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Effects of in vivo CO_2-depletion on electron transport and photoinhibition in the green algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii*

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Abstract

Short-term illumination of the green algae, *Chlamydobotrys stellata* and *Chlamydomonas reinhardtii* in CO_2-depleted cultivation medium under low photon flux density (50 and 150 $\mu mol\, m^{-2}\, s^{-1}$, respectively) resulted in an inhibition of Photosystem II electron transport from water to diaminodurene, but only slightly affected the electron flow from water to 2,6-dichlorobenzoquinone. The intermediary fluorescence level, F_i was raised to the maximum level of fluorescence, F_m. The initial level of fluorescence, F_o was considerably enhanced. The development of the F_o rise was facilitated by low pH, but inhibited in the presence of an acceptor, dichlorobenzoquinone, or by chemical cross-linking of proteins with glutaraldehyde. The uninhibited electron transport and the original F_o level were restored by readdition of CO_2 or by dark adaptation of algae. The observations suggest that in green alga cells CO_2-depletion in the light results in a reversible inhibition of steady-state electron flow between the primary (Q_A) and secondary quinone electron acceptor (Q_B). Following the inhibition of electron transport a long-lived but reversible state of singly-reduced and probably protonated Q_A is formed which manifests itself as an apparent F_o rise. Prolonged photoinhibitory illumination of the CO_2-depleted green alga cells resulted in an irreversible loss of variable fluorescence and electron transport. The photoinactivation developed more slowly in the CO_2-depleted than in the CO_2-containing cells. It is concluded that in the bicarbonate-depleted redox state, which is accompanied with an enhanced F_o level of fluorescence, the Photosystem II reaction center is less susceptible to photoinhibition than in the bicarbonate-containing state.

Keywords: Bicarbonate depletion; Electron transport; Photoinhibition; Photosystem II; Thermoluminescence; (Green alga)

1. Introduction

Bicarbonate is a ligand to the non-heme iron in the Q_A-Fe-Q_B quinone-iron complex of PS II [1–3]. It has been suggested that it is a structural requirement for the native configuration of the reaction center and is involved in the protonation of the secondary quinone electron acceptor of PS II [4–6]. Bicarbonate can be removed from its binding site by several monovalent anions, of which formate is the most effective [1]. In isolated thylakoid membranes depleted of bicarbonate by formate the steady-state electron transport is inhibited [1,2,6,7]. However, it has not been unambiguously clarified whether the inhibition of electron flow by formate treatment is a consequence of the removal of bound bicarbonate or is caused by an inhibitory action of formate [7]. In order to study the in vivo effect of HCO_3^- on electron transport, investigation should be carried out by depletion of bicarbonate in the absence of formate.

Under high photon flux density (PFD) the light energy funneled to the reaction center chlorophyll can exceed the capacity of photosynthetic system to utilize this energy in metabolic reactions or dissipate it in harmless processes. As a consequence, the Photosystem II (PS II)-catalysed

Abbreviations: D1, polypeptide of the reaction center; DBMIB, dibromothymoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_o, initial fluorescence; F_i, intermediate fluorescence level; F_m, maximum fluorescence; F_v, maximum variable fluorescence; P680, primary electron donor chlorophyll of PS II; PFD, photon flux density; PS I, Photosystem I; PS II, Photosystem II; Q_A and Q_B, primary and secondary quinone electron acceptor of PS II; Y_Z^+, redox active tyrosine-161 of the D_1 protein.

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electron transport is inhibited and the variable part of fluorescence emission from PS II is lost [8–10]. In the following the phenomenon will be designated as "reversible" part of photoinhibition if the alterations induced by light stress can be abolished by dark adaptation. If the original state can not be restored by dark adaptation the term "irreversible photoinhibition" will be used.

Interestingly, inhibition of the Q_A to Q_B electron transfer by DCMU-type inhibitors confers protection of the PS II reaction centers against irreversible photoinhibition [11–13]. Photoinhibition was also alleviated when the oxidation of Q_A^- was retarded by low temperature during exposure of *Chl. reinhardtii* cells to high intensity light at 5° C [14]. Moreover, it has been observed that the Q_B-nonreducing PS II reaction centers are highly resistant to photoinhibition [15–17]. These observations suggest that other treatments inhibiting the oxidation of Q_A^- by Q_B may also ease photoinhibition. Consequently, we assumed that bicarbonate/CO_2-depletion which inhibits the oxidation of Q_A^- by Q_B or Q_B^- [1,2,6] would also provide protection against photoinhibition.

Photoinhibitory experiments carried out with CO_2/bicarbonate-depleted samples provided contradictory results. Under CO_2-deficiency the photosynthetic apparatus exhibited enhanced sensitivity to photoinhibition [15,18]. In accordance with this, carbon dioxide dissolved from the atmosphere or addition of extra HCO_3^- provided protection against photoinhibition [19,20]. However, algae deficient in inorganic carbon were less susceptible to photoinhibition than those grown in the presence of inorganic carbon [21]. Thylakoid membranes depleted of bicarbonate by formate treatment also became less susceptible to photoinhibition as compared to non-depleted thylakoid membranes under normal oxygen tension [19,20].

In the present work we observed that in vivo CO_2-depletion of *Chl. stellata* and *Chl. reinhardtii* green alga cells by bubbling the culture medium with CO_2-free air resulted in an inhibition of electron transport from Q_A to the plastoquinone pool demonstrating the requirement of bound bicarbonate in an uninhibited electron flow. It was also found that CO_2-depleted green alga cells, which exhibit high F_o level and accumulate stable reduced Q_A species [22,23] during illumination, are less susceptible to photoinhibition than cells in the non-depleted state.

2. Materials and methods

Chl. stellata, strain 10-1e, Sammlung von Algenkulturen, Pflanzenphysiologisches Institut, Universität Göttingen, Germany and *Chl. reinhardtii* green algae were cultivated at pH 7.0 under continuous light (50 $\mu mol\, m^{-2}\, s^{-1}$ and 150 $\mu mol\, m^{-2}\, s^{-1}$, respectively) by bubbling the cultures with a mixture of 5% CO_2 and 95% air (v/v) [24]. CO_2-depletion was achieved by purging the alga suspensions with CO_2-free air.

The rate of steady-state oxygen evolution was measured in the culture medium by using a Clark-type electrode in a temperature controlled cell at 25° C under saturating white light. The whole-chain basal electron transport rate was measured without any addition. Partial electron-transport rate measurements of PS II were carried out from water to diaminodurene (200 μM) and from water to 2,6-dichloro-*p*-benzoquinone (200 μM). To exclude the influence of CO_2 fixation (Calvin cycle), 1 μM dibromothymoquinone (DBMIB) which blocks electron transport between the plastoquinone pool and the cytochrome *b/f* complex was added to the assay medium. DBMIB not only blocked the electron transport at plastoquinone, but also slightly decreased the rate of O_2 evolution in all Hill reactions. For a most comparable illustration and in order to ignore the inhibitory effect of DBMIB, the data of the individual Hill reactions were plotted as percentages of controls. Before oxygen measurements, the samples taken from the culture vessel were incubated in the dark for 1 min in the presence of the various chemicals.

The alga cultures were centrifuged to a chlorophyll concentration of 125 μg/ml and 0.4 ml aliquots of the suspensions were used for measurements of thermoluminescence. Thermoluminescence glow curves were measured in the temperature region from −80 to +80° C using an apparatus described in [25]. Samples were excited by white light (50 $\mu mol\, m^{-2}\, s^{-1}$) at −80° C for 1 min and heated in the dark at a rate of 20 C°/min.

Fluorescence induction transients were measured in a home-built setup. The cell suspension was illuminated by blue light (Corning 4-96 filter) after a Compur shutter had been opened. The emitted fluorescence was detected through a red filter (Corning 2-94) by an RCA 4463 photomultiplier. The signal was stored in a Nicolet transient recorder and plotted on an *X–Y* recorder.

Photoinhibitory illumination of *Chl. stellata* and *Chl. reinhardtii* alga cultures was carried out at room temperature with white light under photon flux densities of 1500 and 5000 $\mu mol\, m^{-2}\, s^{-1}$, respectively. The heat effect of light was filtered out with a water filter. Following photoinhibitory illumination the CO_2-depleted culture was bubbled for 5 min with CO_2-containing air. Afterwards, both the CO_2-depleted and CO_2-containing cells were kept under the cultivation light for 60 min preceding fluorescence induction and electron transport rate measurements.

3. Results

3.1. Effects of CO_2-depletion on the steady-state electron transport of PS II

Fig. 1 shows the electron transport rates of PS II under various experimental conditions in the course of CO_2 depletion of *Chl. stellata*. Without any addition, the photosynthetic electron transport was almost completely inhib-

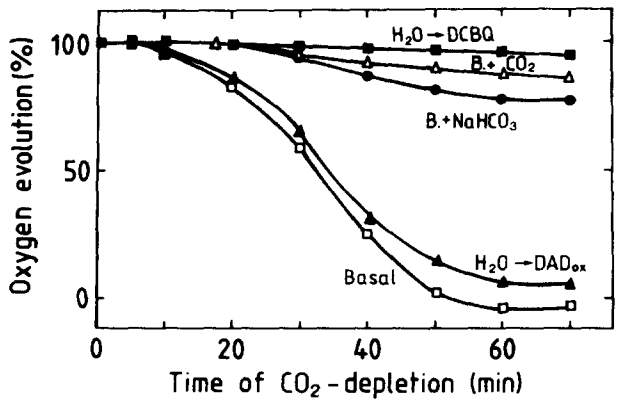

Fig. 1. Partial electron transport rate measurements of the green alga, *Chl. stellata* during CO_2-depletion in the light (50 μmol m^{-2} s^{-1}). □—□, basal electron transport (abbreviated as B.); ▲—▲, $H_2O \rightarrow 200$ μM oxidized diaminodurene (DADox + 1 μM DBMIB); ■—■, $H_2O \rightarrow 200$ μM 2,6-dichloro-*p*-benzoquinone (DCBQ + 1 μM DBMIB); ●—●, basal electron transport + 5 mM $NaHCO_3$ (incubated for 5 min); △—△, basal electron transport + CO_2 (bubbled with CO_2-containing air for 5 min). The absolute rates of O_2 evolution in the autotrophic alga before CO_2-depletion in the same order as the partial electron transport reactions were: 130 μM; 78 μM; 160 μM; 110 μM; 130 μM oxygen evolved/mg Chl per h.

ited in the first hour of CO_2-deprivation. The $H_2O \rightarrow DAD_{ox}$ reaction (oxidized diaminodurene, DAD_{ox}, intercepts electrons at the plastoquinone pool) was also completely inhibited by CO_2 depletion (Fig. 1). However, the $H_2O \rightarrow$ DCBQ Hill reaction (2,6-dichlorobenzoquinone, DCBQ, can accept electrons from Q_A even in the presence of DCMU [26]) was only slightly influenced by the removal of CO_2 showing that the activity of the oxygen-evolving system is not changed by short-term CO_2-depletion. Thus, we can conclude that CO_2 depletion results in an inhibition between the electron accepting site of DCBQ and DAD_{ox} that is between Q_A and the plastoquinone pool.

Bubbling the CO_2-free culture with a strong flow of CO_2-containing (5%) air for 5 min completely restored the original uninhibited rate both in the basic electron transport (Fig. 1) and in the $H_2O \rightarrow DAD_{ox}$ reaction (not shown). Bicarbonate addition was less efficient than CO_2. Incubation of the alga, which was cultivated for 70 min in CO_2-free air, for 5 min in the presence of 5 mM $NaHCO_3$ restored about 80% of the basic electron transport rate (Fig. 1) and 60% of the Hill reaction rate in the $H_2O \rightarrow DAD_{ox}$ reaction (not shown). The electron transport rate measurements demonstrate that, in contrast to [7], in vivo depletion of CO_2 in the absence of formate results in an almost complete inhibition of electron flow.

3.2. Effects of CO_2-depletion on thermoluminescence

Thermoluminescence (TL) is a very sensitive method for localization of the action sites of inhibitory treatments in PS II [27]. Therefore, we applied this method to investigate further the effect of bicarbonate depletion on electron transport. In the CO_2-containing alga (Fig. 2A) the so called B thermoluminescence band at 26° C originates from $S_2Q_B^-$ charge recombination [24]. After DCMU addition the B band was abolished and the Q band, which is associated with $S_2Q_A^-$ recombination, appeared at around +15° C in the glow curve. CO_2-depletion had a DCMU-type effect (Fig. 2B). Similarly, as observed in [24] the B band was diminished with a simultaneous appearance of the Q band. The disappearance of the B band in the TL of CO_2-depleted alga cells suggests an inhibition of electron transport between Q_A and Q_B. Sane et al. [28] also found a DCMU-type effect of bicarbonate depletion on the low temperature TL bands appearing below 0° C. It is of note that in a more elaborate series of TL measurements Govindjee et al. [29] observed a reversible shift of the B band to higher temperatures upon bicarbonate depletion by formate.

In the CO_2-depleted algae the amplitude of the Q band was relatively small. This suggests that after CO_2-depletion only a certain portion of the PS II reaction centers contributes to the TL emission; the rest becomes TL-silent. Moreover, a residual part of the B band always remained in the glow curve, indicating only a partial inhibition of electron flow towards the Q_B acceptor pool. DCMU addition increased the amplitude and accelerated the descending side of the Q band according to a more efficient inhibition of electron transport between Q_A and Q_B. Readdition of CO_2 to the CO_2-depleted alga cells restored the B band (Fig. 2B), suggesting an uninhibited electron flow from Q_A to Q_B. This is consistent with the results of electron transport rate measurements (Fig. 1).

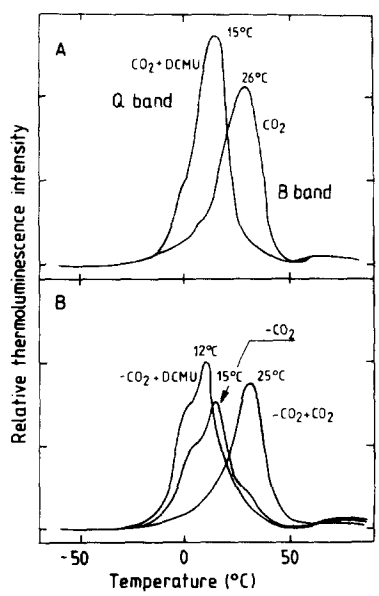

Fig. 2. Thermoluminescence of autotrophic (Part A) and CO_2-depleted *Chl. stellata* (Part B). DCMU was given at a concentration of 10 μM. Chlorophyll content of the samples was 125 μg/ml. Thermoluminescence was excited by continuous white light of 50 μmol m^{-2} s^{-1} at −80° C for 1 min.

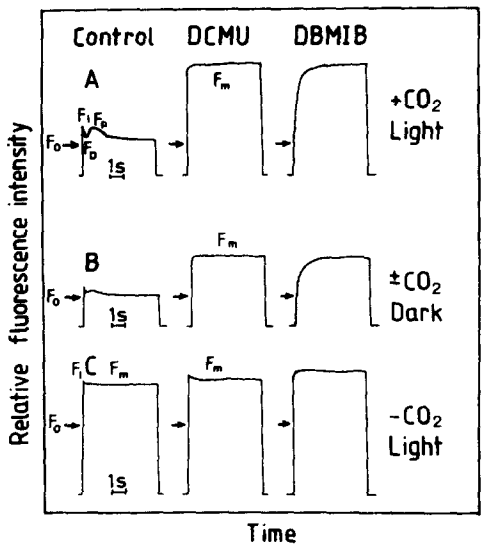

Fig. 3. Effect of cultivation conditions and inhibitors on the fluorescence induction of autotrophic *Chl. stellata*. (A) Alga was cultivated in white light (50 μmol m^{-2} s^{-1}) by bubbling the medium with a mixture of 5% CO_2 and 95% air (v/v). (B) Alga was cultivated in the dark for 1 h either in the presence or absence of CO_2. (C) The same as A except that the cultivation medium had been purged with CO_2-free air for 1 h. Samples were taken directly from the culture and kept in the dark for 1 min before fluorescence measurements. Additions: 10 μM DCMU or 2 μM DBMIB. Designations: F_o, initial; F_i, intermediary; F_m, maximal and F_v, maximum variable fluorescence. F_D is a dip in the fluorescence rise.

3.3. Effects of CO_2-depletion on fluorescence induction

Photoinhibition of the CO_2-depleted alga cells under low PFD

In the fluorescence induction of *Chl. stellata* cultivated in CO_2-containing medium (Fig. 3, upper left corner) the fast rise of fluorescence yield from the initial level, F_o, to the intermediate level, F_i, is followed by a dip, F_D, and a maximum, F_P. DCMU which blocks the electron transport between Q_A and Q_B rapidly raised the F_i level to the maximum level of fluorescence, F_m. Dibromothymoquinone (DBMIB) which inhibits the oxidation of plastohydroquinone had a similar effect. After dark adaptation of alga either in CO_2-containing or CO_2-free medium the F_o level remained the same, but the variable fluorescence, F_v, decreased approximately by half, causing a large decrease in the F_m level (Fig. 3, middle part). Since green algae are in the low fluorescence State II in the dark and convert to the high fluorescence State I upon illumination [30], we can attribute the lowered F_m level to a change in light energy distribution between the two photosystems. In agreement with this, the PS II fluorescence at 77 K was about 50% lower in the dark-adapted alga than in the light-adapted one when the spectra were normalized to the same PS I fluorescence intensity (not shown). It has to be emphasized that no difference could be observed between the fluorescence characteristics of alga dark-adapted either in CO_2-containing or CO_2-depleted medium. This is in agreement with the suggestion that in HCO_3-depleted thylakoids the rate-limiting step in electron transfer is the protonation of Q_B^- and Q_B^{2-}. Thus, the inhibition of electron flow develops only gradually in the light after several turnovers of the reaction center [1,2,5,6]. Since dissociation of bound HCO_3^- is enhanced in the light [1] it can be also assumed that in the dark the firmly bound endogenous HCO_3^- molecules can not be completely removed during the CO_2-depletion procedure. In order to achieve complete removal of bound HCO_3^-/CO_2 in addition to a reduction of partial CO_2 pressure outside the cell, a light-induced photosynthetic utilization of HCO_3^-/CO_2 inside the cell may also be necessary. Moreover, the intrinsic CO_2 production in the dark may inhibit the removal of HCO_3^- from the green alga cells purged with CO_2-free air.

Illumination of the dark adapted CO_2-containing culture with light of moderate PFD (50 μmol m^{-2} s^{-1}) restored the original light-adapted pattern of fluorescence induction (Fig. 3, upper part). However, exposure of the CO_2-depleted culture to light induced large changes in the fluorescence characteristics. Similarly, as after DCMU addition, the F_i level was raised to the F_m level (Fig. 3, lower part) suggesting an inhibition of electron transport between Q_A and Q_B. Moreover, the F_o level considerably increased, simultaneously with about 50% decrease in the extent of maximum variable fluorescence ($F_v = F_m - F_o$). The same phenomenon could be observed when CO_2 depletion of the alga was carried out in the light. The F_o rise also developed in *Chl. reinhardtii* cells during bubbling the alga culture with CO_2-free air under PFD of 150 μmol m^{-2} s^{-1} (not shown). However, the F_o rise was not developed when the alga was cultivated in the presence of electron acceptors, 2,6-dichlorobenzoquinone (see later in Fig. 8) or ferricyanide (not shown). Addition of DCMU or DBMIB to the CO_2-depleted alga increased the F_m level a little further (Fig. 3, lower part), suggesting that neither the Q_A to Q_B nor the Q_B to plastoquinone electron transfer is completely inhibited in the CO_2-depleted alga.

The time-courses of the various fluorescence characteristics measured during CO_2 depletion of illuminated alga are depicted in Fig. 4. By purging the culture medium with CO_2-free air the F_o and F_i levels of fluorescence gradually

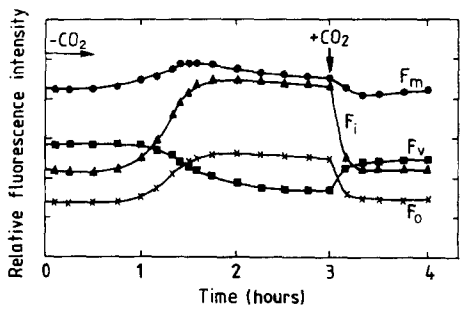

Fig. 4. Time-courses of the fluorescence induction characteristics of *Chl. stellata* during CO_2-depletion in the light. From the moment indicated by the second arrow the cultivation medium was bubbled by a gas mixture containing 5% CO_2 and 95% air (v/v). Designations as in Fig. 3.

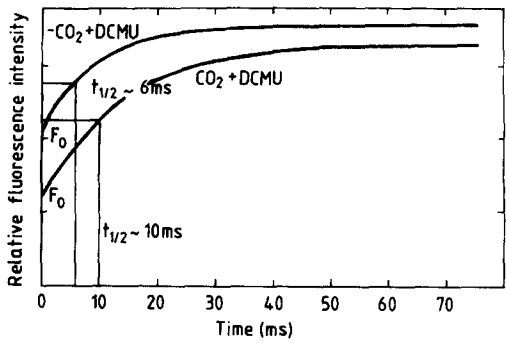

Fig. 5. Time-course of fluorescence rise in autotrophic and in CO_2-depleted *Chl. stellata* in the presence of 10 μM DCMU.

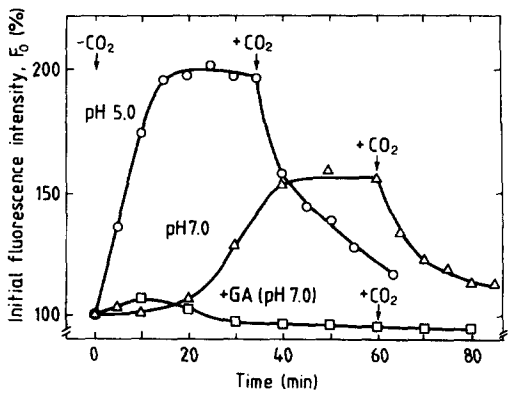

Fig. 6. Effect of pH and glutaraldehyde on the build-up of the enhanced F_0 level in *Chl. stellata*. At the times indicated by the experimental points samples were taken from the alga cultures and the fluorescence was measured after 1 min dark adaptation. First arrow indicates the beginning of CO_2-depletion. Glutaraldehyde treatment (0.002%) was started simultaneously with the CO_2-depletion in the cultivation vessel. From the moments shown by arrows the cultures were bubbled with 5% CO_2 and 95% air (v/v). PFD: 50 μmol m^{-2} s^{-1}.

increased to a maximum and remained relatively constant until CO_2 was resupplied. Simultaneously with the increase of F_0, the F_v part of fluorescence exhibited an almost complementary behavior. Readdition of CO_2 resulted in a drop of F_i and F_0 and in an increase of F_v to approximately the original level. A small irreversible decrease of F_m, F_i and F_0 observed after readdition of CO_2 can be attributed to a second, more severe phase of photoinhibition [31]. If the alga was depleted of CO_2 in the dark before illumination, the enhancement of F_0 and F_i as well as the F_v decrease developed in minutes after the onset of illumination following dark adaptation (not shown).

CO_2-depletion also changed the rise of the F_i level of fluorescence. Fig. 5 shows that the half-rise time of variable fluorescence measured in the presence of DCMU is considerably decreased in the CO_2-depleted alga ($t_{1/2} \sim 6$ ms) as compared to that of CO_2-alga ($t_{1/2} \sim 10$ ms). In the CO_2-depleted alga the fluorescence rise was the same with and without DCMU.

The buildup of enhanced F_0 level was almost the same at pH 7.0 and pH 6.0 (not shown). However, it developed faster and its extent was much higher at pH 5.0 (Fig. 6). At pH 5.0 the enhanced F_0 level was about 60—80% of the F_m level. The original F_0 level could be restored by readdition of CO_2. The increased F_0 yield returned to the control level more slowly at pH 5.0 than at pH 7.0. Since the extent and decay of the elevated F_0 level depends on the pH of the medium we can assume that in the CO_2-depleted alga cells the F_0 rise reflects a protonated state of the reduced Q_A acceptor pool. Another possible explanation is that the faster and stronger increase in F_0 at pH 5.0 have resulted from a faster and more complete CO_2-depletion at low pH. Fig. 6 also shows that chemical cross-linking of proteins by the addition of 0.02% glutaraldehyde completely inhibited the development of the F_0 rise.

The high F_0 level was not stable in the dark. The original dark-adapted level was restored with a decay half-time of about 5 min (Fig. 7). This decay half-time is very long relative to the normal oxidation rate of Q_A^- in forward reaction (~ 400 μs). The long decay half-time indicates a protonated state of Q_A^-. In the presence of DCMU, which inhibits the leakage of electrons from Q_A to Q_B, the decay half-time increased to about 15 min (Fig. 7). Since the backreaction of Q_A^- with the S_2 state should take place with a half-time of about 3 s [25], the long half-time can be accounted for either by a protonation of Q_A^- or by a lack of positive charges on the donor side of PS II.

Our assumption that the elevated F_0 level develops as a consequence of an inhibition of electron transport was confirmed by the observation that in the presence of electron transport inhibitors illumination of the alga culture also induced an F_0 rise (Fig. 8). Illumination of alga in the presence of DCMU increased the F_0 level by a similar extent as in the CO_2-depleted alga. Dibromothymoquinone (DBMIB) which inhibits the oxidation of plastohydroquinone, PQH_2, also had an enhancing but less pronounced effect on the F_0 level. Interestingly, in the presence of a phenolic herbicide, bromoxynil the dark F_0 level of fluorescence was higher at the onset of illumination

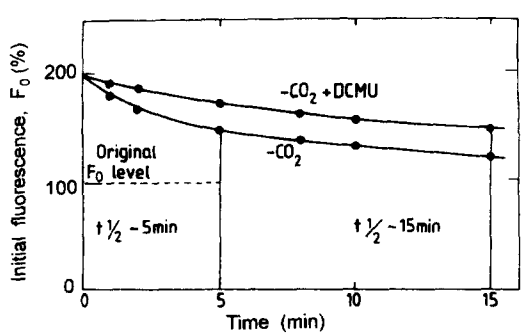

Fig. 7. Dark decay of the initial fluorescence level, F_0 in CO_2-depleted *Chl. stellata* in the presence and absence of 10 μM DCMU. Samples were taken from the darkened alga cultures at times indicated by the experimental points. Scale is given in the percentage of the original F_0 level.

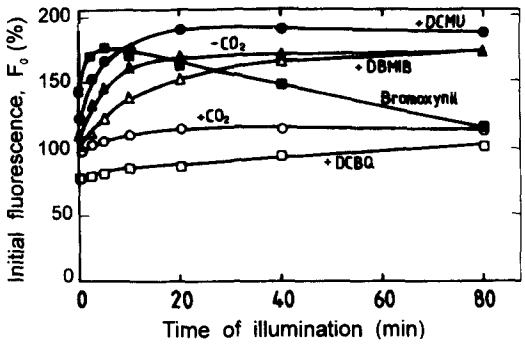

Fig. 8. Development of the enhanced initial fluorescence level, F_0 under various cultivation conditions in *Chl. stellata*. Treatments: ●—●, 10 μM DCMU; △—△, 2 μM DBMIB; ■—■, 200 μM Bromoxynil; □—□, 50 μM 2,6-dichlorobenzoquinone; ○—○, autotrophic alga; ▲—▲, CO_2-depleted alga. Following 1 h dark incubation the cultures were illuminated in the presence of the different chemicals under PFD of 50 $\mu mol\, m^{-2}\, s^{-1}$. Samples were taken at the points indicated. On the vertical axis 100% represents the F_0 level of the untreated and CO_2-containing sample.

than in the DCMU-treated sample, but after a temporary rise it was quickly lost due to a second phase of photoinhibition [31]. It can be relevant that DCMU-type herbicides (atrazine and DCMU), but not ioxynil (a phenolic herbicide), partly protected the PS II reaction center against photoinhibitory degradation [11,13].

3.4. Photoinhibition of the CO_2-depleted alga under high PFD

The first part of photoinhibition which appears as a reversible F_0 rise is followed by a second irreversible part (not restored during dark incubation) which is represented as a gradual decrease of F_m and F_v [31]. In the cultivation light of moderate PFD the F_m level of CO_2-depleted alga decreased very slowly during several hours. The rate of irreversible damage was accelerated by increasing the PFD from 50 $\mu mol\, m^{-2}\, s^{-1}$ to 1500 $\mu mol\, m^{-2}\, s^{-1}$ during cultivation of *Chl. stellata* and from 150 to 5000 $\mu mol\, m^{-2}\, s^{-1}$ for the *Chl. reinhardtii*. A significant part of variable fluorescence was apparently lost due to the rise of the F_0 level and not due to irreversible photoinhibition. In order to determine the real yield of variable fluorescence CO_2 was resupplied to the cultures after photoinhibitory illumination. This restored the original F_0 level and the uninhibited electron flow. The rate of electron transport and the loss of F_v as a function of photoinhibition were measured in the restored cultures of *Chl. stellata* and *Chl. reinhardtii* and the results are depicted in Fig. 9 and Fig. 10. The inhibition of electron transport was the same in the presence and absence of the PS II acceptor, phenyl-*p*-benzoquinone, PpBQ, showing that in our experiments photoinhibition is associated only with PS II and not with PS I. The results demonstrate that both F_v and the electron transport rate decreased slower in the CO_2-depleted alga as compared to the nondepleted alga. If the CO_2-containing

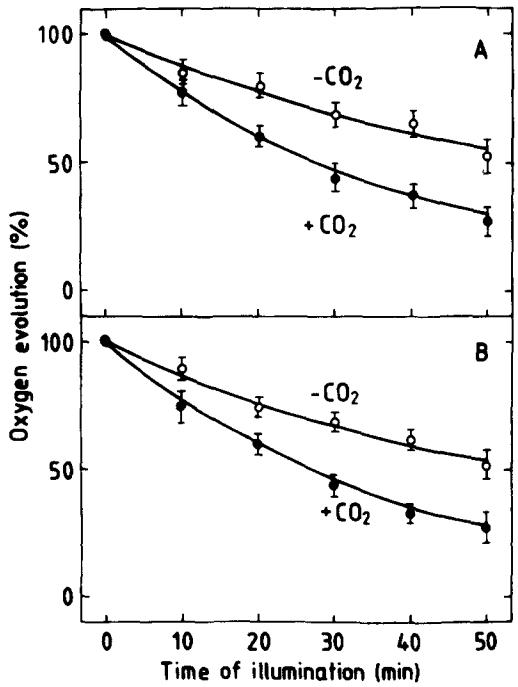

Fig. 9. Effect of photoinhibitory light treatment on the electron transport rate measured from water to 200 μM phenyl-*p*-benzoquinone in control and CO_2-depleted *Chl. stellata* (Part A; PFD: 1500 $\mu mol\, m^{-2}\, s^{-1}$) and in control and CO_2-depleted *Chl. reinhardtii* (Part B; PFD: 5000 $\mu mol\, m^{-2}\, s^{-1}$) alga cells. The points and bars show the means of three measurements with the standard deviations. Other experimental details are described in the Materials and methods.

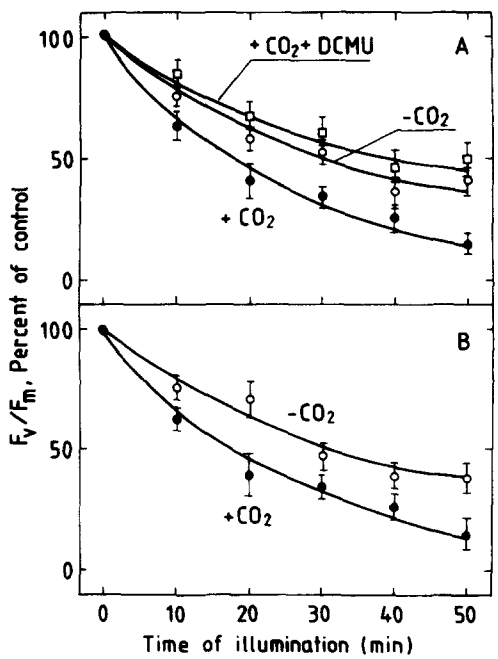

Fig. 10. Effect of photoinhibitory light treatment on the variable fluorescence (F_v/F_m) in control, DCMU-treated and CO_2-depleted *Chl. stellata* (Part A; PFD: 1500 $\mu mol\, m^{-2}\, s^{-1}$) and in control and CO_2-depleted *Chl. reinhardtii* (Part B; PFD: 5000 $\mu mol\, m^{-2}\, s^{-1}$). The points and bars represent the means of three measurements with the standard deviations.

cells were illuminated in the presence of DCMU the photoinhibitory damage was deccelerated and comparable to that observed in the absence of CO_2 (Fig. 10). This is consistent with the protective effect of DCMU-type inhibitors (atrazine, DCMU etc.) against photoinhibition [11–13]. Consequently, inhibition of electron transport between Q_A and Q_B either by CO_2 depletion or by DCMU provided protection against irreversible photoinhibitory damage.

4. Discussion

The present work demonstrates that *Chl. stellata* and *Chl. reinhardtii* alga cells can be depleted of CO_2 without the use of inhibitory anions, simply by purging the medium with CO_2-free air under moderate PFD. We observed that CO_2-depletion completely inhibited the electron transport from water to diaminodurene (an acceptor at the plastoquinone pool), but only slightly influenced the water to dichlorobenzoquinone (acceptor at Q_A) partial reaction. The F_i level of fluorescence was lifted to the F_m level and was not changed further by DCMU addition. In the thermoluminescence glow curve the B band was abolished with a concomitant appearance of the Q band. All of these observations confirm the earlier suggestion [1,2,33] that CO_2/HCO_3^- is a requirement for an in vivo uninhibited electron flow between Q_A and Q_B. Thus, it can be concluded that bicarbonate depletion by formate inhibits the electron transport due to the removal of bicarbonate itself and not due to the inhibitory action of formate as suggested in [7]. In agreement with our conclusions, CO_2 proved to be a necessary cofactor for normal electron transport in wheat and maize leaves as well [34].

It has been reported that in the presence of dithionite, which kept Q_B in a reduced state, similarly as in the CO_2-depleted alga cells, only the Q band (associated with Q_A^-) could be charged in TL [35]. Thus, it can be assumed that inhibition of electron transport in the CO_2-depleted alga is also caused by the development of a reduced state of Q_B. Since bicarbonate is probably involved in the protonation of Q_B^{2-} [1,4–6], in bicarbonate-depleted thylakoids the protonation process determines the rate of electron transport. The retarded protonation process of Q_B^{2-} molecules can result in an "overreduction" of the Q_B pool inhibiting the electron transfer between Q_A and Q_B. A highly reduced state of the acceptor side of PS II can also be accounted for by an enhanced level of NADPH due to inhibition of the Calvin cycle [36].

We have observed that during CO_2-depletion in the light inhibition of electron transport between Q_A and Q_B is accompanied by an enhancement of the F_0 level of fluorescence ("apparent" F_0 level which depends on the redox state of the Q_A acceptor pool). A reversible F_0 rise could also be observed under aerobic conditions [14,15,18,40] especially, when the oxidation of Q_A^- was retarded by low temperature [9,14] and under CO_2 deficiency [15,18]. An increase in the F_0 level can often be observed during photoinhibition, as well [8,9]. This F_0 rise is considered to represent the first and fastest phase of photoinhibition which is very pronounced in anaerobic [22,31,32,41] and in strongly reducing conditions [31]. Since in the CO_2-depleted algae the enhanced F_0 level cannot be observed in the dark-adapted cells but only during illumination we considered the F_0 rise as a phase of photoinhibition of electron transport.

It has been suggested that under anaerobic conditions the enhanced F_0 level represents the accumulation of reduced and protonated forms of the primary quinone acceptor, Q_A [22,31,32]. The question arises what is the reason for the F_0 rise under CO_2-deficiency. It is natural to assume that due to the "overreduced" state of the Q_B pool the steady-state forward electron flow leads to accumulation of electrons in the Q_A pool, as well, even under moderate PFD. Supporting this interpretation the F_0 rise was not developed in alga cells cultivated in the presence of the electron acceptor, dichlorobenzoquinone. Similarly, during anaerobic photoinhibition ferricyanide prevented the development of the F_0 increase [31]. An enhanced level of F_0 was observed in leaves when the CO_2 concentration in the atmosphere was lowered during illumination [15,18] or in the dark after illumination [37]. These phenomena were also interpreted by a partial reduction of the Q_A pool. Reductive titration of fluorescence yield by dithionite led to an increase in the F_0 level of fluorescence, as well [38]. Consequently, the enhanced F_0 level observed in the CO_2-depleted alga cells reflects a partially reduced state of the Q_A acceptor pool.

Continuous illumination of the CO_2-depleted alga cells which keeps a portion of the Q_A pool in a permanently reduced state probably allows a slow protonation of the singly-reduced Q_A molecules to occur. In agreement with [22,23,31] we assume that protonation of the singly-reduced Q_A molecules can be accounted for by the long decay half-time of the enhanced F_0 level. This notion is corroborated by the observation that the extent and decay of the enhanced F_0 level depends on the pH of the medium. At pH 5.0 we observed about 100% increase of the F_0 level (about 60–80% of F_m), suggesting an almost complete reduction and protonation of the Q_A pool. Similarly, in BBY particles under anaerobic conditions (when the reduced Q_A forms were protonated and converted into charge-neutralized state in all of the PS II centers) the F_0 fluorescence reached about 70% of F_m (observed in the untreated control [23]). In the CO_2-depleted green alga cells the development of the enhanced F_0 level was not accompanied by a lowering in F_m. Moreover, it decayed in the dark with a half-time of $t_{1/2} \sim 5$ min. Therefore, the enhanced F_0 level can be ascribed to protonated, singly-reduced Q_A molecules. Double reduction and protonation of Q_A would lead to a more slowly-decaying F_0 level ($t_{1/2} \sim$ 30 min) and in a lowered yield of F_m [23]. We assume that

double reduced protonated and other stable-reduced Q_A species are also formed in the CO_2-depleted green alga cells, but only during prolonged photoinhibitory light treatment similar to what has been observed in chloroplast fragments of higher plants [23].

The reduced plastoquinone pool is oxidized by the atmospheric oxygen with a half-time of about 1 min [39]. The similar decay half-time of the enhanced F_0 level ($t_{1/2} \sim 5$ min) suggests that the reduced acceptor side of PS II is oxidized via the plastoquinone pool by oxygen. Inhibition of the electron transport from Q_A towards the plastoquinone pool can explain the very slow decay of the elevated F_0 level observed in the presence of DCMU.

It has been observed that in spinach thylakoids the increased F_0 level and the reversible state of photoinhibition is produced only by anaerobiosis and not by depletion of bicarbonate [32]. As demonstrated by our present observations, in green alga cells the appearance of the stable, but reversible state of the Q_A pool and the enhanced F_0 level are not restricted to anaerobiosis, but can also be induced by depletion of CO_2 under aerobic conditions. Consequently, the stable reduced state of Q_A is not necessarily abolished by oxygen as suggested in [10,22,23]. In support to our observation an increase in F_0 has also been found under aerobic conditions [14,15,18,40] although it was considerably smaller than that observed under anaerobiosis [22,31,32,41]. We propose that the stable reduced state of Q_A and the F_0 rise can be generated by various treatments which are inhibiting the electron transport and in turn the oxidation of Q_A^-, regardless of the presence of oxygen. In agreement with this, we found that the F_0 rise also developed in alga cells illuminated under aerobic conditions in the presence of DCMU.

It has been established that photoinhibition can be initiated both on the acceptor- and donor-side of PS II [9,10]. In strong light photoinhibition is probably induced by "overreduction" of the acceptor side of PS II ("acceptor-side photoinhibition") [9,10,42]. The reduction of the plastoquinone pool is followed by the formation of stable reduced Q_A species which are manifested as an elevated F_0 level in fluorescence induction [22,31]. Charge neutralization of the singly [23] or doubly [22,43] reduced Q_A forms by protonation or loss of Q_AH_2 from the Q_A site [43] can promote the generation of ^{3}P680 triplet molecules [23]. It has been suggested as a possible mechanism of irreversible photoinhibition that interaction of ^{3}P680 with molecular oxygen yields singlet oxygen [22,44] which can cause rapid abolishment of the stable reduced Q_A species and the F_0 rise [10,22]. Simultaneously the variable fluorescence and electron transport rate of PS II are also rapidly lost [10,22].

The observations of the present work cannot be explained by the above described mechanism of acceptor-side photoinhibition. In contrast to the expectation, in the CO_2-depleted green alga cells the enhanced F_0 level (which probably represents singly-reduced and protonated $Q_A^-(H^+)$ molecules) and the F_m level were constant during several hours of illumination under moderate PFD. The original F_0 and F_m levels and the uninhibited electron transport rate could be completely restored by readdition of CO_2 (or bicarbonate). It can be inferred that formation of charge-neutralized, singly-reduced Q_A species (enhanced F_0 level) which are suggested to be also triplet-promoting [22,23], is not enough to induce irreversible photodamage of the PS II reaction center under moderate PFD.

Under high PFD the loss of electron transport and variable fluorescence was slower in the CO_2-depleted alga cells (possessing enhanced F_0 level) than in the CO_2-containing ones. Therefore, we suggest that CO_2-depletion converts PS II into a state which is less susceptible to irreversible photoinhibition than the CO_2-containing state. This state can be characterized by the inhibition of electron transport between Q_A and Q_B and by formation of stable reduced Q_A species, which are manifested as an enhanced F_0 level. The F_0 rise was also accompanied by partial protection against irreversible photoinhibitory damage in *Chl. reinhardtii* cells illuminated under aerobic conditions at low temperature (5° C) and in spinach thylakoids illuminated under anaerobic conditions [14,32]. Diner et al. [3] also came to the conclusion that dissociation of bicarbonate, like blockage by DCMU [11–13], would protect against photoinhibition. Taken together, we suggest that CO_2-depletion and other treatments which inhibit electron transport between Q_A and Q_B and result in the accumulation of stable reduced Q_A species during illumination, can provide partial protection against irreversible photoinhibition of the PS II reaction center. This conclusion can hardly be reconciled with the suggestion that formation of triplet-promoting stable reduced Q_A species can facilitate the generation of singlet oxygen and in turn damage of reaction center chlorophyll, P680 [22,44]. In agreement with our observations, EPR experiments demonstrated that photoinhibitory illumination which impaired the function of Q_A left the primary charge separation and consequently the reaction center chlorophyll, P680 intact [42].

The F_0 rise does not appear in the presence of a protein cross-linking agent, glutaraldehyde. Therefore, the possibility cannot be excluded that inhibition of electron transfer between Q_A and Q_B is not the consequence of a reduced state of the Q_B pool, but is caused by a conformational change of the quinone-iron complex accompanying bicarbonate depletion. In an altered conformation the Q_A binding region can be exposed to an aqueous phase facilitating protonation of Q_A^-. Moreover, a structural change of the PS II reaction center can provide partial protection against photoinhibition if it prevents the electron flow from reaching the site where the main damaging mechanism of photoinhibition is triggered. The protection against photoinhibition found in the CO_2-depleted algae is consistent with the observations which point toward the Q_B site as being the primary site altered during photoinhibition [9,45–47].

A more probable explanation of our observations can be given by assuming a donor-side mechanism of photoinhibition. Light-induced formation of high ΔpH and acidification of the thylakoid lumen may induce Ca^{2+} release from the water-splitting system [48]. When the water-splitting system is impaired illumination can cause overoxidation of the donor side of PS II and generation of very positive reactive radicals, Y_Z^+ and $P680^+$ [9,10,12]. These radicals may result in the destruction of the PS II reaction center (donor-side photoinhibition). Several observations indicate that, under physiological conditions, donor-side photoinhibition might be predominant [16,49]. In the CO_2-depleted alga cells limited electron flow at the acceptor side of PS II, due to inhibition of electron transport between Q_A and Q_B and formation of stable reduced Q_A molecules, prevents the overoxidation of the donor side. Thus, CO_2-depletion can provide protection against donor-side photoinhibition similarly to the DCMU provided protection in donor-side inactivated systems [9,12]. The protective effect of CO_2-depletion against photoinhibition as observed in the present work may substantiate the in vivo existence of a donor-side mechanism of photoinhibition.

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