Photoinactivation of Photosynthetic Electron Transport under Anaerobic and Aerobic Conditions in Isolated Thylakoids of Spinach

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The effect of exposure to strong white light on photosynthetic electron transport reactions of PS I and PS II were investigated in spinach thylakoids in the absence or presence of oxygen. Irrespective of the conditions used for photoinactivation, the damage to PS II was always much more than to PS I. Photoinactivation was severe under anaerobic conditions compared to that in air for the same duration. This shows that the presence of oxygen is required for prevention of photoinactivation of thylakoids. The susceptibility of water-splitting complex in photoinactivation is indicated by our data from experiments with chloride-deficient chloroplast membranes wherein it was observed that the whole chain electron transport from DPC to MV was much less photoinhibited than that from water. The data from the photoinactivation experiments with the Tris-treated thylakoids indicate another photodamage site at or near reaction centre of PS II. DCMU-protected PS II and oxygen-evolving complex from photoinactivation. DCMU protection can also be interpreted in terms of the stability of the PS II complex when it is in S_2 state.

Introduction

Isolated chloroplast membranes undergo photoinactivation upon exposure to high light intensities. The damage is primarily due to decreased photosystem II activity [1, 2]. Although the D_1 is implicated in photoinhibitory damage [3, 4] there is no agreement on the molecular mechanism of the damage. The breakdown of the 32 kDa D_1 polypeptide to 23.5 kDa polypeptide has been documented [5, 6] and the cleavage site of the D_1 has been proposed to be at phenylalanine 239 adjacent to arginine 238 [7].

Presence of oxygen-promoted photoinhibition of CO_2-dependent oxygen evolution [8]. However, anaerobiosis accelerated photoinactivation [9–11]. The role of oxygen products is, as yet, unclear in photoinhibition. There is also no agreement on the sites of photoinactivation of the uncoupled chloroplasts under different conditions.

Abbreviations: DCCD, N,N-dicyclohexyl carbodiimide; DCMU, 3′-(3,4-dichlorophenyl)-1,1-dimethyl urea; OEC, oxygen-evolving complex; Q_A and Q_B, first and second quinone type PS II acceptors respectively; LHC II, light-harvesting complex associated with PS II; S_0 to S_4, oxidation states of water-oxidizing complex.

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In an attempt to understand the mechanism of photoinactivation of isolated chloroplasts and the role of oxygen in the process, studies on photoinactivation were carried out on chloroplasts under aerobic and anaerobic conditions as well as on thylakoid preparations treated with Tris. HCl and depleted of chloride to inactivate the water-oxidizing complex. The results show that the presence of oxygen reduces the extent of photoinactivation. The sites damaged during photoinactivation are the water-oxidizing complex and the donor side of photosystem II in addition to the acceptor side *viz.* Q_B. Further the protection by DCMU against photoinactivation appears to be due to the prevention of damage to the donor side of photosystem II.

Materials and Methods

Broken chloroplasts were prepared from the market spinach [12] and chloride-free chloroplasts were prepared in absence of chloride salts [13]. The photosynthetic electron transport was measured polarographically with temperature-controlled water jacketed, Clark type 5/6 Oxygraph (Gilson Medical Electronics, U.S.A.). White light from a Kindermann slide projector with a 24 V 150 W lamp was focussed on the chamber of the oxygen electrode assembly through a water-filled round-bottomed flask to absorb heat. The light intensity

could be varied using the neutral density filters. The whole chain electron transport was measured as electron flow from water to 0.1 mM methylviologen (MV) in presence of 0.3 mM NaN_3, PS II activity as electron flow from water to 3 mM parabenzoquinone (PBQ) or 2 mM potassium ferricyanide ($K_3Fe(CN)_6$) while PS I activity was measured as electron transport from reduced dichlorophenol indophenol (DCPIP) to MV in presence of 1 μM DCMU and 3.0 mM sodium ascorbate. The electron donation to the oxidizing side of PS II was measured in the presence of 1 mM diphenyl carbazide (DPC) as electron donor and MV as electron acceptor. The reaction medium was composed of 300 mM sucrose, 50 mM $KHPO_4$ (pH 6.9), 50 mM NaCl and 5 mM $MgCl_2$. The final chlorophyll concentration taken for each reaction was 20 $\mu g \cdot ml^{-1}$.

The electron transport rates were monitored at 30 °C and the photon fluence density (PFD) was 2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The average electron transport activities were as given in Table I.

Table I. Average rates of partial electron transport of spinach thylakoids measured at 30 °C at saturating PFD (2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Electron transport reaction	O_2 consumed or evolved [$\mu mol\ O_2 \cdot mg^{-1}$ chl $\cdot h^{-1}$]
H_2O–PBQ	175 ± 15
H_2O–MV	190 ± 18
H_2O–$K_3Fe(CN)_6$	264 ± 10
DPC–MV	160 ± 10
DCPIP–MV	315 ± 20

The photoinhibitory treatments to the chloroplast membranes (150 $\mu g \cdot ml^{-1}$) were given in the temperature-regulated chamber of the oxygen electrode at the PFD of 2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a temperature of 25 °C unless otherwise mentioned. Percent photoinactivation was calculated as the activity in light-treated sample (L) as percent of the dark-treated control (D), as given below:

$$\% \text{ photoinactivation} = \frac{D-L}{D} \cdot 100.$$

For the aerobic conditions the reaction medium was bubbled with air for 10 min prior to the assay while the anaerobic conditions were created with glucose/glucose oxidase system. For this N_2 was bubbled for 5 min through the reaction medium prior to adding glucose and glucose oxidase.

After the photoinhibitory treatment, the chloroplast suspensions were centrifuged at 10,000 × *g* for 10 min and the pellet was immediately resuspended in the minimum amount of the fresh reaction medium. The chlorophyll concentration was estimated [14] and the chloroplast suspensions were kept in ice and the electron transport rates were monitored immediately.

The trypsinization of the thylakoids (300 μg chlorophyll/ml) was done at pH 7.0 with 200 μg trypsin (Sigma) and 12 μM $MgCl_2$ for 2 min (unless otherwise mentioned) at 20 °C. The reaction was terminated by adding 1 mg trypsin inhibitor (Sigma) and subsequent dilution and centrifugation at 10,000 × *g* for 10 min.

The treatment of the chloroplast membranes (100 $\mu g \cdot ml^{-1}$) with Tris (0.8 M, pH 8.0) was performed at 25 °C for 10 min after which the suspension was diluted and centrifuged at 10,000 × *g* for 10 min.

The chloroplast membranes were preincubated with the inhibitors like DCMU or DCCD for 5 min and the preillumination was carried out in their presence. The inhibitors were removed in the dark by 10-fold dilution with the ice cold reaction buffer followed by centrifugation at 10,000 × *g* for 10 min.

Results and Discussion

Fig. 1 shows that the photoinactivation of whole chain electron transport increased with increase in PFD and the duration of preillumination period.

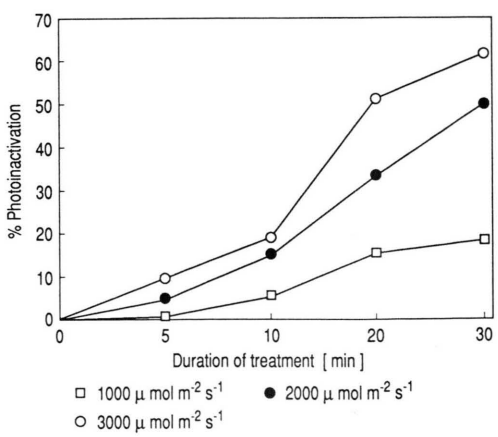

Fig. 1. Time course of photoinactivation of whole chain electron transport (H_2O–MV) on preillumination of chloroplast membranes with the various light PFDs at 25 °C under aerobic conditions.

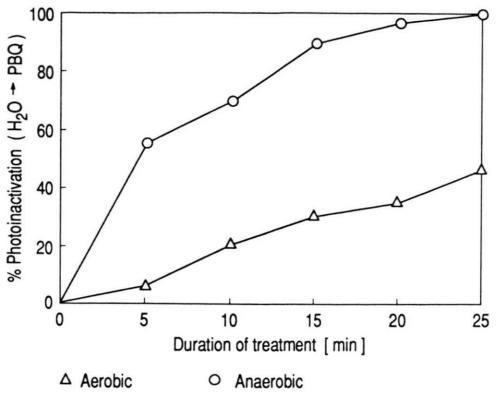

Fig. 2. Time course of photoinactivation of PS II activity (H_2O-PBQ) on preillumination with white light 2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under aerobic and anaerobic conditions.

Figs. 2 and 3 show the effect of anaerobiosis on the extent of photoinactivation. The inhibition of PS II electron transport, studied as electron transport from water to PBQ, was much greater (70%) under anaerobic conditions (Fig. 2) than under aerobic conditions (20%) for the same duration (10 min). This shows that oxygen offered considerable protection against photoinactivation, probably by acting as an electron acceptor.

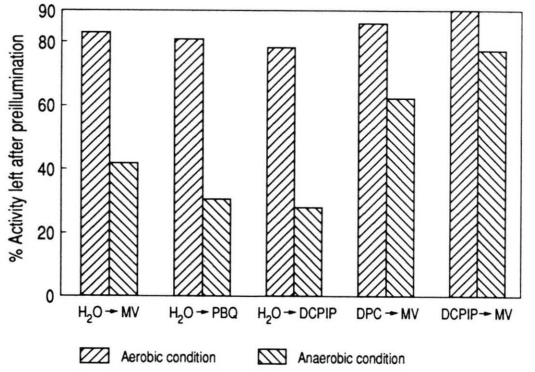

Fig. 3. Photoinactivation of various electron transport reactions on 10 min preillumination with strong white light 2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ under aerobic and anaerobic conditions.

Greater reductions in PS I, and whole chain electron transport activities were observed on illuminating the thylakoids at 2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the absence of O_2 than in its presence (Fig. 3). Similar results were reported earlier [11, 15]. The irradiation of chloroplasts under anaerobic conditions led to "overreduction" of electron transport chain [11] and in the presence of oxygen, this overreduction was relieved probably by oxygen. Photoinactivation observed in presence of oxygen, may be caused by the reactive oxygen species because partial protection could be achieved by simultaneous addition of superoxide dismutase and catalase which are the scavengers of the toxic oxygen products [16]. Studies carried out by us in which oxygen radicals were produced by irradiation of flavin adenine dinucleotide (FAD), showed a significantly greater photoinactivation of whole chain electron transport (Table II). These results also point to a role of oxygen products in causing photoinactivation under aerobic conditions.

PS II activity was inactivated much more than PS I activity showing a lesion at PS II (Fig. 3). All the reactions involving water oxidation were greatly inactivated but electron flow from DPC to MV which does not require water oxidation, was much less inactivated especially so under anaerobic conditions. This points to another possible site of damage at the water-oxidizing complex. In addition, the lumen side of the D_1 protein-housing P680 and the stromal side of D_1-housing Q_B could also be damaged. The photoinhibition primarily destroys the photochemical reactions and the protein degradation leading to the loss of Mn ions follows subsequently [17]. Inactivation of PS II-involving damage to the two opposing sides of D_1

Table II. Photoinactivation in presence of illuminated FAD under aerobic conditions. Duration: 10 min; PFD: 2500 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

	H_2O-MV [$\mu mol\ O_2$ (consumed) $\cdot mg^{-1}$ chl $\cdot h^{-1}$]		
	Before treatment	After treatment	% P.I.
Zero FAD	135 ± 8	112 ± 6	17
0.2 mM FAD	130 ± 10	68 ± 7	48

and the water-oxidizing complex has been previously documented [16, 18].

Photoinactivation of PS I to the extent of 23%, was observed in anaerobic conditions only (Fig. 3). PS I has been reported to be photoinhibited in isolated intact chloroplasts under anaerobic conditions [19, 20]. This was shown to be due to photodestruction of its reaction centre.

The role of Q_B protein was investigated using trypsinized membranes and 1 μM DCMU. Fig. 4 shows the time course of inactivation of PS II activity ($H_2O-K_3Fe(CN)_6$) due to trypsinization at pH 7.0. Trypsin selectively inactivates Q_B [21]. For further experimentation, the time interval of 2 min for trypsinization was chosen where PS II activity was reduced by *ca.* 37% (Fig. 4) and potassium ferricyanide reduction became DCMU insensitive (data not shown).

The trypsinized and untrypsinized membranes were preilluminated in the absence or presence of 1 μM DCMU for 10 min under anaerobic conditions. The results are shown in Fig. 5. The whole chain and PS II activities were equally photoinactivated in trypsinized and untrypsinized membranes in the absence of DCMU. 1 μM DCMU completely protected the whole chain and PS II activities in untrypsinized membranes but it was not so with the trypsinized membranes. This suggests that when Q_B site of D_1 protein is occupied by DCMU, the photoinactivation of PS II associated with the D_1 protein is prevented. In trypsinized membranes such a protection by DCMU is not seen because trypsin selectively digests the DCMU-binding site of the D_1 protein.

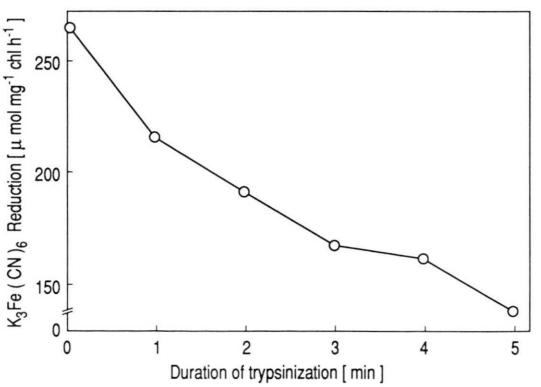

Fig. 4. Time course of inactivation of PS II activity ($H_2O-K_3Fe(CN)_6$) on trypsinization at pH 7.0.

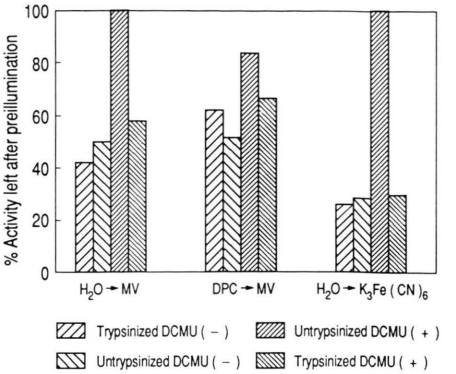

Fig. 5. Photoinactivation of the partial electron transport reactions, on 10 min preillumination with strong white light on trypsinized and untrypsinized thylakoids under anaerobic conditions.

Since the whole chain and PS II activities were equally photoinactivated in trypsinized and untrypsinized membranes in the absence of DCMU (Fig. 4), it can be speculated that Q_A was photoinactivated in trypsinized membranes. This suggestion is based on the report [21] who showed that trypsin selectively inactivates Q_B and Q_A becomes accessible to potassium ferricyanide. Inhibition of DPC-MV electron transport on preillumination of the trypsinized membranes is not significantly different from untrypsinized ones, in the absence or in presence of 1 μM DCMU, showing no involvement of the oxygen-evolving complex (OEC) in this case. The fact that DCMU presence protects $H_2O-MV/H_2O-K_3Fe(CN)_6$ much more than DPC-MV suggests that it offers protection against inactivation of OEC.

DCMU and DCCD both are the inhibitors of photosynthetic electron transport chain in uncoupled thylakoid membranes. DCMU blocks the electron transport from Q_A to Q_B while DCCD blocks the oxidation of Q_B and reduction of PQ through blocking proton movement needed to reduce PQ to PQH_2 [22]. The S_2 state is generated on illumination of DCMU-treated chloroplast membranes due to only one turnover that the reaction center can undergo. However, in the presence of DCCD which blocks the reduction of PQ from Q_B, S_3 state is generated as detected by thermoluminescence technique [23]. The data in Table III show that 1 μM DCMU-treated membranes suffered no photodamage while photoinactivation of

Table III. Photoinactivation of electron transport in chloroplast membranes in (a) absence of inhibitors, (b) presence of 1 μM DCMU, (c) presence of 1 mM DCCD and (d) presence of DCMU and DCCD.

Treatment	% Photoinactivation		
	H_2O-PBQ	H_2O-MV	DPC−MV
(a) O inhibitor	72	55	38
(b) DCMU, 1 μM	0	0	0
(c) DCCD, 1 mM	72	47	45
(d) DCCD + DCMU	71	60	29

the various electron transport activities in the 1 μM DCCD-treated membranes was substantial. An interesting observation made in Table III is that when DCMU was added to those chloroplasts that were treated by DCCD and exposed to light, the protective effect of DCMU was lost. This is conceivable if one realizes that light in the presence of DCCD will generate S_3 and subsequent addition of DCMU will not generate S_2. It thus appears that the protective effect of DCMU may be due to the creation of S_2 state.

The effect of high PFD on the 50 mM NaCl-added chloroplast was similar to that observed in the normal chloroplasts shown in Fig. 5.

The activity in chloride-deficient thylakoids was inhibited by about 50% by light when reactions involving donation by water were studied (H_2O-PBQ or H_2O-MV). The DPC-MV reaction was only slightly inactivated. The chloride sufficient thylakoids behaved like usual thylakoids membranes. These data indicate that water-oxidizing complex is an important component which is inactivated by exposure to light. The data for Tris-treated chloroplast membranes is qualitatively similar except that the percent inhibition is much greater. Since the PS I was not photoinactivated the inhibition of activity when DPC-MV was studied could be attributed to the damage at Q_B site. Apparently Tris treatment rendered this site more susceptible. PS II electron flow (H_2O-PBQ) was 100% inactivated on exposure of the Tris-treated membranes to light. Considerable inactivation of DPC-MV (greater than that of H_2O-MV) points to a damage at DPC donation site near PS II reaction centre. The PS II reaction centre appeared to be the primary site of photoinactivation when NH_2OH-treated chloroplasts were illuminated with strong light [24]. More recently it has been shown that the damage during photoinhibition is not restricted to a single site but depends upon ex-

Table IV. The photoinactivation (PI) of the partial electron flow activities on preillumination for 10 min of (a) chloride-free and chloride-added thylakoids, and (b) Tris pH 8.0-incubated thylakoids at 25 °C. Activities were measured as μmol $O_2 \cdot mg^{-1}$ chl $\cdot h^{-1}$.

Temperature and duration of treatment		H_2O-PBQ	PI [%]	H_2O-MV	PI [%]	DPC−MV	PI [%]	DCPIP−MV	PI [%]
a) Cl-free	D	71 ± 5	52	80 ± 6	50	105 ± 5	12	277 ± 12	12
25 °C, 10 min	L	34 ± 5		40 ± 5		92 ± 7		244 ± 10	
50 mM Cl^-	D	116 ± 9	14	115 ± 10	20	118 ± 11	18	285 ± 15	0
added, 25 °C, 10 min	L	99 ± 7		92 ± 8		97 ± 8		285 ± 15	
b) Tris pH 8.0	D	20 ± 5	100	27 ± 6	37	106 ± 8	55	330 ± 10	0
incubated	L	O		17 ± 5		48 ± 7		330 ± 12	
25 °C, 10 min									

PI, photoinactivation; D, dark-treated; L, light-treated.

perimental conditions including light intensity [2]. Our data confirm such a conclusion.

It can be inferred from the present study that PS II water oxidation is mainly damaged by photoinactivation. The reaction centre and Q_B protein also appear to be the susceptible sites. Stability of S_2 state appears to be important for relief from photoinactivation. Photoinactivation is more pronounced under anaerobic conditions while oxygen, despite its toxicity, provides relief from photoinactivation. The inactivation of PS II by different treatments including chloride depletion renders it more susceptible to photoinactivation.

[1] G. H. Krause, S. Somersalo, E. Zumbusch, B. Weyers, and H. Laasch, J. Plant Physiol. **136,** 472 (1990).
[2] H. J. Eckert, B. Geiken, J. Bernarding, A. Napiwotzki, H. J. Eichler, and G. Renger, Photosynth. Res. **27,** 97 (1991).
[3] D. J. Kyle, I. Ohad, and C. J. Arntzen, Proc. Natl. Acad. Sci. U.S.A. **81,** 4070 (1984).
[4] I. Ohad, D. J. Kyle, and C. J. Arntzen, J. Cell Biol. **99,** 481 (1984).
[5] B. M. Greenberg, V. Gaba, A. K. Mattoo, and M. Edelman, EMBO J. **6,** 2865 (1987).
[6] C. A. Shipton, J. B. Marder, and J. Barber, Z. Naturforsch. **45c,** 388 (1990).
[7] A. Trebst and B. Depka, Z. Naturforsch. **45c,** 765 (1990).
[8] G. H. Krause, M. Kirk, U. Heber, and C. B. Osmond, Planta **142,** 229 (1978).
[9] S. B. Powles and O. Bjorkman, Planta **156,** 97 (1982).
[10] S. Nilsen, R. Chaturvedi, and C. Dons, Acta Horticulturac **162,** 129 (1984).
[11] G. H. Krause, S. Koster, and S. C. Wong, Planta **65,** 430 (1985).
[12] T. Kuwabara and N. Murata, Plant Cell Physiol. **23,** 533 (1982).
[13] S. M. Theg, L. J. Filar, and R. A. Dilley, Biochim. Biophys. Acta **849,** 104 (1986).
[14] D. I. Arnon, Plant Physiol. **24,** 1 (1949).
[15] A. Trebst, Z. Naturforsch. **176,** 660 (1962).
[16] B. Barneyi and G. H. Krause, Planta **163,** 218 (1985).
[17] I. Virgin, S. Styring, and B. Andersson, FEBS Lett. **233,** 408 (1988).
[18] C. Critchley, Plant Physiol. **67,** 1161 (1981).
[19] K. Satoh and D. C. Fork, Photobiochem. Photobiophys. **4,** 153 (1982).
[20] K. Satoh and D. C. Fork, Plant Physiol. **70,** 1004 (1982).
[21] B. Arntz and A. Trebst, FEBS Lett. **194,** 43 (1986).
[22] P. V. Sane, U. Johanningmeier, and A. Trebst, FEBS Lett. **108,** 136 (1979).
[23] P. V. Sane and S. S. Rane, Indian J. Expt. Biol. **23,** 370 (1985).
[24] R. E. Cleland and A. Melis, Plant Cell Environ. **10,** 747 (1987).