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Mechanism of photoinhibition in pea thylakoids: effects of irradiance level and pH

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Abstract

The photosynthetic apparatus can be damaged by light energy in the process of photoinhibition. The target of this photoinhibition is mainly photosystem II (PSII). The mechanism leading to photoinhibitory damage is not yet known. We have characterized photoinhibition by measuring the photoinactivation of electron transport rates using the electron acceptors silicomolybdate and ferricyanide at different irradiance levels and different pH values. The effects of light on the donor side of PSII were measured with silicomolybdate, the effects on the acceptor side were measured with ferricyanide. We observed that photoinactivation of both donor and acceptor side of PSII are light dose-dependent, donor and acceptor side inactivation being independent processes. The donor side of PSII is less sensitive to photoinhibition than the acceptor side. The difference in pH dependence of donor and acceptor side photoinactivation leads us to propose that light-induced release of bicarbonate from PSII is a primary event leading to photoinhibition. In addition, we report that a photoinhibitory treatment increases the proton permeability of thylakoid membranes. This increase seems to be related to the presence of inactivated PSII reaction centers. It is suggested that radicals formed by inactivated PSII reaction centers causing lipid peroxidation are responsible.

Keywords: Acceptor side inhibition; Bicarbonate; Donor side inhibition; Photoinhibition; Photosynthetic control; Silicomolybdate

1. Introduction

The photosynthetic apparatus not only utilizes light but it can also be damaged by light. The process of inactivation of photosynthesis by light is called photoinhibition. Photoinhibition is defined here as the inactivation of photosynthetic electron transport by light. Much research has been directed towards the elucidation of the mechanism by which light inhibits PSII. This process has been studied under several conditions, under aerobic [1,2] and anaerobic conditions (e.g., Vass et al. [3]), as well as at low temperatures (e.g., Huner et al. [4]) and at room temperatures (this study and many others), to mention the most important conditions. Donor-side-induced as well as acceptor-side-induced photoinactivation have been described (for a review, cf. Aro et al. [5]).

Sundby [6,7] obtained indications for a functional role of bicarbonate in the process of photoinhibition by studying the protective effect of added bicarbonate during a photoinhibitory treatment. Experiments with nitric oxide (NO) [8] and with SiMo [9] have shown that these compounds affect the binding of bicarbonate to PSII non-competitively. A non-competitive relation indicates that binding of an inhibitor to the enzyme cannot be reversed by high substrate concentrations. In kinetical terms this means that the inhibitor affects the maximal velocity of the reaction but not the concentration dependence of substrate binding. Thus, considering PSII (water-plastoquinone oxido-reductase) as an enzyme complex and bicarbonate as a substrate, NO and SiMo displace bicarbonate, but once displaced, bicarbonate is not able to displace the inhibitors. This implies that the addition of bicarbonate is not a very effective way to prevent the release of bicarbonate by NO or SiMo. If bicarbonate release from PSII should be a primary event leading to photoinhibition, addition of bicar-

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCPIP, dichlorophenolindophenol; FeCy, potassium ferrihexacyanide; MV, methylviologen, 1,1-dimethyl-4,4′-bipyridylium-dichloride; Q_A, primary quinone electron acceptor of Photosystem II; Q_B, secondary quinone electron acceptor of Photosystem II; SiMo, silicomolybdate; SOD, superoxide dismutase.

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bonate may possibly not prevent photoinhibition. Therefore, we have designed other experiments to study if bicarbonate plays a role in the mechanism of photoinactivation.

Schansker and Van Rensen [9] have shown that silicomolybdate (SiMo) can be used as a probe for the acceptor side of PSII. Further it was found that SiMo-reduction at saturating irradiance was relatively insensitive to the presence of DCMU and bicarbonate. In the presence of the inhibitor DBMIB (1 μM), SiMo accepts electrons at PSII close to Q_A. In the absence of DBMIB, SiMo accepts additionally electrons at a site beyond plastoquinone [9]. Utilizing the differential effects of light and pH on the inhibition of SiMo- and FeCy-mediated electron transport, the mechanism by which light inhibits PSII was studied.

It was reported that photoinhibition causes a loss of the trans-thylakoid proton gradient [10,11]. In the last part of this study the relationship between changes in the permeability of the thylakoid membrane for protons and the photoinactivation kinetics of PSII were studied.

2. Materials and methods

2.1. Isolation of thylakoid membranes

Pea (*Pisum sativum*) leaves were used to isolate thylakoids, as described elsewhere [12]. The thylakoids were resuspended in 2 ml medium consisting of 0.4 M sorbitol, 20 mM tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ and 2 mg ml^{-1} bovine serum albumin. The chlorophyll concentration was determined by the method of Bruinsma [13]. Thylakoids (2 mg Chl ml^{-1}) were stored at −80°C until use.

2.2. Electron transport measurements

Experiments were conducted at a Chl concentration of 25 μg ml^{-1} and a temperature of 25°C. White light was provided by a slide projector. The reaction medium consisted of 0.3 M sorbitol, 5 mM $MgCl_2$ and a buffer. In the experiments in which the pH was between 5.8 and 6.7, MES (50 mM) was used as a buffer, between pH 7.0 and 7.3, Hepes (50 mM) and at pH 7.6 and 7.9, Tricine (50 mM) was used. Thylakoids were uncoupled after the photoinhibitory treatment with 5 μM gramicidine below pH 7.0 and with 5 mM NH_4Cl at pH 7.0 and above. Electron transport was measured as oxygen evolution using a Gilson oxygraph as described elsewhere [14].

The use of the electron acceptors SiMo and FeCy is described in the figure legends. Except when stated otherwise, SiMo was used in combination with 1 μM DBMIB. Electron transport activity from $DCPIPH_2$/ascorbate to MV was assayed in the presence of the following additions: 40 μM DCPIP, 2 mM ascorbate, 20 μM MV, 100 units SOD to accelerate the formation of H_2O_2, 2 mM DTE to prevent the formation of O_2 out of H_2O_2. Thylakoids were uncoupled with 5 μM gramicidin and the buffers were 50 mM MES at pH 6.4 and 50 mM Tricine at pH 7.6. The measuring irradiance was 2200 μmol m^{-2} s^{-1}.

2.3. SiMo preparation

SiMo (M_r = 1970) was dissolved in water. The insoluble fraction was removed by centrifugation and the concentration was adjusted using a molar extinction coefficient of $\epsilon 400 = 1.07$ cm^{-1} mM^{-1} [15].

2.4. Photoinhibition experiments

Thylakoid membranes (25 μg/ml) and reaction medium were added to the reaction vessel of a *Gilson* oxygraph and irradiated with 'white light' of about 5700 μmol m^{-2} s^{-1} provided by a slide projector. The projector was equipped with a water filter and the reaction vessel was cooled by water. For further details see the figure legends.

3. Results

3.1. Primary site of photoinhibitory damage

In Fig. 1, SiMo was used to study the primary site of photoinhibitory damage. As demonstrated by Schansker and Van Rensen [9], there are two sites in the electron transport chain where SiMo accepts its electrons. The first site is located close to the non-heme iron between Q_A and Q_B. The second site is located beyond the cyt b_6f-complex, probably on the acceptor side of PSI. Both sides are

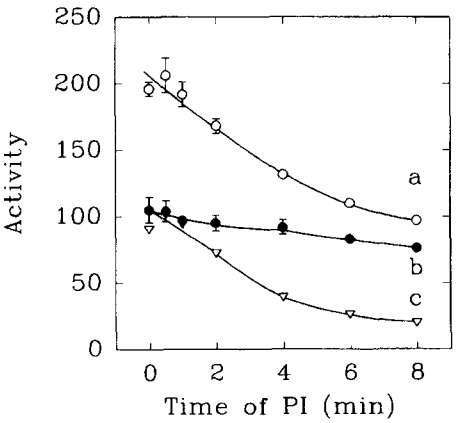

Fig. 1. Electron transport activity from H_2O to SiMo as a function of the time of the photoinhibitory treatment (PI): (a) measured in the absence (open circles), (b) in the presence of 1 μM DBMIB (filled circles) and (c) the difference between a and b (open triangles). Electron transport activity is expressed in μmol(O_2) mg^{-1}(Chl) h^{-1}. DBMIB and NH_4Cl (5 mM) were added after the photoinhibitory treatment; SiMo (0.1 mM) was added at the moment the actinic light was turned on. Bars represent standard error (n = 3–4).

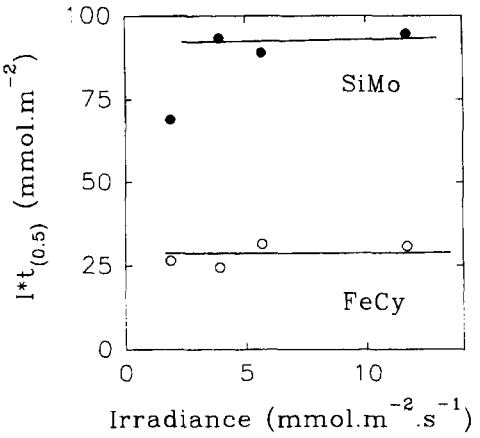

Fig. 2. Effect of photoinhibitory irradiance level on the light dose needed to inactivate the reactions $H_2O \rightarrow$ SiMo (filled circles) and $H_2O \rightarrow$ FeCy (open circles) by 50%. At every irradiance level the halftime of inactivation was determined from plots of electron transport activity versus photoinactivation time. The pH of the medium was 7.6.

mutually exclusive [9]. If SiMo is added immediately after the light is turned on, a mix of whole chain and PSII-mediated electron transport is measured (Fig. 1a and Ref. [9]: Fig. 2). Electron transport to the second acceptor site is prevented by the addition of 1 μM DBMIB (Fig. 1b). Whole chain electron transport was determined by subtracting the rate of PSII-mediated electron transport from the mixed rate (Fig. 1c). This was done under the assumption that two separate populations of electron transport chains were measured. Without photoinhibition (PI = 0 min) the activity of curves b and c are the same. This indicates that under these conditions (and before photoinhibition) 50% of the PSII reaction centers donate electrons to SiMo at a site between Q_A and Q_B (first binding site) and the other 50% of the reaction centers are able to transfer electrons to the plastoquinone pool (second binding site). Whole chain electron transport (Fig. 1c) was affected much more by the photoinhibitory treatment than electron transport to Q_A (Fig. 1b). The kinetics of the inactivation in Fig. 1c are comparable to that using FeCy as electron acceptor (see Fig. 4). Since electron transport between cyt b_6f and the acceptor side of PSI is less affected by the photoinhibitory treatment under our conditions (see later, e.g., Fig. 5), these data indicate that the primary site of photoinhibition is to be found between Q_A and the plastoquinone pool. Electron transport to Q_A as measured by SiMo (Fig. 1b) is also sensitive to photoinhibition, indicating that the donor side of PSII is sensitive to a photoinhibitory treatment though much less than electron transport between Q_A and the plastoquinone pool.

3.2. Irradiance dependence of the treatment

The irradiance dependence of two partial reactions was determined: $H_2O \rightarrow$ SiMo and $H_2O \rightarrow$ FeCy. If the inactivation of these two reactions is determined by the dose, the irradiance level multiplied by the half-time of inactivation ($I \times t_{1/2}$) should be constant. As illustrated in Fig. 2 this is true for both the reaction from $H_2O \rightarrow$ SiMo and the reaction from $H_2O \rightarrow$ FeCy. Only at the lowest inactivation irradiance used, a deviation from linearity was found for the inactivation of the $H_2O \rightarrow$ SiMo reaction, but this is probably caused by a contribution of dark inactivation of the thylakoids due to the long incubation times.

Repetition of the experiment of Fig. 2 at pH 6.7 indicated that lowering the pH had little effect on the relation between irradiance level and light dose in the case of inactivation of SiMo-mediated electron transport (not shown). In the case of FeCy-mediated electron transport, a 20% decrease of the dose needed to inactivate 50% of the electron transport activity was observed. In both cases the relation between the light dose and the half-time of inactivation, indicating a dose–response dependence, was also found at pH 6.7.

The existence of a dose–response relationship for the range of irradiances tested indicates that the use of a high inactivation irradiance (6 mmol m^{-2} s^{-1}) does not lead to deviating results.

3.3. pH-profile of inactivation

It has been suggested [16] that the inactivation of the acceptor side of PSII is brought about by the irreversible loss of bicarbonate from its binding site. The exchange of labeled bicarbonate with the medium appears to be virtually non-existent above pH 7 and becomes progressively facilitated as the pH drops [8,17,18]. As those experiments were carried out in the dark, it is possible that exchange would have occurred at more alkaline pH values in the light. The observed pH dependance [8,17,18] indicates that lowering the pH will facilitate release of bicarbonate. If release of bicarbonate is the cause of photoinhibition, it is expected that lowering the pH below 7 will increase the rate of inactivation of FeCy-mediated electron transport while SiMo-mediated electron transport will remain unaffected. In Fig. 3 the results of this experiment are presented. For the inactivation of SiMo-mediated electron transport a broad optimum peaking around pH 6.7 is found. The pH-profile of inactivation follows more or less the pH-profile of the photosynthetic activity of PSII [19,20]. The relationship between the inactivation of FeCy-mediated electron transport and the pH follows a much more complicated pattern. As hypothesized the rate of inactivation increases sharply below pH 7. The ratio between the inactivation half-times of SiMo- and FeCy-mediated electron transport is maximal around pH 6.4 (Fig. 3b). The shape of this curve is very similar to the pH profile for the bicarbonate effect as reported by Vermaas and Van Rensen [21]. The ratio of the FeCy Hill reaction plus over minus bicarbonate was 1 above pH 7.5, had an optimum at pH 6.5 and approached 1 again at pH 5.5. The result of Fig. 3 is thus a strong indication that the difference in light

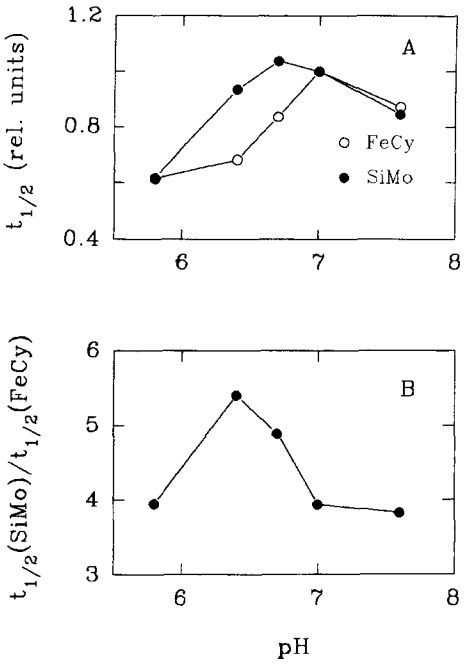

Fig. 3. pH dependence of the half-time of photoinactivation ($t_{1/2}$) of the reactions $H_2O \rightarrow$ SiMo (filled circles) and $H_2O \rightarrow$ FeCy (open circles). In Fig. 3a the measured inactivation half-times in minutes of both reactions were normalized taking the value at pH 7.0 as 1.0 in relative units. In Fig. 3b the ratios between the half-time of inactivation of $H_2O \rightarrow$ SiMo and the half-time of inactivation of $H_2O \rightarrow$ FeCy are plotted. Half-times of inactivation were determined as in Fig. 2. The buffers used were MES pH 5.8–6.7, Hepes pH 7.0 and Tricine for the highest pH value.

sensitivity between the SiMo and the FeCy reaction is caused by release of bicarbonate. This release affects only the FeCy reaction, while it does not affect the SiMo reaction. It could be argued that the pH-profile of the inactivation of the FeCy-mediated electron transport reaction follows the pH-profile of whole chain electron transport activity. However, no relationship between the pH-profile of FeCy-mediated electron transport and the pH-profile of the half-time of inactivation of FeCy-mediated electron transport was found (not shown, see also Ref. [19]).

3.4. Residual activity

As demonstrated in Fig. 4, it is difficult to inactivate the last 20% of control electron transport activity at alkaline pH values. In this experiment FeCy was taken as the electron acceptor because it is more convenient to use than SiMo. In order to estimate the whole chain electron transport rate with SiMo two electron transport rates have to be subtracted (plus and minus DBMIB, cf., Fig. 1) which is less precise than a single measurement with FeCy. In Fig. 4 photoinactivation curves are plotted at different pH values. It appears that the phenomenon of residual activity is pH-dependent and is no longer observed below pH 6.7.

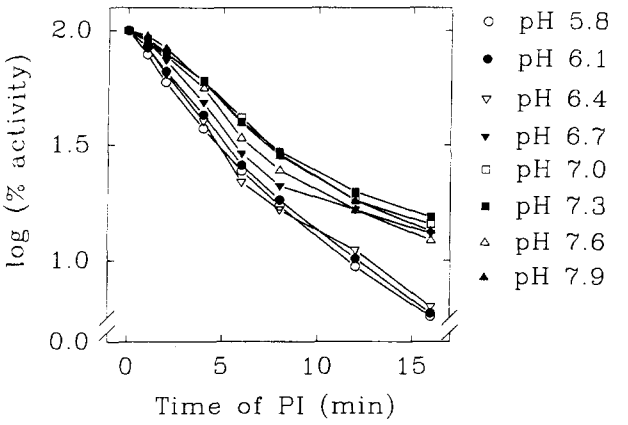

Fig. 4. pH dependence of the relationship between the duration of the photoinhibitory treatment (time of PI) and the relative electron transport activity of $H_2O \rightarrow$ FeCy plotted on a log scale. If the duration of the photoinhibitory treatment was longer than 8 min, a deviation from the exponentional decline of activity is seen at pH 6.7 and above.

3.5. Photoinhibition and proton permeability

To study the effect of photoinhibition on membrane permeability, it is necessary to find a partial electron transport reaction that is only slightly affected by a photoinhibitory treatment. A decline of electron transport activity makes it a priori difficult to separate the effect of a decrease in the build up of the transmembrane pH gradient and leakage of protons through the membrane. For these experiments the reaction from $DCPIPH_2$/ascorbate to MV was chosen. Uncoupled electron transport rate between this donor-acceptor couple decreased during a photoinhibitory treatment but this system was far less sensitive to photoinhibition than PSII-mediated electron transport (compare Fig. 1c and Fig. 5 (diamonds)). The $DCPIPH_2$/ascorbate reaction is inhibited by a low lumen pH and an increased proton permeability of the thylakoid membrane is reflected

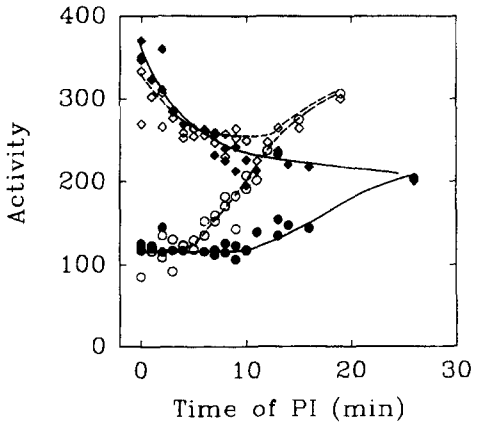

Fig. 5. Effect of a photoinhibitory treatment on photosynthetic control at pH 6.4 and pH 7.6. Electron transport activity ($\mu mol(O_2)\ mg^{-1}(Chl)\ h^{-1}$) was measured from $DCPIPH_2$ /ascorbate to MV in the absence (circles) and presence (diamonds) of 5 μM gramicidin. The experiment was performed at pH 6.4 (open symbols) and 7.6 (filled symbols).

by an increase of the electron transport rate in the absence of an uncoupler. At pH 7.6 the electron transport rate in the absence of an uncoupler starts to increase as a consequence of the photoinhibitory treatment after a lag period of 10 min and after 25 min the rate has almost doubled. At pH 6.4 the lag period is shorter (about 5 min) and the increase of the electron transport rate in the absence of an uncoupler is larger. After a photoinhibitory treatment of 20 min the electron transport rate has almost tripled (Fig. 5). In the presence of gramicidin (diamonds) the electron transport rate decreases during a photoinhibitory treatment at pH 7.6. However, after a photoinhibitory treatment of 15 min at pH 6.4, the uncoupled electron transport rate increases somewhat again. This can be explained by assuming that at this pH the addition of gramicidin did not dissipate the whole pH gradient and that the photoinhibitory treatment is a more effective uncoupler than gramicidin.

4. Discussion

4.1. Comparison of $H_2O \rightarrow$ SiMo and $H_2O \rightarrow$ FeCy measurements

We have compared inactivation of SiMo-reduction with inactivation of FeCy-reduction to study the mechanism of photoinactivation of PSII. This approach is only valid if in both cases PSII is rate-limiting for electron transport. Measurements were done with thylakoids uncoupled by NH_4Cl or gramicidin, a system in which PSII is expected to limit electron transport [22]. A difference is that the reaction rate for FeCy-reduction was two times higher than that for SiMo-reduction. However, as mentioned in Section 3, under our circumstances SiMo binds only to about 50% of the PSII reaction centers (see also Refs. [9,23]). This means that the differences in electron transport rates between both systems are relatively small, making the results more comparable. In neither system was an appreciable lag in the inactivation observed, also indicating that there is no rate limitation other than PSII. In conclusion, the comparison of the FeCy and the SiMo data seems to be valid.

4.2. Primary site of photoinhibition

The data in Fig. 1 indicate that both electron transport from H_2O to Q_A as well as electron transport from Q_A to the plastoquinone pool are sensitive to a photoinhibitory treatment. However, Fig. 1 also indicates that electron transport from Q_A to the plastoquinone pool (curve c) is far more sensitive to light than electron transport from H_2O to Q_A (curve b). The conclusion that the primary site of photoinhibition is located between Q_A and Q_B is supported by in vivo data from Cyanobacteria [24,25], *Chlamydomonas* [26–28] and spinach [29]. In other papers the stabilization of charge separation is pinpointed as the primary site, meaning that Q_A does not function properly anymore [30–33].

4.3. Acceptor and donor side inactivation

A light energy dose–response relationship has been observed for whole chain electron transport in thylakoids [1], cyanobacteria [34,35] and leaves [36,37]. The data in Fig. 2 indicate that a dose–response relationship exists for the inactivation of both FeCy- and SiMo-mediated electron transport. SiMo accepts electrons from Q_A and under saturating irradiances is relative insensitive to changes in the Q_B-region [9], while FeCy-mediated electron transport is sensitive to changes in the Q_B-region. This could mean that inactivation of FeCy-reduction is a measure for acceptor side inactivation and SiMo-reduction is a measure for donor side inactivation. In this case there are two possible interpretations: (1) there is a causal relationship between acceptor side inactivation and donor side inactivation in the sense that inactivation of the acceptor side makes the donor side more sensitive to light or (2) acceptor and donor side inactivation are two independent processes each with their own quantum yield of inactivation.

The pH-dependence of both reactions is not the same (Fig. 3) and Schansker and Van Rensen [23] demonstrated that the presence of DCMU has no effect on the induction of donor side inactivation. These two observations favor the independence of both inactivation reactions. This independence is also supported by the observation of several authors that donors to PSII were able to overcome part of the photoinhibitory damage (maximally 10–20%) [38,39].

In conclusion, inactivation of SiMo-mediated electron transport is a measure for donor side inhibition and inactivation of FeCy-mediated electron transport is a measure for acceptor side inhibition. The data favor independence of donor and acceptor side inactivation.

The experiments do not allow an identification of the site of donor side inhibition. PSII reaction centers with an impaired donor side are very sensitive to light [2,40]. This sensitivity is thought to be caused by increased lifetimes of radicals like $TyrZ^+$ and $P680^+$ [2,40]. However, the electron transport rates under our photoinhibitory conditions were low and it is unlikely that the oxygen evolving machinery could not keep up with it. Moreover, in Ref. [40] it was observed that DCMU could prevent photoinactivation in Cl^--depleted samples. This protection is not observed under our conditions (see above), which makes it less likely that the donor side inhibition we observed in thylakoids is the same as in PSII reaction centers with an impaired donor side.

4.4. Role of bicarbonate in the mechanism of photoinhibition

It was suggested [16,41] that the loss of bicarbonate from its binding site is a primary event in photoinhibition.

The role and presence of bicarbonate in PSII is a very elusive subject of study. At the moment there are no direct unambigual methods to study this phenomenon. On the basis of what is known about the characteristics of bicarbonate, it is possible to make some predictions and test these experimentally. One of these predictions is that loss of bicarbonate should be facilitated below pH 7 [8,17,18]. In Fig. 3 this hypothesis was tested and confirmed. FeCy-mediated electron transport which depends on the presence of bicarbonate (sensitive to a formate treatment [42]) showed a marked increase of the rate of photoinhibition as the pH of the medium was lowered below 7. SiMo-mediated electron transport, that under saturating light is not sensitive to the presence of bicarbonate (not sensitive to a formate treatment [42]), did not show this type of pH-dependence. It showed a broad optimum around pH 6.7, where SiMo-mediated electron transport was the least sensitive to photoinhibition.

Other circumstantial evidence supporting a role for bicarbonate in the primary step of photoinactivation was obtained using a site-directed mutant of *Synechocystis* PCC 6803 [41]. This mutant contained an amino acid alteration close to the non-heme iron. It grows very badly in the absence of glucose and is 4 times more sensitive to photoinhibition than wild-type cells, although the acceptor side was only marginally affected by the mutation. The main effect was a higher sensitivity to formate and the acceptor side was, in addition, inhibited by metabolites related to the presence of glucose. Both results indicate a lower binding affinity of PSII for bicarbonate.

The irradiance dependence of photoinhibition (Fig. 2) indicates that for both SiMo- and FeCy-mediated electron transport there is a dose–response relationship between irradiance level and inactivation. This cannot be associated with the photochemical turnover of PSII as the electron transport rate is very low in the absence of electron acceptors and the highest irradiances used would be supersaturating. One explanation might be that under supersaturating light conditions the structure of PSII is affected, somewhat increasing the probability that a bicarbonate molecule escapes. For example charge separations are thought to cause a contraction of the reaction center [43].

4.5. Effect of a photoinhibitory treatment on the proton permeability of thylakoid membranes

As shown in Fig. 5 photosynthetic control is affected by photoinhibition. After a photoinhibitory treatment of 25 min at pH 7.6 or 12 min at pH 6.4, the addition of an uncoupler no longer stimulates the rate of electron transport from $DCPIPH_2$/ascorbate → MV. Apparently, the lumen pH has lost its regulatory function. Initially, there is a lag time of 10 min at pH 7.6 and 5 at pH 6.4. Lowering the pH from 7.6 to 6.4 halves the time needed to eliminate photosynthetic control and the lag time. In Fig. 4 it can be seen that these lag times in both cases coincide with loss of 70% of PSII activity. It seems that at both pH values a comparable extent of PSII inactivation is needed before proton permeability is affected (the electron transport rate in the absence of an uncoupler is stimulated). This observation is at variance with data of Tjus and Andersson [10] who found no lag phase for stimulation and additionally found that the effect on proton permeability occurred at an even faster rate than inactivation of PSII. The extent of the stimulation found in their study (two-fold) is comparable to our results.

One explanation for the data could be that in thylakoid membranes in which the majority of PSII reaction centers is inactivated the formation of radicals increases. These radicals could cause lipid peroxidation which would lead to an increased permeability of the thylakoid membrane to ions including protons. Alternatively, the photoinhibitory treatment could affect the properties of ATPase and other ion channels, leading to proton slip [44]. However, these authors observed this effect at alkaline pH values, whereas we see an increase of the effect after lowering the pH of the medium. In conclusion, the photoinhibitory effect on the proton permeability of the membrane is suggested to be caused by radical induced lipid peroxidation. The radicals are likely to be produced by the inactivated PSII reaction centers. Radical formation in PSII is in accordance with the measurements of Hideg et al. [45,46] who observed an increased production of singlet oxygen after the inhibition of PSII. Alternatively, work by Miyao et al. [47] suggests that an interaction between H_2O_2 and the non-heme iron is capable of inducing D1 degradation fragments comparable to those observed under in vivo conditions. It was reported that H_2O_2 is produced during illumination by autooxidation at the acceptor side of PSII [48].

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