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Degradation of D2 protein due to UV-B irradiation of the reaction centre of photosystem II

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Abstract

Exposure of isolated reaction centres of photosystem II to UV-B radiation generates specific breakdown products of the D2 protein. When the quinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone is present a 22 kDa fragment containing the N-terminus of the mature protein is generated. Concomitant with the appearance of the N-terminal fragment, two fragments containing the C-terminus of the D2 protein having apparent molecular masses around 10–12 kDa are observed. It is concluded that the primary cleavage occurs in the hydrophilic loop linking putative transmembrane segments IV and V. No such cleavage was observed when silicomolybdate was used as an electron acceptor, suggesting that this UV-B damage is dependent on binding of the added quinone to the Q_A site.

Key words: UVB light; Photosystem II; D2 protein; Photoinactivation

1. Introduction

As result of measuring the action spectrum of photoinhibition of isolated thylakoids, Jones and Kok concluded that UV-B light was more effective than visible light at inhibiting the electron transport capacity of isolated thylakoids [1]. Since then a series of studies of the effectiveness of UV-B light at causing photoinhibition have been reported [2–5] and reinforced the original conclusion of Jones and Kok that the impairment site is photosystem two (PSII). Of particular interest was the finding of Greenberg et al. [6] that the rate of turnover of D1 protein was stimulated by UV-B radiation. Even more striking was the finding from the same laboratory that UV-B light, particularly when supplemented by visible light, induced the turn over of the D2 protein to a rate comparable with that of the D1 protein [7]. Recent studies with isolated thylakoids have shown that both donor and acceptor sides of PSII may be affected by UV-B light [8–10] and degradation products of the D1 protein have been identified [8,11,12]. Although loss of D2 protein after UV-B irradiation of isolated thylakoids has been reported [11] no breakdown fragments were detected. Here we show that when isolated PSII reaction centres are used, UV-B induced fragments of the D2 protein can be detected and characterized.

The isolated reaction centre of photosystem II has already proved to be a useful experimental system to investigate the molecular events that underlie the photoinhibitory process. This complex consists of the D1 and D2 proteins together with the α-subunits of cytochrome b_{559} and the product of the *psbI* gene. Using visible light it has been possible to follow photoinhibitory mechanisms due to either donor or acceptor side damage. The two routes of damage are characterized by specific patterns of irreversible loss of pigments and D1 and D2 degradation products [13–17]. The acceptor induced pathway is favoured when the radical pair $P680^+/Pheo^-$ (where P680 is the primary electron donor and Pheo is a pheophytin *a* molecule, that functions as the primary electron acceptor) is allowed to recombine and generate singlet oxygen via the P680 triplet state. When an exogenous electron acceptor is present, the lifetime of $P680^+$ is increased and the donor side mechanism dominates due to deleterious electron extraction from the local environment by this highly oxidising species.

Given that UV-B light is effective at inducing photoinactivation and in promoting degradation of the D2 protein in vivo and in isolated thylakoids, we have carried out a series of experiments to test the effect of this radiation on isolated reaction centres of PSII.

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Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; PAGE, polyacrylamide gel electrophoresis; pheo, pheophytin; P680, primary donor in photosystem II, PSII, photosystem II; PVDF, polyvinylidenedifluoride; SDS, sodium dodecyl sulphate.

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2. Materials and Methods

Reaction centres were isolated from the PSII membranes of peas according to the procedure described in [18] with modifications introduced by Chapman et al. [19]. UV-B irradiation was performed by a VL-215M lamp (Vilbert-Lourmet) which had a maximum emission at 312 nm. Illumination was performed at 4°C with a light intensity, at the surface of the reaction centre suspension, of about 70 W/m^2. Before illumination, the reaction centres were diluted to 50 μg chl/ml with an ice-cold buffer containing 50 mM Tris-HCl, pH 7.2 and 2 mM dodecyl β-D-maltoside. In some experiments the quinone 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was added from a stock solution 100-fold concentrated than required; ethanol was also added to the control, so that all the samples contained 1% ethanol. SDS-PAGE in the presence of 6 M urea was performed as described earlier [20]. After electrophoresis, gels were either stained with Coomassie blue or prepared for immunoblotting. Gels were blotted onto nitrocellulose (Sartorius, 0.45 μm) for immunodetection of proteins, or onto PVDF membrane (ProBlot, Applied Biosystems) for N-terminal sequencing, which was performed using a 477A model protein/peptide sequencer. It was found that the transfer buffer of Dunn [21] was suitable for both purposes. Immunodecoration of blots was performed using a polyclonal raised against a synthetic peptide corresponding to the C-terminus of the protein and kindly provided by Dr. P. Nixon or by using a polyclonal raised against D2 protein isolated from spinach [22]. The antibody used in different experiments is specified in the figure legends.

Immunoreactions were detected by incubating nitrocellulose filters with biotinylated goat anti-rabbit IgG and then with extrAvidine alkaline phosphatase-conjugate (Sigma). Chromogenic substrates were nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Proteolysis on two-dimensional gels was carried out using the method of Cleveland et al. [23] with modifications described in [24]. Anoxygenic conditions (oxygen concentration less than 2 μM) were achieved by adding to the reaction centre suspension an oxygen chemical trap containing glucose, glucose oxidase and catalase at concentrations of 5 mM, 0.1 mg/ml, 0.1 mg/ml, respectively.

Densitometric analyses of immunodecorated blots were carried out using a Shimadzu CS 930 densitometer.

3. Results

Fig. 1 shows an immunoblot with anti-D2 antiserum of isolated PSII reaction centres illuminated with UV-B light for different periods of time either in the absence (lanes 1–6) or presence (lanes 7–11) of 500 μM DBMIB. The antibody used was a polyclonal to the whole spinach D2 protein [22]. Although in both cases a significant amount of D2 was lost during irradiation, no breakdown fragments were observed in the absence of DBMIB. On the contrary, at least three new cross-reacting bands were detected in the presence of the quinone. A band was observed at 22 kDa after 5 min of illumination and a doublet at around 10 to 12 kDa. To characterise further these UV-B induced breakdown products, they were subjected to proteolytic digestion and analysed by 2-dimensional electrophoresis and immunoblotting.

Fig. 2A shows a 2-dimensional gel of dark control PSII reaction centres subjected to proteolysis with *S. aureus* V8 protease and to immunoblotting with polyclonal antibody raised against the D2 protein. Under the conditions used, the D2 protein is cleaved at least into four fragments by V8 protease. In the UV-B irradiated sample the entire D2 protein is cleaved in just the same way as the control and the 22 kDa fragment, detected

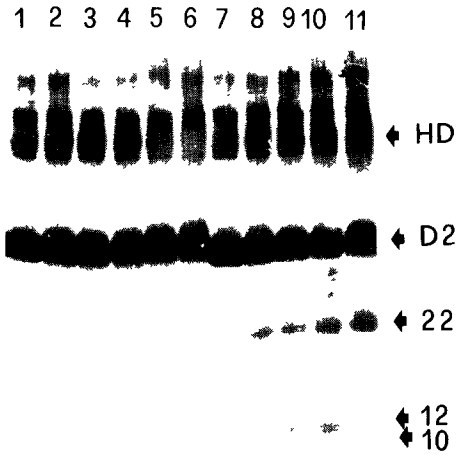

Fig. 1. Immunoblot of UV-B treated reaction centres with polyclonal raised against the whole D2 protein. Isolated reaction centres were illuminated for 0 (lane 1), 2.5 (lanes 2 and 7), 5 (lanes 3 and 8), 10 (lanes 4 and 9), 20 (lanes 5 and 10) and 30 (lanes 6 and 11) min, either in the absence (lanes 1–6) or presence (lanes 7–11) of 500 μM DBMIB. Samples were irradiated at 4°C at a chlorophyll concentration of 50 μg/ml. Each gel lane contained 0.5 μg chl. HD, heterodimer formed by D1 and D2.

along the diagonal of the gel, is also cleaved by V8 protease (see arrow). The fragment produced by V8 digestion of the UV-B generated breakdown product of 22 kDa, gave rise to a 10 kDa off-diagonal spot. As can be observed in Fig. 2B, the same fragment seems to occur among the V8 digestion products of the whole D2 protein. Despite several attempts, it was not possible to obtain a N-terminal sequence of this V8-induced fragment of 10 kDa, even under experimental conditions which allowed N-terminal sequencing of the α-subunit of cytochrome b_{559}, or of co-electrophoresed β-lactoglobulin. We interpret this finding to suggest that this fragment contains the N-terminus of the D2 protein, which is known to be post-translationally modified during its maturation [25].

If this interpretation is correct the UV-B breakdown product of the D2 protein observed at 22 kDa must represent the N-terminal portion of the protein and the companion C-terminal fragment should be located in the 10 kDa region where the double band is observed. To check this hypothesis a gel of irradiated reaction centres was blotted and immunodecorated with a polyclonal antibody raised against a synthetic peptide corresponding to the C-terminus of D2.

Fig. 3 shows that this polyclonal reacts with the 10 kDa double band, indicating the C-terminal origin of these peptides. In contrast, no cross-reaction was observed with the 22 kDa breakdown fragments (lane 2), confirming its N-terminal origin. Based on the apparent molecular mass of fragments we can therefore locate a main cleavage site due to UV-B radiation around amino acid residue 240, in the hydrophilic loop connecting the IV and V putative transmembrane segments of the pro-

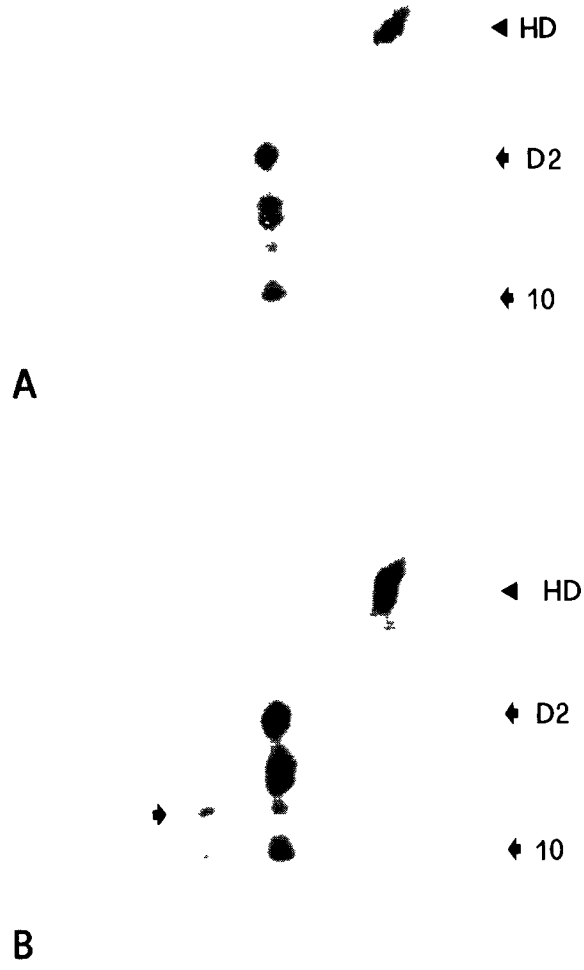

Fig. 2. Immunoblots with anti-D2 antisera of PSII reaction centres kept in the dark (A) or illuminated with UV-B light for 30 min in the presence of 500 μM DBMIB (B). Polypeptides were resolved by SDS-PAGE and a gel lane was loaded onto a second identical gel whose stacker contained the V8 endoproteinase at a concentration of 10 μg/ml. After digestion of polypeptides, gel electrophoresis was carried out and proteins electroblotted onto nitrocellulose membrane for immunodetection.

tein. This is the region of the D2 protein involved in the binding of the first stable plastoquinone electron acceptor Q_A [26,27] and this is clearly linked to the observed necessity of a quinone acceptor for the UV-B radiation to be effective in producing a protein cut at this site. The role of exogenous quinone acceptor in determining the UV-B induced protein cleavage in the region of the Q_A binding site is further supported by results shown in Fig. 4; when irradiation was performed in the presence of the electron acceptor silicomolybdate instead of DBMIB. In this case the UV-B induced degradation of the D2 protein gives rise to a different set of breakdown products (lanes 5–7) as compared with those observed with DBMIB (lanes 1–4).

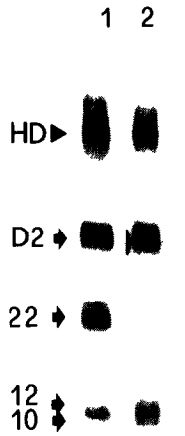

Fig. 3. Immunoblot of UV-B exposed reaction centres with polyclonals raised against the whole D2 protein (lane 1) and a synthetic peptide corresponding to the C-terminus of the D2 protein (lane 2). Samples were treated with UV-B light for 30 min in the presence of 500 μM DBMIB at 4°C.

Further investigation of the UV-B induced DBMIB-dependent fragmentation of the D2 protein showed that the cleavage mechanism was independent of oxygen and was not inhibited by adding a cocktail of protease inhibitors consisting of soybean trypsin inhibitor, *o*-phenantroline, α-macroglobulin and phenyl methyl sulphonyl fluoride (not shown).

Fig. 5 shows the dependence of the relative amount of the 22 kDa fragment (evaluated after 30 min of UV-B irradiation) on the concentration of DBMIB. The effect saturates at a concentration corresponding approximately to a 10-fold concentration of reaction centres.

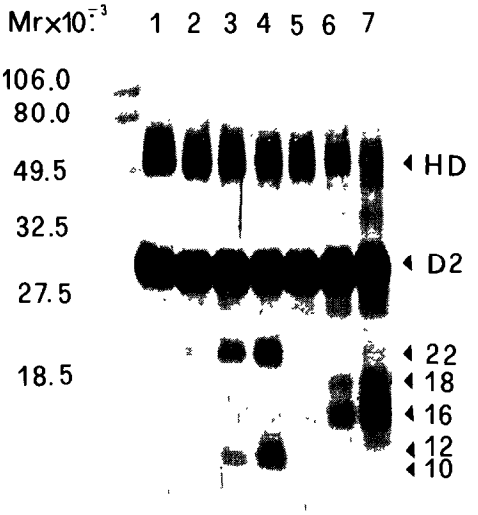

Fig. 4. Immunoblot with anti-D2 polyclonal raised against the entire protein, of reaction centres illuminated with UV-B light either in the presence of 500 μM DBMIB (lanes 1–4) or 250 μM silicomolybdate (lanes 5–7). Samples were illuminated for 0 (lane 1), 5 (lanes 2 and 5), 10 (lanes 3 and 6), and 30 (lanes 4 and 7) min at 4°C.

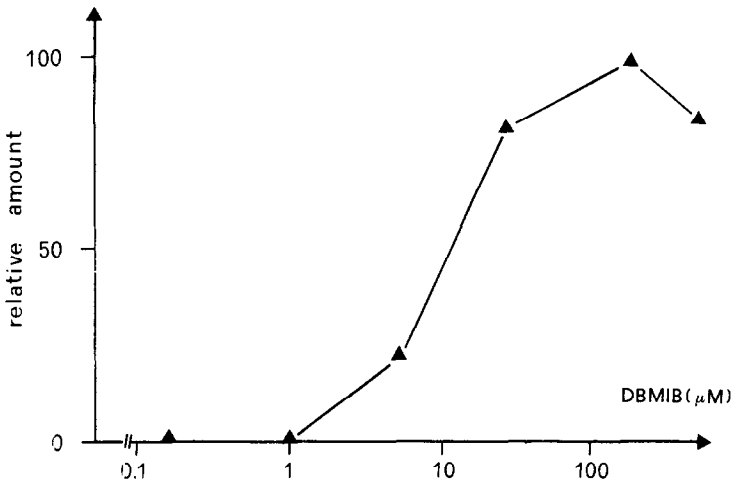

Fig. 5. Relative amounts of 22 kDa N-terminal D2 fragment induced by 15 min of illumination with UV-B light in the presence of different concentrations of DBMIB. Chlorophyll concentration was 30 μg/ml. Amount of fragment was estimated by densitometric analysis of two independend immunodecorated blots.

4. Discussion

Degradation of the D2 protein as a consequence of UV-B irradiation of isolated reaction centres is evident both in the absence (see also [28]) and presence of exogenous electron acceptors. However, only in the latter case have fragmentation products been observed in significant amounts. Fragments are also of a different molecular size depending on the electron acceptor used.

When the artificial quinone DBMIB is used, a specific cleavage is observed in the hydrophilic loop connecting the putative transmembrane segments IV and V. This region of the D2 protein contributes to the binding niche for the first stable quinone acceptor, Q_A [26–27]. In view of the relatively high optical absorption properties of DBMIB in the UV-B region and of the high reactivity of the semiquinone radical, a likely explanation is that the excited semiquinone form of the bound acceptor is the promoter of the peptide bond cleavage. A similar conclusion was also reached by Jensen et al. [7] to explain UV-B induced rapid turn over of the D2 protein in vivo. They propose that the semiquinone form of Q_A is the vulnerable chemical species, and this view is supported by the action spectrum of the UV-B effect and by the increased susceptibility to damage when visible light is superimposed on the UV-B radiation. A similar conclusion was also reached for the UV-B induced damage of the D1 protein [6]. Our results further suggest that the chemistry giving rise to the quinone dependend UV-B induced cleavage of D2 protein does not involve oxygen nor proteolytic activities. From these conclusions it seems that the degradation of D1 and D2 proteins induced by UV-B light is by a different process than that due to an excess of visible light. In the latter, oxygen is necessary for the acceptor side mechanism, and silicomolybdate and DBMIB give similar results for the donor side mechanism [13,15].

The doublet band at 12 and 10 kDa observed on illumination with UV-B light contains the C-terminal of the D2 protein and presumably originates from the primary cleavage. Again, these are only observed when DBMIB is present.

In conclusion is it clear that one of the main mechanisms of UV-B damage of PSII reaction centres involves a process which differs from those observed with visible radiation and is associated with absorption of short wavelength light by the plastoquinones bound to the Q_A and, possibly, to the Q_B sites. Our results therefore support and extend those of Greenberg et al. [6], Trebst and Depka [11] and Jensen et al. [7].

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