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Specific degradation of D1 protein during exposure of thylakoid membranes to high temperature in the dark

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Abstract

Exposure of thylakoid membranes to high temperature in dark leads to the degradation of D1 protein. Maximum degradation of D1 protein occurred at 45 °C. Using N-terminal specific D1 antibody, a 23 kDa fragment of D1 protein was detected. The degradation of D1 protein could be prevented both by radical scavengers and inhibitors of serine protease and metallo-protease. These results suggest that degradation of D1 protein during exposure of thylakoid membranes to high temperature in dark is catalyzed by protease.

Additional key words: antibody; polyacrylamide gel electrophoresis; protease; *Triticum aestivum*; wheat.

Introduction

Photosystem (PS) 2 is a large multisubunit complex that catalyzes the reduction of plastoquinone and oxidation of water to produce molecular oxygen. The reaction centre in PS2 is composed of two highly hydrophobic proteins D1 and D2 which together bind most of the redox components involved in photosynthetic electron transport through PS2. Despite its functional importance, D1 protein is highly unstable in light (Prasil *et al.* 1992). Light-dependent degradation of D1 has been shown in various *in vivo* (Mattoo *et al.* 1984) and *in vitro* photosynthetic membrane preparations (Aro *et al.* 1993).

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide; EDTA, ethylenediaminetetraacetic acid; OEC, oxygen evolving complex; P680, primary electron donor of PS2; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PS, photosystem; Q_A and Q_B, primary and secondary quinone acceptors of PSs; SDS, sodium dodecyl sulphate; TBS, Tris buffer saline; Tyr, tyrosine 161 of the D1 protein.
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Intense irradiation of leaves or isolated photosynthetic membranes results in the specific inhibition of PS2 electron transport chain and eventually leads to the irreversible damage of reaction centre, a process known as photoinhibition (Prasil *et al.* 1992). Both the acceptor side and donor side mechanisms are involved in irreversible damage to PS2 during photoinhibition (Prasil *et al.* 1992, Aro *et al.* 1993). In both mechanisms, damage to PS2 eventually leads to degradation of D1 protein, and the degradation pattern depends on the site of inactivation. During acceptor side inhibition, D1 is degraded into 23, 16, and 10 kDa fragments (De las Rivas *et al.* 1992) whereas 24, 16, and 9 kDa fragments are produced during donor side inhibition (Barbato *et al.* 1992). The molecular mechanism involved in D1 protein degradation is not known, although involvement of both proteolytic and photosensitized cleavage reactions have been suggested by Aro *et al.* (1993), Mishra and Ghanotakis (1994), and Miyao *et al.* (1995).

Inhibition of D1 protein degradation by free radical scavengers and initiation of D1 protein degradation in dark by free radical generating systems have suggested that the D1 protein degradation during photoinhibition is caused by free radicals (Mishra and Ghanotakis 1994, Miyao *et al.* 1995). A number of oxidative reactive radicals are produced in the vicinity of D1 protein.

On the other hand, the observation that serine protease inhibitors can inhibit D1 degradation (Virgin *et al.* 1991) suggests the involvement of protease in D1 protein degradation. However, complete inhibition of D1 protein by serine protease inhibitors has not been shown (Salter *et al.* 1992). Moreover, temperature dependence of light-induced D1 degradation has been taken as an evidence that protease is involved in D1 protein degradation (Aro *et al.* 1990). However, the degradation of D1 protein readily occurs at 4 °C (Mishra and Ghanotakis 1994). In the present investigation, we have studied the effect of high temperature on D1 protein in thylakoid membranes.

Materials and methods

Thylakoid membranes from 8-d-old wheat (*Triticum aestivum* L. cv. HD-2329) seedlings were isolated as described in Mishra and Singhal (1992). Temperature treatment to thylakoid membranes at 100 g(Chl) m^{-3} dissolved in buffer containing 50 mM Hepes, pH 7.5, 15 mM NaCl, and 5 mM $MgCl_2$ was made in a double jacketed glass cuvette under constant stirring. Temperature of glass cuvette was maintained at specified temperature by circulating water around the cuvette with the help of a water-bath. Various additives such as protease inhibitors: PMSF (40 μM), leupeptin (10 μM), pepstatin (1 μM), antipain (2 μM), EDTA (2 mM), 1,10-phenanthroline (2 mM); radical scavengers: histidine (25 mM), rutin (0.2 mM), quercetin (0.2 mM), and DPC (2 mM) were added, whenever required, to thylakoid membranes prior to the treatment.

Thylakoid proteins were electrophoresed on 13 % SDS-PAGE in presence of 6 M urea as described in Laemmli (1970). The proteins were transferred onto

nitrocellulose membrane and were probed with D1 antibody as described in Harlow and Lane (1988). 5 % non-fat dry milk powder in TBS, pH 7.4 was used for blocking the membrane. Chlorophyll (Chl) concentration was determined spectrophotometrically according to Arnon (1949).

Results

Exposure of thylakoid membranes to high temperature in the dark caused degradation of D1 protein as revealed by the appearance of a 23 kDa fragment (Fig. 1). Maximum degradation of the D1 protein occurred at 45 °C (Fig. 1). Further increase in temperature resulted in the loss of degradation product. Concomitant with the loss of 23 kDa fragment, high molecular mass cross-linking products consisting of D1 protein appeared in the lane and at the interface of stacking and separating gels (Fig. 1, 50 to 60 °C). The loss at high temperature could be due to formation of cross-linking products or due to loss of activity responsible for D1 protein degradation. The 23 kDa degradation fragment detected during the temperature treatment of thylakoid membrane originated from the N-terminal because the antibody used in the present study was raised against a synthetic peptide homologue to N-terminal of D1 protein.

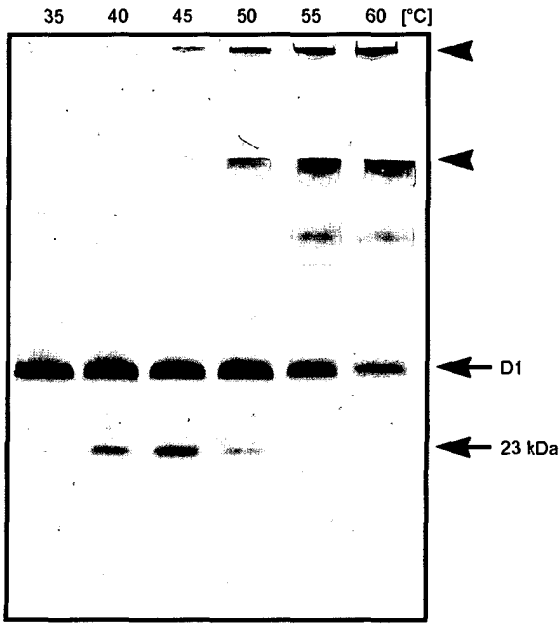

Fig. 1. Immunoblot showing degradation of D1 protein during incubation of thylakoid membrane at high temperature for 30 min in darkness. Thylakoid membranes suspended in buffer containing 50 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 15 mM NaCl at 100 g(Chl) m^{-3} were incubated at respective temperatures (35 to 60 °C). The proteins were solubilized, separated by 13 % SDS-PAGE in presence of 6 M urea, electroblotted onto nitrocellulose membrane, and immunodetected with D1 antibody using alkaline phosphatase conjugated secondary antibody. Equal amount of thylakoid proteins equivalent to 7 μg Chl was loaded. The position of mature D1 protein and 23 kDa degradation product is indicated. The crosslinking component consisting of D1 protein is indicated with arrowheads.

In order to find out the mechanism leading to degradation of D1 protein at high temperature in dark, thylakoid membranes were incubated either with scavengers of

active oxygen species or with protease inhibitors. Immunoblot (Fig. 2) shows the effect of various scavengers of oxygen species (histidine, rutin, and quercetin) on the degradation of D1 protein. These substances protect the PS2 complex by scavenging the active oxygen species formed during the photoinhibitory processes (Wagner *et al.* 1988, Mishra *et al.* 1992, 1994). Rutin had no effect on D1 protein degradation (Fig. 2). Rutin scavenges Chl triplets (Mishra *et al.* 1994), therefore our results suggested that degradation of D1 protein in temperature-treated thylakoid membranes is not due to formation of Chl triplets. As compared to rutin, histidine was effective to some extent in the protection of D1 protein degradation. Histidine scavenges singlet oxygen and also protects the D1 protein degradation in the photoinhibited photosynthetic complexes (Mishra *et al.* 1994). In contrast to histidine and rutin, near complete inhibition of D1 protein degradation was observed with quercetin (Fig. 2). Quercetin scavenges superoxide anions and fatty acyl radicals (Wagner *et al.* 1988). Our study therefore suggests the possible involvement of fatty acyl/superoxide radicals and singlet oxygen in the D1 protein degradation during the high temperature treatment to thylakoid membranes. DPC, an artificial electron donor to PS2, also inhibited D1 protein degradation (Fig. 2).

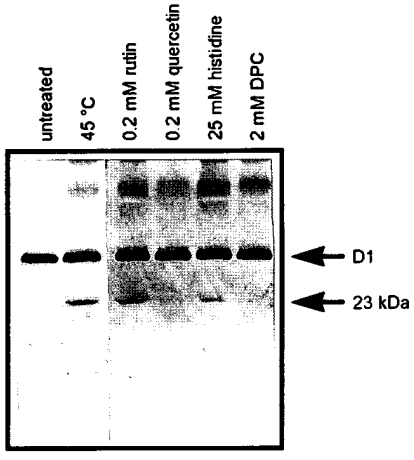

Fig. 2. Effect of scavengers of active oxygen species on the degradation of D1 protein. Thylakoid membranes with different additives were subjected to high temperature treatment at 45 °C for 30 min in dark. The proteins were electroblotted and immunodetected with D1 antibody as described in Fig. 1. The position of mature D1 protein and 23 kDa degradation product is indicated.

Effect of inhibitors of various proteases on D1 protein degradation in high temperature-treated thylakoid membranes was also studied (Fig. 3): Pepstatin, an inhibitor of aspartic proteases, had no effect on D1 degradation, but PMSF (serine protease inhibitor), EDTA, and 1,10-phenanthroline (inhibitors of metal-activated proteases) completely suppressed the D1 protein degradation. Partial inhibition of D1 protein degradation was also obtained with leupeptin and antipain (inhibitors of serine and cysteine proteases). The inhibition of D1 protein degradation by serine-protease inhibitors is known (Virgin *et al.* 1991), however, inhibition by inhibitors of metal-activated proteases is shown here for the first time. Unlike in previous studies,

we found a complete inhibition of D1 protein degradation by inhibitors of serine and metal-activated proteases.

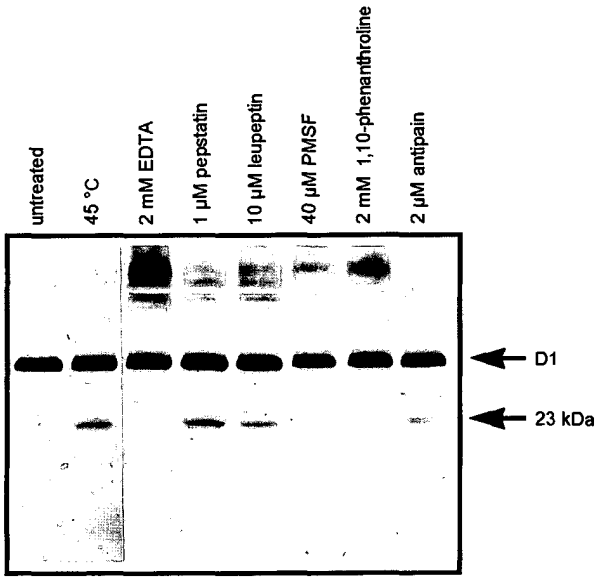

Fig. 3. Effect of protease inhibitors on the degradatian of D1 protein. Experimental details are the same as in Fig. 1. The position of mature D1 protein and 23 kDa degradation product is indicated.

Discussion

We found that exposure of thylakoid membranes to high temperature in the dark resulted in the degradation of D1 protein giving rise to the fragment of 23 kDa. Analysis by using $D1_N$ antiserum suggested that the 23 kDa fragment originates from the N-terminus of D1 protein. On the basis of proteolytic fragment mapping, the 23 kDa fragment detected during *in vivo* photoinhibition of *Chlamydomonas* was suggested to be the primary degradation product derived from the N-terminus of the D1 protein (Greenberg *et al.* 1987). Our identification of the 23 kDa fragment implies that the D1 protein degradation pathway during exposure of thylakoid membranes to high temperature in the dark is similar to that observed during the photoinhibitory process in intact photosynthetic systems.

In order to discuss the results, it is necessary to introduce the process leading to D1 protein degradation during photoinhibition. Inactivation of PS2 leading to the D1 protein degradation during photoinhibition may be brought by two different mechanisms: acceptor- or donor-side photoinactivation (Prasil *et al.* 1992). In the acceptor side mechanism, over-reduction of the plastoquinone pool may lead to formation of stable reduced Q_A, possibly followed by double reduction, protonation, and eventual loss of Q_A quinone (Zer *et al.* 1994). Under these conditions, the radical pair $P680^+/Pheo^-$ recombines rapidly. This may result in formation of $^TP680$ which interacts with oxygen and generates harmful singlet oxygen (Vass *et al.* 1992). In the

proposed donor side mechanism, generation of high ΔpH and acidification of the thylakoid lumen during photoinhibition may lead to the dissociation of Ca^{2+} from the OEC (Ono and Inoue 1989). The inactivation of OEC will lead to the generation of oxidative cation radicals (Tyr_Z^+, $P680^+$) and to irreversible modifications of the PS2 reaction centre (Prasil *et al.* 1992, Aro *et al.* 1993). In both processes, the D1 protein is degraded.

Since we treated thylakoid membranes in the dark, involvement of Tyr_Z^+, $P680^+$, and $^TP680$ in the irreversible modification leading to the degradation of D1 protein was ruled out. Usually, the modification of D1 protein is taken as a pre-requisite for its degradation (Prasil *et al.* 1992, Aro *et al.* 1993). Then, what is the nature of radical(s) involved in the irreversible modification of D1 protein during the treatment of thylakoid membranes at high temperature in the dark? Lipids of thylakoid membranes are peroxidised during high temperature treatment in the dark (Mishra and Singhal 1992). Although the mechanism leading to initiation of lipid peroxidation in this process is not known, it could lead to production of a number of lipid peroxides and radicals such as lipid hydroperoxide, lipid peroxy-radical, and lipid alkoxyl-radical (Asada 1994). These free radicals could react with oxygen to produce the reactive oxygen species involved in the irreversible modification of PS2 reaction centre and hence in the D1 protein degradation. Involvement of these radicals in the damage to PS2 reaction centre in the present condition comes from observation that quercetin inhibited the D1 protein degradation. This could be due to the ability of quercetin to quench the lipid radicals produced during the high temperature treatment of thylakoid membranes. The protection of D1 protein by histidine suggested the involvement of singlet oxygen which could be produced by the decomposition of lipid hydroperoxy-radicals (Youngman 1984).

The D1 protein is proteolytically degraded (Aro *et al.* 1993) under the involvement of a serine protease in thylakoid membranes (Virgin *et al.* 1991). Doubts have been raised because the inhibitors of the proteases were not able to completely inhibit the D1 protein degradation, but our results showed a complete inhibition of D1 protein by inhibitors of serine and metal-activated proteases. Hence the incomplete inhibition of D1 protein degradation by protease inhibitors observed in earlier studies may have resulted from accessibility restrictions due to shielded location of PS2 in the lipid bilayer within the appressed regions of grana stacks. This accessibility restrictions are, however, not likely to interfere in binding of inhibitors to the protease during high temperature treatment because high temperature increases the passive permeability of small molecules through membrane (Süss and Yordanov 1986).

Evidence of involvement of protease in D1 protein degradation further comes from the studies using DPC. Inhibition of D1 protein degradation by DPC could not be due to its ability to scavenge radicals or due to donation of electrons to donor side. The likely explanation of observed inhibition by DPC in present condition could be due to its interaction with D1 protein which affects the protein cleavage domain. Interaction of DPC with the herbicide-binding niche of PS2 has been shown (Purcell *et al.* 1991) as well as inhibition of D1 protein degradation by various inhibitors of electron flow that replace bound plastoquinone (Jansen *et al.* 1993). However, DCMU as an inhibitor of PS2 electron transport replacing Q_B from the D1 protein,

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had no effect on D1 protein degradation (results not shown). The role of DCMU during photoinhibition is not well understood: in the thylakoid membranes under strong irradiation, DCMU had no effect on D1 protein degradation (Mishra *et al.* 1994). However, under irradiation of thylakoid membranes with inactive donor side, DCMU protected D1 protein degradation (Jegerschöld *et al.* 1990).

A new finding presented in this paper is the inhibition of D1 protein degradation by inhibitors of two different types of proteases. At present, we have no explanation for this observation. It is very unlikely that two different types of proteases are involved in D1 protein degradation which yields the 23 kDa fragment. Both EDTA and 1,10-phenanthroline act by chelating essential metal atom of the metallo-proteases. In relation to this, the D1 protein degradation is increased by Mg^{2+} ions (Salter *et al.* 1992).

The D1 protein is degraded following dissociation of the LHC2 complex from PS2 (Georgakopoulos and Argyroudi-Akoyunoglou 1997). The dissociation of LHC2 complex may leave the D1 protein unprotected and vulnerable to proteolysis. Involvement of a similar mechanism in degradation of D1 protein during exposure of thylakoid membranes to high temperature in the dark may not be ruled out because high temperature induces dissociation of the LHC2 complex from PS2 (Gounaris *et al.* 1984). Further studies are required to understand the relationship between dissociation of LHC2 and degradation of D1 protein during high temperature treatment.

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