Plant Physiol. (1982) 70, 1004–1008
0032-0889/82/70/1004/05/$00.50/0

Photoinhibition of Reaction Centers of Photosystems I and II in Intact *Bryopsis* Chloroplasts under Anaerobic Conditions[1]

Received for publication March 30, 1982 and in revised form June 1, 1982

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ABSTRACT

Illumination of intact *Bryopsis corticulans* chloroplasts under anaerobic conditions induced a decline of chlorophyll fluorescence and photoinhibition of Photosystems I and II. The time course of the light-induced decline of chlorophyll fluorescence and the decreases of activities of reactions sensitized by Photosystems I and II were compared. Photosystem I activity decreased in parallel with the disappearance of active P700. The time course of the destruction of the reaction center of Photosystem II was similar to that of photoinhibition of 2,6-dichlorophenolindophenol-Hill reaction.

It appears that the initial events in photoinhibition are the destruction of the reaction centers of Photosystems I and II and that the reaction centers that are inhibited become quenchers of chlorophyll fluorescence.

Effects of inhibitors of electron transfer and of an electron donor to Photosystem I showed that photoinhibition was related to Photosystem I activity.

Recently, we reported that under anaerobic conditions, illumination of intact *Bryopsis corticulans* chloroplasts caused photoinhibition of PSI and II and a decrease in the yield of Chl fluorescence (20). The time course of the decline of Chl fluorescence showed that there were at least two exponential phases. The rapid phase (R-phase) corresponded to the photoinhibition of PSII and the slow phase (S-phase) corresponded to the photoinhibition of both photosystems (20). The fluorescence decline and the photoinhibition were observed with very low light intensities (less than 1 w/m^2) and with very short light exposures (20). Photoinhibition in isolated chloroplasts has been studied intensively by Kok and his co-workers (7, 8, 10) and by Satoh (15–18). However, in these cases (under aerobic conditions, using broken chloroplasts), very strong light was needed to induce the photoinhibition (7, 8, 15). Powles and co-workers (12–14) have studied photoinhibition in leaves of higher plants under low O_2 and CO_2 concentrations. The materials we have been using are intact *Bryopsis* chloroplasts. The advantages of using intact chloroplasts are: (a) more uniform illumination is possible in chloroplasts than in leaves, (b) measurements of activities of PSI and II after the photoinhibition are easier, (c) several reagents can reach the thylakoid membranes more easily, and (d) more complete anaerobiosis can be obtained in intact chloroplasts because O_2 may come out of the chloroplasts and be consumed rapidly by dithionite or by a glucose-glucose oxidase system. O_2 which is evolved by PSII in leaves may stay longer inside the cells and decrease the anaerobiosis. Therefore, the use of intact chloroplasts under anaerobic conditions is well suited to the study of the initial events of photoinhibition.

In this paper, we characterized the photoinhibition further and obtained results which showed that the reaction centers of PSI and II were inhibited by light and that the reaction centers that were destroyed became quenchers of chlorophyll fluorescence.

MATERIALS AND METHODS

The marine green alga, *Bryopsis corticulans* was collected at Monterey Bay, CA. Preparation of intact chloroplasts was done by two mild centrifugations (1,000*g*, 15 s) (9, 25). Concentrations of Chl were determined according to the method of Arnon (1).

Chl fluorescence was measured at 685 nm, using a photomultiplier EMI 9558B (19). For measurements of the fluorescence induction in the presence of DCMU in the ms time scale, the chloroplasts were excited with a low intensity of light (2.2 w/m^2) and a Nicolet 1010 Signal Averager was used as a transient time converter.

Rates of O_2 consumption were measured with a Clark-type O_2 electrode (19).

The extents of P700 photooxidation were measured using a single-beam spectrophotometer. A second photomultipler out of the measuring beam was used to cancel Chl fluorescence (3). Absorbance changes at 560 nm induced by a xenon flash were measured with the single beam spectrophotometer set to have a time constant of 10 ms (5), and eight signals were averaged using a Nicolet 1010 signal averager.

Preillumination of the chloroplasts under anaerobic conditions was done as described previously (20). Anaerobic conditions were obtained by removing O_2 upon the addition of dithionite to the reaction mixture. After the preillumination, the chloroplasts were washed by two centrifugations (1,000*g*, 15 s) to remove the dithionite.

The basal reaction mixture contained 1.0 M sorbitol, 11 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM $NaNO_3$, 50 mM Hepes (pH 7.5) and intact *Bryopsis* chloroplasts containing the indicated amounts of Chl. For the measurements of P700 photooxidation, 1.0 M sorbitol was omitted from the reaction mixture.

All the measurements were carried out at room temperature.

RESULTS

Figure 1 shows the time course of the yield of Chl fluorescence upon illumination of intact *Bryopsis* chloroplasts under anaerobic conditions. The dotted line shows the F_0 level measured separately at a faster time scale using the same chloroplast preparation. The yield of Chl fluorescence decreased strongly and after 5 min of illumination of the chloroplasts had attained a level less than one-fifth of the maximum intensity. Figure 1 shows clearly that the

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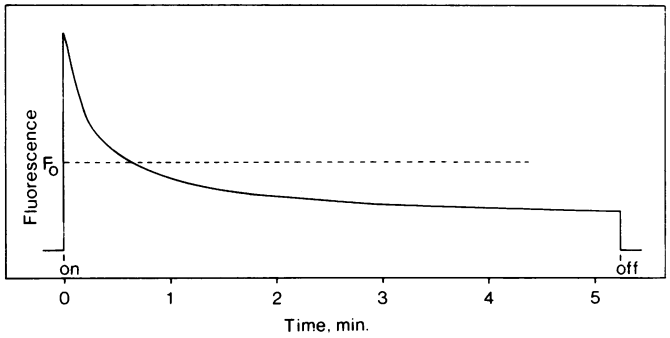

FIG. 1. A time course of fluorescence quenching in intact *Bryopsis* chloroplasts under anaerobic conditions. The reaction mixture contained 1.0 M sorbitol, 11 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 2 mM $NaNO_3$, 50 mM Hepes (pH 7.5), 5 mM dithionite, and intact *Bryopsis* chloroplasts equivalent to 18.6 μg Chl/ml. The intensity of the actinic light was 77 w/m^2. Dotted line shows the F_0 level.

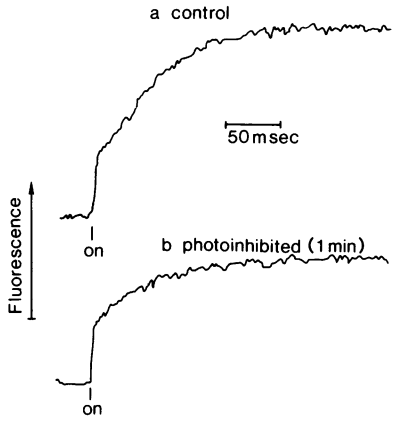

FIG. 2. Time courses of Chl fluorescence in the presence of DCMU before and after the preillumination of the chloroplasts. Time courses were measured after washing the chloroplasts twice by centrifugation (see text) and transferred to aerobic conditions. Where indicated, the chloroplasts were preilluminated for 1 min under anaerobic conditions (in the presence of 5 mM dithionite). Intensities of the preillumination and the excitation light were 77 and 2.2 w/m^2, respectively. Concentrations of DCMU and Chl were 10 μM and 3.2 μg/ml, respectively. Other conditions were the same as in Figure 1.

yield of the Chl fluorescence decreased even below the F_0 level, indicating that not only F_v but also F_0 was quenched during the illumination of the chloroplasts.

Figure 2 shows the induction of Chl fluorescence in the presence of DCMU in control chloroplasts and in chloroplasts preilluminated for 1 min. After the preillumination, the chloroplasts were washed twice by centrifugation to remove the dithionite and transferred to aerobic conditions to make sure that Q was oxidized before the measurements of Chl fluorescence. In the preilluminated chloroplasts, the extent of F_0 level was about the same but F_v was decreased (Fig. 2b). The same phenomenon (the decrease of only F_v) was observed in chloroplasts which had been preilluminated more than 3 min (data not shown). In the preceding paper (20), we showed that there was a partial recovery of the yield of Chl fluorescence if the chloroplasts which had been preilluminated for more than 15 s were transferred from anaerobic to aerobic conditions. This result, together with that shown in Figure 2, suggests that the quenching of F_0 fluorescence is mostly reversible and is recovered quickly by the transfer of the chloroplasts from anaerobic to aerobic conditions.

Figure 2 also shows that, although the extent was lowered, the time course and half increase time of the variable fluorescence were not affected greatly by preillumination of the chloroplasts.

Table I. *Effects of the Preillumination Period on the Rate of Photosystem I Activity and the Extent of Photooxidation of P700*

Chloroplasts were preilluminated in the presence of 5 mM dithionite for the periods indicated in the Table and then washed by two centrifugations to remove the dithionite. For measurements of the rate of methyl viologen photoreduction, 2 mM methyl viologen, 0.2 mM DCIP, 5 mM ascorbate, and 10 μM DCMU were added. The concentration of Chl was 15 μg/ml. For measurements of P700, 1.0 M sorbitol was omitted from the reaction mixture, and 2 mM ascorbate and 10 μM DCMU were added. The Chl concentration was 45 μg/ml. Other conditions, see the text.

Preillumination Time	P700	Rate of Methyl Viologen Photoreduction
s	%	
0	100	100
5	99.5	99.5
20	93.1	94.5
45	86.6	87.2

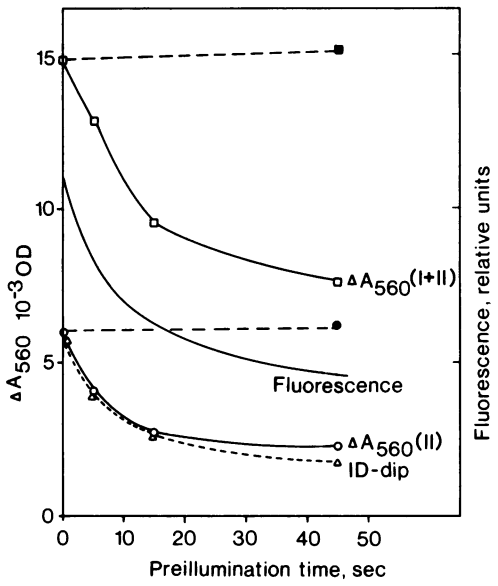

FIG. 3. Extents of Chl fluorescence, the ID-dip and of flash-induced electrochromic shifts of carotenoids after various periods of preillumination of *Bryopsis* chloroplasts. The time course of Chl fluorescence was measured under the same anaerobic conditions as the preillumination of the chloroplasts as described in Figure 1. The extent of the ID-dip was measured as reported previously (22). The concentration of Chl was 265 μg/ml. For measurements of the electrochromic shifts of carotenoids, the chloroplasts that had been preilluminated in dithionite were washed twice by centrifugation and transferred to aerobic conditions and the extents of flash induced absorbance increases at 560 nm were determined. Eight signals were added and averaged as described in the text. To inhibit the reaction center of PSII, 10 μM DCMU and 1 mM NH_2OH were added and the chloroplasts were preilluminated several times with a saturating xenon flash. The concentration of the Chl was 200 μg/ml.

This indicates that the number of antenna chlorophylls per reaction center of PSII was little affected.

It has been suggested that photoinhibition of the electron transfer system might occur near the reaction center of PSII (8, 10, 18) or in some algae at the site of P700 (4). Therefore, we compared the activity changes of the reaction centers of PSI and II with the photoinhibition process. Table I shows the activity of PSI and the extent of light-induced changes of P700 after illumination of the chloroplasts for various periods. The linear relationship between the two clearly shows that photoinhibition of the PSI reaction is due to the photodestruction of the reaction center of PSI.

Figure 3 shows the flash-induced electrochromic shifts of carot-

enoids after various periods of illumination of the chloroplasts. In *Bryopsis* chloroplasts, siphonaxanthin and siphonein act as indicators of membrane potential changes and give rise to absorbance changes around 560 nm (11, 21). The extent of the initial rapid change is attributed to a transmembrane charge separation at the reaction centers of both photosystems (squares, see ref. 24). In the presence of DCMU and NH_2OH, PSII activity is completely inhibited (2). Therefore, the difference of the extents of electrochromic shifts in the presence and absence of DCMU and NH_2OH corresponds to the charge separation produced by the reaction centers of PSII (circles). PSII activity decreased rapidly with illumination of the chloroplasts under anaerobic conditions (with 15 s of preillumination, PSII activity decreased to about 45%). The decrease of the activity became very slow after longer periods of preillumination. The fluorescence time course measured under the same conditions is also shown in Figure 3. In the preceding paper (20), we measured the activities of DCIP[3]-Hill reaction after various periods of preillumination of the chloroplasts under the same conditions. The decrease of the DCIP-Hill reaction activity was the same as the fluorescence decay if the preillumination time was less than 10 s. With longer periods, the rate of decrease of the DCIP-Hill reaction activity became lower than that of the fluorescence decay. The DCIP-Hill reaction activity was about 70% of the control after 15 s of preillumination (see Fig. 4 in Ref. 1). The difference of the magnitudes of the photoinhibition of the DCIP-Hill reaction and of the reaction centers of PSII can be explained by the presence of a rate-limiting step in the DCIP-Hill reaction. Inasmuch as we could not use much higher concentrations of DCIP because of its strong absorption of the actinic light and because DCIP cannot penetrate into the intact chloroplasts very rapidly, the electron accepting reaction of DCIP from electron transport component(s) may become rate-limiting. In this case, the inhibition of the reaction center may induce a lesser decrease of the DCIP-Hill reaction than expected. Therefore, the decrease of the DCIP-Hill reaction can be explained by the photodestruction of the reaction center of PSII. The same kinetics of the photoinhibition of the reaction center of PSII compared to that of the DCIP-Hill reaction support this idea. If photoinhibition of the reaction center of PSII is the main early event of photoinhibition and the inhibited reaction center becomes a quencher of Chl fluorescence, then the time course of F_v quenching and of photoinhibition of system II reaction centers should be the same. If this idea is true, we can explain the similar decreases of fluorescence and system II activity in terms of photoinhibition of system II reaction centers. Under anaerobic conditions, Q remained reduced and we were unable to measure F_v directly. Therefore, we measured changes in the extent of the initial fluorescence transient termed the "ID dip" (Ref. 22; Fig. 3, triangles). Under the conditions where PSI activity was little changed (as was the case in these experiments; see Table I), the extent of the ID dip can be used as a measure of F_v. This is because the ID dip is produced upon photooxidation of Q by PSI and the extent of F_v is parallel to the reduced level of Q (22). It was seen that the time course of the decrease of the ID-dip (parallel to F_v) was similar to the photoinhibition of the reaction center of PSII except after 45 s of preillumination. The difference seen after 45 s of preillumination may be due to a partial recovery of fluorescence under aerobic conditions (20), because we measured fluorescence time courses under anaerobic conditions while reaction center activities were observed under aerobic conditions. No photoinhibition of the reaction centers of either photosystem was observed in aerobic conditions. The flash-induced membrane potential change in chloroplasts preilluminated for 45 s under aerobic conditions was just the same as in control chloroplasts (solid square, induced by both photosystems; solid circle, induced by PSII).

[3] Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; PMA, phenylmercuric acetate.

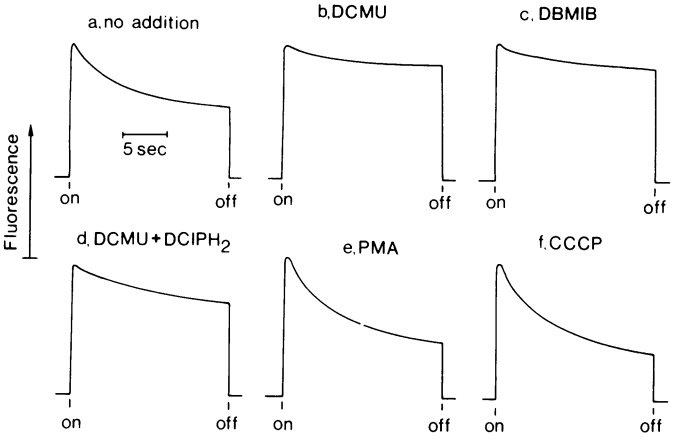

FIG. 4. Effects inhibitors of electron flow, of an electron donor to PSI and of an uncoupler of photophosphorylation on the time course of Chl fluorescence under anaerobic conditions. Where indicated, 10 μM DCMU, 10 μM DBMIB, 0.2 mM DCIP with 5 mM ascorbate, 100 μM PMA or 1 μM CCCP were added. The intensity of the actinic light was 200 w/m^2. The concentration of Chl was 2.7 μg/ml. Other conditions were the same as in Figure 1.

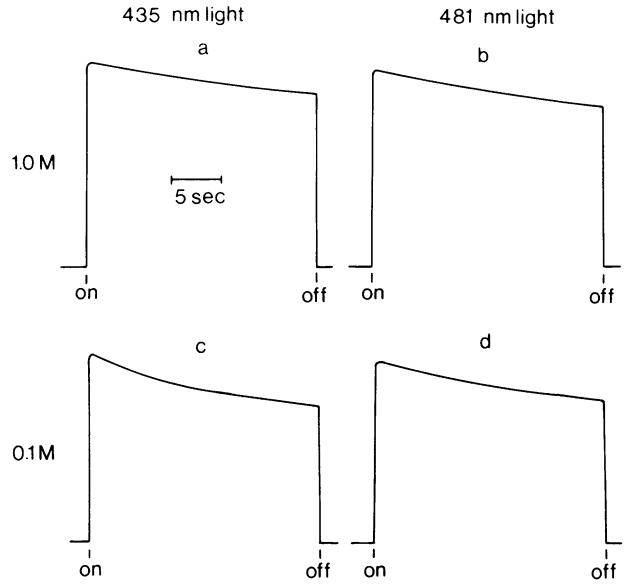

FIG. 5. Effects of DCMU on the time course of Chl fluorescence in the presence of 0.1 M sorbitol under anaerobic conditions. Where indicated, 10 μM DCMU was added. The concentration of sorbitol was lowered to 0.1 M. Other conditions were the same as in Figure 4.

Figure 4 shows effects of inhibitors of electron transfer and of an uncoupler of photophosphorylation on the fluorescence quenching under anaerobic conditions. Inhibitors of electron flow between PSI and II (DCMU and DBMIB) inhibited the quenching (Fig. 4, b and d). PMA which inhibits the electron flow on the reducing side of PSI (at the site of ferredoxin and ferredoxin-$NADP^+$ reductase, see Ref. 6) slightly accelerated the quenching (Fig. 4e). Inhibition of the quenching by DCMU was partly recovered by further addition of $DCIPH_2$ which acts as an electron donor to PSI (Fig. 4d). An uncoupler of photophosphorylation (CCCP) which accelerates the flow of electrons slightly increased the rate of the quenching (Fig. 4f). Similar effects of DCMU and $DCIPH_2$ on the photoinhibition of the reaction center of PSI was also observed (data not shown). These data clearly show that the photoinhibition was induced by electron transport activity through PSI. Inasmuch as PMA had a slightly accelerating effect (Fig. 4e),

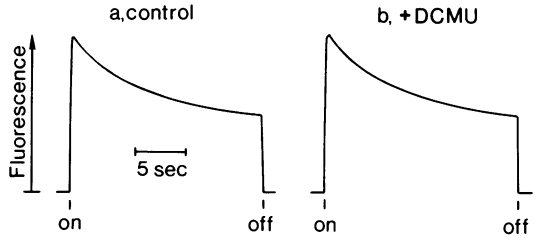

FIG. 6. Time courses of fluorescence quenching with system I (435 nm) and system II (481 nm) lights in the presence of 1.0 M or 0.1 M sorbitol under anaerobic conditions. Where indicated, 1.0 M or 0.1 M sorbitol was used. The 435 nm and 481 nm light was obtained using Balzers interference filters centering at 435 nm or 481 nm, respectively, and attenuated by Balzers neutral density filters. The light source was a 21 v, 150 w tungsten lamp. The light intensities of the 435 nm and 481 nm lights were 15 and 8.7 w/m^2, respectively.

it could be that the reducing power accumulated between ferredoxin and the reaction center of PSI was the cause of the destruction of the reaction centers.

Figure 5 shows the effect of DCMU on the fluorescence quenching when the concentration of sorbitol was lowered to 0.1 M. In contrast to the results in Figure 4b, DCMU failed to inhibit the fluorescence quenching. At the isosmotic concentration of sorbitol (1.0 M), dithionite cannot penetrate into the chloroplasts easily but at 0.1 M, dithionite penetrates into the chloroplasts and is able to donate electrons to electron carriers of electron transport systems (23). This result also supports the idea that the photoinhibition of the reaction centers of both photosystems was induced by the electron transfer activity through PSI.

This idea was further supported by the results of Figure 6 that show the effects of light absorbed by system I (435 nm, Chl *a*) and system II (481 nm, Chl *b*) on the rate of the fluorescence quenching. Both the system I and II lights were attenuated so as to excite pigment-system II equally (to give the same intensity of fluorescence). At 1.0 M sorbitol, no difference was observed with the system I and II lights. However, at 0.1 M sorbitol, the system I light quenched fluorescence more rapidly than the system II light. At 1.0 M sorbitol, there might be only a small amount of an endogenous electron donor to PSI inside the isolated chloroplasts. Therefore, the electron transfer through PSI was limited by the activity of PSII. At a concentration of 0.1 M sorbitol, dithionite was able to donate electrons to PSI (23) resulting in more PSI activity with system I light.

DISCUSSION

The decrease of Chl fluorescence under anaerobic conditions shows that either quencher formation (increase of K_D) or the change in energy distribution to pigment-system II or the increase in energy transfer from system II to I (increase of k_T) occurs during illumination of the chloroplasts. The data contained in Figure 2 show that the rate of the photoreduction of the remaining Q was little altered by the preillumination of the chloroplasts. This result means that energy distribution to pigment-system II and spillover of energy from pigment system II to I was not greatly changed. If quenchers were formed at random among the Chl bed, they should also decrease the quantum yield of PSII activity, and this is obviously not the case. The data of Figure 3 clearly show that the reaction center of PSII was destroyed by the preillumination of the chloroplasts. Therefore, it seems likely that these inhibited reaction centers of PSII become potent quenchers of Chl fluorescence. In this way, we can explain the parallel decreases of PSII activity and fluorescence intensity without significant effects on the quantum yield of the intact reaction centers of PSII. Direct comparison of the extent of F_v and the activity of PSII may give a strict proof to this idea. But in this study, we could not compare the two values directly because there was also a quenching of F_0 fluorescence and it recovered very quickly under the conditions where the reaction center activity was measured (Figs. 1 and 2). Instead, we measured the extent of the ID-dip which has a linear relationship to the extent of F_v (Fig. 3), and we found that the decrease of the extent of the ID-dip was just parallel to that of PSII activity, especially with shorter periods of preillumination of the chloroplasts.

Another important feature of the photoinhibition seen here is that the photodestruction of the reaction centers of both photosystems was induced by the activity of PSI (Figs. 4–6). Inasmuch as PSI and II are located separately within the thylakoid membranes, this result suggests that some mobile substance(s) or particular state of the thylakoid membrane, are related to the destruction of the reaction center of PSII. The nature of the mobile substance (or this membrane state) is not clear. Because antimycin A had no effect on the fluorescence quenching (data not shown), a cyclic electron transport system around PSI cannot be involved in photoinhibition.

Acknowledgments—The authors acknowledge the help of Ms. Celia Smith who aided us in obtaining the *Bryopsis* used in this work.

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