

Photoinduced degradation of the D1 protein in isolated thylakoids and various photosystem II particles after donor-side inactivations

Detection of a C-terminal 16 kDa fragment

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Photoinduced degradation of the photosystem II (PSII) reaction center D1 protein was studied in isolated thylakoids and different PSII subparticles. A 16 kDa fragment corresponding to the C-terminus of the protein is detected in thylakoids when they are inactivated at the donor side before illumination. The same D1 fragment is found in different types of PSII preparations at different integration levels characterized by different polypeptide compositions so long as they have an inactivated donor side and an active electron acceptor for the reduced pheophytin. However, when the PSII particle is equal to or smaller than the 43-less PSII core complex, other fragments are observed which are not found in more integrated systems.

Photosynthesis; Photosystem II; D1 polypeptide; Photoinhibition; *Spinacia oleracea*

1. INTRODUCTION

Over-illumination of oxygenic photosynthetic organisms brings about impairment of their photosynthetic activity which can be experimentally observed in vivo and/or in vitro as decreased activity in carbon dioxide fixation, oxygen evolution and electron transport [1,2]. Photosystem II (PSII) has been indicated as the main target for this phenomenon, which is generally referred to as photoinhibition [1]. In association with reduced photosynthetic performance under photoinhibitory conditions degradation of the D1 protein of the PSII reaction center (RC) is observed [3]. The D1 protein is characterized by unusually fast turnover under normal light conditions [4], becoming even faster with increasing light intensity [5]. The experimentally observed depletion of D1 from isolated thylakoid membranes under photoinhibitory conditions has been thought to represent the in vivo situation when its degradation rate is

faster than its biosynthetic rate [3,5]. Recent evidence suggests that D1 degradation is mediated by serine-type proteolytic activity [6,7], possibly associated with a component of the RC itself [8].

Although considerable efforts have been made to identify the sites where photoinhibition starts and the D1 protein is cleaved the actual mechanisms for light-induced irreversible damaging of the electron transport chain and the triggering event for D1 degradation are still not clearly understood: different cofactors of the electron transport chain have been indicated as protagonists in the reactions leading to impairment of electron transport activity. Two main mechanisms have been proposed: the first, referred to as the 'acceptor-side' mechanism, implies modifications at the level of either Q_B or Q_A plastoquinones [5,9,10] thereby preventing exchange with the plastoquinone pool. The second, or 'donor-side' mechanism, implies the accumulation of highly oxidant species such as Tyr_Z^+ and/or P_{680}^+ [11,12].

Different patterns for D1 degradation have also been reported under different experimental conditions and various PSII preparations [8,13–16,19]. Thus during in vivo light-induced turnover D1 protein is thought to be cleaved at or close to the QEEE sequence [15,17] or at residue 238 [18], both located in the stroma-exposed loop between the fourth and fifth putative transmembrane helices thereby producing an N-terminal fragment with an apparent mol. wt. of 23.5 kDa. In contrast, photoinhibition of isolated RC has been found to produce, among other things, a C-terminal 24 kDa fragment, which must be produced by a cleavage in the

Abbreviations: chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCPIP, 2,6-dichlorophenolindophenol; DPC, 2,2'-diphenylcarbonic dihydrazide; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; LHC, light harvesting complex; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; P_{680} , primary donor of photosystem II; RC, reaction center complex of photosystem II; SDS, sodium dodecyl sulfate; TRIS, tris(hydroxymethyl)aminomethane; Tyr_Z , tyrosine 161 on the D1 protein.

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lumen-exposed loop connecting the first and second transmembrane helices [19]. The two different cleavage sites are possibly correlated to the two different photo-inhibition mechanisms, the former being associated with the acceptor-side and the latter with the donor-side mechanism [15,19].

In the present study we report the identification of a 16 kDa, C-terminal fragment produced by cleavage in the lumen-exposed loop between the third and fourth transmembrane segments. This fragment appears after photoinhibition of a series of PSII preparations with different antenna size, from unsolubilized thylakoids to the purified RC complex.

2. MATERIALS AND METHODS

2.1. Plant material and thylakoid isolation

Seeds of wheat (*Triticum aestivum* L.) were soaked in tap water and grown in vermiculite in a growth chamber with a 12-h photoperiod, 24/20°C day/night, 80% relative humidity, and a light intensity of $400 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. 8–10-day-old plants were harvested and leaves homogenized in 0.33 M sorbitol, 50 mM HEPES, pH 7.2, 5 mM MgCl_2 , 15 mM NaCl. Chloroplasts were pelleted by centrifugation at $1400 \times g$ for 10 min. After resuspension in 50 mM HEPES, pH 7.2, 5 mM MgCl_2 , 15 mM NaCl, thylakoids were recovered by centrifugation and then resuspended in the above buffer supplemented with 0.1 M sorbitol (incubation buffer) at the desired chlorophyll (chl) concentration.

2.2. PSII particles with different polypeptide compositions

PSII membranes were isolated as described in [20] with the modification introduced in [21]. An oxygen-evolving PSII complex lacking the main LHCII and CP24 but still retaining CP29 and CP26 [22] (PSII complex) was obtained by solubilization of PSII membranes with the non-ionic detergent, octyl β -D-glucopyranoside, in the presence of high ionic strength as described in [23]. This preparation was further fractionated either by gel filtration [23] or anion-exchange chromatography [24] to obtain, respectively, a preparation lacking all the chl *a/b*-proteins (core complex) or the same preparation but deprived of CP43 (CP43-less core complex). RC complex, containing D1, D2, cyt b_{559} and *psbI* gene product, was obtained as described in [25].

2.3. Photoinhibition

Photoinhibition of thylakoids, PSII membranes and PSII complex was performed at $200 \mu\text{g}$ chl/ml in incubation buffer. Core complex, CP43-less core complex and RC complex were illuminated at $50 \mu\text{g}$ chl/ml in respective isolation buffer. When appropriate, 0.2 mM DBMIB was added before photoinhibition. To ensure uniform illumination the samples were illuminated under continuous stirring. Light intensity was $4,500 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, temperature was 22°C.

2.4. Other methods

SDS-PAGE and immunoblotting were performed as previously described [19]. The properties of the two polyclonal anti-D1 antisera (anti-D1_C and anti-D1_N, recognizing, respectively, the C- and N-terminal regions of the protein) have previously been described [16,19]. Proteolysis of wheat thylakoids with the Lys-C-specific endoprotease was performed at a chl concentration of $200 \mu\text{g}/\text{ml}$ with 1 U/ml protease for 30 min at room temperature. TRIS washing of thylakoids, PSII membranes and PSII complex was performed by incubation in 0.8 M TRIS-HCl, pH 8.0, for 30 min. Concentration of chl was calculated according to [26]. Electron transport rates were measured from water to DCPIP or from DPC to DCPIP using a home-made spectrophotometer which will be described elsewhere (Giacometti et al., manuscript in preparation).

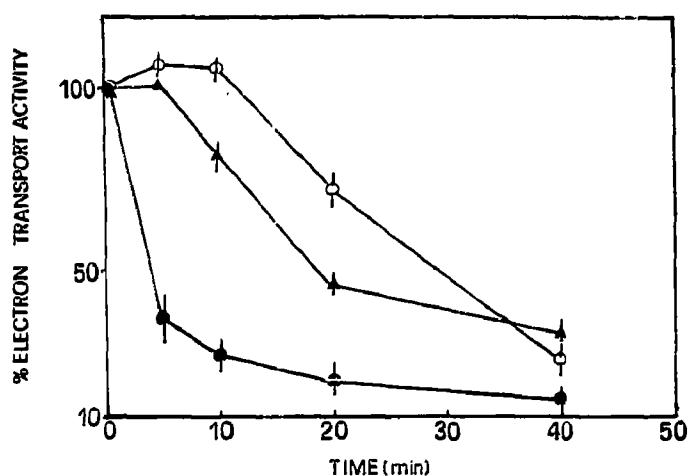


Fig. 1. Time-courses for impairment of electron transport activity of thylakoid membranes exposed to high light illumination. For TRIS-washed samples (filled circles), reaction was measured from DPC to DCPIP. For samples with a functional water-splitting enzyme (open circles), reaction was measured from water to DCPIP. Closed triangles: samples photoinhibited with intact donor side but measured after TRIS-washing using DPC as the electron donor. The reaction was performed in incubation buffer, pH 7.2 (see section 2) at 20°C with the following conditions: DPC 1.5 mM; DCPIP 0.06 mM; chl 20 $\mu\text{g}/\text{ml}$.

3. RESULTS

3.1. Photoinactivation of electron transport activity

In this study we compared the sensitivity to light of thylakoids with a functional donor side to that of thylakoids whose donor side had been inactivated before illumination by TRIS washing. Fig. 1A shows the results of photoinhibition of electron transfer from water to DCPIP in normal thylakoids (open circles) and from DPC to DCPIP in TRIS-washed thylakoids (filled circles). The ability to reduce DCPIP donor is lost faster when the water-splitting enzyme is inactivated by TRIS washing before illumination. The decrease in the electron transport rate with illumination time is also reported for a sample which was photoinhibited in the presence of an active donor side but measured after TRIS washing (triangles). As expected, in the latter case the time-course for photoinhibition was similar to that of control thylakoids, confirming that the differences in photoinhibition time-course depend on the state of the donor side and is independent of the method for measuring electron transport rate (from water to DCPIP or from DPC to DCPIP). We may also notice that, when photoinhibition is performed in the presence of a functional donor side, independently of the method of measurement, a lag phase of several minutes is observed before the effect of over-illumination is reflected in a decreased electron transport rate. We have no explanation for this phenomenon which, being present in both control experiments yet absent in TRIS-washed samples, must be associated with water-splitting activity.

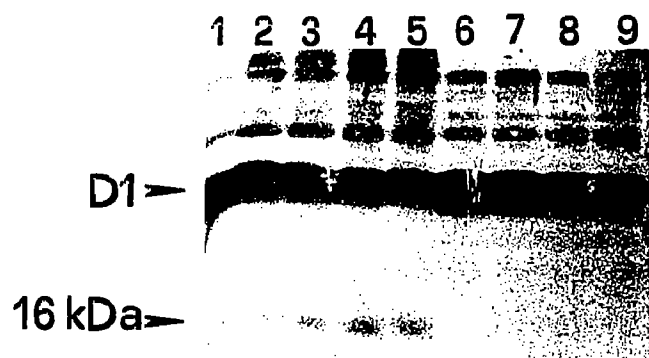


Fig. 2. Immunoblot with anti-D1 polyclonal antiserum of dark control (lane 1) and photoinhibited thylakoids (lanes 2-9). Thylakoids were illuminated for 5 (lanes 2 and 6), 10 (lanes 3 and 7), 20 (lanes 4 and 8) and 30 min (lanes 5 and 9). Samples in lanes 2-5 were TRIS-washed before illumination while samples in lanes 6-9 were not.

3.2. Degradation of D1 protein

Fig. 2 shows the result of an immunoblot with anti-D1 polyclonal antiserum of normal and TRIS-washed thylakoids exposed to light for different times. Inactivation of the donor side results in much faster degradation of the D1 protein with respect to controls: after 30 min illumination the level of the D1 protein still present in the TRIS-washed thylakoid membranes (lane 5) is significantly lower than that observed after the same illumination time in normal thylakoids (lane 9). The fragmentation pattern is also different for the two cases: in TRIS-washed thylakoids an immunodetectable 16 kDa fragment (lanes 2-5) already appears after 5 min illumi-

nation and, when compared to the residual amount of D1 protein, slightly increases with time (lanes 2-5); in contrast, no D1 fragments are detected at 16 kDa by the same antibody in photoinhibited control thylakoids (lanes 6-9), even after much longer illumination times (not shown).

3.3. Characterization of the 16 kDa D1 fragment

The blot shown in Fig. 2 was reacted with our anti-D1c polyclonal antiserum previously shown to recognize the C-terminal region of the protein [16]. Therefore, the 16 kDa fragment we detected must represent the C-terminal region of the protein. Since the D1 protein from wheat contains a single lysine residue at position 238 we may compare the photoinduced 16 kDa fragment with the two polypeptides obtained by digestion of wheat D1 protein with the highly specific Lys-C endopeptidase [16]. Of these two fragments the N-terminal one was recognized by our anti-D1N (Fig. 3, lane 2) and not by the anti-D1c polyclonal antiserum. The opposite was true for the C-terminal fragment (Fig. 3, lane 4). In our gel system the C- and N-terminal fragments have apparent mol. wts. of, respectively, 10-12 and 19-20 kDa. The mol. wt. of the photoinduced fragment, which is only recognized by the anti-D1c polyclonal (lane 5), differs from that of both the Lys-C fragments. These findings suggest that the photoinduced fragment is from the C-terminus and is produced by cleavage in a region of the protein located approx. 40-50 amino acids towards the N-terminus with respect to Lys-238. Lys-C proteolysis of photoinhibited TRIS-washed thylakoids

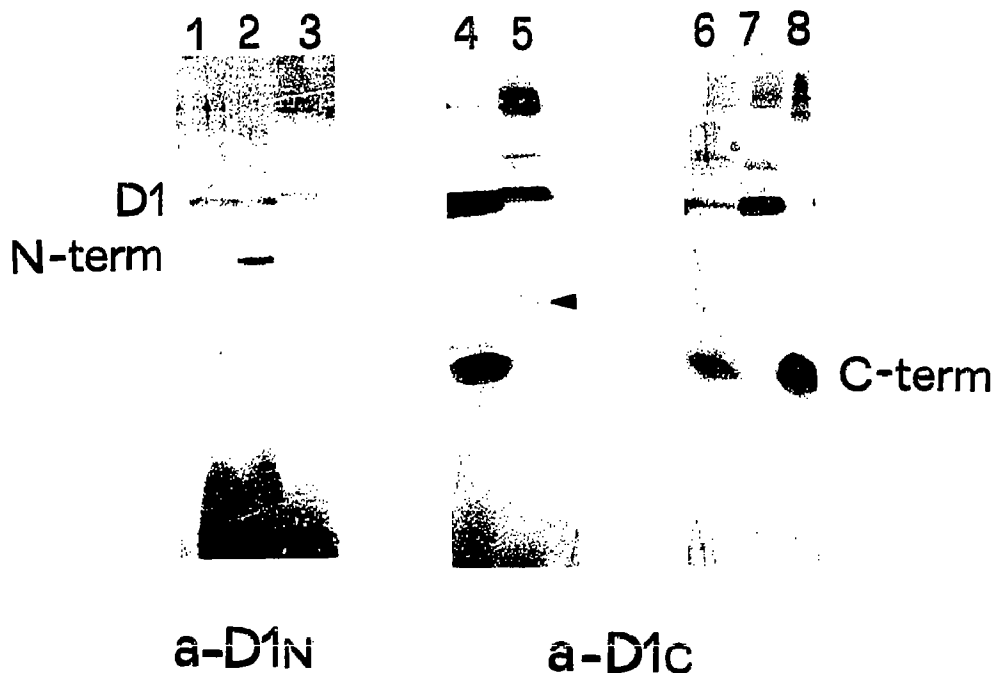


Fig. 3. Characterization of the photoinduced 16 kDa D1 fragment. Dark control thylakoids (lanes 1 and 7), dark control thylakoids digested with Lys-C endopeptidase (lanes 2 and 4), thylakoids photoinhibited after inactivation of donor side (lanes 3 and 5), same as lanes 3 and 5 after digestion with Lys-C (lane 6), isolated C-terminal Lys-C D1 fragment (lane 8) (see section 3.3 for further details).

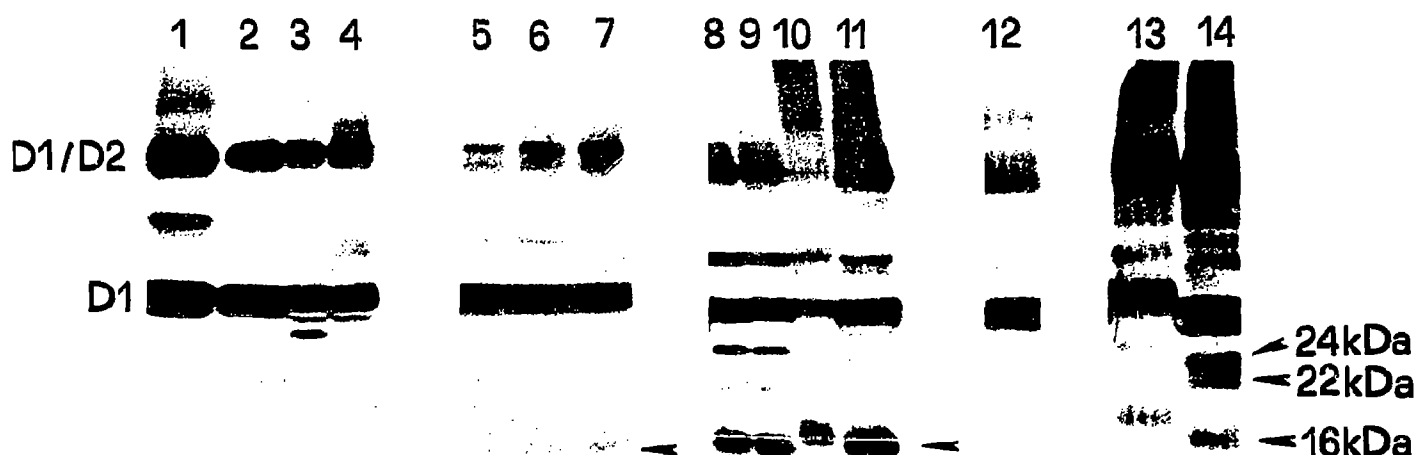


Fig. 4. Immunoblot with anti-D1_C polyclonal antiserum of different PSII preparations. RC (lanes 1 and 14), CP43-less core complex (lanes 2, 12 and 13), core complex (lanes 4, 10 and 11), PSII complex (lanes 3, 8 and 9), PSII membranes (lanes 5-7). Lanes 1-5 are dark control samples (see section 3), lanes 6-14 are photoinhibited samples. Samples in lanes 6, 7, 8, 10 and 12 were illuminated in the absence of DBMIB while samples in lanes 9, 11, 13 and 14 were illuminated in the presence of 0.2 mM DBMIB. PSII membranes were illuminated for 10 min (lane 6) and 20 min (lane 7) and all other samples for 20 min. The small band appearing below D1 in lanes 8 and 9 is not related to photoinduced degradation of D1 since it is present also in the dark control (lane 3).

(lane 6) brings about a fragment of about 10-12 kDa, detected by the anti-D1_C antiserum and identical to that obtained from proteolysis of normal thylakoids (lane 4). At the same time the 16 kDa photoinduced D1 fragment disappears from the blot. This implies that the Lys-C restriction site is contained within the 16-kDa photoinduced fragment observed above. The authentic C-terminal fragment obtained by Lys-C digestion of the isolated protein [19] is shown in lane 8.

3.4. Degradation of different PSII subparticles

D1 degradation during high light illumination has been studied in several preparations, ranging from whole plants [15] to isolated RC complex [8,19]. A number of differently sized degradation products have been detected by immunoblotting with specific polyclonal antisera [8,13-16,19]. Here, we checked for the production of the 16 kDa fragment observed above when preparations having different polypeptide composition and antenna size were subjected to the same type of photoinhibition. The preparations used had their donor side inactivated either by the isolation procedure or by TRIS-washing. Moreover, in those preparations which were depleted of the first stable electron acceptor Q_A (absence of plastoquinone after HPLC analysis, see [27]) we included the artificial electron acceptor, DBMIB. Under such conditions photoinhibition should be of the donor-side type irrespective of the particular preparation used. Immunoblotting of the non-photoinhibited control PSII preparations is shown in Fig. 4, lanes 1-5. In photoinhibited samples a 16-kDa D1 fragment, recognized by anti-D1_C but not by anti-D1_N polyclonal antiserum is observed. This fragment is the only one detected by our polyclonal antiserum after photoinhibition of PSII membranes (lanes 6 and 7), PSII com-

plex (lanes 8 and 9) and core complex (lanes 10 and 11). In contrast, when photoinhibition is performed on the CP43-less core complex (lane 13) and RC complex (lane 14) another fragment of approximately 22-24 kDa appears together with the 16 kDa one. As reported for the case of isolated RC [8] illumination of CP43-less core complex in the absence of DBMIB does not produce any detectable D1 fragment (lane 12).

4. DISCUSSION

Illumination of isolated thylakoids, whose donor side had previously been inactivated by TRIS-washing, results in rapid degradation of PSII reaction center activity. Under the same illumination conditions both electron transport activity and D1 content of the membrane decrease faster when the donor side is inactivated by TRIS-washing than when the water-splitting enzyme is fully functional. This observation, also reported by others [12,28], has been interpreted in terms of an abnormal accumulation of highly oxidizing species such as P₆₈₀⁺ and/or Tyr₂⁺ in the absence of an efficient electron donor [8,12,28].

After donor-side type photoinhibition we could detect a C-terminal breakdown product of D1 showing an apparent mol. wt. of about 16 kDa. Other authors have observed photoinduced fragments of similar apparent wt. [13,14,16,19], and the same fragment has been observed after photoinhibition of a preparation of isolated RC [8,19]. Here we show that the 16-kDa breakdown product contains the C-terminus of the protein and that its cleavage site is located approximately 40-50 amino acids towards the N-terminus with respect to the Lys-C restriction site at position 238. The cleavage site producing this fragment is therefore in the hydrophilic loop

connecting the third and fourth putative transmembrane helices, a highly conserved region of the protein sequence (see [29]). This result indicates that at least three different regions of D1 are involved in its degradation after photoinduced activation of proteolytic activity. The first is located in the hydrophilic loop between the first and second transmembrane helices [19], the other two are, respectively, in the loop between the third and fourth ([19], this work), and fourth and fifth transmembrane helices [15]. We may notice that the first two cleavage sites are activated preferentially by donor-side type of photoinhibition and are exposed to the lumen. The third cleavage site, on the stromatic side of the membrane, is preferentially activated by photoinhibition of the acceptor-side type. Also, uncleaved D1 shows a different apparent mol. wt. in RC complex with respect to the other PSII subparticles. This behaviour, previously described [8,16,19], whose meaning is not clear, is somewhat variable from experiment to experiment but, when present, is also reflected in the apparent wt. of the fragments (see Fig. 4, lanes 13 and 14).

Provided that photoinhibition is of the donor-side type and an electron acceptor, either endogenous or exogenous, is present, the 16 kDa fragment is formed in all the preparations used, ranging from whole thylakoids to isolated RC complex. The 16 kDa fragment is the only one detected by our anti-D1_C polyclonal antiserum in whole thylakoids, PSII membranes, PSII complex and core complex. In the preparation lacking the CP43 internal antenna (CP43-less core and RC complexes) at least another C-terminal fragment appears, of 22–24 kDa, corresponding to a cleavage site in the first hydrophilic loop [19]. This result indicates that interaction between the RC complex and its inner antenna, CP43, plays a role in shielding the cleavage site in the loop between the first and second helices or in modulating the proteolytic activity involved in degradation of the D1 protein.

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