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Characterization of a 41 kDa photoinhibition adduct in isolated photosystem II reaction centres

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When isolated reaction centres of photosystem II are subjected to photoinhibitory illumination, a 41 kDa SDS-PAGE band is observed under all experimental conditions. The same band is also found, together with lower molecular weight fragments of the D1 protein, in whole thylakoids and in all PSII sub-particles investigated up to now. In the case of isolated reaction centres the 41 kDa band is represented by a heterodimer of the D1 polypeptide and the α-subunit of cytochrome b_{559}. The cross-linkage between D1 and α-cyt b_{559} involves a region on D1 between the N-terminal residue and Arg-225, and is an early event in photo-induced damage to the D1 protein.

D1 protein; Cytochrome b_{559}; Photoinhibition; Reaction centre; Photosystem II

1. INTRODUCTION

Irreversible inactivation of the photosystem II (PSII) reaction centre (RCII) is the origin of the complex phenomenon of photoinhibition by which higher plants, algae and cyanobacteria decrease their photosynthetic yield when exposed to an excessive flux of light [1]. It has been clearly established that inactivation of photochemical activity is followed by dismantling of the damaged PSII RC, involving proteolytic degradation of the D1-protein [2,3]. The latter process is supposed to be catalyzed by serine-type protease(s) [4,5] closely associated with the reaction centre complex [6,7]. Recent data suggest that more than one proteolytic activity may be involved in degradation of the D1 protein, acting either on the stroma- or lumenal-exposed hydrophilic loop of the protein. For the former, CP43 has been proposed as the active agent [8], while for the latter autoproteolytic activity of the reaction centre itself has been hypothesized [7]. A number of investigations have been undertaken on the phenomenology of D1 degradation and the characterization of its degradation products. Thus, a 23.5 kDa N-terminal fragment of D1 protein was first detected in vivo by Greenberg et al. [9] during light-induced turnover of D1, and a number of photo-induced fragments of the same protein were detected and characterized in in vitro systems, such as isolated thylakoids [2,10], various PSII preparations [6,11] and also in isolated reaction centres [7,12]. The latter system is particularly interesting as it contains only five polypeptides: D1, D2, the two subunits of cytochrome (cyt) b_{559} and the 4.8 kDa product of the psbI gene [13]. This is the simplest system which maintains the electron transport activity and which is inactivated and degraded by over- illumination. It has been found that in isolated reaction centres irreversible chlorophyll and carotenoid bleaching – are events induced under photoinhibitory conditions which precede degradation of the D1 protein [14]. Moreover, fragmentation of D1 is not the only event following the inactivation of electron transport. Besides the reaction centre polypeptides and D1 fragments, additional bands have been detected in the SDS-PAGE of photoinhibited reaction centres with molecular weights higher than that of the D1 protein. One of the detected bands, also observed in vivo and defined as 32* [15], has been assigned to a phosphorylated form of the D1 protein [16]. A second band at 37–41 kDa appears after photoinhibition of isolated thylakoids [2], as well as in all PSII preparations so far analysed, including isolated reaction centres [6,7,11]. Although this band is usually considered as a D1/D2 heterodimer truncated in D1, its identity has not yet been firmly established. This point is examined here and evidence is provided to show that this band is a cross-linking product of D1 with the α-subunit of cyt b_{559}.

Abbreviations: CP, chlorophyll protein; cyt, cytochrome; DBMIB, 5-bromo-3-methyl-6-isopropyl-p-benzoquinone; PAGE, polyacrylamide gel electrophoresis; PS II, photosystem II; RC II, reaction centre of photosystem II; SDS, sodium dodecylsulphate.

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2. MATERIALS AND METHODS

Isolation of the reaction centre of PSII consisting of D1, D2, α and β subunits of cyt b_{559} and of I protein (RCII), from both spinach and wheat PSII membranes, was performed as described by Chapman et

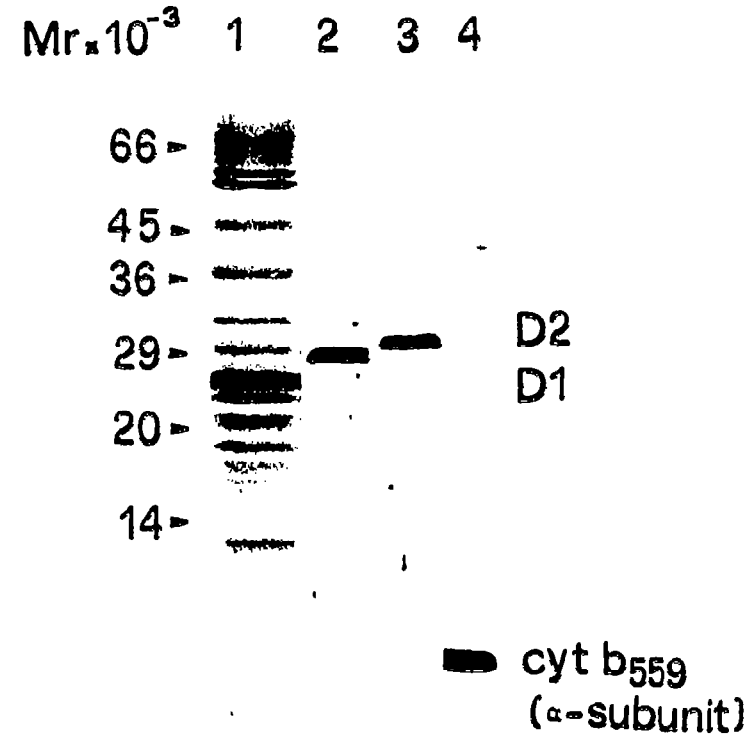

Fig. 1. (Lane 1) Coomassie blue-stained electrophoretic profile of thylakoids. (Lanes 2–4) Immunoblots of the gel shown in lane 1 with a mixture of polyclonals recognizing, respectively, N-terminal (anti-D1N) and C-terminal (anti-D1C) portions of D1 (lane 2), D2 protein (anti-D2) (lane 3) and the α-subunit of cyt b_{559} (lane 4).

al. [17]. Illumination of RCII was carried out as described by Shipton and Barber [7].

SDS-PAGE and immunoblotting procedures were those already described in Barbato et al. [10].

For two-dimensional proteolysis, a modification of the original procedure of Cleveland et al. [18] was used. A gel lane from the first dimension was loaded onto a second identical gel, whose stacking 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 polypeptides. Then electrophoresis was performed as usual.

To raise monospecific polyclonals against the α-subunit of cyt b_{559}, the RCII polypeptides were resolved by preparative electrophoresis and, after a short staining, the band corresponding to α-subunit of cyt b_{559} was cut from the gel and electroeluted. The immunization schedule and bleeding of the rabbit were according to standard procedures [19].

Spectral analysis was performed on an HP 8452 diode array spectrophotometer. Chemically induced difference spectra (ferricyanide oxidized minus dithionite reduced) of cyt b_{559} were obtained as previously described [20].

3. RESULTS

We tested the 41 kDa band resulting from illumination of isolated reaction centres in the presence of 0.2 mM DBMIB with a series of antibodies raised against individual proteins of the PSII reaction centre. The monospecificity of the antibodies used is shown in Fig. 1. A mixture of two antibodies, anti-D1N and anti-D1C, which recognize, respectively, the 1–238 N-termi-

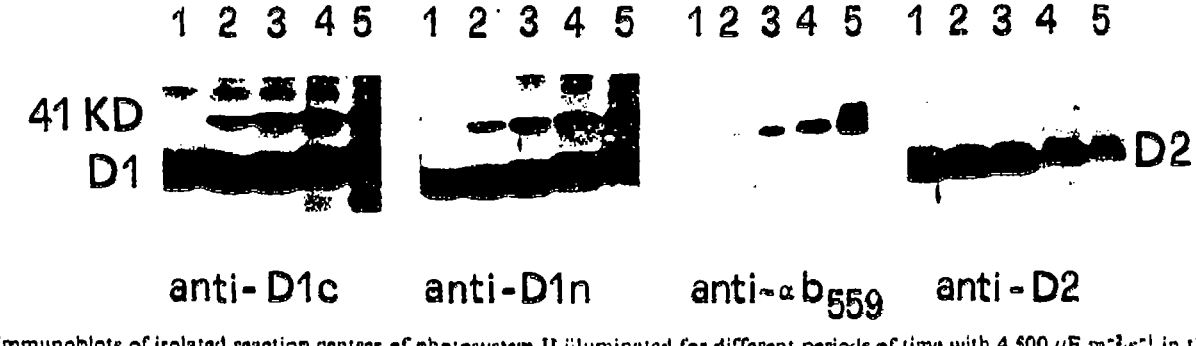

Fig. 2. Immunoblots of isolated reaction centres of photosystem II illuminated for different periods of time with 4,500 μE $m^{-2} \cdot s^{-1}$ in the presence of 0.2 mM DBMIB. Samples were illuminated, respectively, for 0, 10, 20, 30 and 60 min (lanes 1–5).

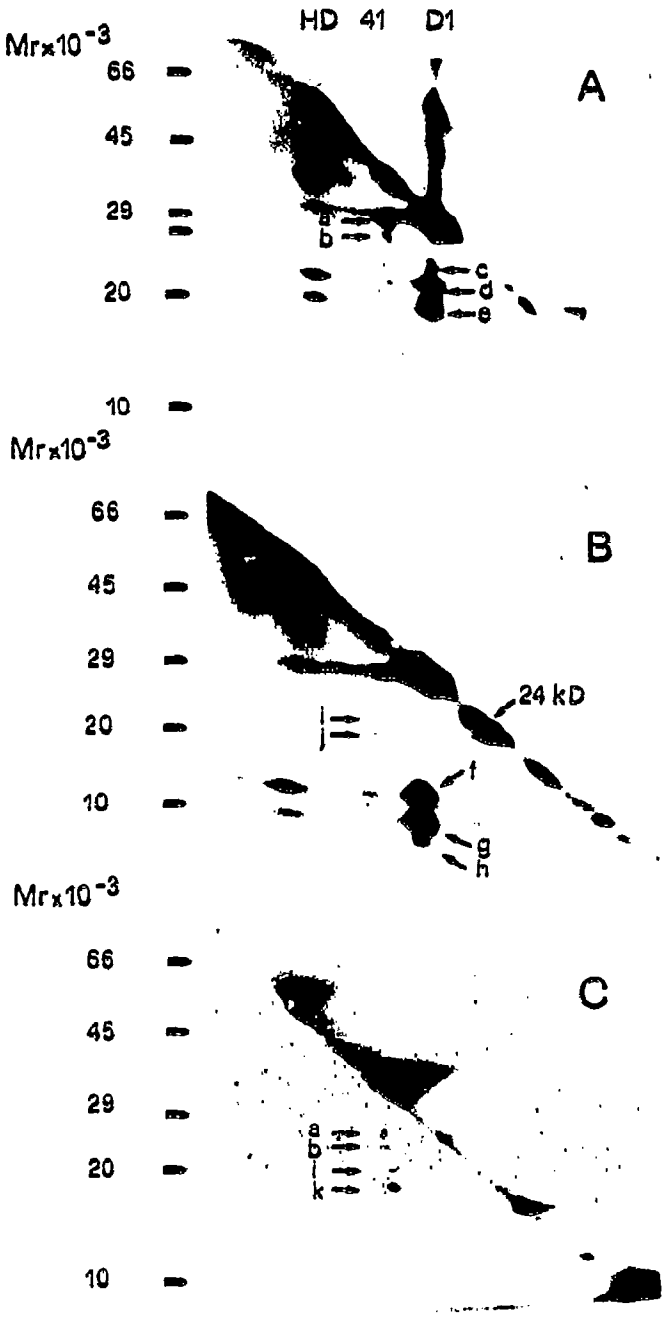

Fig. 3. Immunoblots with anti-D1N (A), anti-D1C (B) and anti-α-cyt b_{559} (C) of photoinhibited reaction centre II subjected to SDS-PAGE and limited proteolysis in the second dimension (see text). Isolated reaction centres were photoinhibited with 4,500 μE $m^{-2} \cdot s^{-1}$ for 30 min.

nal portion and the 239–344 C-terminal portion of D1, were used in lane 2. Both antibodies recognize the entire D1 polypeptide, but each one is specific for a different region of the protein [12]. The anti-D2 (lane 3) and anti-α cyt b_{559} (lane 4) polyclonals are highly specific for their antigens and no cross-reaction with other thylakoid polypeptides is observed.

Fig. 2 shows the 41 kDa band formed by illumination of RCII, probed with the series of antibodies. The 41 kDa band is clearly an early product of photoinhibition, appeared already after 5 min illumination (lane 2), and this photoproduct is recognized by both anti-D1 polyclonals and polyclonals against the α-subunit of cyt b_{559}. In contrast, it is not recognized by the anti-D2 polyclonal, not even when the blotting is sufficiently over-developed to detect some D2 breakdown fragments (not shown). This result allows us to asign the 41 kDa band to a heterodimerization product between the D1 polypeptide and the α-subunit of cyt b_{559}.

A further characterization of the 41 kDa photo-induced band was attempted by limited proteolysis on two-dimensional gels followed by immunodetection of the tryptic fragments. Polypeptides of photoinhibited RCII were resolved in the first dimension and then subjected to tryptic digestion on the top of the second dimension gel (see Materials and Methods).

Fig. 3 shows the results after immunoblotting the gels with the anti-D1N, anti-D1C and anti-cyt b_{559}. The anti-D1N polyclonal (Fig. 3A) recognizes two main tryptic fragments of D1 (marked as d and e) with apparent molecular weights of about 22 and 20 kDa and, to a lesser extent, a fragment (marked c) with a molecular weight of about 24 kDa. Fragments d and e are also found among the tryptic products of the D1/D2 heterodimer and the 41 kDa band. Trypsinization of the 41 kDa band gives rise to two more fragments (a and b) having molecular weights of about 29 and 27 kDa, which are not present either in the proteolysis of the D1/D2 heterodimer or presumably in that of D1. The strong reaction of this antibody with the D1 protein prevents a direct check of the absence of fragments a and b among the proteolysis products of D1, but if they were present they should also appear in the heterodimer proteolysis.

Using anti-D1C on the same blot, a different set of off-diagonal spots was identified (Fig. 3B). In fact, this polyclonal identifies two D1 fragments at about 12 and 10 kDa (spots f and g) and, to a lesser extent, a 7–8 kDa band, marked h. Spots f and g are also detected in the proteolysis of the D1/D2 heterodimer, the 24 kDa photo-induced D1 fragment and the 41 kDa band. In addition, trypsinization of the 41 kDa band gives rise to two additional spots at about 22–20 kDa, which are also detected in the proteolysis of the D1 protein (i and j).

When the immunoblot is reacted with anti-α cyt b_{559} (Fig. 3C), at least four off-diagonal spots with apparent molecular weights ranging from 29 to 20 kDa are obtained from trypsinization of the 41 kDa band. Careful comparison of the three immunoblots, as well as reprobing the same blot with different mixtures of polyclonals (not shown), indicate that the first two spots identified by the anti-α cyt b_{559} are the same as those recognized by the anti-D1N, i.e. spots a and b. The third spot is probably the same as i (detected by anti-D1C),

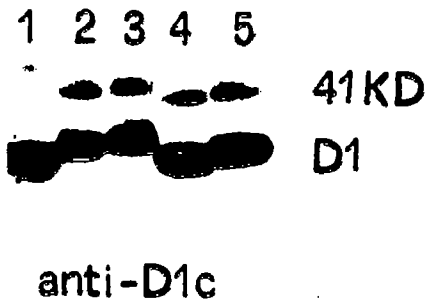

Fig. 4. Immunoblot with anti-D1C of isolated reaction centres under different experimental conditions: (lane 1) dark control; (lanes 2–5) after 30 min illumination in the presence of (lane 2) no addition, (lane 3) 1 mM $MnCl_2$, (lane 4) 1 mM $MnCl_2$ plus 0.2 mM DBMIB, (lane 5) 0.2 mM DBMIB.

while the fourth (k) co-migrates with d, although its identity is uncertain.

In Fig. 4 the appearance of the 41 kDa band upon photoinhibition was checked under different experimental conditions. It is clear that the formation of this cross-linking product is not related to the presence or absence of electron donors and acceptors.

As reported by Telfer et al. [14], photoinhibition is accompanied by a quick bleaching of the reaction centre carotenoids and chlorophyll absorption. Fig. 5 compares these phenomena with the loss of the chemically induced difference spectrum of cyt b_{559} at 560 nm. The bleaching of chlorophyll is approximately linear with the illumination time, whereas the carotenoid band disappears faster and exponentially. The amount of dithionite reducible cyt b_{559} remains constant during the first 30 min of illumination, i.e. during a time period in which the 41 kDa band is completely formed and all the carotenoids are bleached (see insert in Fig. 5). This result suggests that the cross-linkage formation between the D1 polypeptide and the α-subunit of cyt b_{559} is an early event which parallels the bleaching of the two carotenoid molecules in the reaction centre. Moreover, cross-linking with D1 does not involve extensive unfolding of the cytochrome structure since no loss of the reduced-oxidized difference spectrum is observed.

4. DISCUSSION

In vivo and in vitro extended photoinhibition generates aspecific aggregation of thylakoid proteins, which manifests itself as diffuse high molecular weight bands in gels and Western blots. However, some specific bands induced by photoinhibition are reproducibly formed in the region of molecular weights higher than that of the reaction centre polypeptides. Among these, the 41 kDa band attracted our attention because it is constantly present in the gels of the photoinhibited samples and is accumulated during the early phases of the process.

Because of the cross-reactivity of the bands in this region of the gel with anti-D2 polyclonals, it has been suggested that the 41 kDa band represents a D1/D2 heterodimer truncated in D1 [2,4] or in both D1 and D2 [10]. The results reported here demonstrate the existence in this region of the gel of a heterodimer containing D1 and the α-subunit of cyt b_{559}, and that this product is the only one significantly present when isolated reaction centres are photoinhibited.

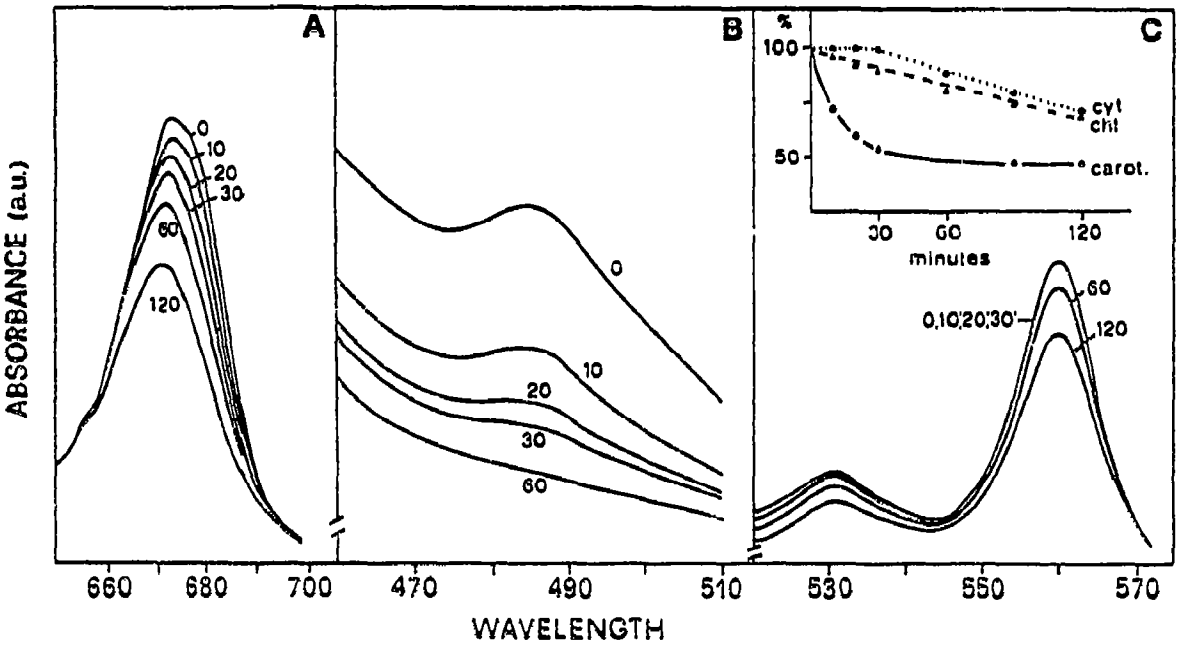

Fig. 5. Optical absorption spectra of a solution of isolated reaction centres after various exposures to photoinhibitory light. (Panels A–C) Absorption regions for chlorophylls, carotenoids and cyt b_{559} respectively. The scales of B and C are amplified, respectively, 2- and 20-fold (Insert) Absorption time-course in different spectral regions.

The fact that both anti-D1N and anti-D1C polyclonals recognize the 41 kDa band, and that all the tryptic fragments obtained from D1 are also found as proteolytic products of the 41 kDa adduct, indicates that the entire D1-protein is contained in this band. Moreover, analysis of partial proteolysis provides some information on the region of D1 interacting with cyt b_{559}. A careful tryptic map of the D1-protein from *Spirodella oligorhiza* has been obtained by Marder et al. [22]. Two N-terminal fragments of 22 and 20 kDa (called T22 and T20, respectively) deriving from cleavage at Arg-225 and Arg-238 were identified. This assignment of the tryptic sites on D1 was confirmed by N-terminal sequencing of the fragments (unpublished results). These two fragments most probably coincide with the two N-terminal fragments which we call d and e (Fig. 3A). If this is so, fragments f and g detected by our anti-D1C must represent the remaining part of D1. Thus, cleavage at Arg-225 gives rise to fragments e and f, and cleavage at Arg-238 to fragments d and g. Since fragments a and d differ in molecular weight by about 9–10 kDa, which is the molecular weight of the α-subunit of cyt b_{559}, and the same is true for fragments b and e, the obvious interpretation is that fragments a and b represent the same trypsinization products as d and e, but with the α subunit of cyt b_{559} attached to them. We may therefore conclude that the site of linkage between the D1 protein and cyt b_{559} involves a region of D1 before Arg-225. Unfortunately, no further mapping of the interaction site between the cytochrome and D1 was possible, since attempts to obtain trypsinization of the α subunit of cytochrome gave no results.

The nature of the interaction between D1 and the α subunit of cyt b_{559} also remains to be established. This band is not sensitive to reducing agents such as mercaptoethanol or dithiotreitol, indicating that no disulfide bridge is involved in its formation. One possibility is that cross-linking take place as a consequence of secondary oxidation of amino acid side chains brought about by the strong oxidants generated at the donor side under these conditions. In any case, the bond formation between these two polypeptides must involve residues which are located at interacting distance in the native structure of the RCII, and the linkage reaction does not perturb the cytochrome structure sufficiently to affect heme absorption at 560 nm.

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