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Two sites of primary degradation of the D1-protein induced by acceptor or donor side photo-inhibition in photosystem II core complexes

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Depending on experimental conditions we have found that photo-inhibitory treatment of photosystem II (PSII) core complexes, isolated from wheat, can generate two fragments of about 23–24 kDa that contain either the C-terminal or N-terminal regions of the D1-protein. A 24 kDa C-terminal fragment appears when the water splitting reaction is not functional and an electron acceptor is present. This 'donor'-side inhibition also generates an N-terminal fragment of about 10 kDa and is suggested to be due to the cleavage of a peptide bond in the region connecting transmembrane segments I and II of the D1-protein. In contrast, an N-terminal 23 kDa D1-protein fragment is detected when the water splitting reactions of the isolated complex are active, and occurs in the absence of an added electron acceptor. This 'acceptor'-side photo-inhibition also generates a C-terminal fragment of about 10 kDa.

Photo-inhibition; Photosystem II; D1-protein

1. INTRODUCTION

It is now widely agreed that the in vivo rapid turnover [1,2] of the D1-protein of the photosystem II (PSII) reaction centre [3,4] is a consequence of the unique and highly reactive redox chemistry of the light-driven reactions of water oxidation and plastoquinone reduction [5–7]. The damaged D1-protein has to be replaced by a new protein copy to re-establish PSII electron transfer activity. Photo-induced degradation of the D1-protein can occur in isolated oxygen-evolving PSII core complexes [8] and even in isolated reaction centres [9]. This has led to the suggestion that the PSII complex harbours its own proteolytic activities [10–12].

From in vivo studies, Greenberg et al. [2] were able to show that an initial breakdown product of the D1-protein had an apparent molecular weight of 23.5 kDa, and proteolytic mapping of this fragment suggested that it contained the N-terminus of the intact D1-protein. This conclusion was consistent with the proposal that the likely site for the initial cleavage is close to an alpha-helix destabilising stretch of amino acids, rich in glutamate, serine and threonine residues [2]. Based on a predicted folding model for the structure of the D1-protein in the membrane [13–15], this cleavage site would be located on the outer surface of the thylakoid membrane in the polypeptide segment linking transmembrane alpha-helices IV and V. Proteolytic mapping indicated that this site may be near residues 241–245 where there is a conserved QEEET motif [16], while N-terminal sequencing of a UV-induced fragment of the D1-protein suggested that the cleavage site is at, or immediately adjacent to, residue 238 [17].

More recent in vitro studies on the origin of the 23.5 kDa D1-protein fragment has created some controversy. Shipton and Barber [9] have shown that a fragment of the D1-protein, having an apparent molecular weight of about 24 kDa, can be observed after illuminating isolated PSII reaction centres in the presence of an electron acceptor. They attributed the generation of this fragment to photo-damage on the oxidising side of PSII due to the prolonged lifetime of $P680^+$. In a follow-up study [18] this 24 kDa fragment was clearly demonstrated to contain the C-terminus of the D1-protein, suggesting that the cleavage was occurring in the stretch connecting transmembrane segments I and II. In an equally convincing set of experiments, Salter et al. [19] have recently demonstrated that a photo-induced 24 kDa degradation fragment observed after photo-inhibitory treatment of oxygen-evolving PSII core particles contains the N-terminus of the D1-protein.

In this communication we describe experiments which clarify this apparent controversy. We show that the appearance of either 24 kDa or 23 kDa fragments containing the C- or N-terminal of the D1-protein, re-

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; LHCII, light harvesting complex II; PAGE, polyacrylamide gel electrophoresis; PSII, photosystem II; SDS, sodium dodecyl sulphate.

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spectively, is determined by whether photo-inhibition is brought about by triggering events on the oxidising or reducing side of the PSII reaction centre.

2. MATERIALS AND METHODS

The PSII core complexes used in this study were isolated from wheat leaves. Thylakoid membrane fragments enriched in PSII were prepared as previously described [20]. Samples of these PSII-enriched membranes (15 mg Chl) were washed in 40 mM MES–NaOH (pH 6.0), 0.5 M sucrose and resuspended in 3.0 ml of a buffer containing 72 mM MES–NaOH (pH 6.0), 1.8 M sucrose, 72 mM $MgCl_2$, 18 mM NaCl. To this was added 2 ml of 10% *n*-heptylthioglucopyranoside to obtain a final detergent concentration of 4%, and a chlorophyll concentration of 3 mg · ml^{-1}. The mixture was homogenized, incubated on ice in the dark for 1 h, subjected to a 2.5-fold dilution with the first buffer, and then centrifuged at 30,000 × *g* for 15 min at 4°C. The supernatants were decanted, re-diluted 2-fold with the first buffer without sucrose, and centrifuged at 175,000 × *g* for 2 h at 4°C to obtain a dark green pellet of LHCII. The pale green supernatant was again diluted twice in the first buffer without sucrose and centrifuged at 150,000 × *g* for 1 h at 4°C to obtain a pellet of the PSII core complex. Polypeptide compositions were analysed by SDS gel electrophoresis in a 10–17% gradient polyacrylamide gel containing 6 M urea [9]. The PSII core complexes consisted of the reaction centre polypeptides (D1- and D2-proteins), apoproteins of cytochrome b_{559}, the *psbI* and *psbH* gene products and apoproteins of CP43 and CP47. In addition, the PSII core complex bound the extrinsic 33 kDa protein and evolved oxygen when assayed in the presence of 5 mM $CaCl_2$ and 1 mM *p*-benzoquinone (added as an electron acceptor) in 50 mM MES–NaOH (pH 6.0) at 25°C.

To obtain PSII proteins labelled in their N-terminal with ^{32}P, the PSII cores were isolated from wheat thylakoids that had been incubated under reducing conditions in the presence of [γ-^{32}P]ATP [22]. After SDS-polyacrylamide gel electrophoresis and electroblotting onto nitrocellulose, the phosphorylated proteins were detected by autoradiography [18,22].

Photo-inhibitory treatment of the PSII isolated cores was carried out according to [9] in a buffer containing either 50 mM Tris–HCl (pH 8.0) or in 50 mM MES–NaOH, (pH 6.0) with 5 mM $CaCl_2$; both buffers also contained 2 mM dodecyl-β-maltoside. Where stated the quinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), was added as an electron acceptor to a final concentration of 0.2 mM. Illumination of samples (100 μg Chl·ml^{-1}) was performed for fixed periods of time at 20°C using heat-filtered (Schott filter, RG1) white light with an intensity of 6,000 μmol photon $m^{-2}\cdot s^{-1}$.

Immunoblotting of proteins after separation by SDS-PAGE was performed as described previously [9,18]. Four different anti-D1 polyclonal antisera have been utilized during this study and are referred to as anti-$D1_T$, anti-$D1_C$, anti-$D1_{CL}$ and anti-$D1_{NL}$. Anti-$D1_C$ and anti-$D1_{CL}$ recognise epitopes located on the C-terminal region of the D1-protein (the former was raised to the isolated D1-protein [18] and the latter to a synthetic peptide based on the C-terminal sequence of the spinach D1-protein, kindly provided by Dr. P.J. Nixon); anti-D_T was raised against the product of the *psbA* gene expressed in *E. coli* [23]; anti-D_{NL} was raised to the N-terminal region of the wheat D1-protein generated by cutting the protein at the unique lysine residue 238 by using the specific endoprotease Lys-C [18]. Following incubation with the primary antibody, immunoreactions were detected by the alkaline phosphatase-conjugated secondary antibody method. Cleveland digests [24] were performed as described previously [18] using the endopeptidase Lys-C purchased from Sigma.

3. RESULTS

Fig. 1 shows the pattern of breakdown fragments generated by illuminating wheat PSII core complexes

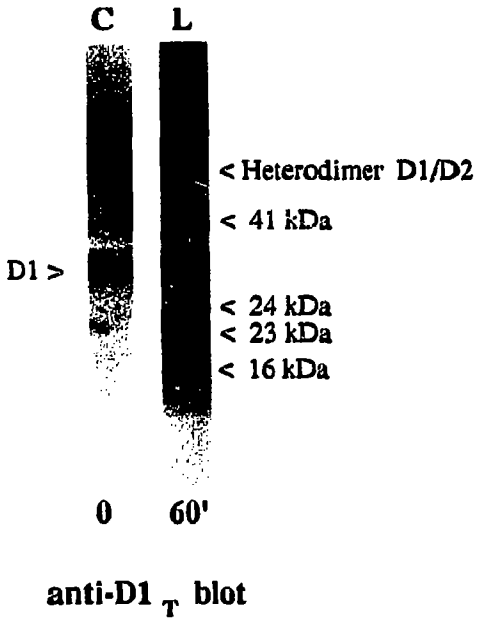

Fig. 1. Immunoblot of isolated PSII core complexes, control and light-treated, isolated from wheat. The PSII particles were maintained in darkness (C) or illuminated (L) for 60 min in 6,000 $\mu Em^2\cdot s^{-1}$ of white light at 20°C at a chlorophyll concentration of 100 $\mu g\cdot ml^{-1}$. The proteins were subsequently solubilized, separated by SDS-PAGE, electroblotted onto nitrocellulose membrane and immunodetected with D1 antiserum (anti-$D1_T$) using alkaline phosphatase conjugate as secondary antibody. The PSII core complexes were suspended in 50 mM Tris–HCl, pH 8.0, in the presence of an electron acceptor (0.2 mM DBMIB). In the light-treated sample three main degradation fragments are detected with apparent molecular weights of 24, 23 and 16 kDa.

for 1 h and detected immunologically using the antibody designated anti-D_T. In this experiment the PSII cores were suspended in Tris buffer at pH 8.0 with no added $CaCl_2$ and with DBMIB added as an electron acceptor. Under these conditions the PSII core complexes no longer evolve oxygen, presumably due to the disruption of the Mn cluster and partial loss of the 33 kDa protein. The D1-protein breakdown fragments detected consisted of bands having apparent molecular weights of about 24, 23 and 16 kDa. This same pattern was obtained previously when isolated PSII reaction centres of wheat were treated in a similar way [18]. In the latter case, it was clearly shown that the 24 and 16 kDa (designated 14 kDa in [18]) fragments contained the C-terminus of the D1-protein.

To investigate further the origin of the two larger degradation fragments, the D1-protein was phosphorylated at its N-terminal threonine [21] and Fig. 2 shows the results of such an experiment. The autoradiograms in Fig. 2a show the usual pattern of phosphorylation obtained with PSII-enriched mem-

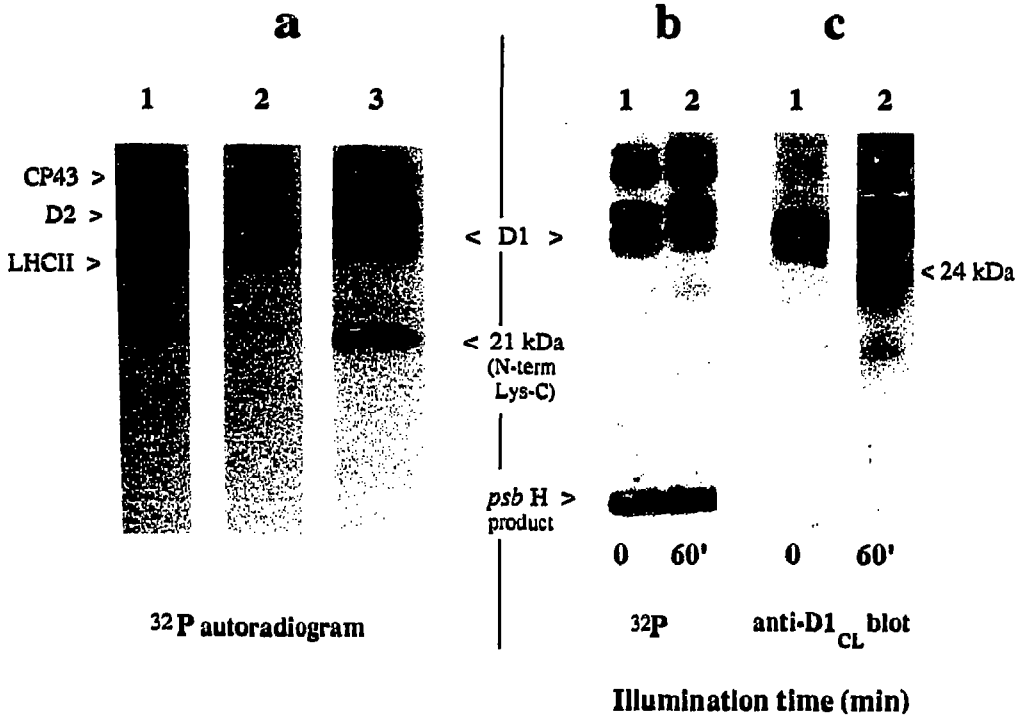

Fig. 2. (a) Lane 1, autoradiogram of ^{32}P-labelled PSII membranes; lane 2, isolated PSII core complexes; and lane 3, isolated PSII core complexes partially digested with endopeptidase Lys-C. (b) Autoradiogram of the PSII core complexes, control (zero time) and light-treated for 1 h. (c) Immunoblots with anti-$D1_{CL}$ antiserum corresponding to lanes 1 and 2 in (b).

branes and PSII core complexes [25] and also detects the 21 kDa N-terminal product of the D1-protein of wheat subjected to proteolysis with the endopeptidase Lys-C (lane 3) [22]. This band is due to specific cleavage of the D1-protein at the unique lysine at position 238 in the wheat sequence [16,18].

Fig. 2b shows an autoradiogram before and after photo-inhibitory illumination of isolated PSII core complexes under similar conditions as in Fig. 1. Again the autoradiogram shows, in the dark control, all the phospho-labelled bands in PSII core complex, including the 9 kDa phosphoprotein which is the product of the *psbH* gene [21]. Immunoblotting using anti-$D1_{CL}$ clearly detected the 24 kDa light-induced breakdown product but no corresponding radiolabelled band appeared on the autoradiogram, indicating that no radiophosphate was associated with this fragment. The action of Lys-C on this fragment is shown in Fig. 5b (lane 3) which also confirms that it is derived from the C- terminal end of the D1-protein since it was cleaved to lower molecular weights.

Fig. 3a again shows the appearance of the C-terminal 24 kDa light-induced D1-protein fragment, detected with anti-$D1_{CL}$ measured in this experiment as a function of the illumination time. As in the case of the isolated reaction centre [11], this fragment accumulates with increasing time of exposure to the photo-inhibitory light. This experiment was carried out under conditions

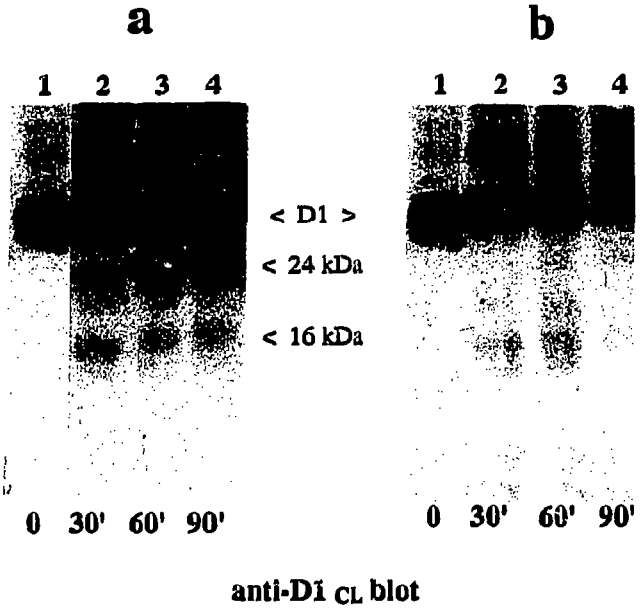

Fig. 3. Immunoblot, with antiserum anti-$D1_{CL}$, of phosphorylated PSII core complexes subjected to photo-inhibitory illumination for different times (indicated in min). (a) In the buffer 50 mM Tris–HCl, pH 8.0, and (b) in the buffer 50 mM MES–NaOH, pH 6.0 with 5 mM $CaCl_2$, both in the presence of the electron acceptor DBMIB (0.2 mM).

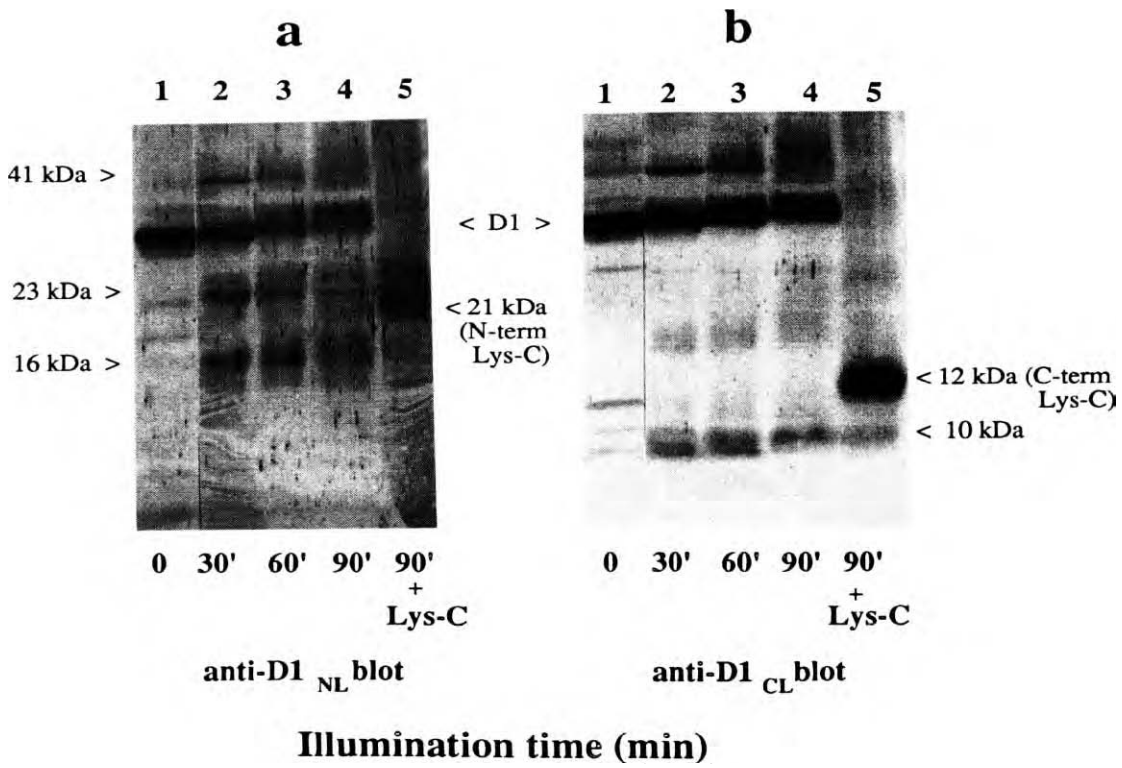

Fig. 4. Immunoblot of PSII core complexes subjected to photo-inhibitory illumination for different times (indicated in min) in buffer 50 mM MES–NaOH, pH 6.0, with 5 mM $CaCl_2$ but without the electron acceptor, DBMIB. The same samples were split into two aliquots and immunodetected with (a) anti-$D1_{NL}$ (N-terminal antibody) and (b) anti-$D1_{CL}$ (C-terminal antibody). Lane 5 in (a) and (b) shows Lys-C digestion of the sample subjected to 90 min light treatment.

which inhibit oxygen-evolving activity (pH 8.0, no $CaCl_2$). However, as shown in Fig. 3b, if the same photo-inhibitory light treatment is given to PSII core complexes, having the quinone DBMIB present but able to split water (pH 6.0, plus 5 mM $CaCl_2$), then the 24 kDa C-terminal fragment does not appear. In contrast, when the same experiment was conducted with ^{32}P-labelled PSII core complexes, a weak phospho-band at 23 kDa was detected after giving photo-inhibitory treatment (data not shown). Conformation of the generation of an N-terminal 23 kDa fragment was obtained by using the N-terminal-specific antibody, anti-$D1_{NT}$, and by not including the quinone acceptor, DBMIB (see Fig. 4). This figure shows an experiment performed under oxygen evolving conditions (50 mM MES–NaOH (pH 6.0), 5 mM $CaCl_2$) in the absence of the quinone, DBMIB. Interestingly, in this case, the N-terminal 23 kDa fragment was most clearly observed during the initial period of the illumination treatment, unlike the C-terminal 24 kDa fragment which accumulated with time (see Fig. 3a). The other observation shown in Fig. 4a is that the N-terminal photo-induced 23 kDa fragment was cut by Lys-C to a slightly lower molecular weight. This suggests that photo-induced cleavage in wheat occurs to the C-terminal side of lysine residue 238. Fig. 4b shows exactly the same experiment as Fig. 4a, except that the immunoblotting was performed with anti-$D1_C$. In this case the antibody, anti-$D1_C$, clearly detects a D1-protein fragment at about 10 kDa which is presumably a C-terminal part of the cleaved protein. Also Fig. 4b shows that the C-terminal fragment of the D1-protein, generated by Lys-C treatment (lane 5), gives a band at a molecular weight (about 12 kDa) just above the 10 kDa C-terminal photo-induced fragment. This confirms the above conclusion that under these experimental conditions a light-induced proteolytic cleavage of the D1-protein has occurred on the C-terminal side of the lysine residue 238, as proposed previously [2,16]. Moreover our results show that this proteolytic cleavage is enhanced when water splitting activity is functional but no exogenous quinone acceptor is present.

Worthy of note is that all four antibodies, to different degrees, detect photo-induced cleavage bands at 10 kDa. Such bands have been noted previously in photo-inhibited PSII oxygen-evolving cores [8,19] and isolated PSII reaction centres [9,18]. In spinach PSII complexes, this 16 kDa band has been attributed to a primary C-terminal fragment [19].

Fig. 5 further clarifies the proteolytic action of Lys-C

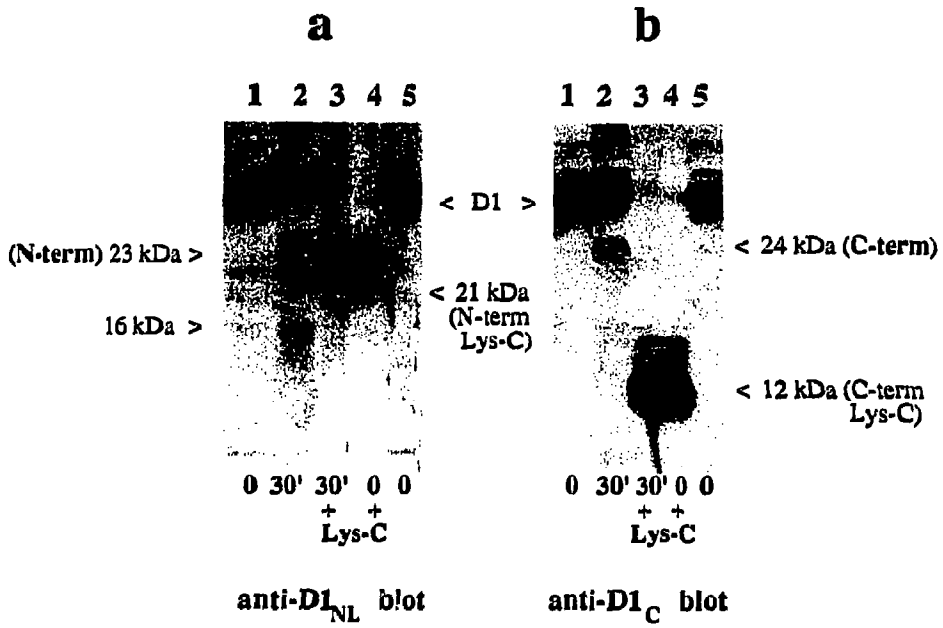

Fig. 5. Immunoblot of control (zero time) and light-treated PSII core complexes (a) in 50 mM MES–NaOH, pH 6.0, with 5 mM $CaCl_2$ detected with anti-$D1_{NL}$ (N-terminal antibody), or (b) in 50 mM Tris–HCl, pH 8.0, with 0.2 mM DBMIB detected with anti-$D1_C$ (specific C-terminal antibody). The samples were subjected to 30 min of photo-inhibitory illumination (lane 2) and subsequently digested with Lys-C (lane 3). Lanes 1 and 5 are controls and lane 4 the control digested with Lys-C.

on the 23 and 24 kDa fragments of the D1-protein. Under conditions of oxygen evolution the appearance of the 23 kDa and 16 kDa fragments can be seen (lane 2 of Fig. 5a) using the N-terminal antibody, anti $D1_{NL}$. Lys-C digestion clearly shows (lane 3 of Fig. 5a) that the 23 kDa band shifts to a slightly lower molecular weight indicating that only a small portion of the fragment is removed on the C-terminal side of lysine residue 238 (see also Fig. 4a). As can be seen by comparing lanes 3 and 4 in Fig. 5a, this Lys-C-cut fragment ran at a slightly higher molecular weight than the Lys-C-cut fragment generated with dark-treated material. This upward whift in the apparent molecular weight after photo-inhibitory treatment is also seen with the intact D1-protein (e.g. compare lanes 1 and 2 in Fig. 5a). Although the 16 kDa fragment was detected with the N-terminal antibody, it was completely lost after Lys-C treatment indicating that the lysine residue is probably located in the middle of the fragment. This suggests that this fragment contains the C-terminus of the D1-protein as argued previously [18,19]. Fig. 5b shows an immunoblot with anti-$D1_C$ after photo-inhibitory treatment of the PSII core complex under non-oxygen-evolving conditions and with DBMIB present as an acceptor. As with anti-$D1_{CL}$, this antibody clearly recognises the 24 kDa photo-induced fragment and further shows that it is digested by Lys-C to lower molecular weight products, as expected if it contains the C-terminus of the D1-protein.

4. DISCUSSION

In this study we have clearly demonstrated using an isolated PSII core complex that, depending on the experimental conditions employed during the photo-inhibitory treatment, the D1-protein undergoes primary cleavage in two different sites. This yields two fragments of about 23–24 kDa that differ in origin, one containing the N-terminus and the other the C-terminus of the D1-protein. The 24 kDa C-terminal fragment is generated under conditions when the donor side of PSII is inhibited and therefore is not only observed in PSII cores having their water splitting reactions inhibited by high pH as shown here, but also in isolated PSII reaction centres that are not able to evolve oxygen [9,11,18]. In both experimental systems the appearance of the C-terminal-containing fragment requires the presence of an electron acceptor so as to allow long-lived oxidised species to accumulate on the donor side of the reaction centre [9]. It is therefore proposed that in some way, possibly by destruction of secondary pigments [26,27], these species trigger the proteolytic hydrolysis of a peptide bond in the polypeptide loop spanning transmembrane segments I and II at the lumenal side

of the thylakoid membrane [18,28]. In so doing fragments of about 24 kDa and 10 kDa are generated, where the former contains the C-terminus and the latter the N-terminus of the D1-protein.

On the other hand, we have shown that when water splitting is active and therefore electron donation to $P680^+$ is occurring (pH 6.0, plus 5 mM $CaCl_2$), our isolated PSII cores give rise to 23 kDa and 10 kDa fragments derived from the N-terminus and C-terminus of the D1-protein, respectively. In this case the degradation is enhanced when no exogenous quinone acceptor is added. The action of Lys-C on these fragments indicates that the photo-induced cleavage is occurring in the region connecting transmembrane segments IV and V on the stromal side of the membrane, as argued previously [2,16,17,19]. The effect of Lys-C proteolysis also shows that this cleavage site is on the C-terminal side of lysine residue 238, probably in the QEEET motif as suggested in [16]. Our results suggest that, in this case, the trigger for light-induced proteolytic cleavage involves 'acceptor-side' inhibition, possibly by enhancing the recombination of the radical pair $P680^+Pheo^-$ and the generation of singlet oxygen via the P680 triplet state [29–31]. Indeed it has been demonstrated previously that 'acceptor-side'-induced photo-inhibition only leads to irreversible damage and D1-protein degradation if oxygen is present [31]. In contrast the degradation of the D1-protein due to 'donor-side' photo-inhibition occurs under both aerobic and anaerobic conditions in isolated reaction centres [9,11] and PSII-enriched membranes [32].

The results and discussion reported above rationalize the problem concerning the origin of the photo-induced D1-fragments of about 23–24 kDa that have been identified previously as a single fragment and attributed to either the C-terminal [18] of N-terminal [19] region of D1-protein. At the same time, the results give experimental support to the concept of a double mechanism of photo-inactivation of the electron transport reactions in PSII, one originating from acceptor-side damage and the other from donor-side damage [6,7,31–33]. It now seems clear that the light-dependent inhibition of electron transfer on the donor- or acceptor-side of PSII can damage the D1-protein and trigger its degradation on either the lumenal or stromal side of the thylakoid membrane. It is highly likely that selective conformational changes activate and dictate the site of the cleavage reactions.

Finally it is appropriate to point out that the yield of either the 23 or 24 kDa fragments will not be 100%. Rather it seems that depending on the experimental conditions there is likely to be a mixture of the two photo-induced fragments. Thus for the in vivo situation the origin of the D1-protein fragments will be dependent on the balance between the two mechanisms.

The work presented here is clearly a significant step forward in our understanding of the degradation processes of the D1-protein but there remains a number of unknowns. Among these are the identification of the precise cleavage sites that require investigation by N-terminal sequencing or analyses by mass spectrometry. Also the details of the enzymology which gives rise to the proteolytic cleavages have to be unravelled.

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