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Light-induced degradation of D2 protein in isolated photosystem II reaction center complex

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When isolated photosystem II reaction centers from spinach are exposed to photoinhibitory light in the presence of an electron acceptor, breakdown products of the D2 protein at 28, 25, 23, 18, 9, 5 and 4.5 kDa are detected by immunoblotting with a monospecific anti-D2 polyclonal antibody. In a time-course experiment the 23 and 4.5 kDa fragments show a transient appearance, whilst the others are photoaccumulated. The regions of the D2 protein containing the cleavage sites for the 28 and 18 kDa photoinduced fragments have been identified. Significant degradation of D2 takes place only in the presence of an electron acceptor, and breakdown of the protein is partially prevented by serine-type protease inhibitors.

D2 protein; Photoinhibition; Photosystem II

1. INTRODUCTION

Light-induced inactivation of oxygenic photosynthesis, a phenomenon referred to as photoinhibition [1], consists of the impairment of the activity of photosystem II (PSII). The reaction center complex of PSII (RCII) contains the D1 and D2 proteins, α- and β-subunits of cyto b_{559} [2,3] and the 4.8 kDa protein encoded by the plastidial *psb*I gene [4]. Four to six chlorophylls *a* (including the primary donor P_{680}), 2 pheophytins *a*, and 2 β-carotene molecules are bound to this protein complex [5,6].

In vivo, the D1 subunit is characterized by very rapid turnover [7] which has been linked with photoinhibition, since D1 is degraded and resynthesized in the PSII repair cycle [8]. Under photoinhibitory conditions, the D2 protein also turns over more rapidly [9], but this aspect of photoinhibition has not been further investigated.

Degradation of D1 has been widely studied in simple systems such as isolated thylakoids [10,11], detergent-derived, oxygen-evolving particles [12,13] or even isolated RCIIs [14–16]. Some light-induced fragments have been isolated and characterized [15] and there is now agreement that degradation of the protein may proceed along different pathways, depending on whether its degradation is triggered by damage at the donor or acceptor sides [17–19]. Some photodegradation products of the D2 protein have also been detected [12,14]. Fragments of 19–21 and 29 kDa have been identified during high light illumination of PSII core [12] and isolated RCII preparations [14]. However, no information is yet available on the region of the protein containing the cleavage sites.

In this study we analyse in more detail the degradation of the D2 protein in isolated RCII. By using a polyclonal antibody against D2 from spinach, we have observed at least 7 photoinduced fragments with apparent molecular weights of 28, 25, 23, 18, 9, 5 and 4.5 kDa. The regions of the protein containing the cleavage sites for the 28 and 18 kDa fragments have been identified. The experimental conditions under which D2 degradation was observed were the same as those leading to fragmentation of D1, namely requiring the presence of an electron acceptor. Degradation of both proteins is partially prevented by PMSF and soybean trypsin inhibitor.

2. MATERIALS AND METHODS

Isolation of RCII was carried out as detailed in [20]. Illumination of isolated RCII, SDS-PAGE and immunoblotting were performed as previously described [15]. The antigen used to obtain a polyclonal antibody directed against the D2 protein was isolated by preparative electrophoresis of isolated reaction center complex purified as described in [21]. Proteolysis on two-dimensional gels was performed as follows: a gel lane from the first dimension was loaded onto a second gel, the stacking of which contained trypsin at a concentration of 100 μg/ml. When the chlorophyll reached the lower limit of the stacking gel, the current was switched off for 45 min to allow trypsinization of

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Abbreviations: cyt, cytochrome; DBMIB, 5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; PMSF, phenyl-methane-sulphonyl fluoride; PSII, photosystem II; PVDF, polyvinylidene difluoride; RCII, reaction center complex of photosystem II.

polypeptides. Then electrophoresis was performed as usual. For N-terminal sequencing, tryptic peptides were resolved by SDS-PAGE, electroblotted to PVDF-type membranes (Pro Blott, Applied Biosystem) according to [22], and subjected to automated Edman degradation using an Applied Biosystem protein/peptide Sequencer, model 477A.

3. RESULTS

Fig. 1 shows the result of an immunoblot with an anti-D2 polyclonal antibody of isolated RCII illuminated with 4,500 $\mu E \cdot m^{-2} \cdot s^{-1}$ for different periods of time in the presence of 0.2 mM DBMIB. In the dark control (lane 1), the D2 protein and a band at about 60 kDa are detected, the latter being composed of a D1/D2 and/or a D2/D2 dimer [23]. Already after 5 min of illumination, a new band appears at 23 kDa (lane 2). Its amount increases with illumination time (lanes 2–5) but, after 60 min of illumination, it disappears from the blot (lanes 6–8). The same behaviour is also observed for a 4.5 kDa peptide (lanes 3–5). Other bands corresponding to D2 fragments appear at 28, 25, 18, 9 and 5 kDa (lanes 3–8), and a band at 44 kDa also becomes evident. Unlike the 23 and 4.5 kDa bands, which show a transient appearance during the time-course illumination, these bands are photoaccumulated during light exposure.

Degradation of D1, whose time-course photoinhibition is shown for comparison in Fig. 2, is thought to be a proteolytic reaction catalyzed by a serine-type [24,25]

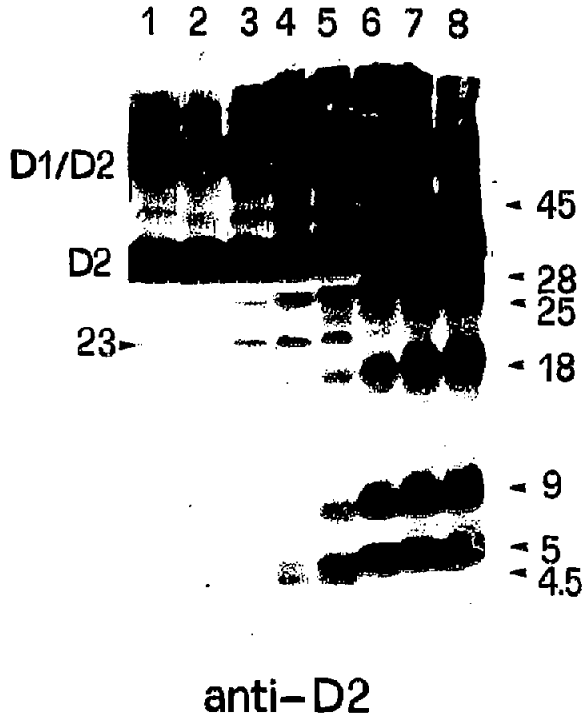

Fig. 1. Immunoblot with an anti-D2 polyclonal antibody of isolated RCII illuminated for different periods of time as described in section 2. Lanes 1–8: samples illuminated respectively for 0, 5, 10, 20, 30, 60, 90 and 120 min. Numbers at the left and right of the panel refer to the apparent molecular weights of photoinduced fragments.

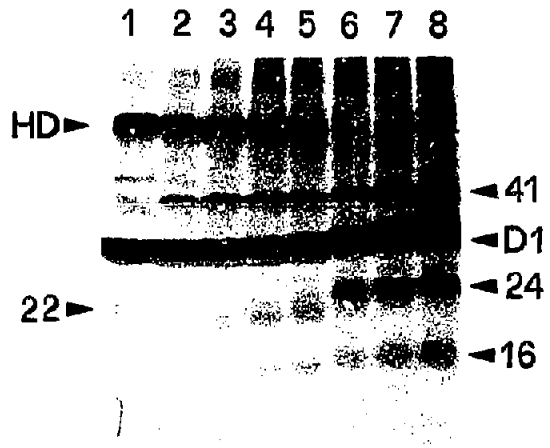

Fig. 2. Immunoblot with an anti-D1 polyclonal antibody of isolated RCII illuminated for different times as in Fig. 1.

intrinsic protease [12,14] and, in the case of isolated RCII, it depends on the presence of an electron acceptor, i.e. on the accumulation of highly oxidizing species at the donor side of PSII [14]. It is therefore of importance to check if D2 degradation also depends on the presence of an electron acceptor and if it is inhibited, at least in part, by the presence of PMSF and soybean trypsin inhibitor. Fig. 3A shows an immunoblot with anti-D1 (C-terminal specific). D1 fragments are barely detected in the absence of the electron acceptor (lanes 2 and 3), but the typical 24 and 16 kDa breakdown products become apparent when DBMIB is present (lanes 4 and 5). When soybean trypsin inhibitor is included during illumination, the amount of detected fragments is fairly low (lanes 6 and 7), confirming the results previously reported in [16]. When an identical blot is reacted with the anti-D2 polyclonal, a very similar result is obtained (Fig. 3B): no fragments are detected when illumination is performed in the absence of electron acceptor (lanes 2 and 3); they become apparent in the presence of 0.2 mM DBMIB (lanes 4 and 5; see also Fig. 1), and their amount is lower when soybean trypsin inhibitor is included during illumination (lanes 6 and 7). Similar results are obtained when soybean trypsin inhibitor is substituted by PMSF (not shown).

The problem of identifying the cleavage sites on D2 was approached by studying trypsinolysis of D2 and its photoinduced breakdown fragments on two-dimensional gels, on which tryptic fragments are expected to appear as off-diagonal spots. Thus, after photoinhibition, RCII polypeptides were resolved by SDS-PAGE and subjected to limited proteolysis on a second gel whose stacking contained trypsin at a concentration of 100 μg/ml. The results are shown in Fig. 4, in which tryptic fragments were detected by immunoblotting with the anti-D2 polyclonal antibody. D2 is cleaved by trypsin, giving rise to four immunodetectable fragments at 24, 20, 12 and 10 kDa. The 12 kDa tryptic fragment

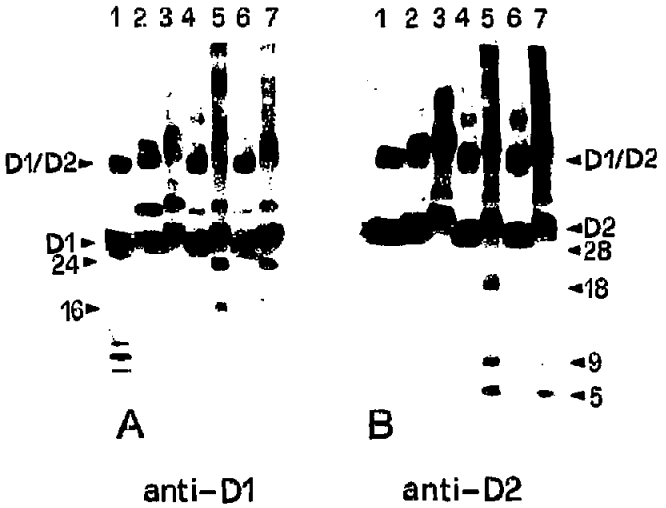

Fig. 3. Immunoblot with anti-D1 (A) and anti-D2 (B) polyclonal antibodies of isolated RCII. Lanes 1, dark control samples; lanes 2 and 3, samples illuminated, respectively, for 10 and 30 min in the absence of DBMIB; lanes 4 and 5, as in lanes 2 and 3 but illuminated in presence of 0.2 mM DBMIB; lanes 6 and 7, as lanes 4 and 5 but illuminated in presence of 1 mg/ml of soybean trypsin inhibitor.

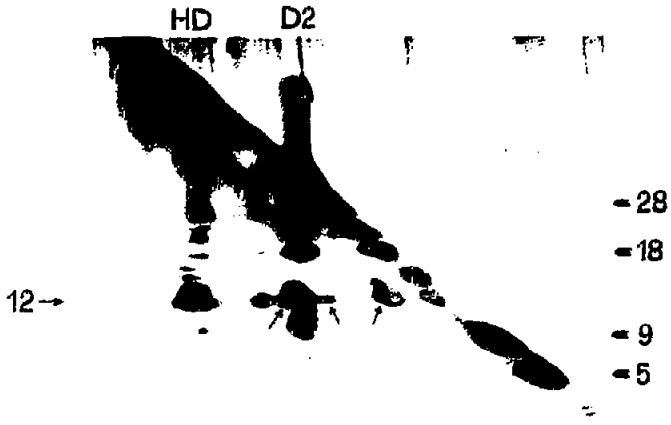

Fig. 4. Immunoblot with an anti-D2 polyclonal antibody of a two-dimensional gel in which polypeptides of photoinhibited RCII resolved in the first dimension (left to right) were subjected to limited proteolysis with trypsin in the second dimension (top to bottom). Some off-diagonal spots appear after trypsinolysis of heterodimer (HD). D2 and some photoinduced D2 breakdown products. The same 12 kDa fragment (indicated as 12 to the left) is detected from digestion of heterodimer, D2, 28 and 18 kDa photoinduced fragments (→). By running isolated D2 protein on the same gel, a 12 kDa tryptic fragment was blotted to PVDF-type membrane, Coomassie stained and subjected to N-terminal sequencing (see Table I).

is also detected as an off-diagonal spot from the proteolysis of the 28 and 18 kDa photoinduced bands. To identify the trypsinolytic site giving rise to the 12 kDa fragment, isolated D2 protein was digested inside the gel, the fragments were electroblotted onto a PVDF-type membrane, and the 12 kDa one was subjected to N-terminal sequencing. The result, reported in Table I, indicates that cleavage involves the Arg-234/Ala-235 peptide bond. Since the photoinduced fragments at 28 and 18 kDa both give the 12 kDa tryptic fragment (see above), we conclude that they represent C-terminal portions of D2.

4. DISCUSSION

Turnover in the light of the D1 polypeptide is a well-established event [26]. Its breakdown products have been identified both in vivo [27] and in vitro [10,11], and the existence of specific pathways for D1 degradation depending on whether donor or acceptor side photoinhibition is involved, has been proven [17–19]. Acceptor side photoinhibition is thought to produce cleavage in the hydrophobic loop connecting the 3rd and 4th transmembrane segments [27], whilst donor side photoinhibition probably leads to a cleavage in the loop connecting the 1st and 2nd and the 3rd and 4th transmembrane segments [15].

Much evidence suggests that, like D1, D2 is also subjected to increased turnover under high light conditions [9], but no breakdown fragments have so far been detected in vivo. On the other hand, by illuminating PSII cores or isolated RCII, 19–21 kDa [12,14] and 29 kDa fragments [14] have been detected. In this work up to seven different degradation products were observed. The photoinduced fragments of 23 and 4.5 kDa are particularly interesting (see Fig. 1, lanes 2–5) due to their transient appearance during the first 30 min of illumination. The time domain for the production of these two fragments is therefore similar to that for electron transport deactivation of PSII particles under similar photoinhibitory conditions [13], and they probably represent the first cleavage products which are later further degraded. In contrast, the fragments at 28, 18, 9 and 5 kDa are photoaccumulated after longer illumination time, i.e. when electron transport activity is already completely lost. This situation is similar to that of the D1 protein, in which a 22 kDa fragment (whose origin is under investigation, but see [17]) is detected in the first few minutes of illumination, whilst the characteristic 24 and 16 kDa fragments are photoaccumulated after longer illumination time, when most of the 22 kDa fragment has already been further degraded (Fig. 2).

Of the six photoinduced fragments detected through this work, two (i.e. 28 and 18 kDa) were further characterized as C-terminals of the protein. In fact both of them contain the 12 kDa tryptic fragment which was proven to be C-terminal. From the difference in their apparent molecular weight with that of the tryptic fragment, it follows that they are produced, respectively, by cleavage at the hydrophilic loops connecting the 1st and 2nd and the 3rd and 4th transmembrane segments, as for D1 [15].

Table 1

N-terminal sequence analysis of a 12 kDa fragment obtained from tryptic digestion of the D2 protein

Cycle	Amino acid	Yield (pmol)
1	Ala	22.9
2	Phe	20.5
3	Asn	19.6
4	Pro	19.4
5	Thr	6.7
6	Gln	9.9
7	Ala	9.8
8	Glu	6.4
9	Glu	7.2
10	Thr	4.7

Also similar to the donor side-induced degradation of D1, the presence of an exogenous electron acceptor is required for cleavage of D2, suggesting a similar triggering mechanism. Again in analogy to that which is observed for D1, D2 photodegradation is significantly lowered in the presence of soybean trypsin inhibitor, suggesting the involvement of serine-type proteolytic activity. In conclusion, the mechanism by which D2 is degraded seems to be quite similar to that which acts on D1. This is not surprising when the strong similarities between the two proteins, at the functional and structural levels, are considered.

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