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Formation of cross-linking between photosystem 2 proteins during irradiation of thylakoid membranes at high temperature

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Abstract

Irradiation of thylakoid membranes at 40 °C resulted in complete inhibition of photosystem (PS) 2 activity measured as 2,6-dichlorophenol indophenol (DCIP) photoreduction either in the absence or presence of 1,5-diphenylcarbazide (DPC). Concomitant with the inactivation of PS2 activity, several thylakoid proteins were lost and high molecular mass cross-linking products appeared that cross-reacted with antibodies against proteins of PS2 but not with antibodies against proteins of other three complexes PS1, ATP synthase, and cytochrome b_6f. Irradiation of thylakoid membranes suspended in buffer of basic pH or high concentration of Tris at 25 °C resulted in the formation of cross-linking products similar to those in thylakoid membranes irradiated at 40 °C. Presence of radical scavengers and DPC during the high temperature treatment prevented the formation of cross-linking products. These results suggest the involvement of oxygen evolving complex (OEC) in the formation of cross-linking between PS2 proteins in thylakoid membrane irradiated at high temperature.

Additional key words: D1 protein; diphenylcarbazide; oxygen evolving complex; scavengers; *Triticum aestivum*; wheat.

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Abbreviations: CBB, Coomassie brilliant blue; Chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; DPC, 1,5-diphenylcarbazide; EDTA, ethylenediaminetetraacetic acid; m.m., molecular mass; OEC, oxygen evolving complex; P680, primary electron donor of PS2; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PS, photosystem; SDS, sodium dodecyl sulphate; TBS, Tris-buffer saline; Tyr_z, tyrosine 161 of the D1 protein.

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Introduction

Exposure of leaves or isolated photosynthetic membranes to irradiance in excess of that required to saturate photosynthesis results in photoinhibition (Powles 1984). The decline in photosynthetic activity during irradiation is profoundly increased in presence of other environmental stresses such as drought or low and high temperature (Berry and Björkman 1980). During high temperature stress, prior to the impairment of other cell functions, the photosynthetic apparatus of chloroplasts is irreversibly damaged. PS2 complex and particularly oxygen evolving complex (OEC) is more sensitive to high temperature stress than other supramolecular complexes of thylakoid membrane (Mamedov *et al.* 1993). Sensitivity of OEC to high temperature is due to the release of two of the four manganese atoms and proteins associated with the complex (Nash *et al.* 1985, Thompson *et al.* 1989). Denaturation of PS2 proteins other than the proteins associated with OEC has also been observed (Thompson *et al.* 1989).

Most of the studies on high temperature induced alteration of photosynthetic apparatus has been made in the dark. Only little information is available on interactive effect of irradiance and temperature. Irradiation of photosynthetic apparatus leads to the degradation of D1 protein, one of the reaction centre proteins of PS2 (Aro *et al.* 1993). Degradation of the D1 protein has been shown *in vivo* (Mattoo *et al.* 1984) and *in vitro* in isolated thylakoid membranes (Shipton and Barber 1991, Aro *et al.* 1993). Inactivation of OEC prior to irradiation leads to the accelerated degradation of D1 protein (Jegerschöld *et al.* 1990).

We studied the effect of high temperature on proteins during irradiation of thylakoid membranes. Our results demonstrate the loss of certain thylakoid proteins accompanied by the formation of high m.m. cross-linking products during high temperature treatment. An attempt was made towards identification of various proteins involved in the cross-linking products. Involvement of OEC and free radicals on the formation of cross-linking products was also studied.

Materials and methods

Plants: Wheat (*Triticum aestivum* L. cv. HD-2329) seeds were soaked in distilled water for 12 h and then germinated on moist filter paper in the dark at 25 °C for 48 h. The etiolated seedlings were transferred to a plant growth chamber maintained at 25 °C and grown under 14/10 h dark-light cycles.

Thylakoid membranes from 8-d-old wheat seedlings were isolated as described in Mishra *et al.* (1992). Thylakoid membranes were washed once with buffer A [50 mM HEPES-NaOH (pH 7.6), 15 mM NaCl, 5 mM $MgCl_2$]. Thylakoid membranes were resuspended in buffer A containing 0.4 M saccharose and stored at -80 °C. Chlorophyll (Chl) concentration was determined according to Arnon (1949).

Temperature treatment: Thylakoid membranes at 100 g(Chl) m^{-3} resuspended in buffer A were kept in a double jacketed glass container. Temperature of sample was controlled by a water bath attached to glass container. During the treatment,

thylakoid membranes were exposed to "white light" (100 μmol m^{-2} s^{-1}) provided by a slide projector. Samples were withdrawn after completion of time and centrifuged immediately at 12 000×*g* for 2 min. The resulting pellet was resuspended in buffer A and used to measure the electron transport activity and for protein analysis.

Electron transport activities were measured spectrophotometrically (*Shimadzu UV-3000*) following the rate of photoreduction of DCIP at 600 nm in the absence or presence of DPC as described in Mishra and Singhal (1992). The assay mixture consisted of 0.4 M saccharose, 15 mM NaCl, 5 mM $MgCl_2$, 20 mM MES-NaOH (pH 6.5), 35 mM DCIP, and thylakoid membranes of 5 g(Chl) m^{-3}. For measurement of DCIP reduction in the presence of DPC, 1 mM freshly prepared DPC was added to the reaction mixture.

SDS-PAGE: Proteins were analysed on SDS-PAGE as described by Laemmli (1970) in the presence of 6 M urea. Thylakoid proteins were solubilized in sample buffer containing 4 % SDS, 50 mM Tris-HCl (pH 6.8), 2 % β-mercaptoethanol, and 10 % glycerol by incubating at 40 °C for 40 min. Proteins were electrophoresed on 13 % polyacrylamide gel at a constant voltage of 100 V. Proteins were either visualised by CBB-staining or were electrophoretically transferred on nitrocellulose membrane. The blotted membrane was immunodecorated with various antibodies as described by Harlow and Lane (1992). 5 % non-fat dry milk powder in TBS containing 0.05 % *Tween-20* was used for blocking the nitrocellulose membrane. Protein-antibody complexes were probed with anti-rabbit IgG conjugated to alkaline phosphatase and visualised using nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Antibody against 33 kDa protein of OEC: PS2 membranes from wheat thylakoid membranes were isolated as described by Berthold *et al.* (1981). PS2 membranes were washed with NaCl to release 23 and 16 kDa proteins. The washed PS2 membranes were collected by centrifugation and subjected to treatment with 0.8 M Tris, pH 8.4 for 30 min on ice to release 33 kDa protein. The supernatant was collected and the 33 kDa protein was precipitated with acetone at -20 °C for 2 h. The protein was recovered by centrifugation at 5 000×*g* for 10 min at 4 °C. Protein was dried, dissolved in water, and subjected to SDS-PAGE. After completion of the run, the 33 kDa protein from gel was purified as described in Harlow and Lane (1992). Antibodies against 33 kDa were raised in New Zealand white rabbit by injecting 200 μg protein emulsified with Freund's complete adjuvant. This preparation was subcutaneously injected into rabbit three times at 10-d intervals. 7 d after the third injection, serum was collected and the antibody specificity was checked using Western blotting.

Results

Exposure of thylakoid membranes to high temperature in dark led to the selective inhibition of OEC (Table 1) as reported by other authors. In contrast, when thylakoid membranes were irradiated at 40 °C, the inactivations of PS2 activities measured as DCIP photoreduction either in the absence or the presence of DPC were similar.

Concomitant with the loss of PS2 activity, irradiation of thylakoid membranes at 40 °C led to the loss of several proteins (Fig. 1). The loss of these proteins was not

Table 1. Electron transport activity of photosystem 2 in thylakoid membranes exposed to 25 or 40 °C either under irradiation (I) or in the dark (D) for 1 h.

	H_2O to DCIP [%]	DPC to DCIP [%]
Control	100	100
25 °C + D	98	100
25 °C + I	82	86
40 °C + D	4	70
40 °C + I	0	0

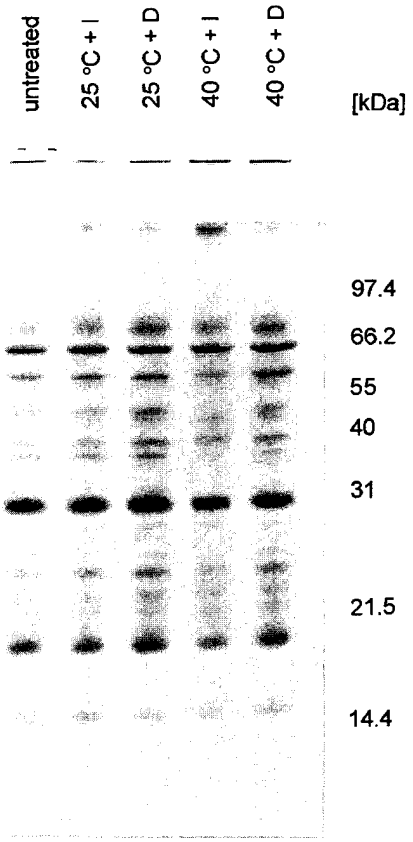

Fig. 1. Effect of high temperature on thylakoid proteins. Thylakoid membranes were exposed to 25 or 40 °C either under irradiation (100 μmol m^{-2} s^{-1}) or in the dark for 1 h. Thylakoid proteins equivalent to 10 μg chlorophyll were loaded per lane.

detected in thylakoid membranes either irradiated at 25 °C or kept in dark at 25 or 40 °C. This result therefore suggested that loss of proteins is due to the synergistic effect of both irradiation and high temperature.

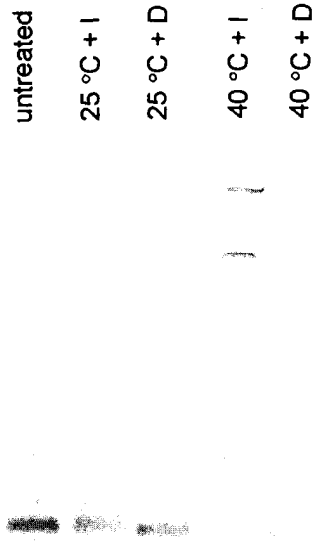

Fig. 2. Effect of high temperature on D1 protein. Thylakoid proteins equivalent to 7 μg chlorophyll were loaded per lane.

Strong irradiation of photosynthetic membranes at ambient temperature results in degradation of D1 protein of PS2 reaction centre. Degradation of D1 proteins has been studied in thylakoid membranes and its sub-complexes (Aro *et al.* 1993). However, the fate of D1 protein in thylakoid membranes irradiated at high temperature is not known. Immunoblot (Fig. 2) shows the degradation of D1 protein in thylakoid membranes under the same treatments: No degradation of the D1 protein was observed in thylakoid membranes irradiated or kept in the dark at 25 °C. This failure to detect any degradation product could be due to the low irradiance used (100 μmol m^{-2} s^{-1}). When the thylakoid membranes were incubated in the dark at 40 °C, a D1 degradation product of 23 kDa was observed. However, when thylakoid membranes were irradiated at 40 °C, no degradation product of D1 was found. Instead, smeared bands of high m.m. cross-linking products were observed at the interface between the resolving and stacking gels. Besides, high m.m. cross-linking products were also observed which could no longer enter the stacking gel (Fig. 2). Formation of cross-linking products was not observed in thylakoid membranes irradiated at 25 °C or kept in the dark at 25 or 40 °C.

The identity of various proteins involved in the cross-linking products was determined using specific polyclonal antibodies against the thylakoid proteins (Fig. 3). All the polyclonal antibodies against proteins of PS2 complex but none of the

antibodies from other three complexes recognised cross-linking products. This result therefore suggested that irradiation of thylakoid membranes at high temperature caused intensive cross-linking of PS2 proteins whereas proteins of other three thylakoid membranes complexes were not affected. Moreover, loss of proteins in thylakoid membranes irradiated at 40 °C was due to the formation of cross-linking products and not to their degradation.

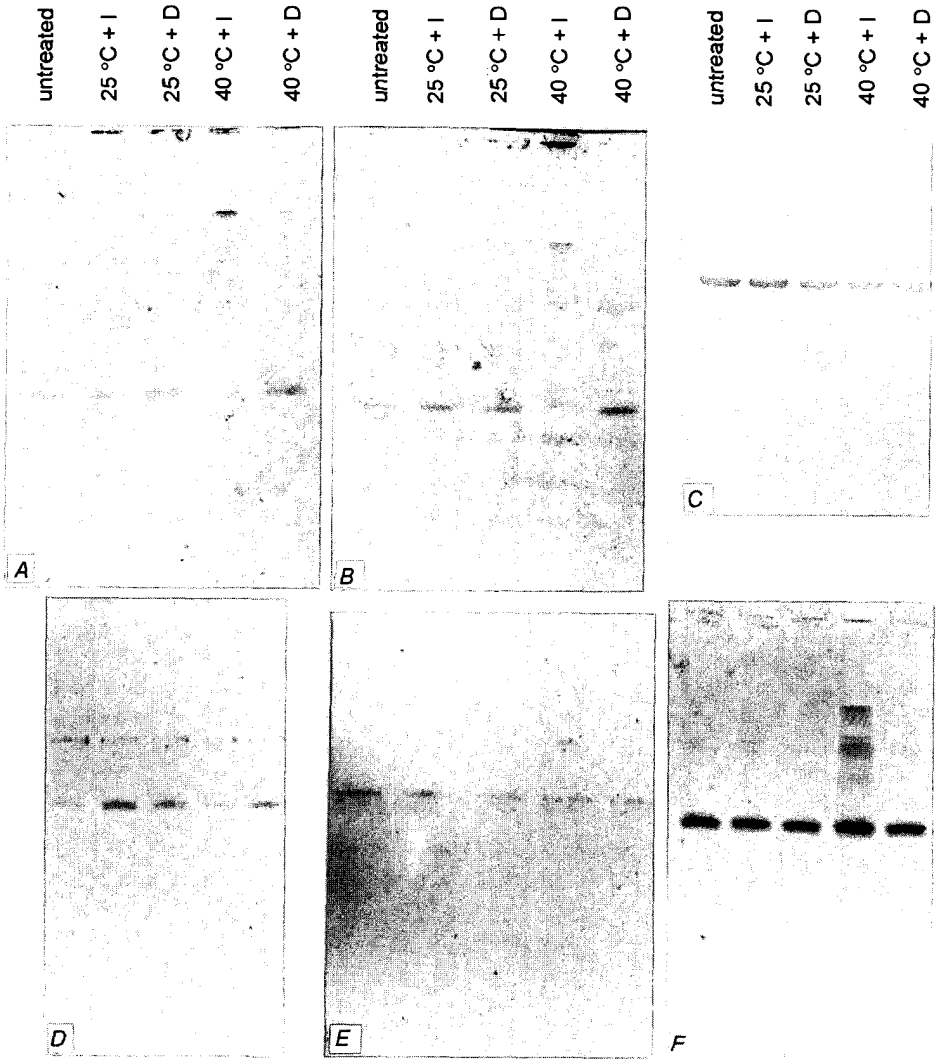

Fig. 3. Effect of high temperature on thylakoid proteins. Western blot analysis of thylakoid membranes with (*A*) D2 antibody, (*B*) CP43 antibody, (*C*) CF1a antibody, (*D*) cytochrome (Cyt) *f* antibody, (*E*) PS1a antibody, and (*F*) 33 kDa antibody. Thylakoid proteins equivalent to 7 μg chlorophyll were used for detecting D2, CP43, Cyt *f*, and PS1a protein. For 33 kDa and CF1a, thylakoid proteins equivalent to 3 μg chlorophyll were used.

Exposure of thylakoid membranes to high temperature leads to the inactivation of OEC (Mamedov *et al.* 1993). Therefore it can be argued that the loss of proteins (Fig. 1) and formation of cross-linking products during high temperature stress (Fig. 2) may be induced by donor side inactivation. Irradiation of Tris-washed thylakoid membranes at 25 °C leads to the loss of proteins (Fig. 4*A*) and subsequent formation

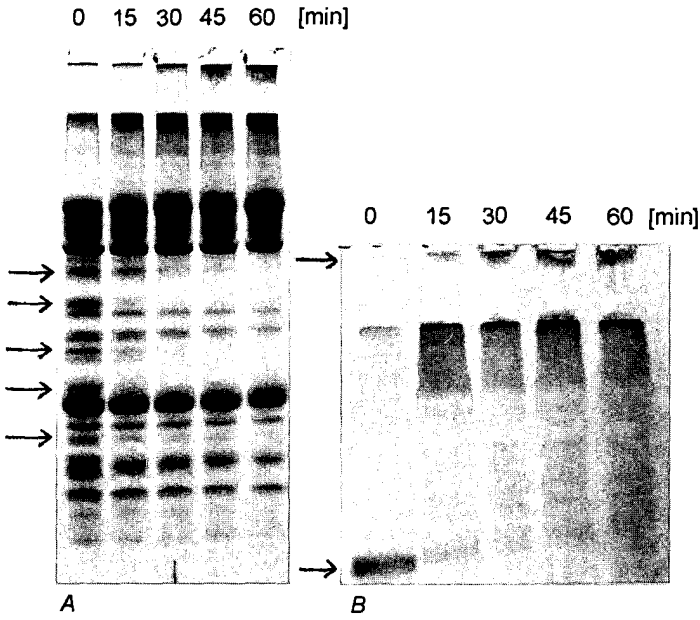

Fig. 4. Effect of Tris washing on (*A*) thylakoid proteins and (*B*) D1 protein. Tris washing was done by imcubating thylakoid membranes in 0.8 M Tris-HCl, pH 8.8 for 30 min at 4 °C. Tris-washed thylakoid membranes were irradiated at 25 °C for 0, 15, 30, 45, and 60 min. Losses of proteins including D1 protein and appearance of high m.m. cross-linking products in the lane are shown by *arrows*.

of cross-linking products consisting of D1 protein (Fig. 4*B*) similar to that observed during irradiation of thylakoid membranes at 40 °C. Similarly irradiation of thylakoid membranes suspended at basic pH also induced formation of cross-linking products during irradiation at 25 °C (Fig. 5). Basic pH selectively inactivates OEC (De las Rivas *et al.* 1992). The formation of cross-linking products occurred only at pH 8.0 (Fig. 5). Lower pH, which had no effect on OEC, did not affect the thylakoid proteins.

Involvement of OEC in the formation of cross-linking products during irradiation of thylakoid membranes was studied further. In Tris-washed thylakoid membranes irradiated at 25 °C in the presence of DPC no loss of proteins was detected (Fig. 6*A*) similar to that which occurred during irradiation of thylakoid membranes at 40 °C in the absence of DPC. The presence of DPC during the treatment also prevented the formation of high m.m. cross-linking products (Fig. 6*B*).

Effect of various radical scavengers (rutin, quercetin, and histidine) was studied on the formation of cross-linking products during irradiation of thylakoid membranes at

et al. 1994) whereas quercetin scavenges superoxide/lipids radicals (Wagner *et al.* 1988). All the three scavengers suppressed the formation of cross-linking products during irradiation of thylakoid membranes at 40 °C. Quercetin was more effective than histidine and rutin.

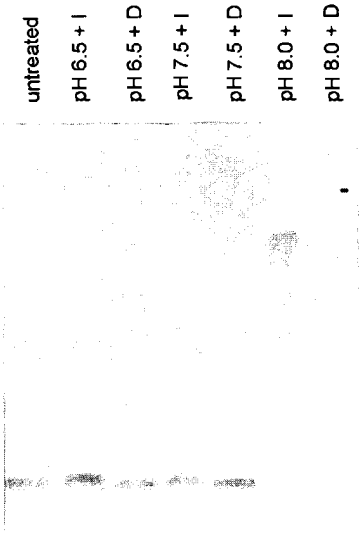

Fig. 5. Effect of basic pH on D1 protein during irradiation (I) or dark treatment (D) of thylakoid membrane for 30 min at 25 °C.

Discussion

Exposure of thylakoid membranes to high temperature in the dark led to the selective inhibition of OEC (Table 1). In contrast, when thylakoid membranes were irradiated at high temperature, losses of PS2 activity from H_2O to DCIP or from DPC to DCIP were similar. The likeliest explanation of this observation is that irradiation of thylakoid membranes at high temperature induces the accumulation of abnormally long-lived, highly oxidising radicals on the donor side of PS2. In the dark, high temperature disrupts the OEC by releasing Mn and/or proteins associated with OEC (Nash *et al.* 1985, Thompson *et al.* 1989). As a result, electron transport from water to DCIP is disrupted but DPC, which donates electron directly to Tyr_Z, is able to restore the activity. Yet irradiation will lead to the transfer of electrons from Tyr_Z and probably from Mn to $P680^+$. If the donor side is inactivated, only two normal reductions of $P680^+$ are possible. In the absence of an active donor side, $P680^+$ and Tyr_Z^+ will accumulate which will further lead to the oxidation of Chl and generation of reactive oxygen species. Thus irradiation of thylakoid membranes at high temperature will inactivate not only OEC but simultaneously also the components involved in electron transport from DPC to DCIP.

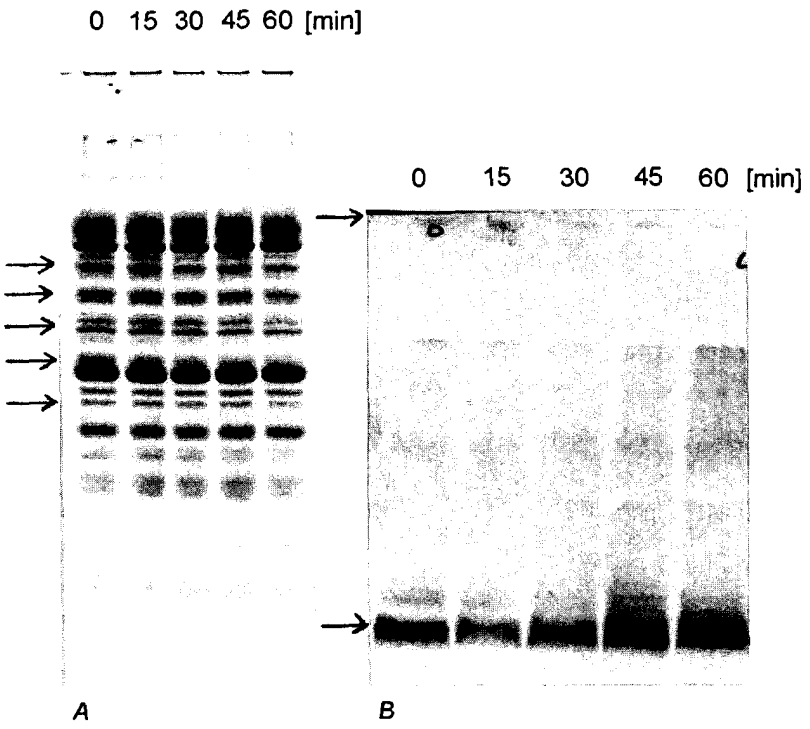

Fig. 6. Effect of DPC on (*A*) thylakoid proteins and (*B*) D1 protein during high temperature treatment. Tris-washed thylakoid membranes were irradiated at 40 °C in presence of 10 mM DPC for 0 to 60 min. *Arrows* indicate the proteins which loss during high temperature treatment was prevented by DPC.

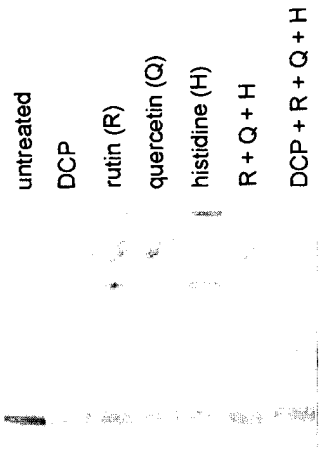

Fig. 7. Effect of scavengers of active oxygen species on damage to the D1 protein during high temperature treatment at 40 °C under irradiation.

We also demonstrated that irradiation of thylakoid membranes at 40 °C results in loss of several proteins. The loss of these proteins is eventually due to the formation of cross-linking products consisting of these proteins. Furthermore, only PS2 proteins were affected by high temperature. At present it is not clear whether the various proteins present in the cross-linking products are photodamaged but undegraded proteins, or photodamaged and decomposed fragments of the proteins. At least in the case of D1 protein, our results suggest that both D1 protein and its degradation fragment are present in the cross-linking products. Loss of D1 degradation fragment could not be due to loss of activity responsible for D1 protein degradation as it readily occurred in thylakoid membranes exposed at high temperature in dark (Fig. 1).

Formation of cross-linking in PS2 proteins is induced by inactivation of OEC. This was inferred since inactivation of OEC by Tris-washing and basic pH also resulted in the formation of cross-linking products formation at 25 °C. Furthermore, protection by DPC suggested that donor side is primarily involved in the formation of cross-linking products. Studies with radical scavengers suggested that a number of different radical species are involved in the formation of cross-linking products.

Based on these results, we can propose the mechanism involved in the formation of cross-linking products during irradiation of thylakoid membranes at high temperature. Since the irradiance used was low (100 μmol m^{-2} s^{-1}), the involvement of acceptor side in the damage to PS2 was ruled out. At ambient temperature both the acceptor and donor side are active. However, at high temperature the donor side is inactivated due to release of Mn and/or proteins associated with the complex. Once the donor side is inactivated, the oxidised Tyr_Z and P680 cannot be reduced and as a result the lifetime of Tyr_Z^+ and $P680^+$ increases. With further irradiation, these reactive species react with Chl and oxygen to generate various reactive oxygen species which would lead to damage to thylakoid proteins. Furthermore, the radicals can also react with the unsaturated lipids of thylakoid membranes to generate lipid alkoxy radicals which together with other reactive oxygen species would induce the cross-linking between proteins.

The formation of high m.m. cross-linking products cross-reacting with 33 kDa protein was specific and did not appear in the dark at either temperature or during irradiation at 25 °C. Detection of smeared bands suggested that several proteins were present in the cross-linking products.

References

Arnon, D.I.: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Aro, E.-M., Virgin, I., Andersson, B.: Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. - Biochim. biophys. Acta **1143**: 113-134, 1993.

Berry, J., Björkman, O.: Photosynthetic response and adaptation to temperature in higher plants. - Annu. Rev. Plant Physiol. **31**: 491-543, 1980.

Berthold, D.A., Babcock, G.T., Yocum, C.F.: A highly resolved, oxygen-evolving photosystem II preparation from spinach thylakoid membranes. EPR and electron-transport properties. - FEBS Lett. **134**: 231-234, 1981.

De las Rivas, J., Andersson, B., Barber, J.: Two sites of primary degradation of the D1-protein induced by acceptor or donor side photo-inhibition in photosystem II core complexes. - FEBS Lett. **301**: 246-252, 1992.

Harlow, E., Lane, D.: Antibodies: A Laboratory Manual. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor - New York 1992.

Jegerschöld, C., Virgin, I., Styring, S.: Light-dependent degradation of the D1 protein in photosystem II is accelerated after inhibition of the water splitting reaction. - Biochemistry **29**: 6179-6186, 1990.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Mamedov, M., Hayashi, H., Murata, N.: Effects of glycinebetaine and unsaturation of membrane lipids on heat stability of photosynthetic electron-transport and phosphorylation reactions in *Synechocystis* PCC6803. - Biochim. biophys. Acta **1142**: 1-5, 1993.

Mattoo, A.K., Hoffman-Falk, H., Marder, J.B., Edelman, M.: Regulation of protein metabolism: coupling of photosynthetic electron transport to *in vivo* degradation of the rapidly metabolized 32-kilodalton protein of the chloroplast membranes. - Proc. nat. Acad. Sci. USA **81**: 1380-1384, 1984.

Mishra, N.P., Fatma, T., Mishra, R.K.: Protection of wheat chloroplasts from lipid peroxidation and loss of photosynthetic pigments by quercetin under strong illumination. - J. Plant Physiol. **140**: 409-413, 1992.

Mishra, N.P., Francke, C., van Gorkom, H.J., Ghanotakis, D.F.: Destructive role of singlet oxygen during aerobic illumination of the Photosystem II core complex. - Biochim. biophys. Acta **1186**: 81-90, 1994.

Mishra, R.K., Singhal, G.S.: Function of photosynthetic apparatus of intact wheat leaves under high light and heat stress and its relationship with peroxidation of thylakoid lipids. - Plant Physiol. **98**: 1-6, 1992.

Nash, D., Miyayo, M., Murata, N.: Heat inactivation of oxygen evolution in photosystem II particles and its acceleration by chloride depletion and exogenous manganese. - Biochim. biophys. Acta **807**: 127-133, 1985.

Powles, S.B.: Photoinhibition of photosynthesis induced by visible light. - Annu. Rev. Plant Physiol. **35**: 15-44, 1984.

Shipton, C.A., Barber, J.: Photoinduced degradation of the D1 polypeptide in isolated reaction centers of photosystem II: Evidence for an autoproteolytic process triggered by the oxidizing side of the photosystem. - Proc. nat. Acad. Sci. USA **88**: 6691-6695, 1991.

Thompson, L.K., Blaylock, R., Sturtevant, J.M., Brudvig, G.W.: Molecular basis of the heat denaturation of photosystem II. - Biochemistry **28**: 6686-6695, 1989.

Wagner, G.R., Youngman, R.J., Elstner, E.F.: Inhibition of chloroplast photo-oxidation by flavonoids and mechanisms of the antioxidative action. - J. Photochem. Photobiol. **B 1**: 451-460, 1988.