PHOTODAMAGE INVOLVES MULTIPLE SITES AND IS DISTINGUISHABLE FROM PHOTOINACTIVATION

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Introduction

Photoinhibition of photosynthesis consists of photoinactivation of RCII and breakdown of the D1 protein (1). Photoinhibition can be strongly affected by O_2 and CO_2 (2). It is alleviated by the presence of CO_2 *in vivo* (2) or by addition of bicarbonate *in vitro* (3).

We find that multiple sites are involved in a photodamage process under CO_2- and O_2-free conditions, which are similar to the anaerobic conditions reported earlier (2-4).

Materials and methods

Axenic cultures of *Lemna gibba* L. G_3 were grown as described (5). Photoinhibitory treatment and measurement of CO_2 uptake were carried out as before (5).

Chloroplasts were prepared (6) immediately after the photoinhibitory treatments. Thylakoid membranes were obtained by suspending the chloroplasts with Tricine buffer (50 mM K-Tricine, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, pH 7.6).

Photosynthetic electron transport was measured by DCIP reduction (7) or O_2 uptake (8).

For the room temperature fluorescence measurements, a PAM system (Waltz, Effeltrich, FRG) with standard accessories and computer analysis was used. The fluorescence decay after a single turnover flash (given by XST-103, Waltz) was recorded in order to detect reoxidation of Q_A^- (9). *Lemna* plants floated on the growth medium in a home-made aluminium cuvette in which light, temperature and gas compositions were controlled. The plants were dark adapted for 10 min before the flash. 77K fluorescence emission was measured using a Perkin-Elmer LS-5 luminescence spectrometer as described (10).

Pulse-chase experiments with ^{35}S methionine were performed as before (5). Thylakoid membrane and RC II-D1 polypeptides were separated by LDS-electrophoresis and autoradiography (5). D1 was quantified densitometrically.

Results and discussion

When *Lemna* plants were illuminated by high PFDs, CO_2 uptake was photoinhibited to a much higher degree in the absence than presence of CO_2 (Fig.1). In pure N_2 the degree of photoinhibition was the highest, and it recovered very slowly (2,5). We describe the high degree of photoinhibition in N_2 as photodamage. In N_2, PSII-dependent electron transport ($H_2O \rightarrow DCIP$) was inhibited by 43% which was also the highest among the other gas conditions (Table 1). This indicates that the photodamage locates at PSII. A similar degree of inhibition of DCIP reduction was obtained using DPC as an electron donor, showing that the water-splitting complex remained unimpaired. During photodamage, inhibition of CO_2 uptake was greater than that of PSII electron transport. The difference may be due to to inhibition of PSI, since in N_2 the rate of PSI electron transport, especially the light-limited, significantly decreased (Table 1).

To probe additional effects on PSI and PSII, we measured 77K fluorescence emission before and after photoinhibition (Table 2). Two emission maxima, at 692nm and 737nm, were observed in all cases. The fluorescence emission at 692 nm, which may reflect the state of the PSII and RCII (11), showed a pattern of changes (Table 2) consistent with those in electron transport (Table 1). Fluorescence emission at 737 nm, which may be ascribed to LHCI and the PSI core (12), decreased by 42% after photoinhibitory treatment in N_2 (Table

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2). This again suggests that PSI may be inactivated during the photodamage process. The size of the PSI antenna may have decreased. This is supported by the decreased 77K fluorescence emission at 737 nm (Table 2) as well as by the inhibited PSI light-limited electron transport (Table 1).

Table 1 Inhibition of electron transport. Thylakoids were prepared from plants photoinhibited for 90 min under PFD of 2800 μmol m^{-2} s^{-1} in the gas phases indicated. The "Control" was at PFD 250 μmol m^{-2} s^{-1} in air. The rates of electron transport are expressed as a percentage of the control rate. The control rate was 268±40 μmol DCIP mg^{-1} chlorophyll hr^{-1} for the PSII reactions, and 167±15 μmol O_2 mg^{-1} chlorophyll hr^{-1} for PSI. HL: measured under saturated light; LL: measured under unsaturated light (PFD of 300 μmol m^{-2} s^{-1}).

		PSII		PSI	
		$H_2O \rightarrow DCIP$	DPC→DCIP	Asc→MV	
		HL	HL	HL	LL
Control		100	100	100	100
Photoinhibition	air	86.7	86.0	101	100
	air minus O_2	88.5	90.6	97.6	99.0
	air minus CO_2	67.9	70.2	92.2	86.7
	N_2	56.8	57.2	91.4	73.0

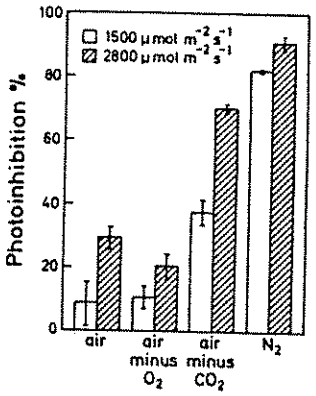

Fig.1 Loss of CO_2 uptake after photoinhibitory treatment. Plants were illuminated for 90 min under the PFD and atmospheric conditions as indicated. Vertical bars represent S.D.

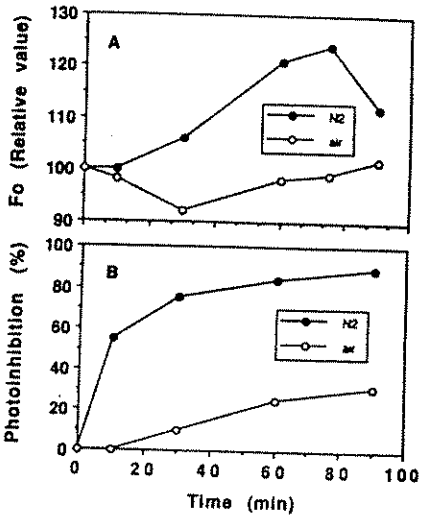

Fig.2 Photodamage occurred earlier than the rise in initial fluorescence (Fo). Plants were illuminated under a PFD of 2800 μmol m^{-2} s^{-1} in air or pure N_2. Fo (A) and CO_2 uptake (B) were measured at the times indicated and are expressed as percentages of photoinhibition (B).

To locate the site of inhibition within RCII during photoinhibition, kinetics of fluorescence decay were examined after giving intact plants a single turnover flash. The fluorescence decay during 40 msec showed three exponential components. The first component reflects the forward electron flow from Q_A^- to Q_B (13). The $t_{1/2}$ of this phase was increased by increasing PFDs (Table 3). Under 2800 mmol m^{-2} s^{-1}, the increase of $t_{1/2}$ of the fast phase was 20% greater in the presence than absence of O_2, and 19% greater in the absence than presence of CO_2 (Table 3). Photoinhibition in pure

Table 2 77K fluorescence emission during photoinhibition. *Lemna* plants were photoinhibited as that in Table 1. Fluorescence spectra were obtained for thylakoid extracts at equal chlorophyll concentration (7 μg ml^{-1}), and were normalised to fluorescence at 550 nm from 1μM fluorescein. The normalised values for total fluorescence emission are given in arbitrary units ±S.D. The "Control" condition was the same as that in Table 1.

		PSII (F_{692})	PSI (F_{737})
Control		0.47±0.03	0.66±0.06
Photoinhibition	air	0.38±0.04	0.58±0.07
	air minus O_2	0.43±0.01	0.62±0.03
	air minus CO_2	0.30±0.02	0.49±0.00
	N_2	0.24±0.04	0.38±0.09

N_2 resulted in a similar degree of inhibition of electron flow from Q_A^- to Q_B as that occurred in air (Table 3). In contrast, the inhibition of CO_2 uptake (Fig.1) and of PSII electron flow (Table 1) was much higher in N_2 than those in air. The higher inhibition of PSII electron transport in N_2 could be due to inhibition of RCII components from P680 to Q_A, since the water splitting component as a donor to PSII seems to have been unimpaired (Table 1).

Table 3 Kinetics of fluorescence decay and D1 degradation after photoinhibition. Intact plants were given a single turnover flash before (Control) and after photoinhibition in the conditions indicated. "Control" condition was the same as that in Table 1.Ymax was the maximal fluorescence following one flash. Fo and Ymax values after photoinhibitory treatment are expressed as percentages of the values before photoinhibitory treatment. The 100% values for Fo and for Ymax were the averages from the controls of each experiment. +CAP: in presence of 500 μg ml^{-1} chloramphenicol.

		$t_{1/2}$ (μs) of fast phase			Fo±S.D. (%)	Ymax (%)	Degradation of D1 (%)
PFD (μmol m^{-2} s^{-1})	Atmosphere	Control	Photoinhibited	Increase in $t_{1/2}$ (%)	Photoinhibited	Photoinhibited	Photoinhibited
1500	air	486	547	13±5	96±2	76	43
	air minus O_2	493	496	1±2	93±7	75	25
	air minus CO_2	459	700	53±8	102±2	77	48
	N_2	508	545	7±15	98±6	78	33
	air (+CAP)	509	587	15	102	61	
2800	air	478	646	35±7	102±5	65	
	air minus O_2	529	563	6±11	102±4	76	
	air minus CO_2	512	937	83±14	107±2	63	
	N_2	460	616	34±2	112±2	74	

After photoinhibitory treatment, intrinsic fluorescence, Fo, increased in N_2 and, to a lesser extent, in air minus CO_2. Fo did not increase in the other gas phases (Table 3). The increased Fo can be due to RCII inactivation by trapping of Q_A^- or by Q_B^- destabilization (1). Fo did not rise in air *in vivo*, although the Q_A reoxidation was inhibited (Fig.2; Table 3). The kinetic studies show that, during photoinhibition in N_2, the rise of Fo occurs later than the inhibition of net photosynthesis (Fig.2). This suggests that the rise in Fo is a secondary event arising as a consequence of photodamage. Moreover, the rise in Fo during photodamage may indicate that PSII charge separation is inactivated. This is supported by the decrease in 77K fluorescence emission at 692 nm (Table 2), which is thought to be fluorescence from the photoactive pheophytin *a* that functions as an electron acceptor in RCII (11).

To test whether the lowered photosynthetic rate (Fig.1) and PSII-electron transport (Table 1) in N_2 result from impaired energy transfer from the antenna to the reaction centre of PSII, we measured PSII electron transport under the actinic light intensities below and above saturation. The results show that inhibition of electron transport could not be improved even if the measuring light intensity was increased by five times (data not shown). This indicates that energy transfer to RCII is unlikely to have impaired in these experiments.

Under high PFD in the atmospheres containing CO_2, photoinactivation (see Introduction), but not photodamage, occurred. The rate of CO_2 fixation (Fig.1) and PSII activity (Table 1 and 2) decreased by a lesser degree during photoinactivation than during photodamage. Kinetics of Fluorescence decay showed that the rate of electron flow from Q_A^- to Q_B was inhibited by 26% as represented by the $t_{1/2}$ after the plants were illuminated under PFD of 2800 mmol m^{-2} s^{-1} in air (Table 3). The inhibition of reoxidation of Q_A^- fitted well with the inhibition of CO_2 uptake and that of PSII electron transport, and was largely prevented in air minus O_2 (Table 3). These results indicate that the photoinactivation is O_2 dependent and largely due to the inactivation of Q_B-site of the D1 protein. Pulse-chase experiments showed that the RCII-D1 protein degraded more rapidly than inhibition of Q_A reoxidation and was

accelerated by presence of O_2 and absence of CO_2 (Table 3). D1 degradation without loss of Q_B reduction could result from efficient D1 synthesis and processing, since loss of Q_B reduction was increased by chloramphenicol, an inhibitor of protein synthesis (Table 3).

The above results indicate that multiple sites are involved in a photodamage process induced by CO_2- and O_2-free conditions. The inactivated sites include the RC II components from P680 to Q_A, the Q_B site, and a RC component of PSI. The donor side of PSII and excitation energy transfer within PSII seem to be unimpaired. We suggest that the photodamage results from the over-reducing conditions and depletion of bicarbonate. Bicarbonate is essential for keeping RCII functional (14). The CO_2- and O_2-free conditions may have caused partial removal of bicarbonate from its D1 binding site, since in *Lemna* the thylakoid has a relatively low pH under physiological condition, and this favours the dissociation of HCO_3^- to CO_2 (14). High light may promote the removal of bicarbonate, since high light drives CO_2 consumption by its assimilation. The removal of bicarbonate results in inactivation of quinone reactions (14), and may lead to inactivation of another component, as indicated in the present work, located between P680 and Q_A. The effect of bicarbonate-depletion is clearly reflected by the rapid degradation of D1 (Table 3), otherwise D1 degradation is largely decreased by O_2-free (1) and by a high ratio of PQH_2/PQ (15) during photoinhibition *in vivo*.

We suggest to distinguish between photodamage and photoinactivation. Photoinactivation occurs in air and the site is within PSII (1). At an early stage *in vivo*, high light drives RCII charge separation in a high rate, resulting in over-reduction of the PQ pool due to rate limitation of NADPH oxidation. When the PSII is gradually photoinactivated, the PQ pool become more oxidized due to a rate limitation of PSII electron flow (1). A high turnover and high ratio of PQ/PQH_2 favour the break down of D1 and thereby promote a degree of inactivation at this stage (15). In contrast, photodamage results from extreme reducing conditions and lack of bicarbonate in the reaction centres. The PQ pool is kept in a fully reduced state consistently. Fo rises during photodamage but not during photoinactivation (Table 3; Fig.2). The rise in Fo reflects over-reduction during photodamage. During photoinactivation such reducing condition may be transient and followed by gradual reoxidation of the PQ pool, and thus not sufficient to cause a rise in Fo. Photoinactivation is strongly temperature-dependent and is enhanced at low temperatures (1,5). In contrast, photodamage is relatively temperature-independent but the following recovery strongly depends on the temperature used during photodamage (5). Higher temperature induced photodamage inhibits subsequent recovery (5). Moreover, oxygen alleviates photodamage but aggravates photoinactivation (Fig.1, Table 1 and 3).

The mechanisms of inactivation of PSI (especially light limited, Table 1 and 2) during photodamage requires further investigation.

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