Degradation of the D-1 Protein Subunit of Photosystem II in Isolated Thylakoids by UV Light

Achim Trebst and Brigitte Depka

Lehrstuhl Biochemie der Pflanzen, Ruhr-Universität Bochum, Postfach 102148, D-4630 Bochum 1, Bundesrepublik Deutschland

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Dedicated to Professor Wilhelm Menke on the occasion of his 80th birthday

D-1 Protein, Photosystem II, Plastoquinone, Thylakoids, UV Irradiation

Thylakoid membranes from spinach were irradiated with UV light of 254 nm. Photosynthetic electron flow driven by photosystem II, but not by photosystem I is fully inactivated in about 2–5 min.

Inactivation of electron flow is prevented by inhibitors of the Q_B site of both the DCMU and of the phenol type. UV light inactivates electron flow both under anaerobic and aerobic conditions. This is in contrast to white light where fast inactivation occurs only under anaerobic conditions and where only inhibitors of the DCMU-type protect. Inactivation of electron flow by UV light is followed on a slower time scale by a specific degradation of thylakoid membrane proteins. As shown by immunoblotting the D-1 protein and to a smaller extent also the D-2 protein subunit – that together form the reaction center of photosystem II – are degraded during UV light irradiation. The disappearance of these proteins occurs only under aerobic conditions. Both types of Q_B site inhibitors prevent the degradation of the two plastoquinone-binding proteins. A degradation product of the D-1 protein is observed at about 8 kDa size.

The results are discussed in their relevance to rapid turnover and photoinhibition *in vivo* and to the topology of the quinone-binding site in the D-1 and D-2 protein.

UV light is long known to inhibit photosynthetic electron flow in isolated thylakoid membranes (see [1]). UV irradiation of isolated thylakoid membranes at 254 nm destroys plastoquinone concomitantly with the function of electron flow through photosystem II [2]. Recent results by Greenberg *et al.* [3, 4] showed that the light-triggered rapid turnover [5] of the D-1 protein subunit of photosystem II can also be induced by UV light in intact *Spirodella* cells. The action spectrum suggests a plastosemiquinone as the UV-activated species for D-1 protein cleavage [3]. We wish to show a correlation of UV light-induced inactivation of photosystem II to the UV light-induced degradation of the D-1 protein in the isolated thylakoid membrane.

For this we extend our earlier experiments [2] on the UV light-induced inhibition of electron flow and destruction of plastoquinone in isolated spinach thylakoid membranes to an analysis of changes in the protein composition, not possible at that time. By immunoblotting we show that indeed the plastoquinone-binding protein D-1 and to a lesser extent also the D-2 protein disappear on irradiation of the membrane with UV light. The degradation of the protein subunits of photosystem II is slower than the inactivation of electron flow through photosystem II. The inactivation of function occurs both under aerobic and anaerobic conditions, but the degradation by UV light of the D-1 protein requires oxygen. Both inactivation of electron flow and degradation of the protein are blocked by inhibitors of the Q_B site.

Abbreviations: DAD, diaminoduren; DCMU, dichlorophenyldimethylurea; DQH_2, duroquinol; MV, methylviologen; Q_A and Q_B, plastoquinone-binding sites in photosystem II; PQ, plastoquinone; PS II, photosystem II; SDS, sodium dodecyl sulphate.

Reprint requests to Prof. Dr. A. Trebst.

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Materials and Methods

Spinach thylakoids were prepared according to standard methods in 0.4 M NaCl and 20 mM Tris buffer, pH 8.0, and the chloroplasts broken in 20 mM Tris buffer, pH 8.0 + 0.15 M NaCl. Their photosynthetic activity (DCMU-sensitive) was 190 μmol O_2/mg chlorophyll/h with methylviologen or K-ferricyanide as acceptor. Irradiation with UV light with spinach thylakoid membranes (1 mg chlorophyll in 20 ml Hepes buffer 40 mM, pH 7.4) was carried out in a quarz glass chamber with a mercury lamp (Zeiss) under air or argon + glucose

oxidase (23 units/ml) + 10 mM glucose + catalase (1000 units/ml). For irradiation at 313 nm a monochromatic interference filter was used. For UV irradiation at 254 nm the thylakoid membranes were illuminated with a UV lamp HL-6-KM (Bachofer). In the visible region the thylakoid membranes were illuminated with a lamp KL 150 B (Schott) using the corresponding cut-off filters.

For the Western blots illuminated spinach thylakoid membranes were dissolved in 5% SDS, 15% glycerin, 50 mM Tris, pH 6.8, 2% mercaptoethanol at room temperature and the polypeptides separated by polyacrylamide gel (15%) electrophoresis at room temperature for 48 h. The gels were blotted for 4 h and 0.4 A at 10 °C on nitrocellulose in 25 mM Tris, 192 mM glycine and 20% methanol. After saturation with 3% gelatine in Tris buffer, pH 7.5, the first antibody was allowed to react over night at room temperature in 1% gelatine. After washing in Tris and 0.05% Tween 20 the second antibody (horseradish peroxidase-conjugated) was allowed to react in 1% gelatine for 1 h, and developed with HPR colour development and 0.005% H_2O_2.

Results

Photoinactivation of photosystem II *in vitro* is long known [6, 7] and thought to reflect photoinhibition *in vivo* (see review [8]) which in turn is related to rapid turnover of the D-1 protein like in *Spirodella* [5] or in *Chlamydomonas* [9]. Electron flow through photosystem II in isolated thylakoid membranes is quickly inactivated by white light – even at low light intensities – if done under strictly anaerobic conditions [6, 10]. The inactivation of electron flow is prevented by certain inhibitors of the Q_B site of the urea/triazine family [6], but not by those of the phenol type [11]. UV light was also shown early to inactivate photosystem II function [2, 7], accompanied by complete plastoquinone destruction [2]. Table I repeats these experiments in the UV, in comparison with white light to show the conditions where inactivation of electron flow is the same or different. Irradiation of thylakoid membranes with UV light at several wavelengths leads to 50% inactivation of photosynthetic electron flow under anaerobic conditions in about 2 min as does white light. Aerobic conditions protect PS II from photoinactivation by white light, but do not prevent of inactivation by UV light (Table I). *o*-Phenanthroline protects from the inactivation of electron flow both in white light and in UV light. Inhibitors of the phenol type, like BNT, ketonitriles and substituted quinolines, do not protect in white light [11], but they do in UV light (Table I). Fig. 1 shows that UV irradiation of thylakoid membranes inactivates particularly reactions driven or including photosystem II, but much less those driven by photosystem I, whether

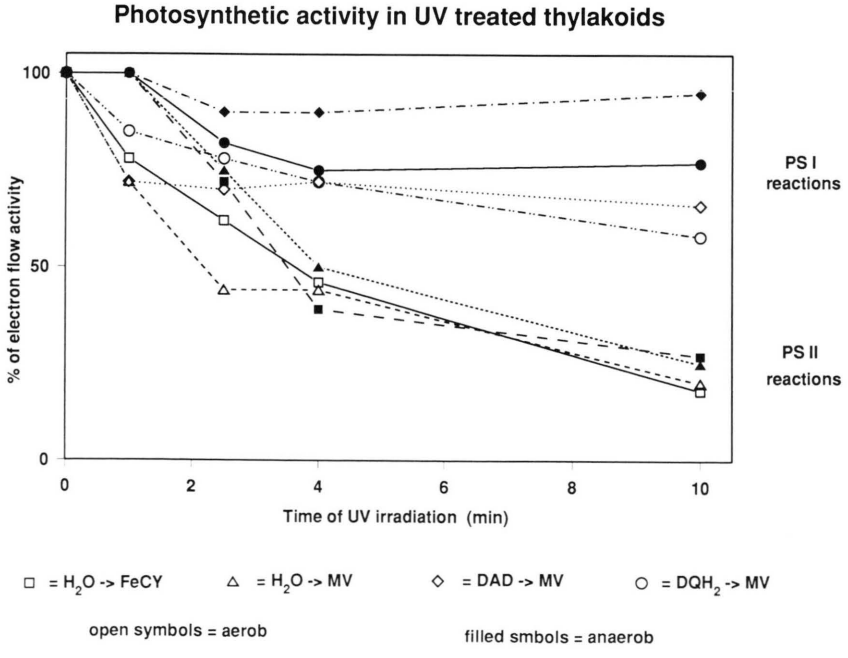

Fig. 1. Inactivation of photosynthetic electron flow by UV light. Conditions as in Table I. After preillumination of the thylakoid membrane under aerobic or anaerobic conditions for the time indicated the membranes were assayed in the different test systems for 10 min under aerobic conditions.

the cytochrome b_6/f-complex is included (DQH_2 as donor) or is not (DAD as donor). Again white light inactivates photosystem II under anaerobic [6], but photosystem I under aerobic conditions [12]. The inactivation of electron flow by UV light is independent of aerobic or anaerobic conditions (activity after irradiation was measured always aerobically for 10 min).

The SDS gel electrophoresis of the thylakoid membrane proteins, stained by Coomassie blue after UV light treatment, shows little effect on the protein bands (Fig. 2). But it is clearly seen by immunoblotting with specific antibodies after SDS gel electrophoresis of the UV-irradiated thylakoids that two bands are changed (Fig. 3–5). Fig. 3 and 4 show that 1 and 2 h of UV light the D-1 protein content is much decreased. Note that the

Table I. Inactivation of photosynthetic electron flow by light in the visible and in the UV.

Wavelength of preillumination	µmol FeCy reduced/mg Chl/h Control	Anaerobic *o*-phe		Aerobic *o*-phe	
	–	–	+	–	+
White light	420	80	420	400	
650 nm cut-off filter	660	84	420	480	
530 nm cut-off filter	600	280	496		
400 nm cut-off filter	492	151	450		
UV light	780	84		20	
313 nm monochrome	636	120	360		
254 nm monochrome	624	36	240	24	276

After preillumination of thylakoid membranes for 5 min under either aerobic or anaerobic conditions and minus or plus 10^{-4} M *o*-phenanthroline the membrane were spun down, washed and assayed for photosynthetic activity in white light under aerobic conditions for 10 min. *o*-phe = *o*-phenanthroline.

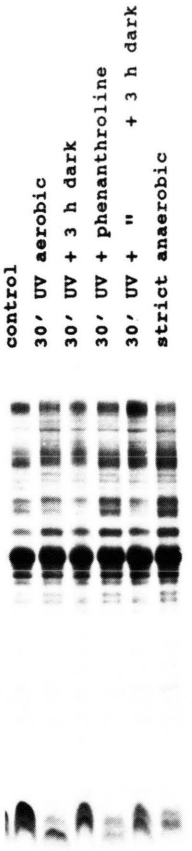

Fig. 2. Protein bands after SDS gel electrophoresis of thylakoid membranes after UV irradiation. The membranes were irradiated for 30 min under the conditions indicated. The control was kept in the dark. After the irradiation some samples were left for further 3 h in the dark.

Fig. 3. Degradation of the D-1 protein by UV irradiation of thylakoid membranes under aerobic conditions and protection by *o*-phenanthroline. Immunoblot with an antibody against the D-1 protein. After SDS gel electrophoresis of thylakoid membranes after irradiation with UV light.

time of UV irradiation in these experiments is much longer than in the inactivation measurements, *i.e.* degradation is much slower than inactivation. Turning off UV light and leaving the samples in the dark does not lead to further degradation (Fig. 3 and 4), *i.e.* there is no indication for a UV light-triggered protease that continues degradation in the dark. The addition of mercaptoethanol (MSH) has no additional effect on degradation, perhaps even rather a protective effect (*i.e.* no indication for a thiol protease) (Fig. 4). UV light irradiation of the membrane under anaerobic conditions does not lead to degradation of the D-1 protein. Fig. 3 indicates that *o*-phenanthroline not only protects photosystem II activity (Table I) from UV light irradiation, but also D-1 protein degradation. Also other inhibitors such as DCMU and BNT have this protective effect (Fig. 4). In Fig. 5 it is furthermore shown by immunoblotting with a D-2 protein specific antibody that also this protein is degraded, though with a slower rate than the D-1 protein.

Propylgallate, shown by Mattoo *et al.* [3] to prevent the UV-induced rapid turnover of the D-1 protein in *Spirodella,* also protected in the isolated thylakoid membrane system (Fig. 6).

Fig. 4. Degradation products of the D-1 protein after irradiation of thylakoid membranes. Immunoblot with an antibody against the D-1 protein. After SDS gel electrophoresis of thylakoid membranes after irradiation with UV light.

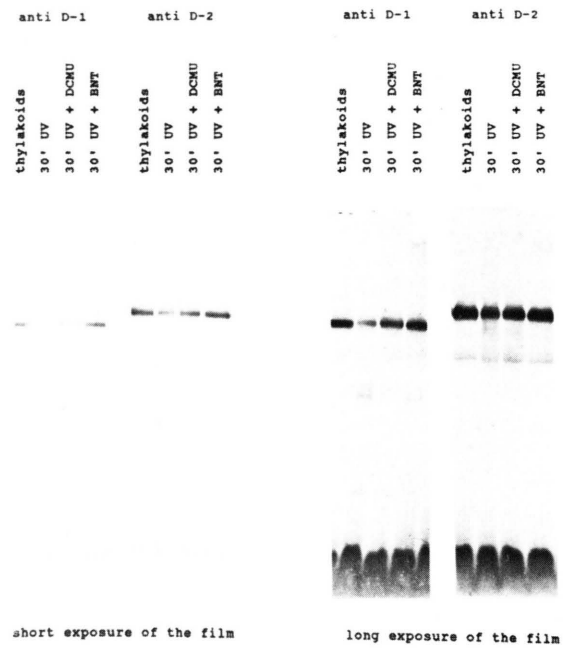

Fig. 5. Degradation of the D-1 and D-2 protein and protection by inhibitors during UV light irradiation of thylakoid membranes. Immunoblot with antibodies against the D-1 and D-2 subunit after SDS gel electrophoresis of membranes irradiated with UV light for 30 min. The same gel is shown with two exposures of the film. BNT = bromonitrothymol.

Fig. 6. Protection by propylgallate of the D-1 protein during UV light-induced degradation. Immunoblot with an antibody against the D-1 protein.

Discussion

The D-1 protein is one of the reaction center subunits of photosystem II (see [11]). This protein, previously called the herbicide or Q_B-binding protein [13], has long been known to have a rapid turnover time in light [5]. The rapid turnover is probably related to photoinhibition [9], in which the D-1 protein damaged by excess light, is degraded and replaced by a newly synthesized protein [9, 14]. A rapid turnover in intact cells can be light-induced at both visible [5, 15] as well as at UV [3, 4]. We have followed an older protocol for the inactivation of photosystem II in isolated membranes by both white [6] and UV light [2]. The protection from the inactivation of photosystem II activity at low light by inhibitors such as DCMU and *o*-phenanthroline was reported in 1961 [6] and [10]. As the results here show, the properties of the light inactivation of photosystem II and its protection by inhibitors in visible light are different from that in UV light. Whereas the first is very much stronger under anaerobic conditions, UV light inactivities under both anaerobic and aerobic conditions. The half time is about 1 to 2 min. Whereas only inhibitors of the DCMU/triazine type protect PS II from inactivation by visible light and those of the phenol type do not [11], in UV light both types of inhibitors of photosystem II protect the protein from inactivation (see Results here).

Mattoo *et al.* [3] have shown that propylgallate, an oxygen radical scavenger, prevents rapid turnover in intact *Spirodella*. The isolated membrane system is also protected from UV light damage by propylgallate.

Greenberg *et al.* [15] have identified the cleavage site in the amino acid sequence of the D-1 protein in rapid turnover. It is likely that the cleavage occurs close to or in the region of glutamic acids 241 to 245. The degradation of the D-1 protein is thought to begin after a hypothetical protease had recognized a PEST sequence between arg 225 and arg 238 [15]. We recently extended the modelling of the topology of the herbicide and Q_B-binding site in the D-1 protein to include amino acids close to tyr 237 and arg 238 that would fold "back" on top of plastoquinone Q_B [11, 16]. These amino acids are situated in a hydrophilic loop between transmembrane helix IV and a parallel helix. These helices are in the D-1 protein of photosystem II, but not in the homologous L-subunit of the purple bacterial reaction center. This loop extending back onto the Q_B site, is situated between the cleavage site and the PEST site that Greenberg *et al.* [15] proposed.

The participation of another short segment in the amino acid sequence of the D-1 protein provides furthermore another contact site between the D-1 and the D-2 protein at respective regions around arg 238. This has the interesting consequence that the trypsin cut of the D-2 protein at arg 237 is prevented in the presence of inhibitors in the Q_B site of the D-1 protein [16]. This can be phrased more generally: The conformation of the D-2 protein is controlled by that of the D-1 protein and this in turn is modulated by the occupancy state of the Q_B site which is dependent on the redox state of the PQ pool. This control by the occupancy state of the Q_B site is relevant also in photoinhibition. The accessibility of certain amino acids of either protein towards the matrix space is modulated as is the sensitivity to photodamage. This can be observed in intact *Chlamydomonas* cells where both the turnover of the D-1 and the D-2 protein under photoinhibition are affected by an inhibitor binding to the D-1 subunit [5, 11, 14, 17]. This interaction is, as again seen in these experiments, with UV light. Inhibitors of the Q_B site on the D-1 protein affect the D-2 protein, which has no herbicide-binding site, in the UV light experiments and furthermore both the D-1 and D-2 protein are degraded. This stresses again the close contact of the two proteins at homologous amino acid sequences. However, the effect of the occupancy state of the Q_B site in photodamage (or photoinactivation) on the function of photosystem II is different from its effect on photodegradation of the protein subunit(s). Photoinhibition therefore consists of two phases, controlled in a different way by the Q_B site. In the first phase the site has to be empty [16], in the second the site is filled. This different effect of inhibitors and the occupancy state in UV and white light in photodamage and photodegradation is summarized in Table II.

The disappearance of the D-1 protein is much slower than the photoinactivation of photosystem II activity and this is followed by a even slower disappearance of the D-2 protein. It is already clear from the earlier experiments with thylakoid membranes [6, 10, 11] and intact *Chlamydomonas* cells

Table II. Two phases in photoinhibition: Different conditions for photoactivation and photodegradation in white *vs.* UV light.

	White light	UV light	Atmosphere	Occupancy state of the Q_B site	Protection by inhibitors DCMU	BNT
Photoinactivation: 50% of electron flow in:	2 min	2 min	anaerobic	empty	yes	no
Photodegradation: 50% of the D-1 protein in:	1 h	20 min	aerobic	occupied	yes	yes

[11, 14, 17], that photoinactivation of function precedes photodegradation. This is opposite to the suggestion that photodegradation is the cause of photoinhibition [9].

We have observed a degradation product of about 8 kDa of the D-1 protein by immunoblotting. This band must contain the main epitope of the antibody which is probably located close to carboxy terminus [18]. Preliminary results on the sequencing of this fragment revealed a .. Q E E E .. sequence in automatic sequencing as of the third sequencing cycle. Although masked by two more unidentified polypeptides and therefore not yet confirmed, it suggests that the cleavage site is at amino acid phe 239. As the sequence does not yield the first two amino acids of the degradation product in the first and second cycle, we conclude that they may be damaged in the UV light-induced degradation and therefore are not picked up in the HPLC run. The cleavage site is three amino acids before the site Greenberg *et al.* suggested [15], but close to the tyr 237, where we propose a interaction between this amino acid sequence and Q_B [17] and which is tagged by photoaffinity labeling with azidomonuron [19].

The degradation of the D-1 protein under UV light proceeds only under aerobic conditions. Under these conditions, as earlier shown, plastoquinone is completely destroyed [2]. It seems possible that the aromatic ring of plastoquinone in the Q_B site is cleaved and/or forms a radical that is unlike the semiquinone radical intermediate in photosynthetic electron flow. The radical induced by UV light might interact with oxygen. Mattoo *et al.* have already put up evidence that the photoreceptor for rapid turnover under white light is different from that in UV light [3]. The UV light-induced degradation of the D-1 protein in the isolated thylakoid system might be a chemical artefact that is not related to *in vivo* degradation. Nevertheless, the results provide further evidence for modelling the topology of the Q_B site in the D-1 protein: plastoquinone in the Q_B site extends towards the amino acid loop that includes tyr 237 and phe 239.

Degradation of the D-1 protein in isolated systems induced by white light, very slow under our conditions [11], has been reported by Styring [20, 21] and more recently by Böger *et al.* [22]. The role of the Q_B site heterogeneity in photoinhibition is discussed by Guenther and Melis [23].

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