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Degradation of Photosystem II reaction center D1-protein induced by UVB radiation in isolated thylakoids. Identification and characterization of C- and N-terminal breakdown products

Giulia Friso [a], Cornelia Spetea [b], Giorgio M. Giacometti [a,*], Imre Vass [b] and Roberto Barbato [a]

[a] *Dipartimento di Biologia, Università di Padova, Via Trieste 75, 35121 Padova (Italy) and* [b] *Institute of Plant Physiology, Biological Research Center, Szeged (Hungary)*

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The effects of UVB radiation on the stability of Photosystem II reaction center D1-protein in isolated thylakoids are investigated. A new C-terminal degradation product has been identified and characterized, produced by cleavage in the second transmembrane segment. Immunological evidence for the presence of N-terminal fragments corresponding to the remaining part of D1-protein is also provided. The appearance of these fragments is not affected by lowering the temperature from 22°C to 4°C, changing the pH from 6 to 8, adding soybean trypsin inhibitor or excluding oxygen from the thylakoid suspension.

Introduction

The effects of light on photosynthetic organisms are complex and involve several phenomena at different levels in the cell. Among these, phytochrome-mediated responses, direct control of gene expression and energy conversion by charge separation in the thylakoid membrane are the most important. However, it has long been known that light may also harm oxygenic photosynthesis, the magnitude of the effect depending on both the quality and quantity of light. Thus, an excess of white light brings about impairment of Photosystem II (PS II) activity, and this event constitutes the starting point for the physiological phenomenon known as photoinhibition [1]. One main consequence of photoinhibition is increased turnover of D1-protein [2], a subunit of the PS II reaction center complex [3,4].

New data have recently been reported on the characteristics of D1-protein degradation under visible light, pointing out: (i) early modifications of D1-protein, triggering its further breakdown [5,6]; (ii) the supposed molecular mechanism for protein cleavage [7–12]; (iii) identification of protein regions containing the cleavage site in vivo during rapid turnover [13] and in vitro after exposition of isolated reaction centers to high light [14,15]; and (iv) rearrangements of the thylakoid membrane following protein degradation [16–18]. The presence of several cleavage sites on D1, all located on the hydrophylic loops connecting hydrophobic transmembrane segments has been hypothesized [13,14] and preferential cleavage at different sites proposed to occur, depending on the particular molecular mechanism by which PS II activity is affected [19].

UV radiation is known to be even more harmful to oxygenic photosynthesis than visible light, and the stratospheric ozone depletion over Antarctica is causing renewed concern about the consequences of increased levels of UVB radiation on phytoplankton communities [20]. Correspondingly, increasing interest is now directed to understanding of the precise molecular targets and mechanisms underlying UVB effects on PS II.

A particularly relevant question is whether or not the same mechanisms hypothesized for photoinhibition induced by visible light are also operative in UV stress. In earlier works, identification of primary target sites was not achieved and, depending on the particular

* Corresponding author. Fax: +39 49 8286374.

Abbreviations: Atrazine, 2-(ethylamino)-4-chloro-6-isopropylamino-*s*-triazine; Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ioxynil, 4-hydroxy-3,5-diiodobenzonitrile; LCH II, light harvesting complex of Photosystem II; OEE, oxygen evolving enhancer; PAGE, polyacrylamide gel electrophoresis; PS, Photosystem; SDS, sodium dodecyl sulfate; UVB, ultraviolet radiation between 280 and 320 nm.

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experimental conditions, either the donor side [21] or the acceptor side [22] were proposed to be affected by UVB radiation. Breakdown products of D1 were also observed [23,24], but a sufficiently clear picture of the phenomenon has not yet emerged.

In this paper we analysed the effects of UVB light on the stability of D1-protein. The identification and characterization of some previously undescribed breakdown fragments generated by the action of UVB radiation on isolated spinach and wheat thylakoids are reported. Our data suggest that cleavage of D1-protein induced by UVB occurs at different sites, as compared to that induced by visible light, and is essentially independent of the presence of oxygen.

Materials and Methods

Leaves from spinach or wheat were homogenized in ice-cold buffer containing 330 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Hepes (pH 7.2) and chloroplasts were pelleted by centrifuging for 1 min at $6000 \times g$. After osmotic shock in the previous buffer lacking sorbitol, lysed chloroplasts were pelleted and resuspended in 100 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 50 mM Hepes (pH 7.2), at 1 mg Chl/ml. Just before light treatment, thylakoids were diluted to 0.25 mg Chl/ml in the same buffer.

UVB illumination was performed using a VL-215M lamp with maximal emission at 312 nm (Vilber Lourmat), giving an intensity of about 50 μE $m^{-2} s^{-1}$. In the case of irradiation in the presence of oxygen, the thylakoid suspension was contained in a Petri dish and illuminated from the top at a distance of 10 cm from the suspension surface, with slow stirring either on ice or thermostated at 22°C. For anaerobic experiments the oxygen-free suspension was contained in a quartz cuvette sealed by a quartz slide. Anoxygenic conditions were achieved by adding to the thylakoid suspension 10 mM glucose, 0.2 mg/ml catalase and 0.2 mg/ml glucose oxidase. Before adding the oxygen chemical trap, buffers were bubbled with argon. Under these conditions the oxygen level was below 2 μM. After SDS-PAGE, which was carried out as previously described [25], proteins were transferred on to nitrocellulose membrane (Sartorius, 0.45 μm) according to Ref. 26 and immunodecorated as described in Ref. 25. Two polyclonal antibodies against D1 were used which recognized the C- (anti-D1C) or N-terminus (anti-D1N) of the protein and whose properties have been described previously [14,25]. The properties of polyclonal antibodies anti-D2, anti-LHCII and anti-OEE2 have been described in Refs. 14 and 18. Limited proteolysis on SDS-gels was carried out as in [27]. Densitometric analysis of immunostained blots was performed using a Shimadzu CS-930 densitometer.

Oxygen evolution was measured using a Clark-type electrode (YSI mod. 53) under saturating light intensity, with 0.4 mM phenyl-*p*-benzoquinone as an electron acceptor at 22°C with 10 μg Chl/ml. Chlorophyll concentration was measured according to Ref. 28. Herbicides ioxynil, atrazine and DCMU and the plastoquinone antagonist DBMIB were added at a final concentration of 2 μM from a 100-fold concentrated stock solution in ethanol. Control samples also contained 1% (by volume) ethanol.

Results

Quantitative determination of D1 by densitometric scan of immunostained blots requires that the amount of antigen is low enough not to saturate the immunostained bands. It was found that, with our method of immunodetection, a chlorophyll level of up to 2.5 μg/lane satisfied this condition. Fig. 1 shows the time course for the inactivation of PS II activity and loss of the D1-protein under aerobic and anaerobic UVB irradiation. Independently of the presence of oxygen, the amount of protein is reduced at around 60% of the control in 40 min. At the radiation dose used, electron transport activity decays faster than the amount of D1-protein in the membrane, as observed for visible light [25]. Elimination of oxygen from the thylakoid suspension does not substantially affect the time course for D1-protein degradation.

Using 3-fold higher amounts of chlorophyll per gel lane (7.5 μg), the breakdown fragments of D1-protein

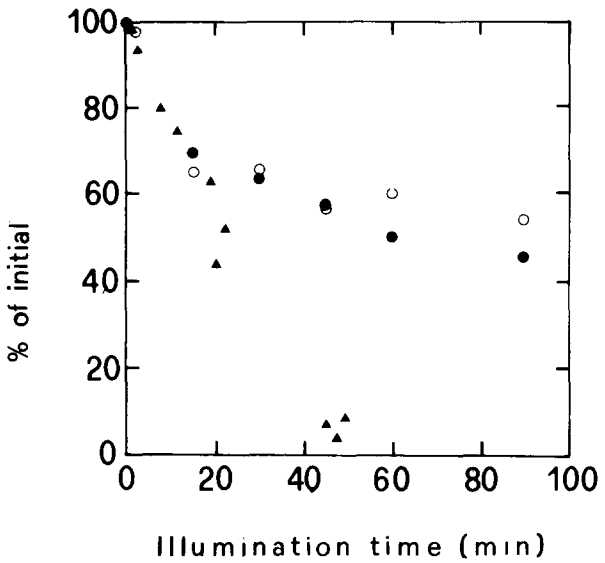

Fig. 1. Kinetics for UVB-induced loss of D1-protein in thylakoids illuminated at 22°C in presence (closed circles) and absence (open circles) of oxygen. D1 levels were estimated by densitometric analysis of anti-D1 immunodecorated blots. 2.5 μg Chl/lane was loaded on gel. Inactivation of PS II (triangles) under the same experimental conditions and correspondingto a half-time of 25 min, is reported for comparison.

became visible in the Western blot, as shown in Fig. 2, panel A where, besides anti-D1, a second antibody against the OEE2 subunit was also included. Besides D1-protein and the OEE2 subunit, a 20-kDa cross-reacting band is clearly detected (lanes 2–5 of panel A). This band, which represents a breakdown product of D1, appears even after only 5 min of illumination (lane 2) and its amount increases with illumination time (lanes 3–5). The same 20-kDa D1 fragment was also observed when UVB-treatment of thylakoids was performed under anaerobic conditions (not shown). To check that UVB-induced degradation of D1 is a process specific for D1, rather than a general consequence of absorption of the UVB light by amino acidic chains of the proteins, blots like those of Fig. 2A were reacted with polyclonals raised against other PS II proteins. Panels B and C of Fig. 2 show immunoblots reacted respectively with antibodies to D2 and LHC II. In both cases, no fragments were detected, in spite of overloading of the blots. The same result was obtained with other polyclonals against CP47, CP43, the 22-kDa protein encoded by the nuclear *psbS* gene and the α-subunit of cytochrome *b*-559. This does not necessarily imply that no degradation of other PS II proteins occurs under UVB irradiation of isolated thylakoids, but simply that no detectable fragments are produced, as in the case of D1-protein. In this context, we may assess that D1-protein is a specific target for proteolytic cleavage induced by UVB radiation.

Degradation products of D1-protein induced by UV radiation have been observed previously [23], and the presence of two fragments has been reported. A 20-kDa fragment was attributed to the N-terminal region of the protein, while a 8-kDa peptide was identified as the C-terminus. The bond between arg-238 and phe-239 was suggested as the cleavage site. In a recent report [24], Melis and co-workers detected by immunoblotting the presence of degradation products of the reaction center proteins at 15, 13 and 5 kDa, but their origin was not further investigated. In our case, the polyclonal antibody used to detect the above 20-kDa fragment was shown to be mainly directed toward epitopes in the C-terminal region of D1 [25], suggesting a C-terminal origin for this fragment. However, since a faint reaction towards the N-terminal region is also documented [25], further characterization of the 20-kDa photodegradation product was undertaken by the following methods: (i) mapping of the fragment with the Lys-C endoprotease [15]; and (ii) 2-D mapping by limited trypsinolysis on SDS-gels, using a modification [27] of the original method described in Ref. 29.

In the first experiment, wheat thylakoids were used, whose D1-protein contains a single lysine residue at position 238 of its amino acidic sequence [30]. Fig. 3 shows that, when control thylakoids (lane 1) are incubated in the dark for 30 min with Lys-C protease, two new immunoreactive bands are detected at 22 and 8 kDa (lane 5). It has previously been established that these bands correspond respectively to the N-terminus (amino acids 2–238) and C-terminus (amino acids 239–344) of the protein [15,25].

UVB-treated thylakoids containing the 20-kDa fragment (lane 2) were then incubated for 30 min in the dark at room temperature either in the presence (lane 3) or absence (lane 4) of 1 unit/ml of Lys-C protease. Lane 3 shows that this treatment gives rise to the 22-and 8-kDa fragments and that the amount of the 20-kDa photoinduced fragment is strongly diminished.

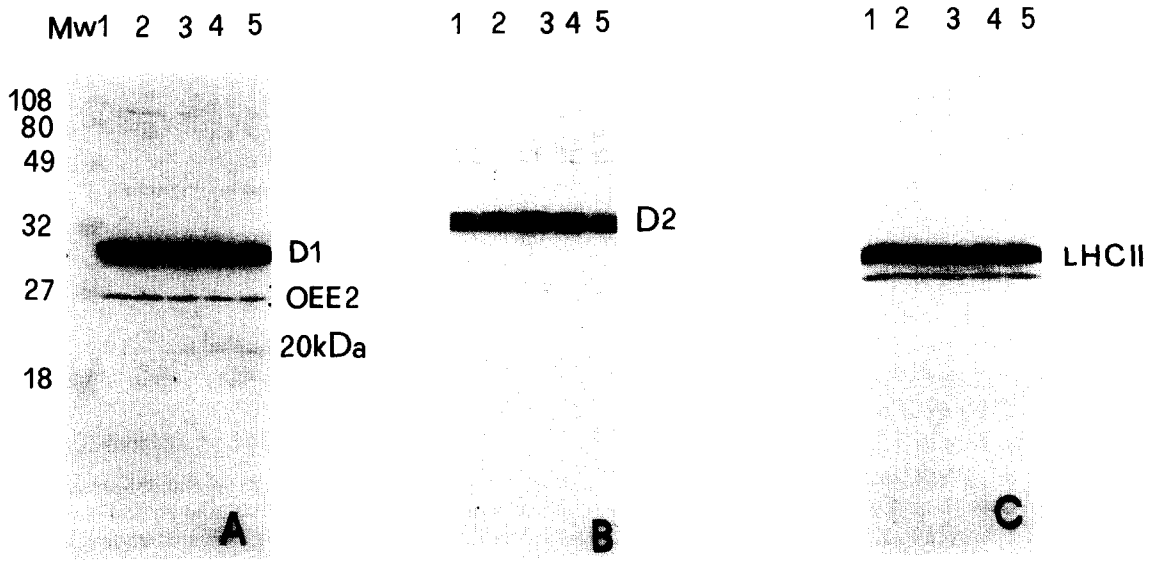

Fig. 2. Immunoblotting of UVB-treated thylakloids (at 4°C) with (A) anti-D1C and anti-OEE2; (B) anti-D2 and (C) anti-LHCII polyclonal antibodies. Mw: prestained molecular markers; lanes 1 to 5 contain samples illuminated respectively for 0, 5, 10, 30 and 60 min. 7.5 μg of chlorophyll were loaded per gel lane.

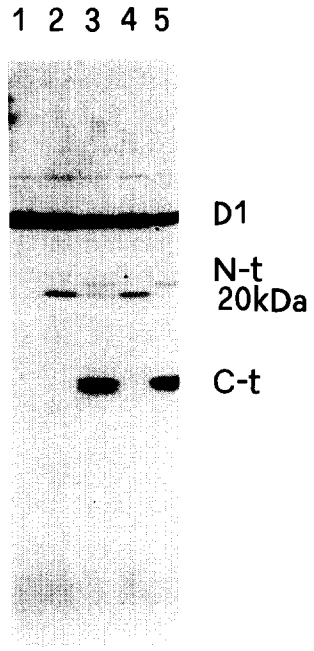

Fig. 3. Immunoblotting of wheat thylakoids with anti-D1C polyclonal. Isolated thylakoids (lane 1) were exposed to UVB light for 30 min at 4°C to generate the 20-kDa fragment (lane 2) and then incubated for 30 min in the dark at room temperature either with (lane 3) or without (lane 4) 1 unit/ml of Lys-C protease. Lane 5 also shows dark control thylakoids digested with Lys-C. 7.5 μg of chlorophyll were loaded per gel lane.

Taking into account that dark incubation of UVB-treated thylakoids at the same temperature but in the absence of protease has no effect on the amount of the UVB-induced fragment, we must conclude that its loss is due to proteolytic cleavage by Lys-C. This observation, together with the absence of cross-reactivity with the N-terminal specific antibody (see Fig. 5), strongly indicates the C-terminal origin of this fragment. However, it should be noted that digestion with Lys-C of UVB-treated thylakoids gives rise to a doublet in the 8-kDa region (lane 3), which is not observed when dark control thylakoids are subjected to the same proteolysis (lane 5). Therefore, the possibility that the 20-kDa fragment contains a modified C-terminus should also be considered.

The same C-terminal origin of the 20-kDa fragment is also supported by the results of the experiment shown in Fig. 4. A gel lane identical to lane 2 of Fig. 3, containing UVB-treated thylakoids, was loaded on the top of a second gel whose stacker contained trypsin at a concentration of 100 μg/ml. Digestion of proteins was carried out inside the gel [29], and tryptic products were detected by immunoblotting. Uncleaved D1/D2 heterodimer, D1-protein and 20-kDa UVB-induced fragment are detected along the diagonal of the gel, whereas the tryptic products are detected as off-diagonal spots. Trypsinolysis of D1-protein in these conditions gives rise to 12-, 10- and 8-kDa C-terminal fragments [27,31]. The 12-kDa fragment has previously been shown to represent the C-terminal portion of D1 [27]. Since this fragment appears as a tryptic product of the 20-kDa UVB-induced fragment, the C-terminal origin of the latter is also proved.

The above 20-kDa D1-fragment was detected with the polyclonal which mainly recognizes epitopes located on the C-terminus of D1. In order to detect N-terminal degradation products, nitrocellulose filters containing UVB-treated wheat thylakoids were also

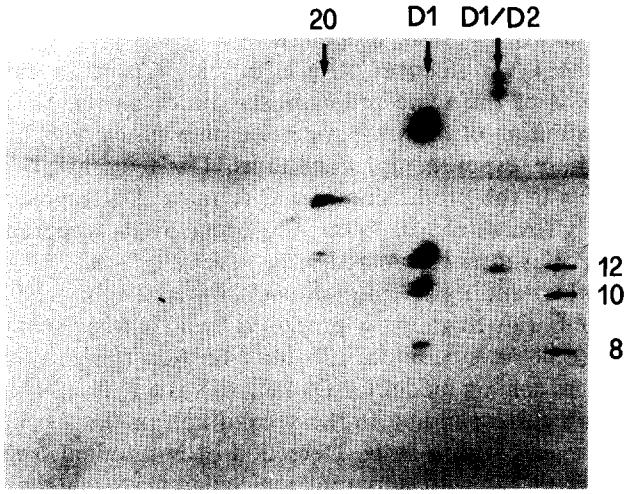

Fig. 4. Immunoblotting with anti-D1C of a 2-D gel in which UVB-treated thylakoids were subjected to SDS-PAGE in first dimension (right to left) and to trypsinolysis in second dimension, as described previously [26]. 7.5 μg of chlorophyll were loaded per gel lane.

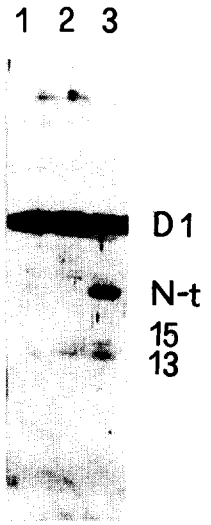

Fig. 5. Immunoblotting with anti-D1 polyclonal specific for N-terminal region of D1 protein of dark control (lane 1) and UVB-treated (lane 2) wheat thylakoids illuminated at 4°C. In lane 3 thylakoids treated like those of lane 2 were further incubated in the dark at room temperature with 1 unit/ml Lys-C protease. 7.5 μg of chlorophyll were loaded per gel lane.

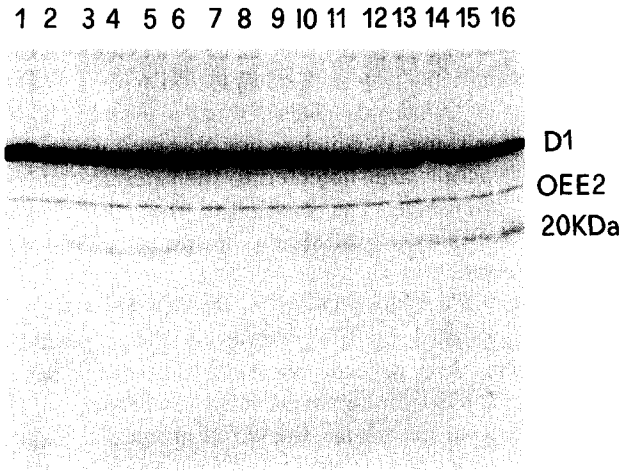

Fig. 6. Immunoblotting with anti-D1C polyclonal of wheat thylakoids illuminated with UVB light for 0 (lane 1); 5 (lanes 2, 7 and 12); 10 (lanes 3, 8 and 13); 20 (lanes 4, 9 and 14); 30 (lanes 5, 10 and 15); 60 min (lanes 6, 11 and 16). Temperature was 22°C for samples on lanes 1–6 and 4°C for all other samples. Samples on lanes 7–11 were illuminated in presence of 2 μM ioxynil. 7.5 μg of chlorophyll were loaded per gel lane.

probed with the anti-D1N polyclonal, which was raised against the 2–238 segment of the wheat D1-protein [14]. Two immunoreactive bands with apparent molecular masses of about 13 and 15 kDa were detected (Fig. 5, lane 2), very likely representing N-terminal portions of the protein. As expected, these fragments do not contain the Lys-238 residue, as shown by their insensitivity to Lys-C protease (lane 3).

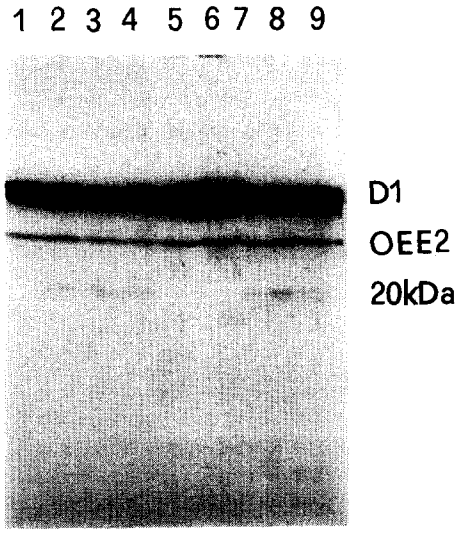

Fig. 7. Immunoblotting with anti-D1C polyclonal of wheat thylakoids illuminated with UVB light in presence (lanes 1–4) or absence (lanes 6–9) of 5 μM soybean trypsin inhibitor. Samples were illuminated for 0 (lanes 1 and 6); 5 (lanes 2 and 7); 10 (lanes 3 and 8) and 30 min (lanes 4 and 9). Lane 5 contains thylakoids incubated in the dark for 30 min. Temperature was 22°C. 7.5 μg of chlorophyll were loaded per gel lane.

Fig. 6 shows that lowering the temperature from 22°C (lanes 2–6) to 4°C (lanes 12–16) does not significantly modify the extent of D1 photodegradation as far as may be inferred from its fragmentation pattern. Instead, addition of 2 μM ioxynil (lanes 7–11) partially inhibits the appearance of the UVB-induced fragment characterized above. Other herbicides such as DCMU and atrazine or the plastoquinone antagonist DBMIB did not significantly prevent the appearance of the fragment.

As shown in Fig. 7, no significant effect was observed by including 5 μM soybean trypsin inhibitor (compare lanes 1–4 with lanes 5–9) or by changing the pH (not shown).

Discussion

UVB radiation adversely affects the functional and structural integrity of PS II in a suspension of isolated thylakoid membranes. Assuming a linear relation between level of damage and radiation dose, it may be estimated that UVB light is roughly 100-fold more efficient than white light at a comparable photon fluence, both in impairing electron transport from water to the plastoquinone acceptor pool and in inducing degradation of the reaction center D1-protein.

In the case of visible light, various mechanisms have been proposed to describe the succession of events by which an excess of excitation energy may damage the electron transport chain, with a decrease in overall photosynthetic activity [32]. Although complete elucidation of these mechanisms has not yet been achieved, it has been clearly established that, once electron transport activity is lost and D1-protein has been damaged, temperature-sensitive proteolytic degradation of D1 starts [7], the protein is turned over and replaced by a newly synthesized one [2].

The UVB fluence (50 μE $m^{-2} s^{-1}$) used in this work is of the same order of magnitude as the UVB contribution of the solar spectrum on a sunny day [33]. In these experimental conditions, UVB illumination results in the production of a C-terminal D1 fragment of approximately 20 kDa. According to its apparent molecular mass (estimated, from several gels, as 19.9 $\pm$ 0.8 kDa) and its orientation with respect to the whole protein, this fragment is produced by a cleavage somewhere between residues 120 and 140. According to the current folding model for D1 [34], this is a hydrophobic region corresponding to the second half of the second transmembrane α-helix. Detection of N-terminal fragments of D1 with apparent molecular mass matching the remaining part of the protein supports this view. Based on the similarity of molecular sizes and on the fact that no additional cross-reacting bands are detected by immunoblotting with anti-D2 polyclonal,

these 15–13-kDa fragments may be the same as those detected in [24].

It has been reported [23] that UV light induces the appearance of 20- and 8-kDa breakdown fragments produced by cleavage of the arg-238/phe-239 peptide bond, which coincides with the single cleavage site for the Lys-C protease in maize or wheat in which arg-238 is replaced by lysine. Given the high sensitivity of our polyclonal towards the C-terminus of D1, the presence of an 8-kDa C-terminal photodegradation product would have been detected. It therefore seems likely that we do not have this fragment in our blots, suggesting that our experimental conditions were different from those of Ref. 23.

It is worth noting that a cleavage site inside a transmembrane α-helical segment has not been observed in the degradation pattern of D1-protein when induced by visible light. All the fragments characterized up to now are produced by cleavage of the hydrophylic loops connecting different transmembrane helices [13,14,35]. In this regard, degradation of D1-protein induced by UVB light is different from that induced by visible light, as no effect on the production of the 20-kDa fragment was found on lowering the temperature to 4°C, changing pH in the range 6–8, adding soybean trypsin inhibitor or excluding oxygen from the reaction mixture. This behaviour is reminiscent of donor-side induced degradation of D1 [11,36].

At variance with previous reports [23], no major protective effect of DCMU on D1 degradation was observed in our experimental conditions. No protective effect was observed with atrazine or with the plastoquinone antagonist DBMIB. Instead, the phenol herbicide ioxynil was found to exert some effect in preventing D1 from degradation, at least as far as cleavage to the 20-kDa C-terminal terminal fragment is concerned. Disagreement with the previous results of Greenberg et al. [33] and Trebst and Depka [23] is only apparent, since our results refer to different molecular events as compared to those reported by the above authors. Greenberg's experiments relate to in vivo turnover under a very low irradiance regime and deal with D1 degradation rate rather than with the appearance of a particular fragment, whereas Trebst's experiments refer to the appearance of a different D1 fragment (20-kDa N-terminal) and therefore to a different cleavage site.

Although it is quite clear that bound quinones Q_a and Q_b are certainly involved in UVB absorption, induced loss of PS II activity and degradation of D1-protein, other protein components such as tyrosine residues may also be responsible for phototrapping of UVB, with the consequent damage. This may explain the fact that, in our experimental conditions, the observed cleavage site is located in a protein region quite far from the Q_b niche. This may also explain why we do not observe any protective effect by Diuron and other herbicides except for ioxynil, which is known to bind D1-protein in more than one site, possibly also in the donor-side region [37].

In conclusion, we suggest that degradation of D1-protein triggered or driven by UVB radiation is associated with molecular mechanisms different from those observed with visible light, and that different photosensitizers may be involved in different regions of the D1 molecule. It is now interesting to address the question of whether the succession of events leading to the dismantling and repair of damaged PS II centers, which have been proposed to be operative after photoinhibition by visible light [17,18], is also valid for UVB stress. Experiments to clarify this point are now in progress.

Acknowledgements

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