ELSEVIER

Biochimica et Biophysica Acta 1186 (1994) 177–185

BBA Biochimica et Biophysica Acta

The rate constant of photoinhibition in vitro is independent of the antenna size of Photosystem II but depends on temperature

Esa Tyystjärvi *, Reetta Kettunen, Eva-Mari Aro

Department of Biology, University of Turku, Laboratory of Plant Physiology, BioCity A, 6th floor, Tykistökatu 6, FIN-20520 Turku, Finland

Received 15 November 1993; revised manuscript received 24 February 1994

Abstract

Photoinhibition of Photosystem (PS) II was studied in thylakoid membranes, inside-out and rightside-out thylakoid vesicles derived from appressed and non-appressed membrane regions, respectively, in detergent fractionated PS II membranes and in oxygen evolving PS II core complexes. The preparations were illuminated without added electron acceptors, and care was taken to keep the oxidizing side of PS II in a functional condition during the experiments. The first-order rate constant of photoinhibition, measured under given photon flux density, was similar in all preparations derived from appressed thylakoid regions and independent of the antenna size. This antenna size independence indicates that under photoinhibitory conditions in vitro, when most PS II traps are closed, the probability of finding an exciton in the reaction centre is not much larger than the probability of finding it in the antenna. Spillover of excitation energy from PS II to PS I may be an important factor protecting the PS IIβ of stroma thylakoids from photoinhibition. Photoinhibition in vitro is a first-order reaction even at low temperatures where the degradation of the D1 protein is slow, which demonstrates that the photoinhibited PS II centres do not protect the remaining active ones from photoinhibition, at least not in vitro. The activation energy of photoinhibition in pumpkin thylakoids, as measured between 6 and 25°C, was 15 kJ/mol; the rate constant of photoinhibition in pumpkin thylakoids increased both below 6 and above 25°C.

Key words: Activation energy; Energy transfer; Exciton trapping; Light-harvesting; Low temperature; Photosynthesis

1. Introduction

Illumination of isolated chloroplasts, thylakoids or thylakoid membrane subfractions results in inhibition of PS II electron transport. This reaction, photoinhibition, follows first-order kinetics (Tyystjärvi, E., Mäenpää, P. and Aro, E-M., submitted to Photosynth. Res.). Photoinhibition also occurs in vivo in plant leaves, algae and cyanobacteria (for review, see [1]). Photoinhibition of PS II involves an irreversible damage to the reaction centre and leads to the degradation of the D1 protein [1–4]. Consequently, recovery from photoinhibition requires de novo synthesis of the D1 protein [5].

The current understanding of the molecular mechanisms of photoinhibition is based on so-called acceptor and donor side photoinhibition mechanisms [6,7]. The acceptor side mechanism is thought to function when the oxidizing side of Photosystem II is functional while donor side photoinhibition occurs when the donor side has been inhibited by a suitable pretreatment before exposure to light.

The first-order rate constant of photoinhibition is directly or almost directly proportional to the photon flux density during illumination [8,10]. The absorption cross-section of PS II has been argued to play an important role in the susceptibility of PS II to photoinhibition [11–,13]. In this study, we show that the size of the light-harvesting antenna does not affect the rate of acceptor-side photoinhibition in vitro in PS II preparations with different antenna size.

We have shown that not only the light intensity but also the temperature during the high light treatment affects the rate of photoinhibition even in isolated thylakoids [14]. Protection against photoinhibition by

Abbreviations: CP43 and CP47, internal chlorophyll *a* antennae of PS II; k_{PI}, first-order rate constant of photoinhibition; LCH II, light-harvesting chlorophyll *a*/*b* protein complex of PS II; Q_A, the primary quinone acceptor of PS II.

* Corresponding author. Fax: +358 21 6338075.

SSDI 0005-2728(94)00043-5

low temperature has also been demonstrated in intact systems under conditions where the repair of photoinhibited PS II centres through de novo synthesis has been inhibited [10,15,16]. We show here that the low temperature protection against photoinhibition is an intrinsic feature of PS II, as the protection can be demonstrated in PS II core preparations as well as in intact isolated thylakoids. We also show that the rate constant of photoinhibition in pumpkin thylakoids follows the Arrhenius equation between 6 and 25°C, with an activation energy of 15 kJ/mol.

2. Materials and methods

Thylakoid membranes were isolated from spinach (*Spinacia oleracea* L.) and pumpkin (*Cucurbita pepo* L.) leaves according to [17]. Rightside-out and inside-out thylakoid vesicles representative of non-appressed and appressed membranes, respectively, were isolated by aqueous polymer two-phase partition following Yeda press fragmentation of thylakoids [18]. PS II membranes and PS II core complexes were prepared by detergent fractionation according to [19].

Randomization of the protein complexes was done by incubating the thylakoid membranes in low salt medium [20] for 20 min at 20°C.

For photoinhibitory illumination the thylakoids and rightside-out vesicles were suspended in 50 mM Tricine (pH 7.6), 0.1 M sorbitol and 20 mM NaCl. 5 mM $MgCl_2$ was included except for the randomized membranes. Inside-out membrane vesicles, PS II membranes and PS II core complexes were suspended in 50 mM Mes-KOH (pH 6.4), 0.4 M sucrose, 10 mM NaCl, 10 mM $CaCl_2$ and 5 mM $MgCl_2$. The chlorophyll concentration was 50 μg/ml. Photoinhibitory illumination was performed either at 20°C or at 2°C with heat-filtered white light using a 250 W projector as a light source. The photon fluency rate was adjusted with neutral density filters. After different times of illumination (0 to 35 min), aliquots were withdrawn for measurements of oxygen evolution.

Light saturated rates of oxygen evolution of the thylakoid membranes and rightside-out membrane vesicles were measured in Hansatech oxygen electrode at 20°C in 50 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 10 mM NaCl and 5 mM $MgCl_2$ using phenyl-*p*-benzoquinone (1 mM) as an electron acceptor. The oxygen evolution of inside-out vesicles, PS II membranes and PS II core complexes was measured in the same buffer as used for the high light treatments.

The activation energy of photoinhibition was measured by using the F_V/F_{MAX} of isolated pumpkin thylakoids as a measure of photoinhibition. The thylakoids were illuminated in 40 mM Hepes-KOH (pH 7.6), 0.33 M sorbitol, 5 mM $MgCl_2$, 5 mM MnCl, at different temperatures, and the F_V/F_{MAX} value was measured periodically with a modulated fluorometer (PAM 101, Heinz Walz, Effeltrich, Germany). The chlorophyll concentration was 100 μg/ml and the sample volume 400 μl. The treatment consisted of 11 cycles starting with a saturating flash (5000 μmol photons $m^{-2}s^{-1}$, 3 s) followed by 3 min of illumination (1800 μmol photons $m^{-2}s^{-1}$), a second saturating flash and 3 min of darkness. The first saturating flash was used to measure changes in F_V/F_{MAX} and the second one to analyze the reduction state of Q_A during illumination. The dim measuring beam of the PAM fluorometer was switched on 10 s before the end of each dark period to check F_0. The rate constant of photoinhibition was calculated from the decrease in F_V/F_{MAX}, corrected for the light-independent decrease in F_V/F_{MAX} at each temperature. Similar experiments were done at 20°C in the presence of 1–10 μM DCMU. The temperature dependence of light-independent decrease in F_V/F_{MAX} (aging) was measured with a similar set-up without the photoinhibitory illumination. The FIP fluorescence software (Q_A-Data, Turku, Finland) was used for fluorometer control and data collection.

The D1 protein content of thylakoids used for the activation energy measurements was analyzed immunologically from three samples treated at 3.5 and 20°C. Thylakoid proteins were solubilized [21] and SDS-PAGE was performed with a 4% stacking gel followed by a 14% separation gel, both with 4 M urea. Polypeptides were transferred to PVDF membrane and the immunodetection of the D1 protein was carried out with the BioRad chemiluminescence kit. The D1 protein antibody (Research Genetics, Huntsville, Alabama) was raised against amino acids 234–242 of *Synechocystis* 6803 D1 protein. The immunoblots were scanned with a laser densitometer.

Chlorophyll–protein complexes of different PS II preparations were solubilized by mild SDS-treatment [22] and electrophoretically separated in non-denaturating gels consisting of a 4% stacking gel and 14% of acrylamide in the separation gel. The gels were scanned at 675 nm to determine the relative amounts of chlorophyll-protein complexes.

Chlorophyll was determined in 80% acetone according to [23].

Curve fitting was done with the Fig.P software (Biosoft, England).

3. Results

3.1. Chlorophyll *a*/*b* ratios and antenna sizes of the preparations

Chlorophyll *a*/*b* ratios and the ratios of chlorophyll associated with the light-harvesting chl *a*/*b*–protein

Table 1
Chlorophyll a/b ratios, the ratios of chl associated with light-harvesting chlorophyll a/b–protein complex to that associated with the internal chl a antennae (CP47 and CP43) of PS II, and control rates of PS II oxygen evolution (μmol O_2/mg chl per h) in different PS II preparations; I.s.o. and R.s.o. = inside-out and rightside-out thylakoid membrane vesicles, respectively

PS II preparation	Spinach	Pumpkin		
	chl a/b	chl a/b	LHCII/(CP47+CP43)	O_2 evolution
Thylakoids	3.0	3.3	3.7	259
I.s.o.	2.3	2.2	4.3	219
R.s.o.	6.4	–	–	43
PS II membranes	2.2	2.2	3.9	353
PS II core	11.9	11.2	0.4	627

complexes (LCH II) to that associated with the internal antenna system of PS II core complex (CP 47 + CP 43) are given in Table 1. In PS II core preparations, only traces of the light harvesting chl a/b protein complex was present when compared with intact thylakoids, inside-out vesicles and PS II membranes with a complete LCH II antenna system.

3.2. The rate constant of photoinhibition of PS II electron transport is independent of antenna size

The rate constant of photoinhibition of oxygen evolution was the same in isolated thylakoids, inside-out membrane vesicles, PS II membranes and oxygen evolving PS II core complexes, provided that the photon fluency rate, the chlorophyll concentration of the sample, optical path length of the cuvette and temperature were the same (Fig. 1). However, the size of the PS II light-harvesting antenna of thylakoids, inside-out vesicles and PS II membranes is almost 4-times that of the PS II core complexes (Table 1). Similar kinetics of inhibition of oxygen evolution was not due to light saturation of photoinhibition, as the rate constant of photoinhibition in different preparations was proportional to the photon fluency rate during the experiment (Fig. 2).

The type of the PS II preparation was unimportant to the rate of photoinhibition, if the preparation was derived from appressed membrane regions. One could argue that the preparation methods of PS II samples affect their sensitivity to light. Lesions on the donor side of PS II, in particular, are known to induce donor side photoinhibition [7,24,25], which has a much larger quantum yield than the acceptor-side photoinhibition [26]. Precautions were taken to avoid donor side photoinhibition by adding Cl^- and Ca^{2+} ions to the incubation media and by doing the experiments in low pH in order to increase the affinity of these ions on the donor side of PS II [27]. Further, the fractionation procedure does not seem to affect the results, since the PS II membranes made by Triton X-100 fractionation (carefully washed afterwards) and inside-out membrane vesicles prepared by mechanical fractionation behaved similarly (Figs. 1 and 2).

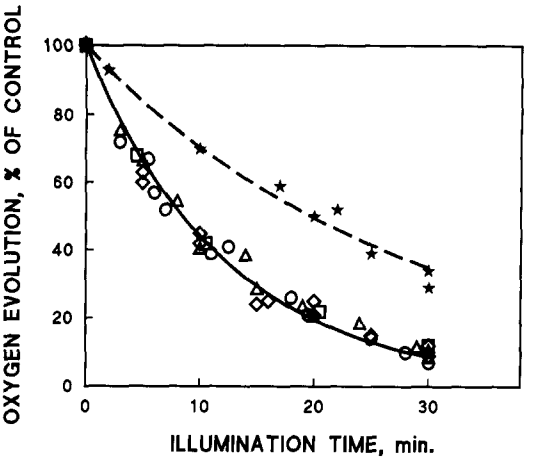


Fig. 1. Photoinhibition of PS II oxygen evolution during strong illumination (3500 μmol photons $m^{-2}s^{-1}$) at 20°C of different PS II preparations isolated from spinach leaves. Thylakoid membranes (◇), inside-out vesicles (△), PS II membranes (□), oxygen evolving PS II core complexes (○), rightside-out vesicles (★, dashed line). The curves are best fits to a first-order equation. For clarity, only one curve (solid line) is shown for thylakoids, inside-out vesicles, PS II membranes and PS II core complexes because the curves are virtually indistinguishable. The k_{PI} constant was 0.035 min^{-1} for rightside-out vesicles and 0.08 min^{-1} for all the other preparations.

3.3. Randomization of thylakoids protects against photoinhibition

In the rightside-out membrane vesicles which contain mainly PS IIβ [28], the rate constant of photo-

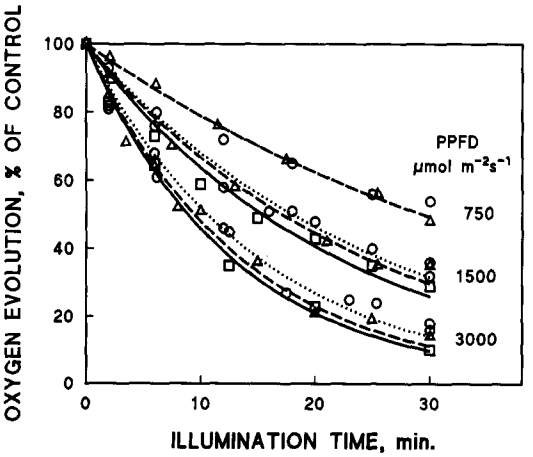


Fig. 2. Time course of photoinhibition of PS II oxygen evolution under different photon flux densities at 20°C. Inhibition of oxygen evolution was followed in inside-out thylakoid membrane vesicles (△, dashed line), PS II membranes (□, solid line) and oxygen evolving PS II core complexes (○, dotted line) isolated from pumpkin leaves. The control rates are listed in Table 1. The curves are best fits to a first-order equation. The k_{PI} constant of all preparations was 0.024 min^{-1} at 750 μmol $m^{-2}s^{-1}$, 0.038–0.045 min^{-1} at 1500 μmol $m^{-2}s^{-1}$ and 0.065–0.078 min^{-1} at 3000 μmol $m^{-2}s^{-1}$.

inhibition was about one-half of that found in intact thylakoids or in other thylakoid subfractions (Fig. 1). Because the smaller antenna of PS IIβ apparently cannot protect from photoinhibition, we tested the possible role of the spillover of excitation energy from PS II to PS I [29] in protecting PS II from photoinhibition. To elucidate the role of spillover, the lateral segregation of PS II and PS I complexes was decreased by incubating the intact, unfractionated thylakoids in a low salt buffer. The rate constant of photoinhibition in randomized thylakoid membranes was only about one-half of that found in stacked membranes (Fig. 3). No ATP was added to the photoinhibition medium, and therefore there was no difference in the phosphorylation states of the proteins between the cation-depleted and control thylakoids. To ensure that the protection against photoinhibition in randomized thylakoid membranes was due to spillover, not to disconnection of LCH II from PS II [30], the above experiments were repeated with PS II membranes which are derived from appressed thylakoid regions and contain almost no PS I. In this case, cation depletion did not induce any protection against photoinhibition (results not shown).

3.4. Low temperature protects against photoinhibition even in PS II core preparations

Photoinhibition was clearly a first-order reaction even at 2°C both in thylakoids and in all other PS II preparations (Fig. 4), which allowed a quantitative measurement of the effect of lowering the temperature on the rate constant of photoinhibition, k_{PI}. Fig. 4A shows that when the temperature was lowered from 20 to 2°C, k_{PI} decreased to about one-half both in unfractionated spinach thylakoids and thylakoid subfractions derived from the appressed thylakoid regions. Right-side-out membrane vesicles behaved differently: lowering the temperature from 20 to 2°C had no effect on the kinetics (Fig. 4B)

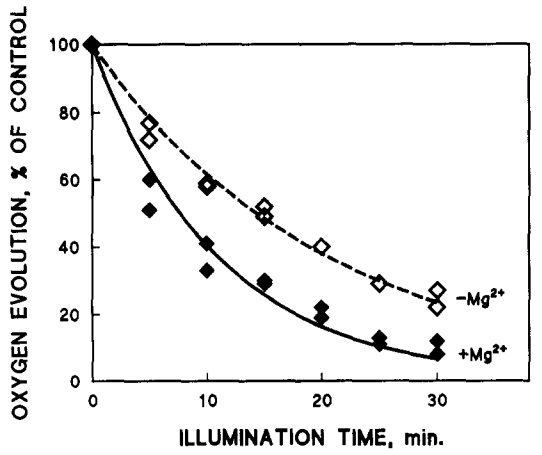


Fig. 3. Time course of photoinhibition of PS II oxygen evolution in spinach thylakoid membranes during illumination at 3500 μmol photons $m^{-2}s^{-1}$, 20°C. Control thylakoids (♦) and thylakoids randomized and illuminated in the absence of divalent cations (◇). The curves are best fits to a first-order equation. The k_{PI} constant was 0.091 min^{-1} in the presence of Mg^{2+}, and 0.048 min^{-1} in the absence of Mg^{2+}.

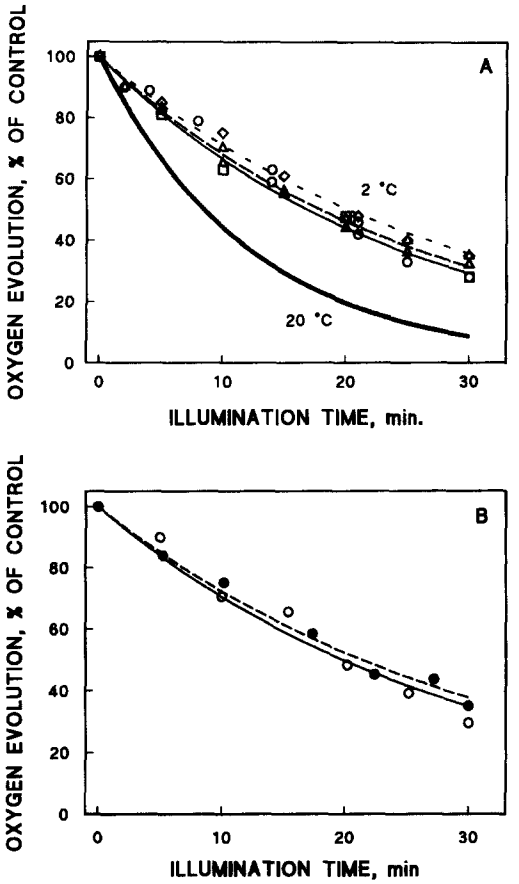


Fig. 4. Effect of temperature on photoinhibition of oxygen evolution in thylakoids and PS II preparations derived from the appressed membranes (A) and in rightside-out thylakoid vesicles (B). All preparates were isolated from spinach leaves. In A, Thylakoid membranes (◇, short-dashed line), inside-out membrane vesicles (△, long-dashed line), PS II membranes (□, solid line) and oxygen evolving PS II core complexes (○, dotted line) were illuminated at 2°C, 3500 μmol photons $m^{-2}s^{-1}$. The curves are best fits to a first-order equation, and the curve (wide line) describing photoinhibition of the same preparations under the same PPFD at 20°C (see Fig. 1 for the complete data) is shown to facilitate comparison. The k_{PI} values of all preparations were 0.034–0.041 min^{-1} at 2°C and 0.08 min^{-1} at 20°C. In B, the rightside-out membrane vesicles were illuminated at 3500 μmol photons $m^{-2}s^{-1}$ at 20°C (○, solid line) and 2°C (●, dashed line). The k_{PI} values were 0.035 min^{-1} at 20°C and 0.033 min^{-1} at 2°C.

3.5. Photoinhibition is a first-order reaction both at low temperature and in the presence of DCMU

The temperature dependence of photoinhibition was measured in more detail with isolated pumpkin thylakoids. In these experiments, F_V/F_{MAX} was used as

a measure of photoinhibition (Fig. 5A). The good correlation between light-saturated PS II oxygen evolution activity and F_V/F_{MAX} during photoinhibition [10,31,32] was confirmed by measuring PS II oxygen evolution activity in the course of some experiments (data not shown). The decrease in F_V/F_{MAX} during illumination resulted mainly from the lowering of F_{MAX} but also from an increase in F_0 (Fig. 5A).

To test whether the kinetics of photoinhibition are affected by slowing down the degradation of the D1 protein, we measured the kinetics at low temperature and in the presence of DCMU. Low temperatures are known to inhibit the degradation of the damaged D1 protein [14], and DCMU has been reported to inhibit the low-light turnover of the D1 protein in vivo in *Spirodela* [33]. Fig. 5B shows that photoinhibition is a first-order reaction both at low temperatures and in the presence of DCMU. Immunological quantification of the D1 protein content of the thylakoids revealed a smaller loss of the D1 protein at 3.5°C than at 20°C (Fig. 5B). However, we did not see any inhibition of D1 protein degradation by DCMU during photoinhibition of isolated thylakoids at 20°C (data not shown). Even low concentrations of DCMU induced an increase in the rate constant of photoinhibition, k_{PI} (Fig. 5C).

3.6. The activation energy of photoinhibition is 15 kJ/mol

As the temperature dependence of photoinhibition suggests that photoinhibition has an activation energy, we proceeded by fitting the temperature dependence of k_{PI} in pumpkin thylakoids to the Arrhenius equation

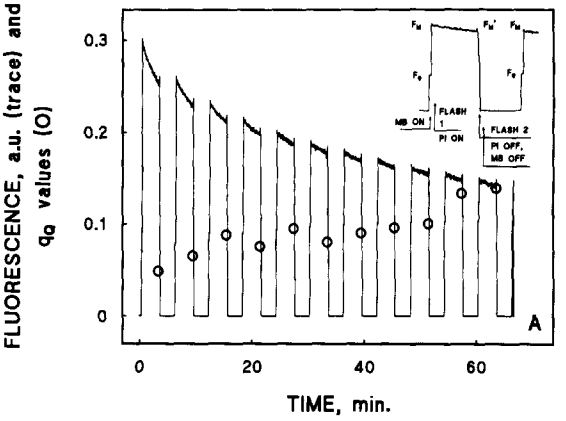
$$k_{PI} = Ae^{-E_a/RT} \quad (1)$$

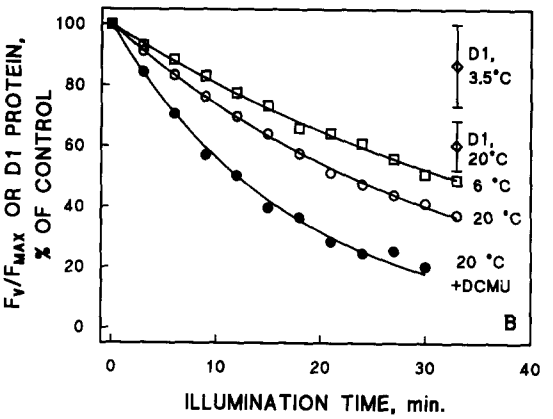
where R = gas constant and T = absolute temperature. An acceptable fit was obtained between 6 and 25°C, with E_a (activation energy) of 15 kJ/mol (Fig. 6). The rate constant of dark inactivation, which also was a first-order process, was subtracted; however, the dark inactivation only affected the kinetics significantly above 25°C (Fig. 6). Even above 25°C, the dark inactivation was much slower than the decrease of F_V/F_{MAX} in the light.

4. Discussion

4.1. Antenna size independence of photoinhibition

The first-order rate constant of photoinhibition, k_{PI}, upon illumination of isolated thylakoids or PS II membrane preparations is mainly determined by the photon fluency rate, which in turn determines the exciton density in the PS II antenna bed. One would expect





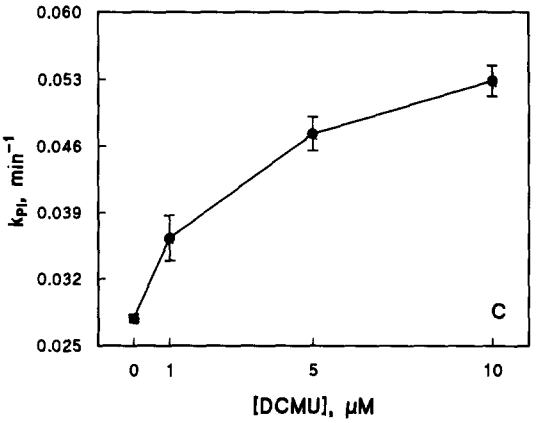


Fig. 5. (A) A fluorescence tracing of isolated pumpkin thylakoids showing how the activation energy measurements were done. The circles (○) show the values of the q_Q quenching parameter at the end of each light period. The insert shows how the light sources (MB = measuring beam of the fluorometer, PI = photoinhibitory light, FLASH = saturating flash) were manipulated during the light/dark cycles. Each FLASH 1 was used to calculate F_V/F_{MAX}, and each FLASH 2 to calculate the q_Q quenching parameter. The F_0 checkpoints show out as shoulders in the trace. (B) Decrease in F_V/F_{MAX} during photoinhibition of pumpkin thylakoids (1800 μmol photons $m^{-2}s^{-1}$) at 20°C (○, ●) and 6°C (□) in the absence (○, □) and presence (●) of 10 μM DCMU. The D1 protein content of thylakoids (◇), measured after the treatment (% of untreated control ± S.E. of 3 determinations) is shown for 3.5 and 20°C treatments. (C) The dependence of k_{PI} on the concentration of DCMU.

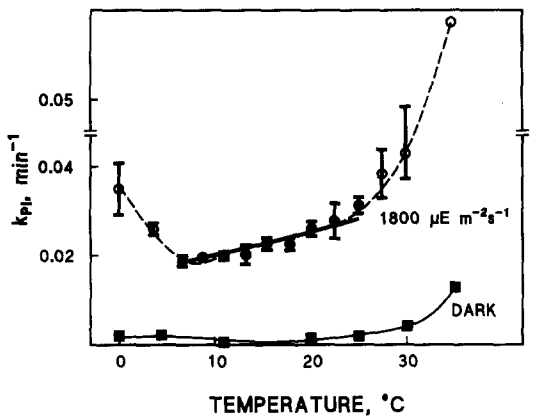


Fig. 6. The rate constant of photoinhibition, k_{PI}, as a function of temperature in isolated pumpkin thylakoids (●, ○). The solid curve is the best fit to the Arrhenius equation between 6 and 25°C (●). The data points beyond the 6–25°C limits (○) are not included in the fit. Each point is a mean of three to four independent experiments, and the bars show the standard deviation. The lower curve (■, one experiment at each temperature) shows the decrease in F_V/F_{MAX} in darkness at the different temperatures; these values have been subtracted from the original k_{PI} values.

that k_{PI} is largest in PS II preparations with largest light-harvesting antenna, i.e., in inside-out membrane vesicles and PS II membranes. These preparations absorb the highest number of light quanta per PS II reaction centre in unit time. However, the kinetics of inhibition of oxygen evolution was independent of the size of the light-harvesting antenna of PS II (Figs. 1, 2). As shown in Fig. 2, the photon fluency rates used in the experiments, although probably saturating for the linear electron transfer reaction in the thylakoids, were not saturating for photoinhibition. In fact, the rate constant of photoinhibition was directly proportional to PPFD in the photoinhibitory PPFD values used (see values in Fig. 2).

Antenna size independent kinetics of photoinhibition in vitro is not limited to experiments with different PS II preparations. We have shown that thylakoids isolated from the same plant species grown either under extremely low light conditions or in strong light were photoinhibited with the same kinetics during strong illumination [34] despite of a 2-fold difference in the size of the light-harvesting antenna.

To understand this quite unexpected result, the exciton movement and trapping in the photosynthetic system must be considered. According to the widely accepted shallow trap model [35], excitons are in equilibrium between antenna and reaction centre chlorophylls, the reaction centre of PS II constitutes a shallow trap for an exciton in the antenna bed, and the rate of trapping limits the lifetime of the exciton. In the following, it is assumed, for simplicity, that the reaction centre is an extremely shallow trap (the probability that an exciton is transferred back to the antenna from the reaction centre is the same as the forward transfer probability).

Let N = number of pigment molecules in an exciton-equilibrated system (reaction centre and antenna), a = number of photons absorbed by one pigment molecule in time Δt, τ = exciton lifetime.

The probability of having an exciton in the system is

$$P_1 = aN\tau/\Delta t \tag{2}$$

and the probability of having an exciton in the reaction centre, if there is one in the system, is

$$P_2 = 1/N \tag{3}$$

From Eqs. (1) and (2) it follows that the probability P of having an exciton in the reaction centre is

$$P = P_1P_2 = aN\tau/(N\Delta t) = a\tau/\Delta t \tag{4}$$

which is independent of N.

It could be argued that the above reasoning is wrong because, in a trap-limited system, the exciton lifetime τ is a linear function of N [36]. The reason for the antenna size dependence of τ is obvious: if N is larger, the exciton has fewer opportunities to be trapped in unit time and therefore it lives longer. Actual measurements [35,37] have shown that this indeed is true for the major fast component of fluorescence decay. The rate of trapping, however, does not affect the overall exciton lifetime if trapping is too slow. When isolated thylakoids or PS II preparations are illuminated with high light, most of the Q_A is reduced for most of the time, i.e. the traps are closed. It has been shown that when Q_A is reduced, the charge separation is slowed down by a factor of six as compared to F_0 conditions [35,38], and consequently the exciton lifetime is governed by nonphotochemical decay channels. The validity of the assumption that the exciton lifetime does not depend on antenna size in F_{MAX} conditions was verified by a review of published fluorescence decay measurements. As the exciton lifetime in photosynthetic systems is not a single exponential, the time average of the lifetime, M_0 [36,38], was calculated from the published data. M_0 is calculated as

$$M_0 = \sum_{i=1}^{N} A_i e^{-k_i t} \Big/ \sum_{i=1}^{N} A_i, \tag{5}$$

where A_i is the amplitude and k_i is the lifetime of the ith component, t = time and N is the number of exponentials.

The data presented in Table 2 confirm that there is no antenna size dependence in M_0 measured in 'F_{MAX} conditions' where all PS II traps are closed. The same result is obtained if only the two fastest lifetime components are used in calculating M_0 (not shown), indicating that the independence of M_0 on antenna size in F_{MAX} conditions is not due to artifacts which may affect the longest lifetime component. It must be ad-

Table 2
M_0, the average exciton lifetime, calculated from published lifetime data

Material	Estimated PS II antenna size	M_0, ns
D1/D2/cyt *b*-559 preparation	8 [a]	1.7 [a]
D1/D2/cyt *b*-559/CP47 preparation	20 [a]	1.4 [a]
Spinach PS II particles	80 [b]	1.2 [b]
PS II core complexes		1.5 [c]
PS II membranes		1.4 [c]
Chlorella in vivo		1.5 [d]
Chlamydomonas in vivo		1.0 [d]
Spinach thylakoids		2.2 [e]
Pea thylakoids		1.7 [c]
Spinach thylakoids		2.0 [c]

The lifetime data were collected from published measurements done in vivo and in vitro in 'F_{MAX} conditions' (PS II traps closed). The PS II antenna of intact thylakoids contains around 250 chlorophyll molecules, depending on the plant material.
Sources of experimental data: [a] [37], [b] [47], [c] [39], [d] [48], [e] [49].

mitted, however, that there are controversies in the relationship between antenna size and exciton lifetime even in F_0 conditions (see e.g. [35,37,39]).

The finding that the rate constant of photoinhibition is independent of antenna size does not mean that a large antenna is irrelevant for light harvesting in photosynthesis. An exciton can only cause photosynthetic electron transfer in PS II if it finds an open trap. The rate of electron transfer in PS II at non-saturating PPFD can therefore have a clear antenna size dependency, although the rate of photoinhibition is independent of antenna size. It is also clear that photoinhibition can be alleviated by efficient electron transfer through PS II [40,41]. The protective effect of photosynthetic electron transfer is also larger if the light-harvesting antenna is smaller, because electron transfer lowers the exciton density in a small antenna bed more than in a large one. Therefore, although the rate constant of photoinhibition in vitro in the absence of linear electron flow is independent of antenna size, plants may acquire some protection from photoinhibition by reducing the size of the light-harvesting antenna. However, measurements of the rate constant of photoinhibition in vivo reveal that this protective mechanism is not very efficient [9].

The acceptor side photoinhibition mechanism which involves double reduction of Q_A [6] was initially shown to function in anaerobic conditions. The antenna size independence of k_{PI} is compatible with the idea that this mechanism also functions in aerobic conditions. A prerequisite for the double reduction of Q_A is the single reduction of this acceptor, and therefore the acceptor side mechanism requires that the reaction centre is closed before photoinhibition. We also feel that the DCMU-induced enhancement of photoinhibition (Fig. 5C) supports the hypothesis that photoinhibition occurs via an acceptor side mechanism in aerobic conditions in vitro.

4.2. Does spillover protect PS IIβ?

The data presented here do not support the hypothesis that the lower susceptibility of PS IIβ than PS IIα centres to photoinhibition is due to the smaller size of the light-harvesting antenna [11,13]. Besides their antenna size, PS II α and β centres also differ in the molecular properties of the reaction centre complex [38,42,43], and these properties might make the PS IIβ centres intrinsically less susceptible to high light. Spillover of excitation energy from PS II to PS I [29] may also protect the PS IIβ centres from photoinhibition, as the PS IIβ centres are located in the non-appressed thylakoid regions in the close vicinity of PS I. Protection by spillover is supported by the finding that the rate constant of photoinhibition decreased by about 50% when unfractionated thylakoids were suspended in low-salt medium (Fig. 3). Low-salt treatment increases spillover by bringing the PS II units into closer contact with PS I [20]. Spillover may also be an important factor in the low susceptibility to photoinhibition in the chlorophyll *b*-less barley mutant [12], which is devoid of LCH II. The finding that lowering the temperature to 2°C did not protect the rightside-out vesicles from photoinhibition (Fig. 4B) may be an indication of a different molecular mechanism of photoinhibition in PS IIβ. However, it is possible that the complexity of the temperature dependency of the photoinhibition of PS IIβ is not fully realized in the experiments done at two temperatures.

4.3. Photoinhibited PS II centres do not protect the remaining active ones

It has been shown that photoinhibition both in vivo, in the presence of inhibitors of chloroplast protein synthesis [10,15,16] and in isolated thylakoids in vitro [14] is slower at lower temperature when the effect of the PS II repair cycle is eliminated. It has been suggested that the protection is possibly due to photoinhibited reaction centres from which the D1 protein has not yet been degraded [14,16,44,45]. Such a protection could be caused by harmless trapping of a fraction of absorbed excitation energy by the photoinhibited centres.

Trapping of excitation energy by a photoinhibited centre can protect a remaining active centre only if there is significant excitation energy coupling between the two centres. However, the protection by low temperature was as efficient in PS II core complexes as in unfractionated thylakoids (Fig. 4A), although it is unlikely that the connectivity of PS II centres is similar in both cases. Protection by the photoinhibited PS II

centres would also distort the first-order kinetics of photoinhibition, and this deviation from first-order kinetics would be most pronounced when the degradation of the D1 protein is slow. However, the photoinhibitory decrease in PS II oxygen evolution activity is a perfect first-order reaction in isolated spinach thylakoids and PS II preparations at 2°C (Fig. 4), and the same observation was made for the photoinhibitory lowering of F_V/F_{MAX} in isolated pumpkin thylakoids at all temperatures between 0 and 35°C (data not shown but see Fig. 5B). As shown in Fig. 5B, a large fraction of PS II centres were photoinhibited but still contained a D1 protein unit after 33 min illumination at low temperature. If the protection by photoinhibited centres were of any importance, we would have seen the distortion of the first-order kinetics of photoinhibition at low temperature. Unfortunately, we could not see any inhibition of D1 protein degradation by DCMU at 20°C; however, even at 20°C in the absence of DCMU (Fig. 5B), the degradation of the D1 protein was so slow that the protective effect of the photoinhibited PS II centres would have been evident if it existed.

4.4. The activation energy of photoinhibition is 15 kJ / mol

After excluding the protection by photoinhibited centres as an explanation for the temperature dependence of photoinhibition, we made experiments to find out if the temperature dependence is due to an activation energy of the rate determining step of photoinhibition. Between 6 and 25°C, k_{PI} followed the Arrhenius equation fairly well (Fig. 6), suggesting that the temperature dependence is simply due to activation energy of photoinhibition of about 15 kJ/mol. We presume that the reason for the deviation from the Arrhenius equation above 25°C is the temperature-induced damage to the oxygen evolving complex, which may lead to a significant contribution from donor side photoinhibition. The reason for the increase in k_{PI} by lowering the temperature between 6 and 0°C is less obvious. This increase was also seen in the rate of photoinhibition of the oxygen evolution activity of the thylakoids (data not shown). It is possible that the increase in k_{PI} between 6 and 0°C is related to the chilling sensitivity of pumpkin. A transition in the thylakoid structure around 6°C was recently suggested to explain a discontinuity in the Arrhenius plots of PS II oxygen evolution activity in *Euglena* [46].

We do not yet know what is the rate-limiting step in photoinhibition and therefore cannot make a precise assignment of the reaction for which the activation energy was measured. Anyway, it is clear that photoinhibition is not an activationless photophysical reaction like the primary photochemistry in PS II. The proposed mechanism of acceptor-side photoinhibition [6] contains several reactions, many of which might be temperature dependent. We considered the most obvious possibility that differences in the reduction state of Q_A during the photoinhibitory illumination, which has been shown to correlate with the kinetics of photoinhibition in vivo [41], could vary and explain the temperature response. However, the q_Q parameter of fluorescence quenching was around 0.1 during photoinhibition at all temperatures (a typical experiment is shown in Fig. 5A), indicating that most of the Q_A was reduced all the time during illumination at the different temperatures.

Acknowledgements

This study was supported by the Academy of Finland. The authors thank Virpi Paakkarinen for excellent technical assistance.

References

[1] Aro, E.-M., Virgin, I. and Andersson, B. (1993) Biochim. Biophys. Acta 1144, 113–134.
[2] Arntz, B. and Trebst, A. (1986) FEBS Lett. 194, 43–49.
[3] Virgin, I., Styring, S. and Andersson, B. (1988) FEBS Lett. 233, 408–412.
[4] Kanervo, E., Mäenpää, P. and Aro, E.-M. (1993) J. Plant Physiol. 142, 669–675.
[5] Greer, D.H., Berry, J.A. and Björkman, O. (1986) Planta 168, 253–260.
[6] Vass, I., Styring, S., Hundal, T., Koivuniemi, A. and Aro, E.-M. (1992) Proc. Natl. Acad. Sci. USA 89, 1408–1412.
[7] Chen, G.-X., Kazimir, J. and Cheniae, G.M. (1992) Biochemistry 31, 11072–11083.
[8] Wünschmann, G. and Brand, J.J. (1992) Planta 186, 426–433.
[9] Tyystjärvi, E., Ali-Yrkkö, K., Kettunen, R. and Aro, E.-M. (1992) Plant Physiol. 100, 1310–1317.
[10] Tyystjärvi, E. (1993) Photoinhibition – Struggle between Damage and Repair of Photosystem II. Doctoral thesis, University of Turku, Turku.
[11] Cleland, R.E., Melis, A. and Neale, P.J. (1986) Photosynth. Res. 9, 79–88, 1986.
[12] Cleland, R.E. and Melis, A. (1987) Plant Cell. Env. 10, 747–752.
[13] Mäenpää, P., Andersson, B. and Sundby, C. (1987) FEBS Lett. 215, 31–36.
[14] Aro, E.-M., Hundal, T., Carlberg, I. and Andersson, B. (1990) Biochim. Biophys. Acta 1019, 269–275.
[15] Kirilovsky, D.L., Vernotte, C. and Etienne, A.-L. (1990) Biochemistry 29, 8100–8106.
[16] Greer, D.H., Ottander, C. and Öquist, G. (1991) Physiol. Plant. 81, 203–210.
[17] Anderson, J.M., Waldron, J.C. and Thorne, S.W. (1978) FEBS Lett. 92, 227–233.
[18] Andersson, B. and Åkerlund, H.-E. (1978) Biochim. Biophys. Acta 503, 462–472.
[19] Ghanotakis, D.F., Demetriou, D.M. and Yocum, C.F. (1987) Biochim. Biophys. Acta 891, 15–21.
[20] Staehelin, L.A. (1976) J. Cell. Biol. 71, 136–158.
[21] Laemmli, U.K. (1970) Nature 277, 680–685.

[22] Andersson, B., Åkerlund, H.-E. and Albertsson, P.-Å. (1976) Biochim. Biophys. Acta 423, 122–132.
[23] Arnon, D.I. (1949) Plant Physiol. 24, 1–15.
[24] Theg, S.M., Filar, L.J. and Dilley, R.A. (1986) Biochim. Biophys. Acta 849, 104–111.
[25] Jegerschöld, C. and Styring, S. (1990) FEBS Lett. 296, 45–48.
[26] Eckert, H.-J., Geiken, B., Bernarding, J., Napiwotzki, A., Eichler, H.-J. and Renger, G. (1991) Photosynth. Res. 27, 97–108.
[27] Baumgarten, M., Philo, J.S. and Dismukes, G.C. (1990) Biochemistry 29, 10814–10822.
[28] Anderson, J.M. and Melis, A. (1983) Proc. Natl. Acad. Sci. USA 80, 745–749.
[29] Butler, W.L. (1978) Annu. Rev. Plant Physiol. 29, 345–378.
[30] Baker, N.R. and Webber, A.N. (1987) Adv. Bot. Res. 13, 2–66.
[31] Krause, G.H., Somersalo, S., Zumbusch, E., Weyers, B. and Laasch, H. (1990) J. Plant Physiol. 136, 472–479.
[32] Öquist, G., Chow, W.S. and Anderson, J.M. (1992) Planta 186, 450–460.
[33] Mattoo, A.K., Hoffman-Falk, H., Marder, J.R. and Edelman, M. (1984) Proc. Natl. Acad. Sci. USA 81, 1380–1384.
[34] Tyystjärvi, E., Koivuniemi, A., Kettunen, R. and Aro, E.-M. (1991) Plant Physiol. 97, 477–483.
[35] Schatz, E.H., Brock, H. and Holzwarth, A.R. (1988) Biophys. J. 54, 397–405, 1988.
[36] Pearlstein, R.M. (1982) Photochem. Photobiol. 35, 835–844.
[37] Freiberg, A., Timpmann, K., Moskalenko, A.A. and Kuznetsova, N.Yu. (1992) in Research in Photosynthesis, Vol. II (Murata, N., ed.), pp. 65–68, Kluwer Academic, Dordrecht.
[38] Roelofs, T.A., Lee, C.-H. and Holzwarth, A.R. (1992) Biophys J. 61, 1147–1163.
[39] Govindjee, Van de Ven, M., Cao, J., Royer, C. and Gratton, E. (1993) Photochem. Photobiol. 58, 438–445.
[40] Demmig, B. and Björkman, O. (1987) Planta 171, 171–184.
[41] Öquist, G., Hurry, V.M. and Huner, N.P.A. (1993) Plant Physiol. Biochem. 31, 683–691.
[42] Hideg, É. and Demeter, S. (1988) Z. Naturforsch. 43c, 596–600.
[43] Guenther, J.E., Nemson, J.A. and Melis, A. (1990) Photosynth. Res. 24, 35–46.
[44] Öquist, G., Anderson, J.M., McCaffery, S. and Chow, W.S. (1992) Planta 188, 422–431.
[45] Schnettger, B., Critchley, C., Santore, U.J., Graf, M. and Krause, G.H. (1993) Plant Cell Env. 17, 55–64.
[46] Zhong, S., Nolan, W.G. and Shi, P. (1993) Plant Sci. 92, 121–127.
[47] Holzwarth, A.R., Brock, H. and Schatz, G.H. (1987) in Progress in Photosynthesis Research, Vol. I (Biggins, J., ed.), pp. 61–65, Martinus Nijhoff, Dordrecht.
[48] Haehnel, W., Holzwarth, A.R. and Wendler, J. (1983) Photochem. Photobiol. 37, 435–443.
[49] Berens, S.J., Scheele, J., Butler, W.L. and Magde, D. (1985) Photochem. Photobiol. 42, 51–57