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UV-B-induced degradation of the D1 protein in isolated reaction centres of Photosystem II

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Abstract

Illumination of isolated Photosystem II reaction centres by low-intensity UV-B light in the absence of added quinones does not result in any detectable breakdown products of the D1 protein but instead induces the formation of a 41 kDa adduct of the D1 protein and a α-subunit of cytochrome *b*-559, and changes its electrophoretic mobility on SDS-PAGE. If, however, DBMIB is present, many breakdown fragments of the D1 protein are detected, indicative of several cleavage sites in its amino acid sequence. Low temperature or the presence of a cocktail of proteinase inhibitors did not prevent the UV-B-induced degradation, nor did anaerobic conditions. Other quinones were also found to be effective, but to a lesser extent, giving rise to the same degradation pattern. Since the pattern of D1 protein fragmentation was not observed when non-quinone electron acceptors, such as silicomolybdate, were present, it was concluded that degradation of this protein involved UV-B-induced formation of quinone radicals.

Keywords: Ultraviolet B; D1 protein degradation; Protein degradation; Photosystem II; Reaction center

1. Introduction

When photosynthetic organisms are exposed to elevated levels of UV-B radiation, a number of different physiological processes are affected [1]. Among these is the process of photosynthesis, with particular sensitivity focused on Photosystem II (PS II). Jones and Kok [2] were the first to show that UV-B radiation was much more effective than visible light, in terms of absorbed photons, at inactivating oxygen evolution from isolated thylakoids. More recently, Greenberg et al. [3] reported that UV-B radiation was very efficient at inducing the rapid turnover of the D1 protein, a key component of the PS II reaction centre. By measuring the action spectrum for the turnover of this protein, it was concluded by Greenberg et al. that a quinone molecule was involved in the sensitisation of the phenomenon, while in the case of visible light the turnover of D1 protein was sensitised by chlorophyll. The presence of breakdown products of the D1 protein ascribed to UV-B-induced damage of quinones has been detected by Trebst and Depka [4] and later by Melis et al. [5].

In addition to the above findings, there is also a line of evidence indicating that the donor side of PS II is an important target for UV-B-induced damage [6,7]. Renger et al. [6] have shown that a UV-B-induced impairment of water oxidase activity can be correlated with a modification of the relaxation kinetics of the oxidised primary donor of PS II (P680) which reflects changes in the ability to re-reduce $P680^+$. Recently, Friso et al. [7] have shown that UV-B irradiation of isolated thylakoids leads to the detection of a 20 kDa C-terminal and 12 kDa N-terminal breakdown fragment of the D1 protein. These fragments are generated by a single peptide cleavage in or near the second transmembrane segment of the D1 protein. Partial characterisation of the breakdown products showed that their appearance was largely independent of temperature and proteinase inhibitors. In support of this, a very recent

Abbreviations: Chl, chlorophyll; DBMIB, 2-5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; $(OH)_2$BQ, 2,5-dihydroxy-*p*-benzoquinone; DMBQ, 2,5-dimethyl-*p*-benzoquinone; PBQ, *p*-benzoquinone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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study by Barbato et al. [8] has shown that the presence of functionally active Mn is required for the appearance of the 20 kDa and 12 kDa breakdown fragments.

Studies designed to elucidate the molecular processes involved in the light-induced degradation of the D1 and D2 proteins have been much aided by the use of the isolated reaction centre of PS II as an experimental system. Such studies have clearly distinguished between the donor and acceptor side mechanisms induced by visible light [9–13]. This isolated complex has also been used to study the action of UV-B light on the D2 protein [14]. In this paper, a complementary study has been undertaken to identify the mode of action of UV-B light on the integrity of the D1 protein within the isolated reaction centre of PS II. We have observed D1 protein fragments due to UV-B irradiation when an electron acceptor is present, but the degradation patterns are affected by the type of acceptor employed.

2. Materials and methods

Isolation of reaction centres of Photosystem II (PS II RC complexes) was carried out as described by Nanba and Satoh [15] with modifications introduced by Chapman et al. [16]. UV-B irradiation was administered to samples contained in a standard 3 ml quartz cuvette which was sealed with a Teflon stopper and placed horizontally below the UV source. The light intensity at the surface of the reaction centre suspension was 50 μE m^{-2} s^{-1} and the pathlength was 3 mm. The sample contained 30 μg Chl ml^{-1} and was kept at 4°C. The reaction centres were suspended in a buffer containing 50 mM Tris-HCl (pH 7.2) and 2 mM n-dodecyl β-D-maltoside. The UV-B irradiation was generated from a Vilber-Lourmat lamp (model VL-215M) with a peak emission at 312 nm. When 2-5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) or other quinones were present, they were added from a stock solution concentrated 100-fold. All samples, including the control, contained 1% ethanol by volume.

SDS-PAGE in the presence of 6 M urea was performed as described previously [17] using a 12–18% linear acrylamide gradient or a 15% single concentration gel, as specified in figure captions. For immunoblotting, the resolved proteins were transferred to nitrocellulose filters (Sartorius, 0.45 μm) according to Dunn [18] and filters probed with different polyclonal antibodies. In this study three different polyclonal antibodies against the D1 protein have been used and hereafter referred to as anti-D1C1, which mainly recognises epitopes located on the 239–344 C-terminal region of the protein; anti-D1C2 raised against a synthetic peptide corresponding to the last 19 amino-acid residues at the C-terminus of the protein, kindly provided by Dr P. Nixon; anti-D1N, raised against the 2–238 N-terminus of D1 protein obtained by Lys-C digestion of the purified wheat protein [10]. The immunological reactions were visualised by a colorimetric method employing alkaline phosphatase-conjugated antibodies. Chromogenic substrates for alkaline phosphatase were nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Anoxygenic conditions were obtained by adding a chemical trap containing 5 mM glucose, 0.1 mg ml^{-1} catalase and 0.1 mg ml^{-1} glucose oxidase.

3. Results

Fig. 1 shows an immunoblot with anti-D1C1 polyclonal of isolated PS II RC which, prior to solubilisation and SDS-PAGE, had been illuminated for various periods of time in the presence or absence of different levels of the artificial electron acceptor DBMIB. Lane 10 shows a dark control sample. When DBMIB was not present (lanes 1–3), illumination with 50 μE m^{-2} s^{-1} of UV-B light resulted in a slowing down of the electrophoretic mobility of the D1 protein (lane 3) and in the appearance of a new band at 41 kDa. Occasionally, some very faint immunoreactive bands were also detected in the 14–20 kDa region. When 50 μM DBMIB was added to PS II RC before illumination the result of UV-B treatment was different (lanes 4–6). The upward shift of D1 was less pronounced and the immunodetectable band at 41 kDa was much fainter. In contrast, a set of breakdown fragments was clearly detected in the 14–25 kDa range. As the DBMIB concentration was increased to 500 μM (lanes 7–9), formation of the 41 kDa band was almost completely inhibited, while the level of the 14–25 kDa breakdown products increased.

A light-induced 41 kDa band containing the D1 protein was first identified by Shipton and Barber [9] and charac-

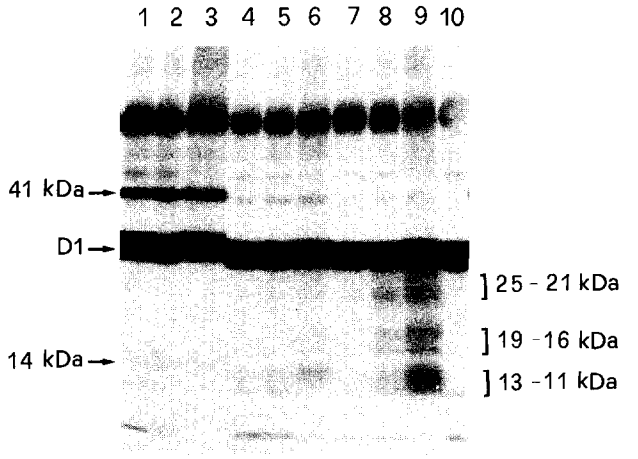

Fig. 1. Immunoblot with anti-D1C1 polyclonal antibody of isolated PS II RC complexes illuminated for 5 (lanes 1, 4 and 7), 10 (lanes 2, 5 and 8) and 20 min (lanes 3, 6 and 9) with 50 μE m^{-2} s^{-1} UV-B light. DBMIB concentrations were: lanes 1–3, none; lanes 4–6, 50 μM, lanes 7–9, 500 μM. Sample in lane 10 is a dark control, kept in the dark for 20 min with 500 μM DBMIB. Each gel lane contains 0.5 μg Chl.

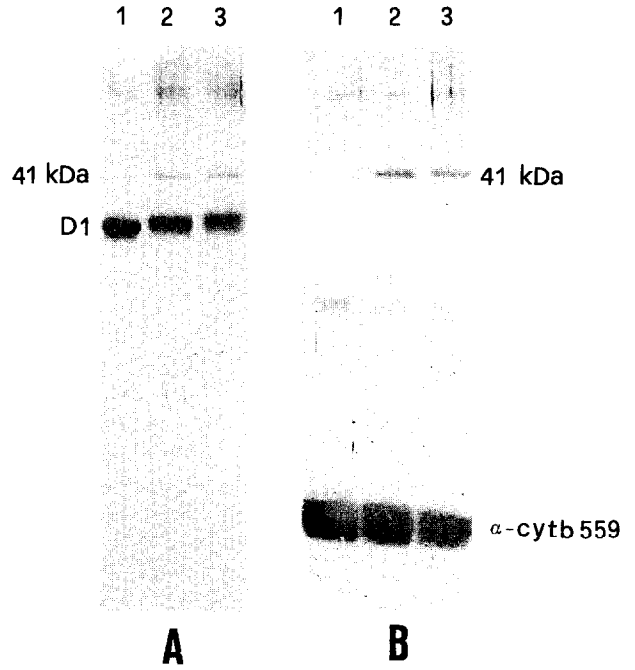

Fig. 2. Immunoblot with polyclonals to D1 (panel A) and cytochrome *b*-559 α-subunit (panel B) of dark control (lanes 1A and 1B) and UV-B-treated samples, respectively, for 5 (lanes 2A and 2B) and (lanes 3A and 3B) 10 min. Each gel lane was loaded with 0.5 μg Chl.

terised during illumination of isolated PS II RCs as an adduct formed by D1 and the α-subunit of cyt *b*-559 by Barbato et al. [19]. In order to understand whether or not the 41 kDa observed after UV-B irradiation was the same photocrosslinked product, immunoblots with anti-D1 (Fig. 2A) and anti-α-cyt *b*-559 (Fig. 2B) polyclonals were performed. Lane 1A and 1B contain dark control samples where the polyclonals recognise their respective antigens. After 5 and 10 min of illumination with UV-B light (lanes 2A and 2B and 3A and 3B, respectively) the 41 kDa was detected by both antibodies. Therefore, we may conclude that, as was observed in visible light treatment, the 41 kDa band represents a photocrosslinking product between D1 protein and the α-subunit of cyt *b*-559.

In addition to identifying the origin of the 41 kDa band, we have also partially characterised some of the observed breakdown fragments. This was achieved by immunoblotting with D1 protein polyclonals having different specificities. In these analyses two polyclonals were used, the first raised against the 2–238 N-terminal segment of the wheat D1 protein [10], the second raised against a 19 amino acid synthetic peptide corresponding to the C-terminus of the D1 protein (denoted anti-D1N and anti-D1C2, respectively). Fig. 3 shows immunoblotting of PS II RCs which, prior to solubilisation and SDS-PAGE, had been illuminated with 50 $\mu E\ m^{-2}\ s^{-1}$ of UV-B light for 30 min with 500 μM DBMIB present. The pattern of cross-reaction shown in lane 1 is for anti-D1C2 (D1C), while lane 2 is that for anti-D1N (D1N). The two antibodies detected some breakdown fragments having the same apparent molecular masses, while other fragments of around 20–25 kDa are clearly different. Taken as a whole, these data indicate that D1 protein is cleaved by the action of UV-B light at many different points in its amino acid sequence and that some of the detected bands represent internal fragments, possibly produced by further cleavage of primary breakdown products.

The effects of some physical and chemical factors on the DBMIB-dependent UV-B-induced breakdown of the D1 protein were investigated. It was found that lowering the temperature from 20°C to 4°C does not affect the UV-B-induced degradation of the D1 protein. Similarly, the UV-B-induced degradation proceeded at 20°C in the presence of a cocktail of proteinase inhibitors (consisting of 10 mM phenylmethylsulfonyl fluoride, 10 mM *o*-phenanthroline, 10 mM soybean trypsin inhibitor and 0.875 unit/ml α-macroglobulin). Therefore, as previously described for the D2 protein [14], the UV-B-induced degradation of the D1 protein does not seem to involve any proteolytic enzyme or related mechanism.

The effect of different pH levels on the degradation of the D1 protein as a consequence of UV-B irradiation of PS II RC in the presence of 250 μM DBMIB is shown in Fig. 4, where the immunoblotting employed the anti-D1C1 polyclonal. It is evident that at pH 6 (lane 1) there seems to be an enhanced level of breakdown fragments as compared with illumination at pH 7 (lane 3) and pH 8 (lane 5).

As shown in Fig. 1, the presence of DBMIB is required for the appearance of UV-B-induced degradation products of the D1 protein. In contrast, when silicomolybdate was used as an electron acceptor, degradation products of D1 proteins were still found, although with different molecular sizes and to a weaker extent as compared to those seen with DBMIB present (see Fig. 5). The effect of other quinones, such as 2,5-dimethyl-*p*-benzoquinone (DMBQ),

Fig. 3. Immunoblots with anti-D1C2 (anti-D1C) (lane 1) and anti-D1N (lane 2) of isolated PS II RC illuminated for 20 min in the presence of 500 μM DBMIB.

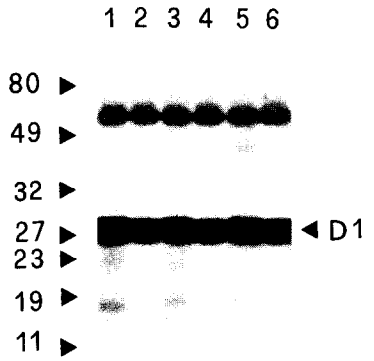

Fig. 4. Immunoblot using anti-D1C1 of isolated PS II RC illuminated with UV-B light for 20 min (lanes 1, 3 and 5) or kept in the dark for the same time (lanes 2, 4 and 6) in the presence of 250 μM DBMIB at pH values of 6.0 (lanes 1 and 2), 7.0 (lanes 3 and 4) and 8.0 (lanes 5 and 6). Each gel lane contains 0.3 μg Chl.

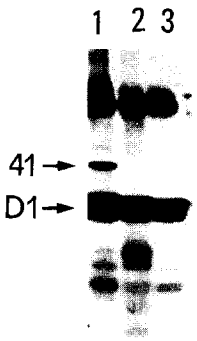

Fig. 6. Immunoblot with anti-D1N polyclonal of PS II RC illuminated with UV-B light for 20 min in the presence of 200 μM DBMIB either under aerobic (lane 1) or anaerobic (lane 2) conditions. Lane 3 contains an anaerobic dark control sample. Each gel lane was loaded with 0.4 μg Chl.

2,6-dichloro-*p*-benzoquinone (DCBQ), *p*-benzoquinone (PBQ) and 2,5-dihydroxy-*p*-benzoquinone $(OH)_2BQ$, on UV-B-induced degradation of D1 protein was also investigated. All the quinones were able to catalyse UV-B-induced degradation of the D1 protein giving rise to the appearance of breakdown fragments of similar size but with different levels of intensity. The approximate order of this effect was (DBMIB >) DMBQ > PBQ > DCBQ > $(OH)_2BQ$ (data not shown). Taking into account the fact that DMBQ and PBQ are known to accept electrons mainly at the Q_B site while DCBQ accepts electrons at Q_A as well as Q_B [20], it is possible that binding of quinone at the Q_B site on the D1 protein plays some role in sensitising the breakdown of the protein, and that the efficiency of binding may affect the ability to catalyse UV-B-induced degradation of D1 protein. Alternatively, the differential effect of the quinones tested may reflect different efficiencies of UV-induced radical formation and variations in their reactivity.

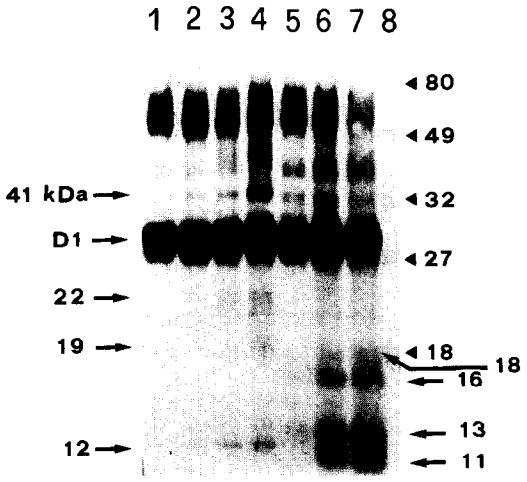

Fig. 5. Immunoblot wih anti-D1C1 polyclonal of isolated PS II RC complexes illuminated with UV-B light in the presence of 250 μM SiMo (lanes 2–4) or 250 μM DBMIB (lanes 5–7) for 5 (lanes 2 and 5), 10 (lanes 3 and 6) and 20 min (lanes 4 and 7). Lane 1 is not illuminated. Lane 8 contains molecular markers which were visualised using Coomassie blue.

It has previously been shown that oxygen is not strictly necessary to induce breakdown of the D2 protein during irradiation of isolated PS II RC with UV-B light in the presence of DBMIB [14]. In order to check further whether the same lack of dependence for oxygen held true for the UV-B-induced breakdown of the D1 protein, we irradiated PS II RCs in the presence of DBMIB under aerobic and anaerobic conditions. The results from this experiment are shown in Fig. 6. Illumination under aerobic and anaerobic conditions results in the appearance of breakdown fragments when DBMIB is present, indicating that the mechanism of cleavage does not involve active species of oxygen. However, the pattern of breakdown fragments and their relative anti-D1N detectable levels did differ between anaerobic and aerobic conditions.

4. Discussion

We have shown that the exposure of isolated reaction centres of PS II to UV-B radiation produces a complex pattern of D1 protein degradation products. This complex pattern is observed when a quinone is present, particularly DBMIB, as an electron acceptor and is affected to some extent by whether oxygen is present or not. Although breakdown fragments of the D1 protein were observed when silicomolybdate replaced DBMIB as the added electron acceptor, the pattern was different and less intense. The origin of the DBMIB-dependent UV-B-induced D1 fragments has not been fully mapped but includes both

C-and N-terminal domains and possible internal regions of the protein. The results are quite different when compared with those obtained from in vivo systems [8] and isolated thylakoids [7] where the main UV-B-induced breakdown products are less in number and more easily defined. Illumination of intact spinach leaves with UV-B light gave rise to 20 kDa C-terminal and 12 kDa N-terminal fragments of the D1 protein indicative of a cleavage in the second transmembrane segment of the D1 protein [8]. Characterisation of this UV-B-induced cleavage suggested that it resulted from a UV-B-sensitised reaction involving the Mn cluster of the water splitting enzyme. These findings were consistent with the earlier work of Friso et al. [7], who observed the same breakdown fragments on illumination of isolated spinach thylakoids with UV-B light.

Although the Mn cluster could be implicated in the UV-B-induced inactivation of PS II and in the consequential degradation of the D1 protein, another line of evidence suggests that quinone acceptors, Q_A and Q_B, could be the target for damage by UV-B light. Trebst and Depka [4] reported that after irradiating isolated thylakoids with UV-B, breakdown of the D1 protein to immunologically detectable fragments occurred and concluded, on the basis of N-terminal sequencing, that the cleavage of the protein took place at residue 238 near to the Q_B binding region. Greenberg et al. [3] also came to the conclusion, based on the measurement of an action spectrum, that the UV-B-induced turnover of the D1 protein in *Spirodella oligorhiza* involved quinone. The possible involvement of bound plastoquinone in the UV-B sensitivity of the D1 protein was further implicated by the work of Melis et al. [5]. They reported that UV-B irradiation of isolated thylakoids induced a lowering of the plastoquinone content. Therefore, plastoquinone or one of its reduced forms also seems to be a sensitiser for UV-B-induced degradation of the D1 protein, at least under some circumstances.

The isolated PS II RC does not contain manganese or water-splitting activity, nor does it have quinones bound to the Q_A and Q_B sites on the D2 and D1 proteins, respectively. However, partial reconstitution with quinones is possible [21], with DBMIB being the most effective species identified to date [22]. The results presented in this paper reinforce the possibility that under some conditions (i.e., when Mn is not bound at the donor side of PS II) UV-B-induced degradation of the D1 protein may involve sensitisation via the quinone bound to the Q_B site. However, since the extent of D1 protein fragmentation is enhanced by increasing DBMIB levels, it is likely that the effect is mediated by quinone radicals generated in the suspension. Such radicals could also account for the instability of the 41 kDa cross-linking product of the D1 protein and the α-subunit of cytochrome *b*-559. Alternatively it is possible that the lack of appearance of this adduct is due to shielding resulting from UV absorption by added quinones. The suggestion that we have identified a quinone-radical-induced breakdown of the D1 protein is reinforced by the fact that the fragmentation is photochemically induced and does not seem to involve proteolytic action. Our work also indicates that the DBMIB-dependent action of UV-B light on the D1 protein is photochemical and does not seem to involve proteolytic events (Vass, I., Spetea, C. and Petrouleas, V., unpublished data).

In the absence of DBMIB, little or no fragmentation is detected, although the D1 protein is modified as shown by the decrease in its electrophoretic mobility on SDS-PAGE. Of particular interest is that under these conditions the adduct between the D1 protein and the α-subunit of cytochrome *b*-559 is readily formed by exposure to UV-B radiation. This cross-linkage is also induced by visible light [19] and is worthy of further investigation.

In conclusion, experiments using the isolated reaction centre of PS II do not give rise to a simple pattern of UV-B-induced D1 protein fragmentation as observed with more intact isolated systems able to evolve oxygen [7] or with intact leaves [8]. Rather, no extensive fragmentation is observed until electron acceptors are present. In the case of quinone acceptors, particularly DBMIB, the pattern of breakdown products is complex and can not be related with those seen previously. Given the complexity of the quinone-dependent UV-B-induced breakdown pattern in reaction centres as compared with the in vivo situation [8], it seems likely that our observations are due to quinone radical formation in the suspension medium. Nevertheless, the results do indicate that quinone-mediated UV-B damage could occur in vivo as suggested previously [3–5], where the active species would be Q_A and/or Q_B. Despite this, there is increasing evidence that UV-B damage of PS II can occur without the involvement of quinones and is localised on the donor side in the vicinity of the Mn cluster [8].

Acknowledgements

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