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A novel degradation pathway of the D1 protein of photosystem II via covalent cross-linked adducts under illumination *in vivo*

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Introduction

The D1 protein of the PSII reaction center is selectively damaged under strong illumination that causes photoinhibition of photosynthesis. The molecular mechanisms of damage to the D1 protein have been intensively studied *in vitro* while those *in vivo* have not been fully understood yet. In PSII preparations under photoinhibitory illumination, the D1 protein is either cleaved mainly at the loop connecting membrane helixes D and E (DE loop) or covalently cross-linked with other reaction center protein, the D2 protein or the α-subunit of cytochrome *b*559, to generate the heterodimer or the 41-kDa adduct (Aro et al. 1993). By contrast, studies *in vivo* have suggested that the D1 protein is cleaved in a site distinct from that *in vitro* and that cross-linking rarely occurs (Kettunen et al. 1996). To bridge a gap between observations *in vivo* and *in vitro*, we compared damage to the D1 protein under photoinhibitory illumination in three materials, namely, leaf discs, intact chloroplasts, and thylakoids. We found that the initial damage to the D1 protein occurs in almost the same way *in vivo* and *in vitro* and that cross-linking is a process involved in complete degradation of the D1 protein *in vivo*.

Materials and Methods

Intact chloroplasts were isolated from spinach leaves by Percoll density gradient centrifugation, treated with thermolysin, and repurified by Percoll density gradient centrifugation (Mizusawa et al. 1999). Thylakoids and stromal proteins were isolated from the chloroplasts by hypotonic rupture followed by differential centrifugation (Mizusawa et al. 1999). The thylakoids were illuminated at 0.1 mE $m^{-2}s^{-1}$ in the presence of 0.4 mM ATP to phosphorylate thylakoid proteins (phosphorylation treatment; Mizusawa et al. 1999). The stromal proteins were dialyzed against 10 mM Hepes-NaOH (pH 7.8) at 4°C for 4 h. PSII complexes were prepared from wheat PSII membranes with *n*-heptyl-β-D-thioglucoside (Miyao 1994).

Photoinhibitory illumination of chloroplasts and thylakoids was performed at 25°C as described previously (Mizusawa et al. 1999), except that a suspending medium for chloroplasts contained 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, 0.33 M sorbitol, and 30 mM Hepes-KOH (pH 7.8). A final chlorophyll concentration of each suspension was 0.2 mg/ml. Photoinhibitory illumination of spinach leaf discs in the presence of lincomycin was performed according to Rintamäki et al. (1996). After illumination, the leaf discs were rapidly frozen in liquid nitrogen and thylakoids were isolated from the frozen discs for SDS-PAGE and immunoblot analysis.

SDS-PAGE and immunoblotting were performed essentially as described previously (Mizusawa et al. 1999). Antisera used were anti-D1 (a generous gift of Dr. M Ikeuchi) raised against the entire D1 protein of spinach, anti-$D1_C$ (a generous gift of Dr. T Ono) and anti-$D1_{DE}$ raised against a synthetic polypeptide that corresponded to the residues of 225-249 and 333-344 of the D1 protein of spinach, respectively.

The D1 protein and the heterodimer were isolated from wheat PSII complexes that had been illuminated at 0.1 mE $m^{-2}s^{-1}$ and 25°C for 30 min by SDS-PAGE and electroelution.

Results

Damage to the D1 protein under strong illumination was compared among thylakoids, intact chloroplasts and leaf discs under conditions in which the PSII activities were decreased to about 20% of original levels. Since substantially all the D1 protein is phosphorylated under illumination *in vivo* (Rintamäki et al. 1996), the thylakoids used in this study had been subjected to phosphorylation treatment prior to photoinhibitory illumination to phosphorylate the D1 protein. As seen in Fig. 1, the fragments and cross-linked adducts of the D1 protein were generated in almost the same way in all the three materials, although their levels were highest in thylakoids and greatly decreased in the chloroplasts and leaf discs. Fragments of the D1 protein derived from cleavage in the DE loop (the N-terminal fragment of 22 kDa and the C-terminal fragments of less

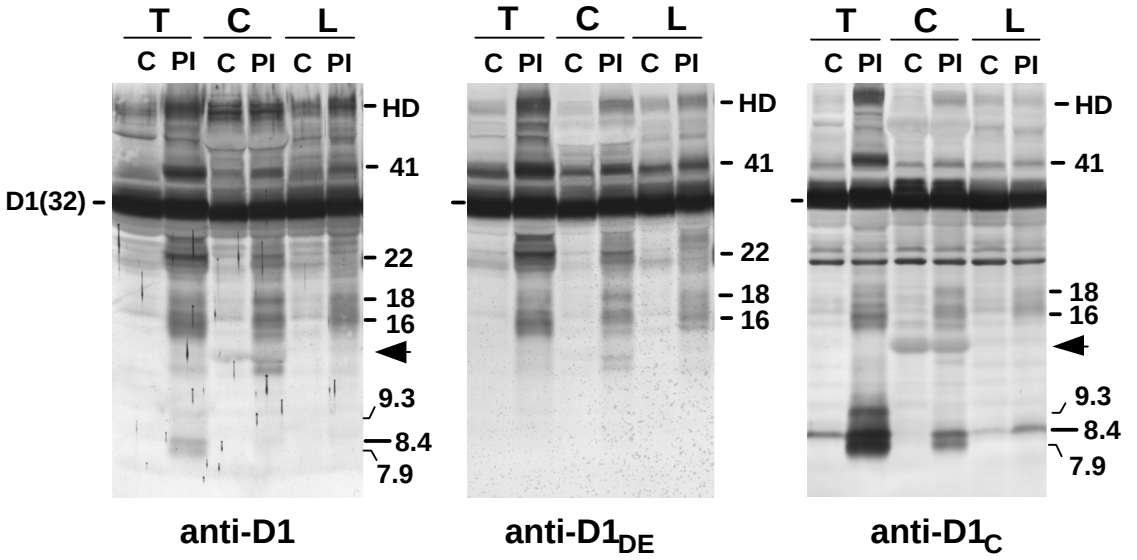

Fig. 1 Damage to the D1 protein under photoinhibitory illumination. Phosphorylated thylakoids (T), intact chloroplasts (C), and leaf discs (L) were illuminated at 25°C with white light at 1, 2.5 and 2.5 mE $m^{-2}s^{-1}$, respectively, until the PSII activities were declined to about 20% of original levels. Immunoblot profiles with three different antisera are shown. D1 and HD denote the D1 protein and the heterodimer, respectively. Arrowheads indicate a band of the small subunit of Rubisco, which cross-reacted with anti-D1 and anti-$D1_C$.

than 10 kDa) and those derived from cleavage inside or in the vicinity of helix D (the 16-18-kDa fragments) were detected, as were two different cross-linked adducts (the heterodimer and the 41-kDa adduct). These results indicate that the initial damage to the D1 protein occurs in almost the same way *in vivo* and *in vitro*.

The very low levels of fragments and adducts observed in chloroplasts and leaf discs (Fig. 1) suggested that they were rapidly digested and could not accumulate in these materials. To test this possibility, leaf discs were subjected to photoinhibitory illumination at 2°C, at which temperature protease activities were greatly suppressed. It was found that both fragments and adducts accumulated in leaf discs under illumination at 2°C to reach levels even higher than those at 25°C (data not shown). When leaf discs illuminated at 2°C were incubated in darkness at 25°C, the levels of fragments and adducts increased slightly within 10 min, subsequently decreased and finally disappeared after prolonged incubation for 1-2 h (data not shown). These results support the above possibility and suggest that the fragments and adducts were generated at 25°C but subsequently disappeared through digestion by proteases *in vivo*.

Since the fragments and adducts accumulated in thylakoids lacking the stroma, it is possible that a stromal protease(s) participates in digestion of fragments and adducts. This possibility was examined by incubating illuminated PSII complexes with stromal proteins in darkness (Fig. 2, PSII). By the incubation, the levels of the two adducts were reduced with concomitant generation of degradation products of 45-50 kDa, whereas the level of the D1 protein of 32 kDa was decreased only slightly. The preferential disappearance of the adducts but not the monomeric D1 protein in the presence of the stromal proteins was confirmed in the isolated D1 protein and heterodimer (Fig. 2, D1, HD). These results clearly indicated that a protease(s) present in the stroma selectively digested the heterodimer and the 41-kDa adduct but not the monomeric D1 protein. In the isolated D1 protein, a smeared band of 60-66 kDa was generated due to artificial cross-linking of two molecules of the D1 protein (the homodimer). The homodimer was also a substrate of the stromal protease (Fig. 2, D1).

This protease seems to be novel since its activity was not affected by either ATP or protease inhibitors (data not shown). Although preliminary, we also observed that an ATP-dependent protease(s) bound to the thylakoid membrane selectively digested the heterodimer (data not shown).

The cross-linking sites of the D1 protein with the D2 protein were examined by peptide mapping of the heterodimer. It was revealed that the cross-linking with the D2 protein occurred in residues 226-244 in the DE loop of the D1 protein (data not shown), which includes the cross-linking site with cytochrome *b*559 (Barbato et al. 1995).

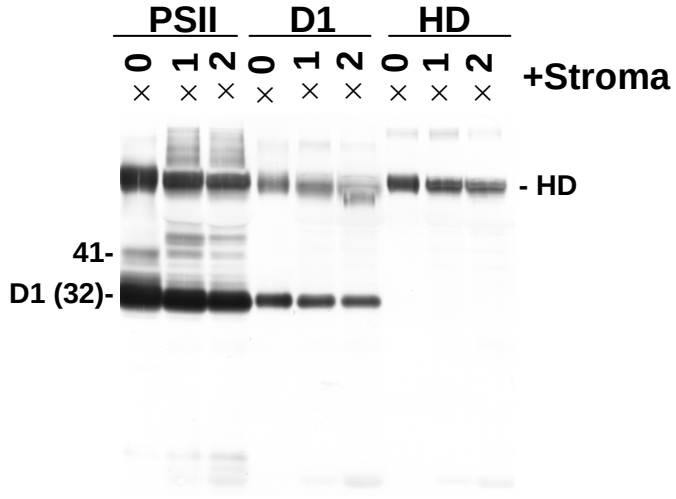

Fig. 2 Digestion of cross-linked adducts by the stromal proteins in darkness. Illuminated PSII complexes (PSII), the isolated D1 protein (D1) and the isolated heterodimer (HD) were incubated in the presence or absence of stromal proteins in darkness at 25°C for 30 min. Immunoblot profiles of anti-$D1_C$ are shown.

Table I. Protease activities to digest fragments and cross-linked adducts of the D1 protein detected in the stroma and the thylakoids

	Substrate					
	HD	41kDa	22 kDa	16-18 kDa	8.4 kDa	7.9 kDa
Stroma	+	+	±	+	–	–
Thylakoids	±	±	**?**	±	–	–
Thylakoids +ATP	+	–	**?**	–	–	–
Thylakoids +Zn^{2+}	–	–	**?**	–	+[a]	–

a: the activity to degrade the 8.4-kDa fragment to be 7.9 kDa
Protease activities detected in isolated thylakoids in the presence or absence of the stroma are listed.

Discussion

This study clearly demonstrated that complete degradation of the D1 protein under illumination proceeds in a stepwise manner *in vivo*. The first step is initial damage, namely, either cleavage or cross-linking that occurs in almost the same way as *in vitro* (Fig. 1). The sites of cleavage and cross-linking were both located in the DE loop of the D1 protein. Studies with isolated PSII preparations suggested that both cleavage and cross-linking are chemical processes caused by active oxygen species generated under illumination in PSII (Barbato et al. 1995; Miyao 1994; Miyao et al. 1995; Okada et al. 1996). It is also proposed that the DE loop has some sequences specifically susceptible to attack by active oxygen species since even when solubilized with SDS the cleavage by active oxygen occurs preferentially in this region (Okada et al. 1996). We presume that the initial damage to the D1 protein *in vivo* might also proceed chemically.

The second step involves digestion of the fragments and cross-linked adducts by proteases. As described above, at least two different proteases specifically digested the cross-linked adducts (see Table I). To our knowledge, this is the first evidence for involvement of specific intermolecular cross-linking reactions in degradation of proteins. It appears that the fragments are also digested by multiple proteases. The 16-18 kDa fragments were digested by the stromal protease(s), and the C-terminal 8.4 kDa fragment was degraded to be 7.9 kDa in a zinc ion-dependent manner in thylakoids (data not shown; see Table I). Proteases responsible for digestion of other fragments, however, remain unidentified. It is likely that the fragments and adducts are digested by a set of proteases that digest abnormal proteins.

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