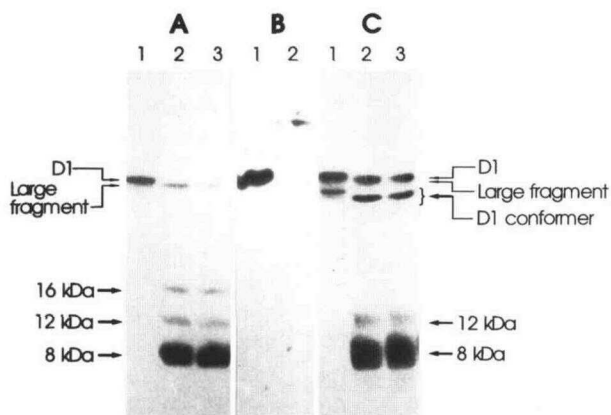
Urea is known to affect the mobility of the reaction center polypeptides D1 and D2 in SDS-PAGE in relation to standard polypeptides. Because both polypeptides have an apparent molecular mass of 32 kDa, the difference in mobility must be related to different conformations rather than to a difference in 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-kDa, 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 kDa in the absence and presence of urea, respectively. The magnitude of this urea effect depended on the concentration of urea in the SDS-PAGE gels (data not shown). Similar difficulties in the estimation of the size of D1 fragments have also recently been reported by Kim et al. (1994).

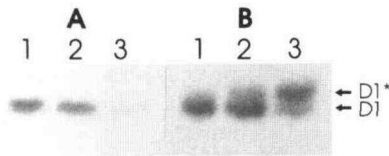
Because it still remained marginally possible that the 20-kDa, C-terminal fragment (Figs. 2 and 3A) could be produced by a cleavage in the DE loop, we decided to compare

the migration of photoinhibition-induced and trypsin digestion-produced C-terminal fragments of the D1 protein. Trypsin cleaves D1 protein primarily in the DE loop at Arg residues Arg<sup>225</sup>, Arg<sup>238</sup>, and, to some extent, also at Arg<sup>257</sup>, and yields C-terminal D1 fragments of 12, 10, and 7 kDa (Marder et al., 1984; Friso et al., 1993). If the (photo)damaged D1 protein is to be cleaved in the DE loop, the resulting D1 fragments, recognized by anti-D1<sub>C</sub>, should have a migration pattern in SDS-PAGE quite similar to that of the C-terminal trypsin fragments.

The three small trypsin digestion fragments of the D1 protein, recognized by anti-D1<sub>α</sub>, had apparent molecular masses of 16, 12, and 8 kDa in urea-SDS-PAGE (Fig. 5A). The 12- and 8-kDa D1 protein fragments are C terminal in origin (Fig. 5C). If urea was omitted from SDS-PAGE, the 12-kDa, C-terminal D1 fragment induced by trypsin digestion migrated together with the 9-kDa 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-kDa, 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-D1<sub>α</sub> (Fig. 5A) and anti-D1<sub>C</sub> (Fig. 5C) but not by anti-D1<sub>N</sub> (Fig. 5B). The epitopes, therefore, recognized by anti-D1<sub>N</sub>, 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-D1<sub>N</sub> (Fig. 6), as also reported by Rintamäki et al. (1995).



**Figure 5.** Immunoblot from trypsin digestion of D1 protein in intact pumpkin thylakoids. Trypsin (50  $\mu\text{g}/\text{mL}$ ) digestion was performed at  $18^\circ\text{C}$ . Polypeptides were separated by urea-SDS-PAGE, electroblotted to a PVDF membrane, and analyzed by immunoblotting with anti-D1<sub>α</sub> (A), anti-D1<sub>N</sub> (B), and anti-D1<sub>C</sub> (C). Lane 1, Thylakoids before trypsin digestion; lane 2, trypsin digestion for 15 min; lane 3, trypsin digestion for 30 min. Chl (4.5  $\mu\text{g}$ ) 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 kDa in C is the D1 conformer and is indicated by the brace.



**Figure 6.** Immunoblot demonstrating that anti-D1<sub>N</sub> does not recognize (A) but that anti-D1<sub>DE</sub> 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<sub>N</sub> (A) and subsequently with anti-D1<sub>DE</sub> (B). Lane 1, Dark-incubated leaves; lane 2, moderate-light-grown leaves illuminated at 1000 μmol photons m<sup>-2</sup> s<sup>-1</sup>, 20°C, for 10 min; and lane 3, illuminated for 1 h. Chl (0.75 μg) was loaded in each well.

**DISCUSSION**

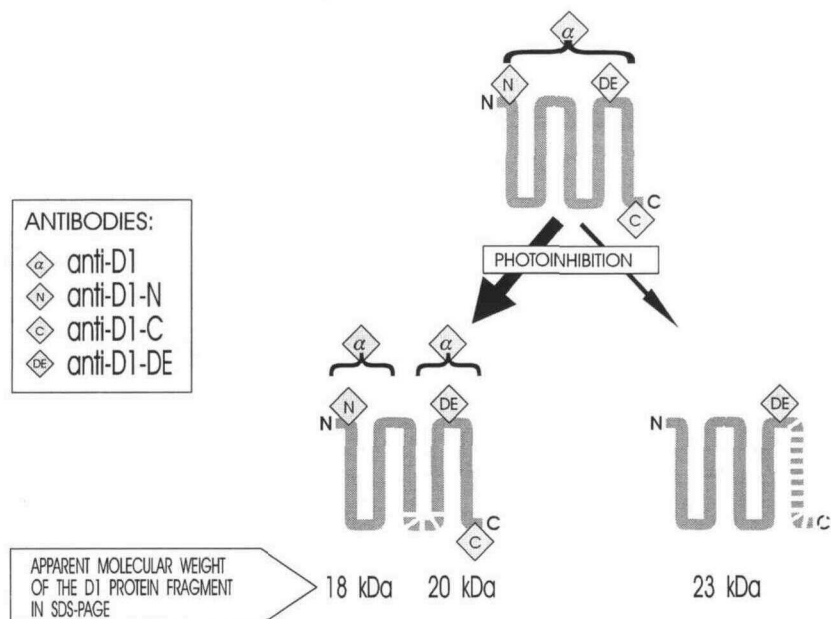
The survival strategy of high-light-grown plants is the high capacity to repair photodamaged PSII by a fast turnover of the D1 protein during exposure to high light (Kettunen et al., 1992; Tyystjärvi et al., 1992). The finding that increasing amounts of D1 degradation products are observed only with 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 α 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<sup>242</sup> and produces the minor 23-kD fragment (recognized by anti-D1<sub>DE</sub>; 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<sub>N</sub>. To solve this discrepancy, we further tested the specificity of anti-D1<sub>N</sub> 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<sub>N</sub> (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<sub>N</sub>. The same reasoning is valid for anti-D1<sub>α</sub>, even though it cross-reacts with both intact proteins, D1 and D1\*, by recognizing the more C-terminal epitopes. Anti-D1<sub>α</sub> was raised against the D1 protein iso-

**Figure 7.** Hypothetical model of the origin of D1 degradation fragments during illumination of intact pumpkin leaves. This model is based on the recognition of the D1 degradation fragments by D1 antibodies specified in the figure.



lated from algae, and lower plants generally do not phosphorylate the D1 protein (De Vitry et al., 1991; Rintamäki et al., 1995). However, the possibility that the 23-kD, N-terminal D1 fragment is too faint to be seen in immunoblots decorated with anti-D1<sub>N</sub> and anti-D1<sub>α</sub> 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 unphosphorylated the D1 protein (Aro et al., 1992), which partly explains why photoinhibitory illumination of PSII core particles isolated from thylakoids phosphorylated with [<sup>32</sup>P]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 degradation fragments. The finding that, after high-light illumination, anti-D1<sub>N</sub> recognized both the intact D1 protein and the N-terminal 18-kD D1 fragment (Fig. 2B) suggests that D1 dephosphorylation had occurred before the proteolytic degradation of the D1 protein.

Considerable amounts of the 23-kD N-terminal degradation product of the D1 protein can be detected by radioactive-labeling experiments after only short pulse periods (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 synthesized D1 protein copies with aberrant folding and incorrect conformation for proper assembly of the PSII complex. It is questionable, however, whether this degradation mechanism is directly related to that induced by PSII photoinhibition and photodamage of the D1 protein *in vivo*. The photoinhibition-induced *in vivo* degradation fragments of the D1 protein, reminiscent of acceptor-side photoinhibition, 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, PNO8, 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 phosphorylated D1 protein and releases a phosphorylated 23-kD, N-terminal fragment. More frequently, however, the photodamaged 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 degradation 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 immunodetection of 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 regulatory aspects of D1 turnover.

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