

Detection of a 10 kDa breakdown product containing the C-terminus of the D1-protein in photoinhibited wheat leaves suggests an acceptor side mechanism

Pedro M. Cánovas and James Barber

AFRC Photosynthesis Research Group, Wolfson Laboratories, Biochemistry Department, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK

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Photoinhibition of intact leaves of wheat generates a 10 kDa breakdown product which is clearly observed both at 4°C and 25°C. Selective immunoblotting has shown that the 10 kDa fragment contains the C-terminus of the D1-protein and, under the conditions employed, supports an acceptor side mechanism for photoinhibition *in vivo*. Although a corresponding 23 kDa N-terminal D1-fragment was not detected our results are consistent with the argument that the primary cleavage site is in the loop joining putative transmembrane segments IV and V.

Photo-inhibition; Photosystem II; D1-protein

1. INTRODUCTION

It is generally believed that the rapid turnover of the D1-protein is linked to the vulnerability of photosystem II (PSII) to photoinduced damage [1,2]. When the rate of damage is not matched by the rate of repair, the apparent efficiency of photosynthesis declines and the physiological state, known as photoinhibition, is observed [3]. Many factors effect the balance between damage and repair (e.g. temperature) and detailed schemes of the various stages of disassembly and reassembly of PSII have been postulated [4,5].

Greenberg et al. [6] have detected a breakdown product of the D1-protein *in vivo* with an apparent molecular mass of 23.5 kDa. Based on proteolytic mapping, these workers concluded that the cleavage occurred in the loop joining putative transmembrane segments IV and V. As compared with the L-subunit of purple photosynthetic bacteria, this loop is excluded and contains a 'PEST' like region [6]. Such a region is thought to destabilize α -helices and is common to proteins which turn over rapidly [7]. Some doubt as to the origin of the 23.5 kDa band detected by Greenberg et al. was raised, however, when Mullet et al. [8] showed that in the absence of chlorophyll synthesis, the D1-protein RNA message was not fully read and a pause site gave rise to

a truncated D1-protein of about 23 kDa. A translation intermediate of similar size has also been detected recently by Taniguchi et al. [9]. Since Greenberg et al. [6] conducted their key experiments in the presence of cycloheximide, to block synthesis of nuclear encoded proteins, it is possible that the band they observed was not due to the breakdown of the D1 protein but rather to its incomplete translation.

Work with isolated PSII complexes has indicated that the primary cleavage of the D1-protein can occur in the region identified by Greenberg et al. [10–13] when subjected to photoinhibitory light. In these studies a 23 kDa fragment containing the N-terminus of the D1-protein was identified, as was a 10 kDa C-terminal fragment. It was further shown by proteolytic mapping that the cleavage giving rise to these fragments was to the C-terminal side of residue 238 [11,13] in agreement with the suggestion of Greenberg et al. [6] and Shipton et al. [14]. These fragments were observed under 'acceptor side' photoinhibitory conditions [15,16] when overreduction, or absence, of Q_A facilitates the recombination of the radical pair $P680^+Pheo^-$. This recombination generates a $P680$ triplet state which, under aerobic conditions, forms singlet oxygen [17,18]. It seems to be this highly toxic species that triggers the proteolytic cleavage of the D1-protein so as to generate a 23 kDa N-terminal fragment and a corresponding 10 kDa C-terminal fragment. In addition to the acceptor side mechanism of photoinhibition there is also a damaging process associated with the donor side of PSII [19–21]. This donor side mechanism occurs when the water splitting reactions are inhibited and electron donation to the oxidised primary donor $P680^+$ is restricted. It is therefore believed

Correspondence address: P.M. Cánovas, AFRC Photosynthesis Research Group, Wolfson Laboratories, Biochemistry Department, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK. Fax: (44) (71) 581 1317.

Abbreviations PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; SDS, sodium dodecyl sulphate.

that the increased lifetime of $P680^+$ under these conditions leads to secondary oxidation processes which are deleterious to the reaction centre and induce the proteolytic cleavage of the D1-protein on the donor side [22,23]. This results in the generation of a 24 kDa C-terminal fragment and corresponding 9 kDa fragment of the D1-protein indicating that the cleavage occurs in the polypeptide loop joining putative transmembrane segments I and II on the luminal side of the reaction centre.

Given the results obtained with in vitro systems, the question arises whether both 'donor' and 'acceptor' side photoinhibition can occur in vivo. In this study we have used immunological blotting to detect breakdown products of the D1-protein in wheat leaves photoinhibited at room temperature and at 4°C.

2. MATERIALS AND METHODS

2.1. Plant growth

Wheat plants were grown for 7 days in controlled environment cabinets at 25°C and a day length of 14 h. Incident irradiation was provided by cool white fluorescent lamps at a photon flux density (PFD) of $200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Seeds were soaked overnight, before being planted in vermiculite. Plants were watered regularly during the growing period.

2.2. Photoinhibitory treatments

Plants were cut and placed in Eppendorf tubes with their petioles immersed in the appropriate solution. Leaves were exposed to a PFD of $2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, provided by an apparatus equipped with a 1 kW halogen lamp and a heat filter. Temperature was controlled by a cooling plate on which the leaves were placed facing the light source.

2.3. Measurements of photosynthetic activity

Room temperature variable fluorescence (F_v/F_m) was measured using a pulse fluorimeter (PAM 103, Walz), which is described in detail by Schreiber et al. [24]. After the light treatment, leaves were incubated for five minutes in the dark before measurement. Leaves were then exposed to a two second saturating light flash and the chlorophyll fluorescence yield measured using a weak modulated light source.

2.4. Pulse-chase experiments

Plants were labelled with [^{35}S]methionine ($> 37 \text{ TBq/mmol}$, Amersham) under normal light conditions ($200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and then transferred to Eppendorf tubes containing a solution of cold methionine (10 mM) before the photoinhibitory treatment. Thylakoid samples were prepared according to Marder et al. [25] and protein content estimated by a method of Esen [26]. Polypeptides were resolved by SDS-PAGE, using 15% polyacrylamide gels in the presence of 6 M urea and the discontinuous buffer system of Laemmli [27]. Gels were stained with Coomassie blue R250, dried and autoradiographed by the usual procedures.

2.5. Western blotting

Thylakoid proteins were prepared as described above except that a cocktail of protease inhibitors (Boehringer) containing 10 different compounds (antipain, APMSF, aprotinin, bestatin, chymostatin, E-64, EDTA, leupeptin, pepstatin and phosphoramidon) was included in the homogenisation buffer. After electrophoresis, polypeptides were transferred to nitrocellulose membranes (Schleicher and Schuell, pore size $0.22 \mu\text{m}$) according to a modification of a method by Towbin et al. [28]. Transfer was carried out in a perspex box with plate electrodes, using a solution of 3 mM Na_2CO_3 , 10 mM NaHCO_3 in 20% methanol as the buffer. After the transfer, the membrane was blocked with a

10% solution of skimmed milk powder in 0.1% Tween 20 TBS buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). Incubation with the D1 antibody proceeded at room temperature overnight. This antiserum was raised against a synthetic peptide corresponding to the C-terminal 29 amino acids of the D1-protein sequence of pea (a kind gift from Dr P. Nixon). The primary antibody solution was prepared by diluting the stock 1,000-fold in TBS buffer. Parallel competition experiments were carried out in order to identify non-specific cross-reactions due to other proteins in the preparation. For this purpose, the 29 amino acid peptide used to raise the D1 antiserum was included in the primary antibody incubation step at a concentration of approximately $10 \mu\text{g} \cdot \text{ml}^{-1}$. After overnight incubation, blots were washed several times with TBS buffer and then incubated with the secondary antibody (anti-rabbit IgG, alkaline phosphatase conjugate, Sigma) prior to being developed.

3. RESULTS

As Fig. 1 shows, when wheat leaves were exposed to light of $2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, photoinhibition occurred as judged by a decrease in the ratio of variable chlorophyll fluorescence (F_v) and the maximum fluorescence attainable (F_m). The extent of the photoinhibition was greater when the leaf temperature was dropped from 25°C to 4°C. No photoinhibition occurred when the light intensity was ten times less. The enhancement of photoinhibition by lowering the leaf temperature from 25°C to 4°C was found to correlate with a decreased rate of D1-protein turnover as judged from pulse-chase experiments using [^{35}S]methionine (see Fig. 2). Pulse-chase experiments also showed that at both temperatures the

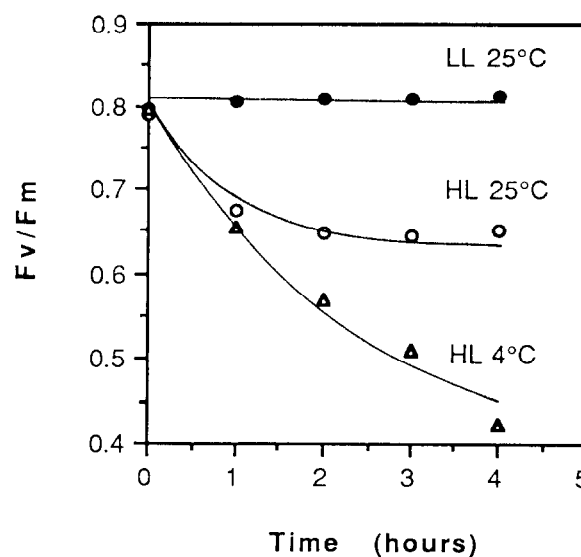


Fig. 1 Decrease of F_v/F_m ratio as a consequence of photoinhibition. 7 day-old wheat plants were cut and placed in distilled water under low light conditions ($200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 25°C). Following this, leaves were transferred to the high light ($2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at different temperatures. Plants exposed to $200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 25°C, LL 25°C (closed circles), plants exposed to $2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 25°C, HL 25°C (open circles), plants exposed to $2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 4°C, HL 4°C (open triangles).

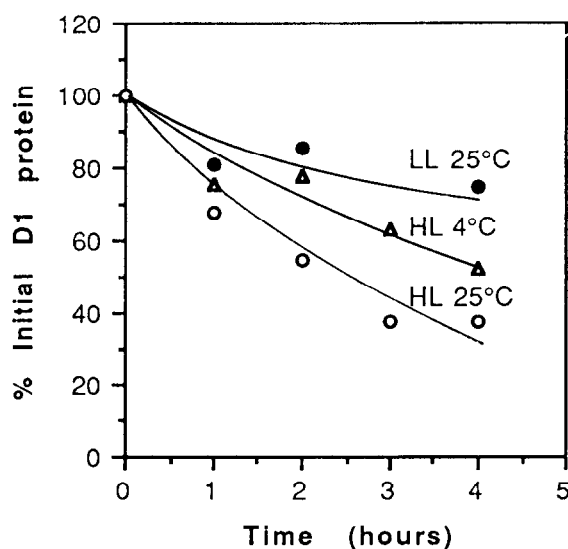


Fig. 2 Loss of incorporated [35 S]methionine from the D1-polypeptide, following pulse labelling of wheat leaves. The same experimental conditions applied as in Fig. 1, except that the plants were given a pulse of [35 S]methionine and then placed in a solution of 10 mM non-radioactive methionine during the photoinhibitory light treatment. Plants exposed to $200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 25°C , LL 25°C (closed circles); plants exposed to $2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 4°C , HL 4°C (open triangles); plants exposed to $2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 25°C , HL 25°C (open circles).

turnover of the D1-protein is greater under photoinhibitory, compared with non-photoinhibitory conditions.

Immunological blotting, using the polyclonal antibody raised to the C-terminal portion of the D1-protein as described in section 2, allowed us to detect a 10 kDa band in thylakoids isolated from illuminated leaves.

The intensity of this band was more clearly observed with leaves exposed to photoinhibitory light (see Fig. 3). This immunosensitive band was not observed, however, in leaves subjected to dark treatment (Fig. 4). In order to check whether the 10 kDa band was derived from the D1-protein and not due to an artefactual cross-reaction, western blotting was performed in the presence of the synthetic peptide used to raise the C-terminal specific antibody (see section 2). As can be seen in Fig. 4, under these conditions not only is the D1-protein band not detected, nor is the 10 kDa band. In contrast, the detection of a weak band of unknown origin at about 20 kDa, observed in the dark as well as the illuminated sample, was not inhibited by the presence of the synthetic peptide and presumably, therefore, is due to a non-specific cross-reaction with the serum.

4. CONCLUSION

We suggest that the 10 kDa band observed in thylakoids isolated from photoinhibited leaves is a C-terminal cleavage product of the D1-protein. This conclusion is based on the fact that the band is only detected in illuminated leaves with a C-terminal specific antibody of the D1-protein and is not observed when immunoblotting is conducted in the presence of the synthetic peptide used to raise the antibody. It therefore seems likely that this fragment is due to a cleavage in the loop linking transmembrane segments IV and V resulting from 'acceptor' side rather than 'donor' side photoinhibition.

Although experiments were conducted to detect the corresponding 23 kDa N-terminal fragment, no such

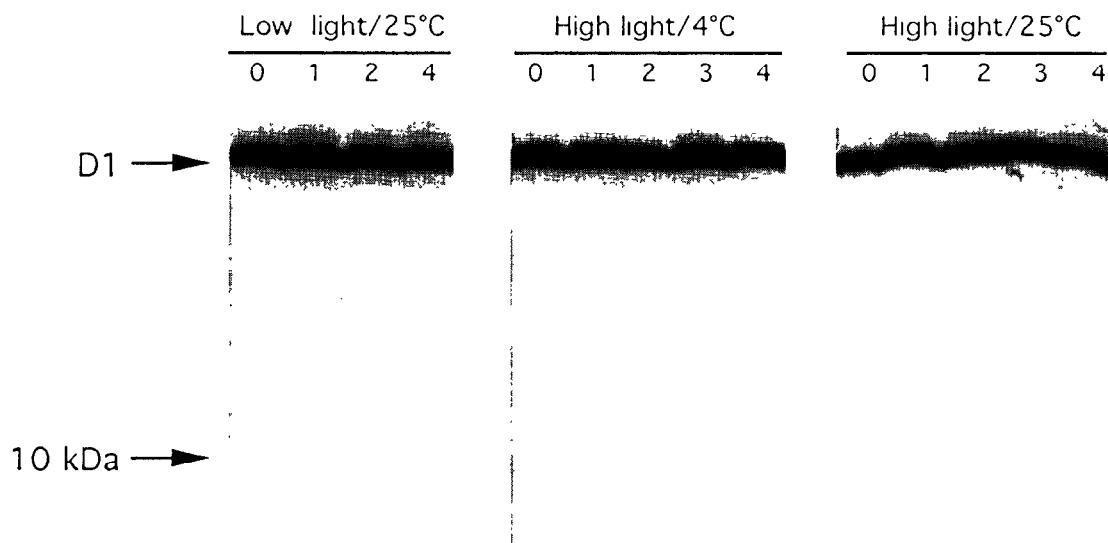


Fig. 3. Detection of a 10 kDa band in thylakoids isolated from light treated leaves. Plants were either exposed to low light ($200 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) or high light ($2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at different temperatures. After the light treatment, leaves were immediately frozen in liquid nitrogen and then homogenized in the presence of protease inhibitors after which crude extracts were washed several times and membrane proteins solubilized. Thylakoid polypeptides were resolved by SDS-PAGE and protein profiles electroblotted onto nitrocellulose membranes. Blots were incubated with a C-terminal D1 antibody (see section 2). Lane 0, control leaves before treatment; lanes 1-4, leaves illuminated for 1, 2, 3 or 4 h, respectively.

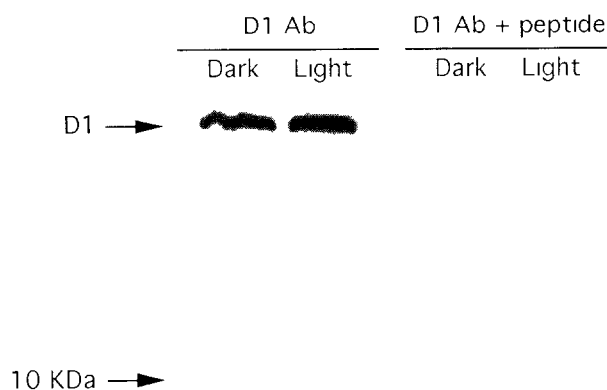


Fig. 4. Identification of the 10 kDa band as a light-induced fragment from the C-terminus of the D1 polypeptide. Leaves were either exposed to photoinhibitory light ($2,000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) or dark-adapted, after which samples were prepared as in Fig. 3 and immunoblotting performed. A parallel competition experiment was carried out in order to assess non-specific binding (see section 2). D1 Ab indicates the C-terminal antibody raised against the D1-protein used in this experiment.

fragment was found using the experimental protocol adopted here. Experiments with isolated PSII particles have also detected a light induced 10 kDa C-terminal fragment of the D1-protein and shown that the corresponding 23 kDa N-terminal fragment is unstable when the illumination period is prolonged [11,12].

Studies with isolated PSII preparations have also detected a 24 kDa C-terminal fragment due to 'donor' side photoinhibition [11–13,22,23]. No such fragment was detected in this work indicating that the acceptor side mechanism seems to be the cause of the photoinhibitory damage. It is possible, however, that the donor side mechanism giving rise to the 24 kDa C-terminal fragment may become operative if the water splitting system is not fully functional. Further work is required to test this prediction as well as to characterise further the condition that gives rise to the 10 kDa C-terminal fragment.

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