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On the role of the Q_B protein of PS II in photoinhibition

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Preillumination of spinach thylakoid membranes under strictly anaerobic conditions (i.e. in the presence of glucose oxidase) and in the absence of an electron acceptor inactivates specifically photosystem (PS) II. Inhibition can be complete within 3 min depending on the glucose oxidase concentration and light intensity. Artificial donor or acceptor systems do not restore PS II. Atrazine binding affinity is not diminished. No degradation of peptide subunits is observed. Trypsinized PS II preparations, in which the Q_B-binding site has been destroyed, can also be inactivated by light. It is concluded that photoinhibition of thylakoid membranes under anaerobic conditions inactivates the reaction center of PS II. This photoinactivation does not depend on the degradation of a peptide subunit. Not only the Q_B site but also a modified (trypsinized) Q_A site can induce photoinhibition.

Photoinhibition *Photosystem II* *Atrazine* *Q_B protein* *Thylakoid peptide*

1. INTRODUCTION

In photosynthesis the reaction center of photosystem (PS) II oxidizes water to oxygen and reduces plastoquinone via protein-bound plastoquinones, called Q_A and Q_B. Five integral and three peripheral peptide subunits are involved in the PS II complex and oxygen evolution [1]. A 47 kDa subunit is assumed to carry the reaction center chlorophyll [2]. A 32 kDa subunit has been assigned the role of a Q_B-binding protein because this peptide has been shown to bind specific inhibitors (herbicides like atrazine) that act by displacing plastoquinone Q_B from the membrane [3]. This peptide, coded in the chloroplast genome by a photogene D-1, turns over rapidly in the light [4,5]. It has recently been proposed from photoinhibition experiments with the algae *Chlamydomonas* [6,7] that this property of rapid turnover is related to the well-known [8] physiological phenomenon of photoinhibition of photosynthesis. This photoinhibition is known to inactivate PS II, but there is no general agreement as to its causes, nor as to the exact site that is damaged (for a review see [8]).

The role of the D-1 coded 32 kDa peptide might extend beyond that of a Q_B-binding protein, because together with another 34 kDa peptide of PS II, coded by the D-2 gene, it may be part of the reaction center chlorophyll of PS II itself [9]. This has been deduced [9,10] from the homology of the two D-1 and D-2 coded peptides in sequence and membrane folding to two subunits L and M of the reaction center peptide of the bacterial photosystem [10]. If so, it seems unlikely that, as proposed by Ohad et al. [6,7] in photoinhibition of PS II, only the Q_B site is damaged and a central peptide subunit of the reaction center itself can be easily and quickly exchanged without loss of primary photochemistry. We have therefore reinvestigated the photoinhibition of isolated thylakoid membranes by preillumination in the minute range. We find that photoinhibition under anaerobic conditions and in the absence of an electron acceptor inactivates the whole PS II complex and precedes degradation of any subunit. Also,

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both an active Q_B or an altered Q_A site can act as a receptor for photoinhibition. However, there is no specific inactivation of the 32 kDa protein, nor is there a loss of herbicidal binding affinity.

2. MATERIALS AND METHODS

For photoinhibition spinach thylakoid membranes or PS II particles at 300 μg chlorophyll/2 ml in 7 mM Hepes buffer, pH 7.0, 50 μg glucose and 0.1 mM NaN_3 were flushed for 3 min with argon or nitrogen. Then at the start of preillumination with white light (Philipps Atralux), glucose oxidase (Merck, 8 U/mg) was added.

Photosynthetic activity was measured as 2,6-dichlorophenolindophenol (DCPIP) reduction at 600 nm in an aliquot of preilluminated membranes with 15 μg chlorophyll in 20 mM Hepes buffer, pH 7.0, 300 mM sucrose, 5 μg gramicidin, 30 mM NaCl, 0.05% serum albumin and 0.06 mM DCPIP in 1 ml. 0.4 mM diphenylcarbazide (DPC) was added where indicated in table 1. Ferricyanide was measured at 420 nm, 0.03 mM methylenedioxydimethylbenzoquinone being added where indicated. The methyl viologen system was measured in an oxygen electrode: 30 mM Tricine buffer, pH 8.0, 0.02 mM NaN_3, 2 mM $MgCl_2$, 15 μg gramicidin, 0.1 mM methyl viologen, membranes at 50 μg chlorophyll/3 ml and 0.5 mM duroquinol or 0.2 mM DAD + 2 mM ascorbate and 2 μM dichlorophenyldimethylurea (DCMU) where indicated. [^{14}C]Atrazine binding was measured according to Johanningmeier et al. [11] by incubating an aliquot of preilluminated membranes at 100 μg chlorophyll/2 ml with 20 mM $MgCl_2$, 20 mM Hepes buffer, pH 7.0, at several concentrations of [^{14}C]atrazine from 0.1 to 5 mM for 6 min at 20°C. Then the membranes were centrifuged down and the radioactivity measured in both pellet and supernate.

Trypsinized membranes were prepared by incubating spinach thylakoid membranes at 300 μg chlorophyll/2 ml in 40 mM Mes buffer, pH 6.5, or Tricine buffer, pH 8.0, and 12 mM $MgCl_2$ with 200 μg trypsin for 1 min at 20°C. Then 1 mg trypsin inhibitor was added.

PS II particles were prepared according to Berthold et al. [12] with an activity of 500 μE/mg Chl per h in the photoreduction of methylenedioxydimethylbenzoquinone. For trypsinization these particles at 300 μg chlorophyll in 30 mM Mes buffer, pH 6.3, and 20 mM $MgCl_2$ were treated with 200 μg trypsin for 20 min at 20°C according to Völker et al. [13] and stopped with 1 mg trypsin inhibitor. Photosynthetic activity in trypsinized membranes was measured in DCMU-insensitive ferricyanide reduction. The reaction in trypsinized PS II particles depended on the addition of 10 mM $CaCl_2$ [9,13].

3. RESULTS

Photoinhibition of photosynthetic electron flow in isolated thylakoid membranes was observed more than 20 years ago [14,15]. The conditions for inactivation in just a few minutes were no acceptor added and anaerobiosis. Even traces of oxygen would protect the system, probably by acting as an acceptor. Inhibitors of the acceptor side of PS II such as *o*-phenanthroline would prevent photoinactivation [14,15].

In figs 1, 2 and table 1 it is again shown that strictly anaerobic conditions are required for photoinhibition of PS II activity in isolated thylakoids; the addition of glucose oxidase and glucose greatly enhances sensitivity to light, even if the reaction is run under nitrogen or argon. No particularly high light intensities are required for photoinhibition; at the particular chlorophyll concentration used 20 klux are sufficient (fig.1). Increasing the glucose oxidase (and glucose) concentration shortens the time required for complete in-

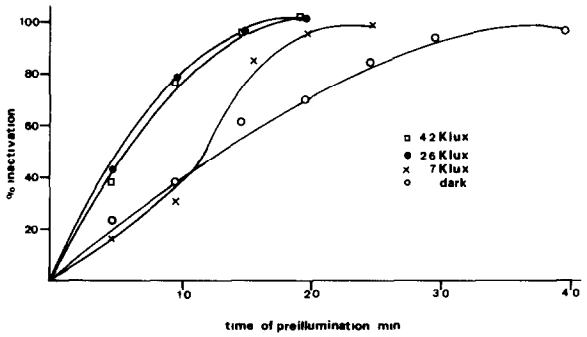

Fig.1. Time course of inactivation of electron flow by preillumination of thylakoid membranes under anaerobic conditions at different light intensities. Conditions: argon + 50 μg glucose and 50 μg glucose oxidase. Activity in a DCPIP reaction.

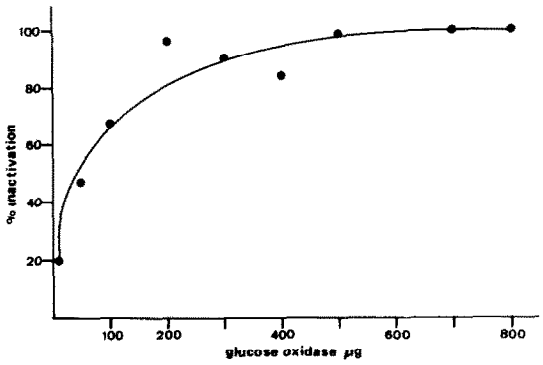

Fig.2. Dependence of the inactivation of electron flow by preillumination on the concentration of glucose oxidase. Conditions: argon + 200 µg glucose and the amount of glucose oxidase as indicated. After 5 min illumination with white light of 42 klux an aliquot was tested for photosynthetic activity in a DCPIP reaction.

activation of photosynthetic activity (fig.2). Damage to the photosynthetic system by preillumination for 5 min in the absence of an acceptor is confined to PS II, which is inactivated to about 90%. PS I activity measured via a donor system (diaminodurene, DAD) is only diminished by about 10%, electron flow through the cytochrome *b/f* complex (duroquinol as donor) being affected about 30% (table 1). PS II activity cannot be restored by an artificial donor (DPC), therefore damage is not due to inactivation of the oxygen-evolution system (table 1). Not shown among the data is that addition of DCMU delays and DBMIB enhances sensitivity to photoinhibition under anaerobic conditions. The variable fluorescence of PS II is diminished in thylakoids preilluminated under anaerobic conditions and reaches maximal level immediately even without DCMU (fig.3), as reported by Kyle et al. [7].

The thylakoid membrane binds herbicides like atrazine, an inhibitor of PS II at the Q_B site on the 32 kDa peptide subunit [2]. Table 2 indicates in two experiments that preillumination under strictly anaerobic conditions for 5 min does not change this affinity, nor the number of binding sites for [^{14}C]atrazine. Not shown are SDS electrophoresis gels because they indicate no change in the subunit pattern after preillumination of the membrane for 5 min.

Trypsinization of thylakoid membrane vesicles specifically inactivates the Q_B-binding site and

Table 1

Inactivation of PS II by preillumination of thylakoid membranes in the absence of an acceptor under strict anaerobic conditions

Photosynthetic activity tested		Control rate	Inactivation (in % of control after preillumination)	
Donor	Acceptor		Nitrogen	Nitrogen + glucose oxidase/glucose
H_2O	→ DCPIP	303	13.6	75.5
DPC	→ DCPIP	274	19.7	73.5
H_2O	→ FeCy	315	16.6	90.5
H_2O	→ methylenedioxy-dimethyl-BQ	270	12.5	98.9
H_2O	→ methylenedioxy-dimethyl-BQ	675		88.9
H_2O	→ methyl viologen	547		75.5
DQH_2	→ methyl viologen	516		30.9
DAD	→ methyl viologen	398		14.7

Conditions for photoinhibition: nitrogen with or without 50 µg glucose and 50 µg glucose oxidase, 5 min preillumination at 42 klux. Photosynthetic rates in the control are those in air in µE/mg chlorophyll per h

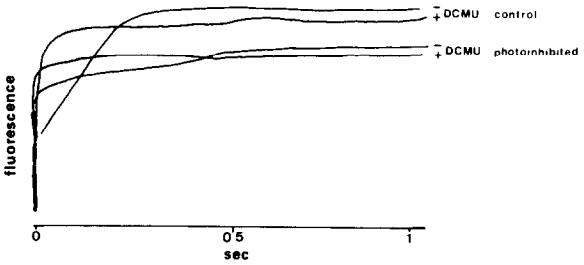

Fig.3. Fluorescence induction in photoinactivated thylakoid membranes. Spinach thylakoid membranes at 30 μg chlorophyll/3 ml in 20 mM Hepes buffer, pH 7.0, were illuminated for 1 s with a 15 mW He/Ne laser with or without 1 μM DCMU. For photoinactivation the membrane had been preilluminated for 7 min with 100 μg glucose oxidase and 100 μg glucose.

Table 2

Binding of [^{14}C]atrazine after preillumination of the membrane under anaerobic conditions

	K_B	pK_B	nmol/mg Chl	Molecules Chl/ molecules inhibitor
No preillumination	0.093	7.03	1.3	850.55
5 min preillumination	0.096	7.02	0.96	1163.3
No preillumination	0.066	7.2	1.51	734.2
5 min preillumination	0.053	7.3	1.28	865.35
10 min preillumination	0.068	7.2	1.10	1006.45

Conditions: spinach thylakoid membranes at 300 μg chlorophyll were photoinhibited with 75 μg glucose oxidase + 75 μg glucose and preillumination at 42 klux

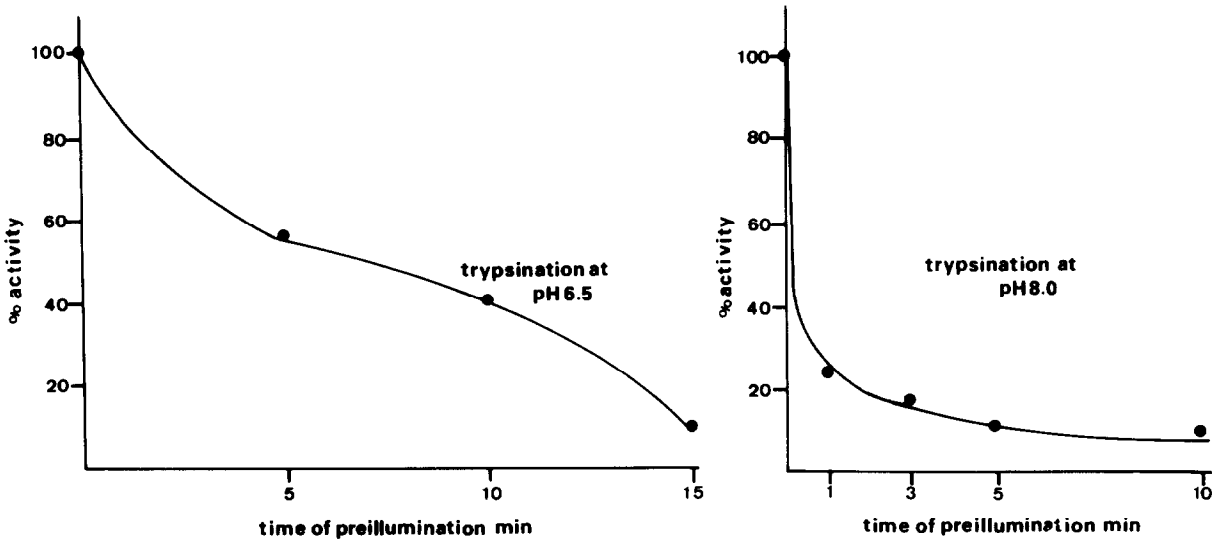

Fig.4. Photoinactivation of PS II of spinach thylakoid membranes after trypsinization at pH 6.5 or 8.0. Conditions: 50 μg glucose oxidase, 50 μg glucose and 3 mM KCl and 42 klux white light for the times indicated. Photosynthetic activity was measured in an aliquot with ferricyanide as acceptor at pH 7.0. The rate in the control kept in the dark was 510 μmol/mg chlorophyll. This rate was no longer stimulated by methylenedioxydimethylbenzoquinone and was insensitive to 3 μM DCMU.

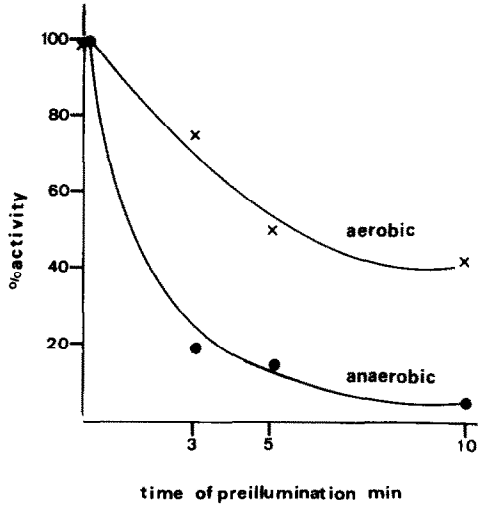

Fig.5. Inactivation of trypsinized PS II particles by preillumination. Particles at 150 μg chlorophyll/ml were preilluminated with 42 klux white light. Photosynthetic activity was measured in a ferricyanide reduction in the presence of 10 mM $CaCl_2$.

opens the Q_B site for access of ferricyanide, i.e. it allows a DCMU-insensitive Hill reaction [16]. Such trypsinized thylakoid membranes are also inactivated by preillumination, the time (in minutes) required being dependent on the pH at which trypsinization has been done (fig.4). PS II particles can still reduce ferricyanide via Q_A after trypsinization at low pH, if $CaCl_2$ is now added to compensate for the loss of two peripheral peptides [9,13]. Such trypsinized PS II particles are also sensitive to photoinhibition under anaerobic conditions (fig.5). Not shown are data indicating that trypsinization of the membrane after photoinhibition of PS II does not restore activity, as expected if the photoinhibition had destroyed just the Q_B site.

4. DISCUSSION

Photoinhibition is a well-known physiological phenomenon and occurs in oxygen-evolving photosynthetic organisms under strong and excess light in the absence of an electron acceptor, i.e. depletion of CO_2. The primary event of photodamage has been localized in PS II (reviewed by Powles [8]). However, the exact site damaged in PS II and the mechanisms involved remain controversial. The early results obtained by Jones and Kok [17], then by Critchley [18], Powles and Björkman [19] and Satoh and Fork [20] and recently by Barényi and Krause [21] indicated destruction of the reaction center itself of PS II or of its donor side. All peptide subunits of PS II were shown to become degraded after photoinhibition had occurred [22]. Recently, it was observed in the algae *Chlamydomonas*, however, that the primary attack of excess light seemed to be on the acceptor side of PS II [6] and on the 32 kDa subunit of PS II; this herbicide and Q_B-binding peptide was preferentially degraded after it had lost atrazine-binding affinity [7]. The attractive hypothesis was advanced that under acceptor limitation plastoquinone radicals accumulate at the Q_B site and destroy the Q_B-binding peptide [6,7]. This sensitivity of the 32 kDa peptide to light was seen as the physiological explanation for the known rapid turnover of the peptide, coded by the photogene D-1 [4,5]. Recently, Ohad et al. [23] have suggested that also after photoinhibition in isolated pea thylakoid membranes the 32 kDa peptide is degraded more rapidly than other PS II subunits. Krause et al. [24], however, report that photoinhibition of chloroplasts damages the reaction center itself.

In evaluating the contrasting observations from different laboratories, it is clear that there are two different mechanisms of photoinhibition under aerobic and anaerobic conditions [8,24]. Furthermore, we observe that the inactivation of function and the degradation of subunits occur on a different time scale. Our photoinhibition experiments with spinach thylakoid membranes performed a long time ago [14,15] had shown that PS II inactivated in a matter of minutes, if illuminated in the absence of an electron acceptor and under anaerobic conditions, but not if traces of oxygen were present. The further results reported here show again that 3 min of relatively low light intensity inactivates PS II, if the system is made strictly anaerobically by glucose oxidase/glucose and no acceptor is present. The inactivation was stimulated by the inhibitor DBMIB (an inhibitor after Q_B) and prevented partly by DCMU (an inhibitor before Q_B) in agreement with several reports on such effects in the literature [6,7,14,15,20]. However, we could not observe that photoinhibition under these conditions specifically damages the Q_B protein. We find that

under our conditions neither the atrazine binding affinity of this peptide has been changed, nor that it is degraded. Both Q_A and Q_B can induce photoinhibition. An operative Q_B site is possibly required in intact thylakoid membranes, but inactivation of PS II occurs also in trypsinized thylakoid membranes or trypsinized PS II particles, in which the Q_B and herbicide-binding affinity of the 32 kDa peptide has been removed. In these particles Q_A has assumed the properties of Q_B, as it can now accept two electrons and is accessible to ferricyanide [16]. An altered Q_A might therefore also be able to induce photodamage. Our experiments are in agreement with those recently reported by Krause et al. [24], where they compare photoinhibition of chloroplasts under anaerobic and aerobic conditions. Our data are different from those of Ohad et al. [23], but not necessarily in disagreement, as the conditions (anaerobic vs aerobic) and the time required for photoinhibition are different. It seems clear now that photoinactivation of PS II activity precedes degradation of the peptide subunits.

There is a particular reason to insist that photoinhibition is not primarily due to degradation of specifically the 32 kDa peptide that nevertheless would leave the reaction center of PS II intact. The reason is that the 32 kDa peptide may be actually part of the reaction center itself. This proposal [9,10] stems from the homology in amino acid sequences and of hydropathy plots of four peptides: the 32 and 34 kDa (products of the D-1 and D-2 gene, respectively) subunits of PS II and the L and M subunits of the bacterial reaction center. Functional amino acids, particularly histidines, involved in Fe, quinone and chlorophyll binding, are conserved at the same equivalent positions in all four subunits. As the X-ray structure of the bacterial center shows, the two subunits form a highly symmetric dimer which together are involved in bacteriochlorophyll and both Q_A and Q_B binding [10]. According to the homology of the 32 kDa peptide of PS II it is equivalent to the L subunit and the 34 kDa subunit to the M subunit [10]. They would together, in a highly symmetric folding through the membrane, bind the Fe and the two quinones on one side of the membrane and the reaction center chlorophyll on the other. This hypothesis contradicts the assignment of the reaction center function to the 47 kDa subunit of PS II [2], as pointed out elsewhere [9]. One would expect from the hypothesis that photodamage under anaerobic conditions induced via Q_B or Q_A (as shown here) interferes with the reaction center itself, which is then followed by degradation of the reaction center peptides that may require oxygen.

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