HIGH LIGHT-INDUCED INHIBITION OF PHOTOSYNTHESIS AND RECOVERY: PHOTOOXIDATIVE INACTIVATION AND TURNOVER OF THE D_1 PROTEIN

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I. Introduction

Plants uptake photon energy, almost exclusively from sunlight, and efficiently convert it through photosynthesis into chemical energy, that is subsequently transduced into all forms of biological energy via a variety of cellular processes. Light is therefore an absolute prerequisite for the autotrophic growth of photosynthetic organisms. However, light is not always beneficial to plant, being potentially harmful under certain circumstances. Although the debilitating effects of light, as have frequently been observed particularly in low light-adapted plants upon exposure to full sunlight, are largely blamed on UV radiation of short wavelengths, *i.e.* UV-B and UV-C, there is a copious body of evidence that even an interaction between visible light and organisms can result in loss of numerous cellular functions to a varied extent. The deleterious effects of bright light in cells are the most evident in the photosynthetic apparatus of plants.

A sharp decrease in the capacity of photosynthesis in green plants, when subjected to irradiation at high light intensities, is termed as photoinhibition of photosynthesis.[1] There is now a general consensus that the primary target for high light-induced damage to chloroplasts is the photosystem II(PS II) complex.[2-5] The term photoinhibition was originally defined by Osmond[6] as a phenomenon in plant cells resulting from overexcitation of light harvesting pigment assemblies in excess of the potential of photosynthetic electron transport system to orderly dissipate the excitation energy. The molecular mechanisms, which have been either proposed with supporting experimental results or simply hypothesized, were in fact based on this concept of Osmond,[6] focusing upon how overexcitation of the pigment assemblies can lead to the damage-initiating processes in PS II.

Photoihibition is an extremely complex phenomenon, comprising various phases, some of which overlap and are thus difficult to resolve: a number of hypotheses and schemes describing the underlying mechanisms for photoinhibition have been proposed,[3] reflecting many different views in detail. According to various experimental data and proposed explanations in literature, photoinhibition damage to PS II seems to occur through a variety of processes.[3,5] The diversity of view as to which process predominantly takes place in PS II might arise largely from the difference in experimental conditions, such as whether the system under study was of plant cells, chloroplasts, thylakoids, oxygen-evolving PS II particles or isolated PS II reaction core complexes, whether the system was illuminated aerobically or anaerobically, and whether an environmental stress-inducing factor other than

light (for example, low temperature) was imposed upon the system. Nonetheless, it is now accepted that photoinhibition of PS II is induced, at large, by two different mechanisms, either from the acceptor side or from the donor side of P_{680}.[3-5]

Because photosynthetic cells are always exposed to the risk of light-caused stress situations, plants have evolved several strategies to cope with it, that is, to avoid or at least to minimize photoinhibition.[7] Thermal dissipation of the excessive excitation energy, that may occur in the antenna bed or in the reaction center complex of PS II (RC II), could be an important protective mechanism.[8-10] Other prophylactic measures are also taking place, which involve dynamic reversible changes in antenna size of PS II.[11] However, the major strategy adapted by plant cells is, without doubt, the repair of PS II *via* replacement of the damaged reaction center subunits.[3,12,13] Indeed, *in vivo* photoinhibition has been observed only when the rate of repair cannot keep pace with the rate of photodamage to PS II. Although the detailed processes of the damage and repair at molecular level have yet to be elucidated and are still debated, recent achievements regarding biochemical knowledge of photoinhibition are remarkable, owing mainly to understanding the structure and function of RC II by analogy to the reaction center of the photosynthetic purple bacteria,[14-16] whose three-dimensional structure has been determined.[17]

In this paper, it has by no means been intended to write a comprehensive review on various aspects of photoinhibiton of photosynthesis and its recovery, which have appeared in the vast number of papers published mostly in the last decade. Instead, the author will briefly review the plausible mechanisms for the damage to PS II and repair processes under high light conditions, which have been proposed by the leading groups in the related field and seem to receive a rather wide recognition from many researchers approching the photoinhibition problems at molecular level. Then, the latter part of this article will be allocated to the description of the experimental results from our laboratory, obtained in the recent years,[18-22] and their implications which apparently address a new mechanistic aspect of photoinhibition damage to plant cells.

II. Photoinhibition and Repair of PS II – an overview

1. The structure and function of PS II

In order to have access to the events involved in photoinhibition of photosynthesis, one would need to comprehend the current state of our knowledge on PS II. The PS

II complexes are embedded in thylakoid membranes and are located mainly in the appressed regions, while only a fraction is found in the non-appressed regions where the majority of PS I and the ATP synthetase are located.[11] PS II is a multisubunit complex, to which at least 23 (or probably 25) polypeptides have been assigned during the last 10-15 years.[7] The primary event in PS II is the capture of light energy by chlorophyll-binding proteins either through the direct absorption of the photosynthetically active radiation (PAR) or through the energy transfer processes. The main portion of the light-harvesting antenna is made up of several chlorophyll a/b-proteins, designated as LHC II, but there are also two major chlorophyll a-proteins, denoted as CP47 and CP43, which are tightly associated with the reaction center and thus often regarded as the components of RC II. Biochemically functioning RC II consists of 8 (or 9) polypeptides (Fig. 1). Three of these assemble as a part of PS II on the inner surface (the lumenal side) of the thylakoid membrane, functioning as the oxygen-evolving complex: the other five subunits, including two plastoquinone-binding proteins called the D_1 and D_2 proteins, which bind the secondary quinone (Q_B) and the primary quinone (Q_A) respectively, a low molecular weight cytochrome (cyt b_{559}) and the afore-mentioned CP47 and CP43, are intrinsic membrane proteins (Fig. 1). Another polypeptide encoded by the psb I gene has also been suggested to be a component of the RC II complex[23] although its function is not yet understood.
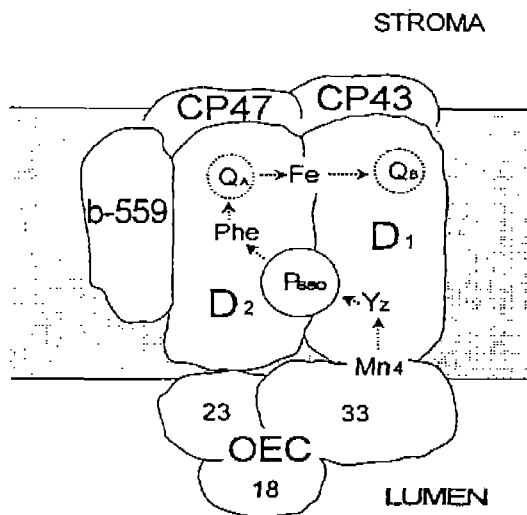

Fig. 1. A model for the functioning reaction center complex of PS II.

The photon energy captured by the antenna is transferred to the primary electron donor P_{680}, producing its singlet excited state ($^1P_{680}$) which possesses an exceptional reducing power strong enough to reduce pheophytin, the primary acceptor, by one electron transfer reaction. This primary charge separation is stabilized by fast electron transfer to Q_A, resulting in forming the radical pair $P_{680}^+ - Q_A^-$. The electron is further transferred from Q_A^- to Q_B, forming the semiquinone radical of the secondary plastoquinone (Q_B^-). After a new light-induced charge separation Q_B^- uptakes a second electron from Q_A^- and then protonated, forming plastoquinol (PQH_2) which immediately leaves the binding site of Q_B to intermix with the plastoquinone pool. On the other hand, P_{680}^+ acts as a very strong oxidant and is thus readily able to extract electron from water[24,25] via a redox intermediate tyrosyl residue Tyrz (also known as Yz) and the manganes cluster (Mn_4) cycle. Of all electron transfer steps in PS II, $H_2O \rightarrow Mn_4 \rightarrow Yz \rightarrow P_{680} \rightarrow Phe \rightarrow Q_A \rightarrow Q_B$, the first half (from H_2O to P_{680}) is known as the donor side reaction and the latter half (from P_{680} to Q_B) is called as the acceptor side reaction, respectively.

Despite the very large number of subunits and their complicated array in PS II, recent biochemical data and the close analogy in structure and function between PS II and the reaction center of photosynthetic bacteria suggest that only the D_1 and D_2 proteins appear directly involved in performing most of the steps of PS II electron transfer,[3,5] $i.e.$ those from Yz to Q_B. P_{680} is ligated to both the D_1 and D_2 proteins together; Phe and Yz, along with the Q_B docking site, are situated in the D_1 protein; and Q_A has its binding site on the D_2 protein.

2. Biophysical and biochemical basis for PS II photoinhibition

Photoinhibition of chloroplasts has been implicated by severe modification of chlorophyll fluorescence emission characteristics,[26-28] which are often used to diagnose a photoinhibitory stress. Fluorescence measurements at liquid nitrogen temperature have revealed that the pigment bed of PS II is markedly disrupted by the photoinhibitory treatment of leaves, algal cells, isolated intact chloroplasts and thylakoids, while only a slight perturbation occurs in the pigment bed. Furthermore, kinetic studies, in which the rate of whole chain (PS II→PS I) electron transport and the rates of partial electron transport through PS II and PS I were measured by using strongly irradiated leaves, chloroplasts and thylakoids, have produced results clearly demonstrating that, while the activity of PS I remains almost uninhibited, the PS II activity is substantially reduced and the extent of this reduction is nearly in parallel with that of the reduction of whole

chain electron transport activity.[29-32] These facts provide a rationale for consensus that PS II is the primary target for photoinhibition damage to the photosynthetic organelles.

The electron transport activity of PS II can be partially fractionated by employing various biophysical techniques and biochemical manipulations.[33,34] Also frequently used are artificial electron donors and acceptors targeting specific components within the complex.[35,36] Fractionation of the partial activities conducted by several groups has produced contradicting results regarding the initial specific site of damage within the PS II complex. As for this site of the primary lesion in the damaged PS II, some groups favor the reaction center chlorophylls[37,38] but the others do the Q_B binding site of the D_1 protein.[39,40] Although the differing views have yet to be reconciled, many studies which have been carefully conducted in order to minimize every possible artifacts arising in such complicated experiments strongly support the latter view.[2,22] Also worthy of note is the fact that the former view has its grounds solely on spectroscopic data obtained with isolated reaction center core complex, consisting of the D_1 and D_2 proteins, cyt b_{559} and the psb I gene product, subjected to the photoinhibitory treatment: such system obviously suffers lack of the physiological relevance because the very primary event which leads to photoinhibition processes of PS II damage occurs in some other components of thylakoids ratherthan the core complex itself.

Clear evidence has consistently been provided since the first observation of Arntzen et al.[41] that only the D_1 protein is significantly degraded, producing a fragment with the assigned molecular weight of 23 kD and other smaller polypeptides in strongly irradiated leaves, isolated chloroplasts and thylakoids.[39,40,42] To some workers,[43,44] therefore, the degradation of the D_1 protein has been viewed as a real' photoinhibition damage and the earlier events leading to D_1 degradation are regarded as reversible processes immediately disappearing when the overenergized state of PS II is dissipated upon the cessation of illumination. However, many kinetic data both in vivo and in vitro show that D_1 turnover follows rather slowly than the inactivation of PS II electron transport, indicating that functional loss of PS II, which is irreversible of course, actually occurs before the fragmentation of the Q_B-binding protein starts to proceed.[28,45]

Another intriguing question regarding photoinhibition damage to PS II is: what is the reactive chemical species initiating a sequential process leading to disturbance of functional and structural integrity of RC II, regardless of whether the initial specific site of damage is the reaction center pigments or the Q_B docking site? Based

on biochemical and biophysical data available from literature,[2-4] it seems that there are two classes of plausible reactive species which have been either implicated by experimental results or simply hypothesized: one involves the radicals of redox intermediates of the light-mediated water-plastoquinone oxido-reductive reaction, such as Yz^+, P_{680}^+ and Q_B^-.[41,46-48] The other comprises activated oxygen species, such as hydroxyl radical (OH ·), superoxide (O_2^-), hydrogen peroxide and singlet oxygen ($^1O_2$) formed in the presence of molecular oxygen by excited pigments or as side-products of redox reactions occurring in thylakoids.[49-52] Whatever the nature of the damage-initiating species is, it appears that the action of this species on RC II results in a conformational change in the reaction center subunits, triggering proteolytic cleavage of the D_1 protein and, to some extent, the D_2 protein also.

3. Turnover of the D_1 protein and repair of photodamaged PS II

Although photoinhibition damage to PS II is closely correlated to the degradation of the D_1 protein *in vivo*[53,54] as well as *in vitro*,[7,55] this is most likely not the primary cause for photoinhibition. In line with this notion, D_1 degradation does not occur despite of a pronounced loss of the electron transport activity upon the exposure isolated thylakoid membranes to bright light at low temperature.[43] Now it seems a general agreement that the light-induced D_1 degradation is regarded as the initial step of the repair process of the already-impaired PS II complex. Circumstantially supporting this, rapid turnover and synthesis of the D_1 protein have consistently been observed whenever the rate of photodamage is high[3] Under most conditions, the rate of the on-going repair mechanism almost matches the rate of D_1 damage and thus there is little net loss of photosynthetic capacity.

Biochemical nature of D_1 degradation still remains under hot debate. The damage-initiating reactive species which are supposedly formed in the overenergized PS II, as discussed in the above section, could cause a direct cleavage of peptide bonds in the D_1 protein. However, it is not very conceivable that such photochemical cleavage' does all the works to cut the whole 32 kD protein into so many polypeptides and even amino acids. The photochemical process, if any, could be responsible for the initial breakdown of the D_1 protein, producing a large (23-24 kD) fragment, even though there is no direct evidence supporting this notion. At present there are abundant biochemical data which definitely favor the enzymatic degradation of the damaged D_1 protein. For example, D_1 degradation, particularly the initial breakdown, is inhibited to a large extent by proteinase inhibitors.[37,44,56] The observation by Aro *et al.*,[43] that D_1 degradation occurs in complete darkness at

room temperature once samples were subjected to photoinhibitory treatments in the cold, is also pertinent to the concept of proteolytic nature of D_1 degradation. For the present the identity and characteristics of the proteinase(s) catalyzing D_1 fragmentation are not well understood, but some important aspects of the responsible proteinase(s) have recently been revealed, thanks to the elaborative works by several groups approaching to this problem. Many experimental results[37,57-59] implicate that the proteinase involved at least in the initial step of the degradation is an integral part of the PS II complex itself and that the component of PS II which acts as D_1-proteinase' can be categorized as a serine-type protease.

Because the degradation of the D_1 protein is most effectively suppressed by diisopropyl fluorophosphate (DFP),[59] which covalently binds to serine residues of proteins[60] and is thus able to inactivate an enzyme if it involve a serine at its active site, radioisotope-labelled DFP has been used in order to identify the subunit of PS II acting as the proteolytic D_1-proteinase and carrying the active serine: the results were that a single, discrete polypeptide with an apparent molecular weight of 43 kD binds both ^{14}C-DFP and ^{3}H-DFP and that the labelled band co-migrates with CP43.[4] This observation suggests that CP43, apart from its main role as a light-harvesting protein, may catalyze the hydrolysis reactions of the D_1 protein. Based on other experimental results,[57,61] however, it can be also suggested that D_1 degradation may be autoproteolytic: this suggestion is in line with the observation that the D_1 protein is cleaved, producing several fragmented peptides, in isolated PS II reaction center particles which consist only of D_1, D_2, cyt b_{559} and the psb I-gene product. The presumed absence of CP43 in isolated reaction center core preparations from PS II could speak against CP43 as the D_1-proteinase involved in the degradation; but the possible involvement of CP43 contamination in the measuring samples would not be totally excluded because CP43 is often a contaminant of the preparation of the RC II core particles and because the contamination has not thoroughly been quantified before the measurements. Although the differing views on the putative PS II component responsible for the enzymatic D_1 degradation have yet to be reconciled, but this may be resolved by accepting the concept that the proteolytic cleavage of the D_1 protein involves more than one proteinase activity of PS II.

Also just speculative are the explanations how the chemical modification of the D_1 protein by the reactive intermediates, formed in thylakoids under high light conditions, turns the protein into a substrate for proteolysis. The most popular model at present is that a marked conformational change of the modified protein brings it into contact with neighboring proteolytic site(s) in the PS II complex.[4,5]
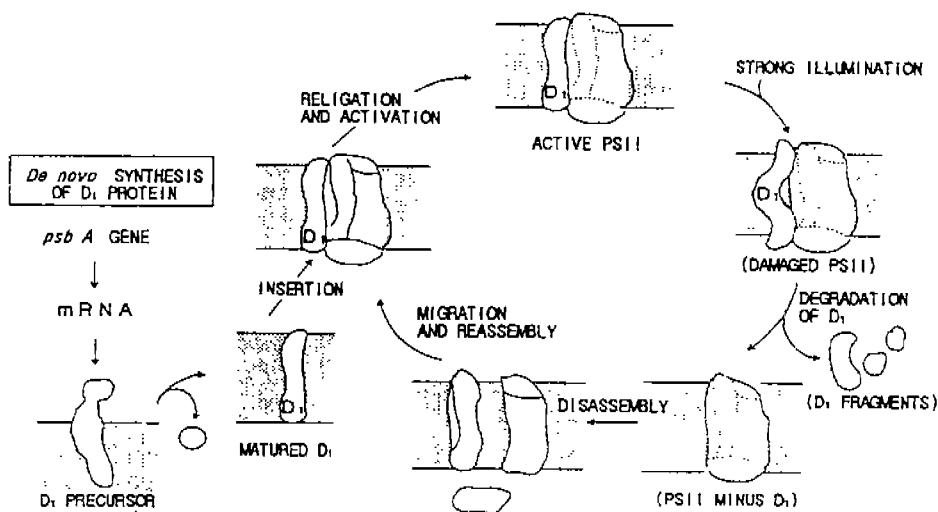
Fig. 2. Schematic representation of the photoinhibition repair cycle.

Recovery of photosynthetic capacity, which occurs only *in vivo* but not in vitro, can be achieved by the replacement of the damaged D_1 and the subsequent reactivation of the whole PS II. The recovery naturally involves a rather complex dynamic process, which may be schematically represented as in Fig. 2. The PS II is repaired after a proteolytic breakdown of the damaged protein and removal of the fragments from the membrane. This D_1 turnover leads to the disassembly and migration of the PS II components. The dissociation reactions for the disassembly involve the disconnection of RC II from the chlorophyll a/b antenna, the removal of the extrinsic protein subunits from the membrane and the release of the Mn cluster.[62] A lateral migration between the appressed and non-appressed regions of the stacked thylakoid membranes is required for the process of the repair because the insertion of the newly synthesized D_1 precursor is restricted to the stromally exposed domains of the membrane to which ribosomes have access, whilst most of PS II are, as mentioned before, shielded in the appressed thylakoid regions.[63] Reformation of active PS II involves the de novo synthesis and membrane insertion of a new copy of the D_1 precursor, followed by the reassembly and activation stage, in which the processing of the D_1 precursor occurs and the cofactors bound to the D_1 protein are re-ligated, fully restoring the PS II function.

III. A New Aspect of Photoinhibition Mechanism

Now, recent experimental results from our own laboratory and their mechanistic implications will briefly be described, which have given rise to a fundamental question against the proposition of Osmond[6] on the definition of photoinhibition and provided a rationale for skepticism on a number of the proposed mechanisms based principally on the Osmond's concept.

1. Action spectra for photoinhibition

Overexcitation of the pigment assemblies of the photosystem, which is, according to the Osmond's concept, the prerequisite for the primary processes leading to the phenomenon of photoinhibition, is achieved most efficiently by absorbing photosynthetically active radiation(PAR). Therefore, one would intuitively expect that the quantum efficieny for photoinhibition damage is substantially higher in the PAR region than in rather short wavelength region of the sunlight spectrum.
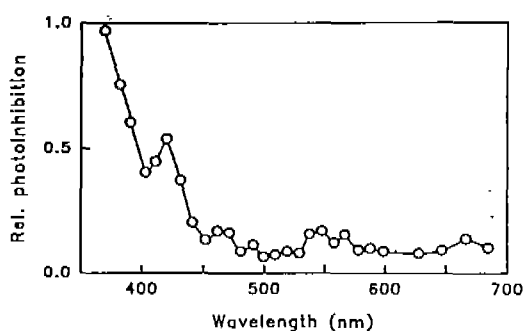

Fig. 3. Action spectrum for the photoinhibition of the photosynthetic electron transport in chloroplasts. The photoinhibition at each irradiating wavelength was measured from the rate of DCPIP photoreduction in the reaction mixture irradiated compared to that in the nonirradiated. Samples were irradiated at 15°C under aerobic condition. From Jung and Kim.[18]

However, such is certainly not the case, as has been evidenced by the reports of two laboratories including ours. Greenberg et al.[64,65] measured the in vivo action spectrum over a broad spectral range covering UV, visible and far red for the degradation of the D_1 protein in Spirodela plants, showing that, while turnover of the D_1 protein being promoted over an unusually wide spectral range in both the presence and the absence of photosynthetic activity, the quantum yield of D_1 degradation was significantly higher in shorter wavelengths (250–450 nm) than in longer wavelengths (550–750 nm). Our laboratory reported the action spectrum for inactivation of photosynthetic electron transport in isolated spinach chloroplasts in the ranges from 360 to 690 nm.[18] demonstrating that the photoinactivation is induced much more efficiently by near UV to blue light than by green to red light (Fig. 3). While there is a considerable similarity in these two spectra, they bear only a little, if any, resemblance to the absorption spectra of PS II pigments. These

observations may be taken as an implication that, apart from the photosynthetic pigments, some other chromophores take part in the primary processes of photoinhibition, acting as photosensitizing agents in thylakoids. Taking an assumption that photoinhibition may result from photodynamic sensitization reactions occurring in the electron transport system of thylakoids, we have conducted a series of studies aiming at the identification of the putative sensitizing chromophores and the major reactive intermediate associated with the harmful effects of near UV and visible light in chloroplasts.

2. Fe-S centers as major photosensitizing chromophores generating $^1O_2$ in thylakoids

Photosensitized reactions in biological systems proceed mainly *via* either type I (free radical mechanism) or type II processes (singlet oxygen mechanism),[66,67] but it is sometimes difficult to establish the reaction mechanism of a photosensitized process. Most of the diagnostic approaches that have been used are designed to determine whether $^1O_2$ is produced in the reaction and whether this species is responsible for the photodamage to the biological system.[66] One approach is to measure the photooxidation of trap′ compounds which efficiently react with $^1O^2$. Simple as it may be in general, however, the trap method often suffers lack of specificity, for many such compounds can also be oxidized to the same products by free radical processes. Together with this, therefore, other techniques are usually employed to provide the supporting data for the involvement of $^1O_2$. One widely used techinque is to examine the effects of $^1O_2$ quenchers on the rate of the photosensitized reaction under investigation. Comparison of the rate of the sensitized reaction in an aqueous medium with that in a deuterated medium is also frequently done in another kinetic method.

In order to determine the photogeneration of $^1O_2$ from thylakoids, we used a trap system consisting of imidazole and N,N-dimethyl-4-nitrosoaniline (RNO), in which imidazole reacts with $^1O_2$ to produce a *trans*-annular peroxide, an unstable intermediate, which in turn reacts with RNO, bleaching it.[68]

By employing the RNO bleaching technique, whose usability in monitoring the relative levels of $^1O_2$ production in biological systems has been discussed elsewhere,[18,69,70] we measured the spectral dependence of $^1O_2$ photogeneration from thylakoid menbranes (Fig. 4). Interestingly enough, the resulting spectrum resembles to some extent the absorption spectrum of the oxidized Fe-S centers, as does the action spectrum for the generation of $^1O_2$ from mitochondrial membranes.[69] This tempted

us to speculate that the thylakoid Fe–S centers may be the chromophores involved in the production of $^1O_2$. In order to prove this, the effect of mersalyl acid (MA) treatment of thylakoids on the bleaching of RNO was studied. As expected, the treatment resulted in a substantial diminution of RNO bleaching in the thylakoid-imidazole–RNO system. Because MA destructs the Fe–S centers in thylakoids,[71] the remarkable reduction of the RNO bleaching is a good indication that the Fe–S centers are most likely involved in the generation of $^1O_2$ from thylakoids.
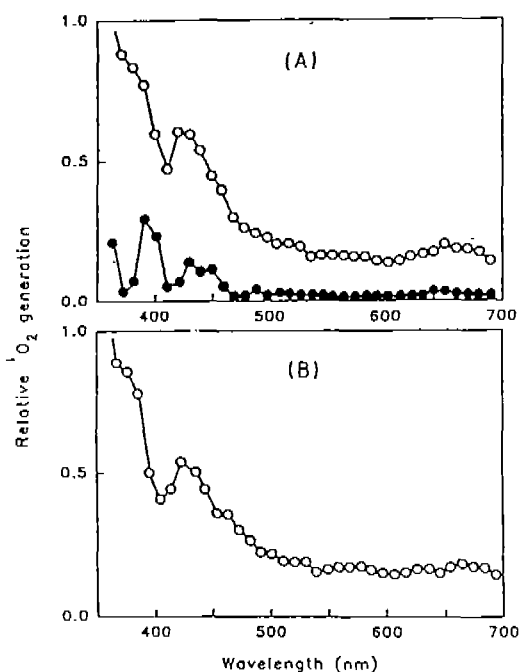

Fig. 4. Spectral dependence of the photogeneration of $^1O_2$ from thylakoid membranes. (A): $^1O_2$ generation from intact thylakoids (○) compared with that from the MA treated-thylakoids (●). (B): $^1O_2$ generation from the thylakoid Fe–S centers estimated from the data presented in (A). Irradiation conditions are same as in Fig. 3. From Jung and Kim.[18]

Subtracting the RNO bleaching in the MA treated thylakoid–imidazole–RNO system from that in the untreated thylakoid–imidazole–RNO system, we estimated the contribution of thylakoid Fe–S centers in the RNO bleaching at various wavelengths and constructed a spectrum which demonstrates the participation of the Fe–S center in $^1O_2$ photogeneration from thylakoids. Although the spectroscopic properties of thylakoid Fe–S centers have not been well characterized yet, except for ferredoxin, the photosynthetic Fe–S centers all seem to absorb at around 430 nm, as with other Fe–S centers.[72] Hence, the action spectrum for $^1O_2$ generation was compared with the absorption spectrum of spinach ferredoxin measured by Tagawa and Arnon.[73] The peaks at 420 nm coincide; another peak of a ferredoxin in the visible region at 463 nm seems to find the counterpart in this spectrum, a shoulder

around 460 nm. In all respects, it would not be unreasonable to suggest that thylakoid Fe-S centers, acting as endogenous photodynamic sensitizers, play an important role in the generation of $^1O_2$ from thylakoid membranes.

3. Correlation between the content of nonheme iron in thylakoids and susceptibility of chloroplasts to photoinhibition

If thylakoid Fe-S centers are indeed the photosensitizing chromophores producing $^1O_2$, it would be rather natural to expect that the larger the content of nonheme iron of thylakoids, the more vulnerable the photosynthetic apparatus are to the detrimental effects of light; for $^1O_2$ is such a reactive species that it can readily react with a variety of biological molecules, causing their structural and functional impairment. Attempting to scrutinize this point, we prepared a set of thylakoid samples, whose nonheme iron content ranges from 27 to 45 nmol per mg chlorophyll, from mung bean leaves grown in the presence of iron at varied concentrations in nutrient solutions, and then measured the relative efficiency of the photoproduction of $^1O_2$ as well as the photoinactivation rates of the electron transport system; also measured were the net photosynthetic rates of attached leaves.[20] As it turned out (Table 1), regardless of their nonheme iron content of thylakoids, chloroplasts retain the photosynthetic activity uninhibited in the leaves, if grown under low light conditions. However, a brief exposure of the low light-adapted leaves to bright sunlight disclosed that there are substantial differences among them in the intrinsic sensitivity of chloroplasts to high light intensities, the susceptibility to photoinhibition clearly being accompanied by an increase in the nonheme iron content of thylakoids (Fig. 5). In line with this observation, the leaves did not show any significant symptom identifiable as an iron toxicity disorder even though they accumulated considerable amounts of iron in the presence of high Fe^{2+} concentrations, when they were grown in room light at a fluence rate far lower than that for proper growth. Although some changes in gross morphology and a decrease to some extent in growth activity, as compared with mung bean grown in room light with an adequate supply of iron, were observed in those supplied with Fe^{2+} at concentrations as high as 1.0 mM, these were apparently discerned from the toxicity symptoms produced in the plants grown in sunlight with an excess iron supply during the growth period. These results probably suggest that the iron accumulation in leaves *per se*, at least to a certain extent, is not the immediate cause of the occurrence of iron overload-induced pathological phenomena in leaves that have frequently been observed with a variety of crop plants,[74,75] such as rice,

sugar cane, navy bean, pea and tobacco.

Table 1. Accumulation of iron in thylakoids and its effect on photosynthetic electron transport (PET) activity and net photosynthesis of mung bean leaves grown for 14 days in room light

Excess Fe^{2+} (mM)	Fe in Thylakoids			PET activity			Net photosythesis		
	Total	NHI	Fe-S		sun	Rel.		sun	Rel.
0	37	27	23	121.6	98.5	19	4.59	4.02	12
0.1	42	31	27	122.1	91.3	25	4.20	3.52	16
0.2	45	33	29	122.0	88.7	27	4.16	3.32	20
0.5	47	38	32	122.6	85.1	31	4.09	3.14	23

*Prior to the isolation of chloroplasts, the plant were subjected to a 3 h exposure to sunlight. Data are averages of triplicate measurements. From Kim and Jung.[20]
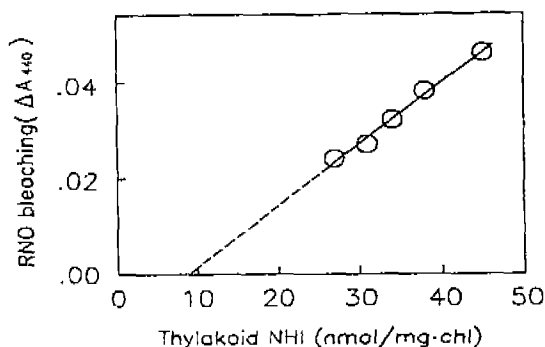

Fig. 5. Dependence of the bleaching of RNO (ΔA_{440}) on NHI content of thylakoids. Data are averages of triplicate measurements. Samples containing thylakoids (10 μg Chl/mL) from mung bean leaves grown in room light, imidazole (8 mM) and RNO (10 μM) in the suspension medium were subjected to photoinhibitory treatments (500 W/m^2) for 10 min at 15°C under aerobic conditions. From Kim and Jung.[20]

Table 2. Effects of overloaded Cu^{2+} and Zn^{2+} on PET activity of isolated chloroplasts from 14 days old mung bean leaves grown in room light

Excess supply level(mM)	PET activity (μmol O_2/mg Chl · h)		
	Control (A)	sunlight (B)*	B/A (%)
None	121.6	98.5	81
Cu^{2+} 0.1	92.7	75.0	81
0.5	81.0	65.2	80
Zn^{2+} 0.1	100.8	81.1	80

*Prior to isolation of chloroplasts, the plants were subjected to a 3 h exposure to sunlight. Data are averages of triplicate measurements. From Kim and Jung.[20]

From a comparative standpoint, chloroplasts from mung bean grown in excess Cu^{2+} or Zn^{2+} at different concentrations under low light conditions were used for measurements of thylakoid electron transport. Unlike iron overload, copper and zinc overloads remarkably affected the electron transport activity of chloroplasts adapted to low light while present in leaves but did not bring about any significant change in the sensitivity of the electron carrier system to light. Note that all chloroplasts equally lost *ca.* 20% of their PET activity during a 3 h exposure of the plants to sunlight (Table 2).

4. The exceptional vulnerability of PS II to $^1O_2$-mediated inactivation

Once $^1O_2$ is produced in thylakoid membranes upon exposure to bright light, the activated oxygen species is likely to diffuse out of its formation sites, attacking a variety of functional target which may include at least a component of the PS II complex. Therefore, if this PS II component happens to be very sensitive to $^1O_2$, not effectively protected by the intrinsic $^1O_2$ quenching systems, and critically important in operating the electron transporting function of thylakoids, it can be readily susceptible to photooxidation, immediately leading to the inhibition of PS II activity as well as whole chain electron transport. In order to provide evidence supporting this conjecture,[21] the partial and whole chain electron transfer activities

Table 3. The partial and whole chain PET activity of spinach thylakoids subjected to different treatments

PET	Control		Rose bengal-incorporated	
	dark	light	dark	light
Whole chain ($H_2O \rightarrow MV$)	113.6	86.2	119.7	44.5
PS I ($DCPIP \rightarrow MV$)	171.9	167.8	180.2	178.1
PS II ($H_2O \rightarrow DCPIP$)	68.3	52.4	70.9	24.5
PS II ($DPC \rightarrow DCPIP$)	72.9	54.1	67.3	24.8

Values, in units of μmole O_2/mg chlh for PS I and whole chain activities, and μmole DCPIP/ mg Chlh for PS II activity, are averages of triplicate measurements. Thylakoid samples were irradiated with white light ($\lambda \rangle$ 320 nm and light fluence rate=500 W/m^2) for 10 min at 20℃ and assayed for the electron transport activity at the same temperature. Data are averages of duplicate measurements. From Kim *et al.*[21]

were measured by using spinach thylakoid samples illuminated in the presence and absence of rose bengal, an efficient type II photosensitizer.[66] As it turned out (Table 3), while irradiation of thylakoids resulted in an impairment of photosynthetic electron transport and this impairment was totally ascribed to the inhibition of PS II activity, in agreement with the general observation, the presence of the exogenous sensitizer in thylakoid samples brought about a further drastic decrease in the rate of the electron transport through the PS II complex, which appears fully responsible for an increase in the extent of the inhibition of whole chain activity; but PS I activity remained almost intact during the period of such photoinhibitory treatments. Because the sensitizer molecule *per se* did not affect thylakoid electron transport but exerted toxic effects on it only in light, these results can be an implication that photoproduction of $^1O_2$ is closely associated with the initiation of photoinhibition which occurs in the PS II complex. Furthermore, the use of diphenyl carbazide (DPC), an artificial electron donor to PS II, enabled us to demonstrate that certain component(s) independent of the water-oxidizing function of the PS II complex are predominantly deteriorated by strong illumination. The involvement of $^1O_2$ in photoinactivation processes of PS II was further substantiated[22] by the obvious effects of histidine, an $^1O_2$ scavenger, and D_2O, which extends the lifetime of $^1O_2$, in thylakoids isolated from green pea leaves: histidine protected PS II from photoinactivation to a significant extent and the medium deuteration resulted in enhancing the extent of PS II inactivation by light (Table 4).

Table 4. Effects of histidine and D_2O on PS II activity of thylakoids from green pea leaves grown in room light, subjected to strong irradation

Treatment	PET activity	
	Electron transfer rate	Relative inactivation(%)
Dark control	83.0 ± 4.9	0
	45.6 ± 3.0	
Light/histidine*	62.2 ± 3.7	25
	24.9 ± 2.1	

* Thylakoids were preincubated with histidine (10 mM) for 30 min at 20℃ before irradiation. † Thylakoids were suspended in the suspension medium containg D_2O by 50%. Samples were irradiated with bright white light ($\lambda > 320$ nm: 700 W/m^2) for 10 min at 20℃ and then immediately assayed for the electron transfer from H_2O to DCPIP through PS II at the same temperature. Data are given as mean ±SE (n=3). Values in parentheses are the percent of the rate of dark control. From Chung and Jung.[22]

As discussed before, thylakoid Fe-S centers are likely the most important endogenous photosensitizers, in so far as type II processes of photosensitized reactions proceeding predominently in the PS II complex with relation to photoinhibition are concerned. This concept might be inconsistent with our knowledge on the distribution of Fe-S centers among the electron transport complexes in thylakoids; for PS I, involving three Fe-S centers, denoted as F_A, F_B and F_X, would be exposed to higher local concentrations of $^1O_2$ generated by its own chromophores than PS II which does not contain Fe-S centers. Based on this inference, one could be skeptical about the proposition that $^1O_2$ photogenerated from the Fe-S centers is the reactive intermediate responsible for initiating the primary processes of photoinhibition damage to PS II. This skepticism would have a rationale, only if the two photosystems possessed comparable sensitivities to $^1O_2$. However, the exceptional vulnerability of PS II to photosensitized inactivation by rose bengal clearly demonstrates that this complex is far more sensitive to $^1O_2$ than PS I, likely resolving such skepticism. This fact may lead one to infer that the activated oxygen species mediating photoinhibition processes is not necessarily produced in PS II but rather generated from other components of thylakoid membranes, such as cyt b_6/f complex and PS I, and diffuses into PS II. In fact, the concentration of $^1O_2$ is not the only factor which determines the rates of reactions between $^1O_2$ and substrates; instead, the rates may be more dependent on the intrinsic reactivities of the substrate molecules towards $^1O_2$. Any complex of photosynthetic electron transport system can therefore be the primary target for $^1O_2$-mediated oxidative damage regardless of local concentrations of $^1O_2$, if this activated oxygen species is overproduced in thylakoid membranes under certain conditions. Furthermore, it should be taken into account that $^1O_2$ has a much extended lifetime in an apolar environment as in the aprotic interior of membranes and hence it can reach the target molecules even located at a distance within its lifetime.

5. The initial, specific site of O_2-mediated damage within PS II

Despite different views as to the initial, specific site of photodamage within the PS II complex, it seems apparent that certain chemical events closely associated with the Q_B binding site are involved in the initiation of photodamage to PS II. It is noteworthy that the primary structure of the D_1 protein carries two unique sequences containing His-Met pairs,[76] which are found only in other proteins interacting with quinones. Furthermore, Wolber and Steinback[77] were able to

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covalently bind azidoatrazine, a herbicide which competes with Q_B for the binding site, to the D_1 protein, confirming that the herbicide was linked to Met-214 of one of the His-Met regions: these facts indicate that the active site of the D_1 protein involves at least one of these pairs. The involvement of histidine and methionine residues in the Q_B binding site is quite interesting in respect that, as is well known, these amino acid residues are readily vulnerable to $^1O_2$-mediated oxidation.[78]
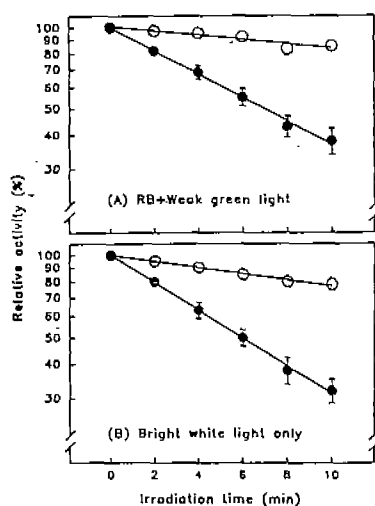

Fig. 6. Inactivation of the Q_B-dependent and the Q_B-independent electron flow through PS II. (A): pea thylakoid suspensions (50 μg Chl/mL) were preincubated with 0.2 mM rose bengal (RB) for 10 min and irradiated with weak green light (400-500 nm; 80 W/m^2). (B): the thylakoid suspensions were irradiated with strong white light (λ > 320 nm; 1200 W/m^2) for 10 min in the absence of the exogenous photosensitizer. After the respective irradiations, the samples were assayed both for the rate of electron transfer from DPC to DCPIP (Q_B-dependent activity; closed circles) and for that from DPC to SiMo (Q_B-independent activity; open circles). The rates of control were 51.7 μmol/mg Chlh and 68.8 μmol/mg Chlh at 20℃, respectively. Averaged results from three experiments are presented with error bars representing SE. From Chung and Jung.[22]

Supposing that $^1O_2$ primarily attacks the labile amino acid residues of the Q_B binding site, causing the oxidative inactivation of the D_1 protein, this point was examined by the partial electron transport activities of PS II,[22] i.e. the Q_B-dependent activity, as accessed by the rate of electron transfer from DPC to dichlorophenyl indophenol (DCPIP) which accepts electron from the secondary quinone, and the Q_B-independent activity, as determined by the rate of electron transfer from DPC to silicon molybdate (SiMo) which is believed to accept electron directly from the primary quinone. For this experiment, pea leaf thylakoids were preincubated with rose bengal and then exposed to weak green light which is in itself too weak to cause photoinhibition damage to PS II but strong enough to generate $^1O_2$ from rose bengal under aerobic conditions. The result obtained was that the Q_B-dependent electron flow was much more severely impaired by the presence of rose bengal plus green light than the Q_B-independent transfer, the inactivation rate constant of the DPC → DCPIP transfer being about 4 times as large as that of the DPC → SiMo transfer. This kinetic pattern of PS II inactivation under the conditions identified with the production of $^1O_2$ at high levels in

thylakoid membranes is neatly in parallel with the inactivation pattern observed in thylakoids subjected to photoinhibitory treatment in the absence of the exogenous sensitizer (Fig. 6), supporting the contention that the Q_B binding domain of the D_1 protein is the primary site for the initiation of photodamage within the PS II complex which is mediated by $^1O_2$ produced in thylakoid membranes under high light stress conditions.

6. D_1 turnover induced by $^1O_2$ from the outside of PS II

Many biochemical data have implicated that the oxidative modification of the active site of a protein can result in not only inactivating the protein but also making it more susceptible to proteolytic hydrolysis.[5,39,79] In this respect, it may be reasonably assumed that the oxidation of the Q_B site residues of the D_1 protein by $^1O_2$ and the ensuing conformational change can bring about a biochemical event in PS II that leads to triggering the proteolytic degradation and loss of the D_1 protein.

Because D_1 turnover is widely accepted as the pivotal step overlaped by the final stage of inactivation and the initial stage of repair during in vivo photoinhibition processes occurring in plant cells, any proposition regarding the mechanistic aspects of photoinhibition should be inevitably evidenced by experimental results which show the presumed pattern of D_1 loss in line with the proposition. In order to comply with this need, the loss of the D_1 protein from thylakoids subjected to treatments under varied conditions was measured, using thylakoids isolated from isotope-labeled green pea leaves.[22] When the labeled thylakoids, preincubated with rose bengal, were briefly illuminated with weak green light and thereby exposed to high internal concentrations of $^1O_2$, they showed a substantial loss of the D_1 protein. The quantification of D_1 loss by densitometry revealed that the rate of the loss, showing first order kinetics, is significantly lower than the inactivation rate of the Q_B-dependent electron transport activity of PS II (Fig. 7), as is the case where thylakoids are subjected to intensive photoinhibitory treatments. Also observed were that both histidine and DCMU, when incorporated into thylakoid membranes, effectively suppressed the loss of the D_1 protein, as did N_2-purging of samples, and that the deuteration of thylakoid suspensions resulted in an increase to some extent in D_1 loss

From the above results discussed thus far, it seems now clear that the inactivation, degradation and loss of the D_1 protein are all closely related to the overproduction of $^1O_2$ in thylakoid membranes under adverse circumstances. In fact, the involvement of $^1O_2$ in the light-induced degradation of the D_1 protein, caused by

lesions on the acceptor side of PS II, has been proposed,[4,38,50] in that $^1O_2$ is postulated to be produced by the energy transfer reaction between the triplet state of P_{680} ($^3P_{680}$), which is formed *via* the recombination of the primary charge pairs of PS II, and the triplet ground state of oxygen. Supporting this, the formation of a chlorophyll triplet and the decrease in its lifetime in the presence of oxygen have been observed in isolated PS II reaction centers by flash absorption studies.[80] Further, recently Macpherson et al.[81] and Hideg et al.[82] were able to directly detect $^1O_2$ from isolated reaction centers and from thylakoid membranes, respectively. The production of $^1O_2$ presumably leads to either the oxidative modification of the reaction center polypeptides[4] or the selective destruction of pigments including P_{680} chlorophylls,[37,80] resulting in inducing a conformational change of the reaction center, which in turn may trigger the proteolytic cleavage of the polypeptides, preferentially the D_1 protein.
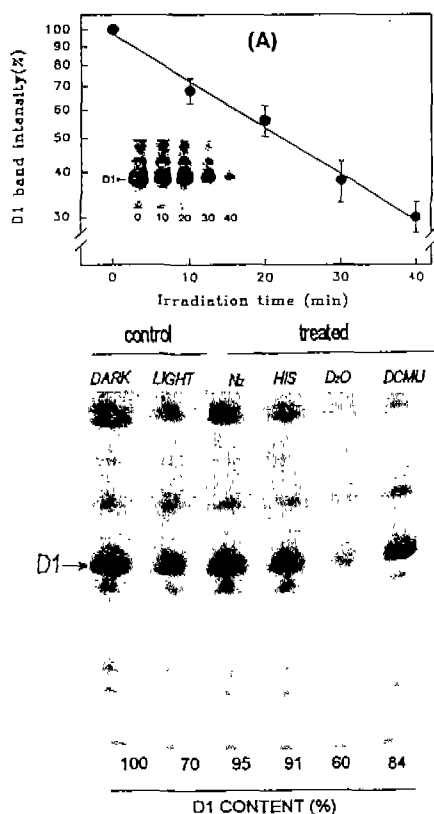

Fig. 7. D_1 loss induced by rose bengal-dependent photosensitization of pea thylakoids. For the kinetic data in (A), [^{35}S]-Met labeled thylakoid suspensions (50 µg Chl/mL), preincubated with rose bengal (0.2 mM), were irradiated with the light for 10 to 40 min. For checking the effects of various treatments of the isotope-labeled thylakoids, as in (B), the suspensions preincubated with rose bengal (0.2 mM) were either purged with N_2, admixed with histidine (10 mM) or DCMU (0.2 µM), or partially deuterated with D_2O (50%), and then exposed to the light at 20℃ for 10 min. The irradiated samples were kept at 20℃ for 15 min in the dark, pelleted, resuspended in the sample buffer, and subjected to protein separation by SDS- PAGE. D_1 band was visualized by autoradiogram. The average of three measurements was used at each point: error bars represent SE. Data for D_1 content are averages of duplicate measurements; the relative value of the spread was not greater than 15%. From Chung and Jung.[22]

However, the concept of $^1O_2$ production through the $^3P_{680}$-O_2 reaction being the primary event for the processes of the acceptor-side photoinactivation of PS II does

not appear pertinent to our results;[18,20-22] that is, the rate of photoinactivation of electron transport in thylakoid membranes under aerobic conditions increases linearly with the increased content of NHI (Table 1), which is in parallel with the NHI content dependence of the rate of $^1O_2$ photogeneration from the membranes (Fig. 5). Because, for the present, no information is available that can provide an explanation how NHI can promote either the formation of $^3P_{680}$ or the energy transfer from $^3P_{680}$ to oxygen, resulting in an increase in the rate of $^1O_2$ production within PS II, and because the quantum yields of inactivation of photosynthetic electron flow as well as D_1 degradation are significantly lower in wavelengths of the red light region of PAR, which should more efficiently energize chlorophylls, than in wavelenths of near UV to blue light (Fig. 1), it does not seem very reasonable to consider that $^1O_2$ responsible for initiating photodamage to PS II is produced mainly *via* the reaction between $^3P_{680}$ and oxygen.

7. Roles of the Fe-S centers and the Q_B-binding site in high light-induced D_1 degradation

If one can specifically destroy thylakoid Fe-S centers, which are located in cyt $b_6/$f complex and PS I but not in PS II, without affecting the function of PS II by a proper chemical treatment of samples, the treated thylakoids may be used as a convenient system that provides experimental evidence for the role of the labile sulfur-containing iron groups in the primary processes of photoinhibition of PS II. Incubation of thylakoids in the presence of low concentrations of MA appears to almost fulfill the requirements for such chemical manipulation: a substantial loss of PS I activity with the concomitant impairment only to a much lesser extent of PS II activity is regarded as being due to somewhat specific destruction of Fe-S centers by MA.

Accordingly, a marked reduction in the susceptibility of PS II to photoinhibition, as manifested by a decrease in the extent of inactivation of the electron transport activity (Table 5) as well as by a suppression of the loss of the D_1 protein (Fig. 8) in thylakoids subjected to the mild MA treatment, further corroborates the concept that $^1O_2$ photoproduced from the outside of PS II by Fe-S centers diffuses into the PS II complex and primarily attacks the D_1 protein, resulting in an oxidative modification of this reaction center subunit which may generate a sort of biochemical signal triggering the proteolytic degradation of the protein. The effect of histidine in protecting the D_1 protein from high light-induced degradation of the D_1 protein is also well in accordance with this concept; for it seems hard to expect

that histidine molecules incorporated into thylakoid membranes have access to the PS II core and scavenge $^1O_2$ assumably formed by the reaction between $^3P_{680}$ and molecular oxygen.

Table 5. PS I and PS II activities of the MA-treated thylakoids.

Electron transport		Control	MA treated[*]
PS I activity	Nonirradiated	120.2 ± 6.3	40.7 ± 3.6
(DCPIP → MV)	Irradiated	119.0 ± 5.5	39.8 ± 4.1
	Relative	0.9%	2.2%
PS II activity	Nonirradiated	83.0 ± 4.9	74.5 ± 5.0
(H$_2$O → DCPIP)	Irradiated	21.8 ± 2.4	43.6 ± 3.9
	Relative	74.0%	41.0%

[*] Thylakoid suspensions were diluted into 10 vol of 0.5 mM MA in the suspension medium, stored for 5 h at 4°C in the dark, and dialyzed against the same medium. Values are in units of μmol O$_2$/mg Chl · h for PS I activity and μmol DCPIP/mg Chl · h for PS II activity. Irradiation and assay conditions were same with those of Table 1 except that irraditation time was 20 min. Data are presented as mean ± SE (n=3).[22]
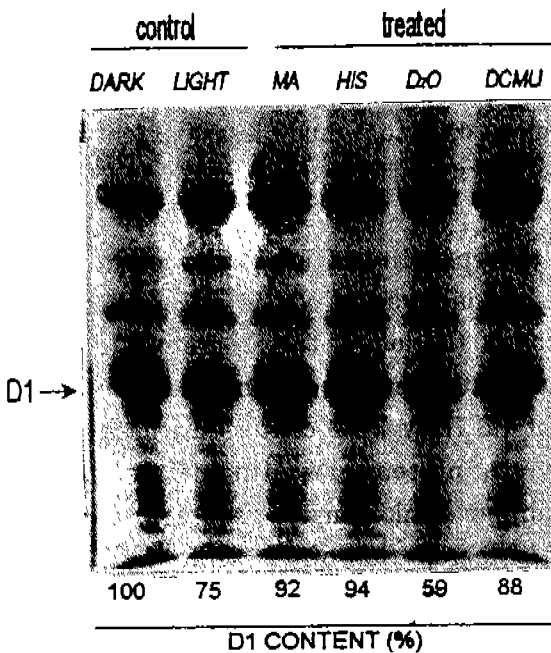

Fig. 8. Effects of various treatments of [^{35}S]-Met labeled thylakoid suspensions on D$_1$ loss induced by high light intensities in the absence of an exogenous photosensitizer. Thylakoid suspensions were either subjected to the MA (0.5 mM) treatment, admixed with histidine (10 mM) or DCMU (0.2 μM), or partially deuterated with D$_2$O (50%), and then exposed to bright white light (λ · 320 nm; 700 W/m^2) for 20 min at 20°C. As to the separation procedure of thylakoid proteins, refer to the legend for Fig. 7. Data for D$_1$ content are averages of duplicate measurements; the relative value of the spread was not greater than 11%. From Chung and Jung.[22]

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It has long been observed[2,42] that some herbicides such as DCMU and azidoatrazine, which are known to displace a liganded plastoquinone (Q_B) from the D_1 protein, protect the D_1 protein against degradation even in severe light-stress situations. It is not yet understood how these herbicides, when bound to PS II, retard the process of photoinduced D1 degradation. Considering that the Q_B-binding site, which is believed to be the domain for the herbicide binding, involves histidine and methionine residues[77] and is presumed to be the specific initial site for photoinhibition damage within the PS II complex, however, an explanation for the protection effect of the herbicide binding can now be derived from the results shown in Figs 7 and 8 taken together: that is, the herbicide DCMU effectively limits the accessibility of $^1O_2$ to the active oxygen-sensitive residues at the Q_B site of the D_1 protein.

IV. Concluding Remarks

There are several common features in the pattern of photoinhibition damage observed with systems of undisturbed thylakoid membranes ranging from isolated intact thylakoids to cells. Citing a few, (i) among the electron transporting components of thylakoids only PS II has a substantial susceptibility to photoinactivation, (ii) the Q_B-dependent electron transfer activity of PS II is always lost prior to the Q_B-independent activity, (iii) the loss of PS II electron transport is accompanied by the loss of the D_1 protein from the membrane, but the rate of former being significantly higher than that of the latter, and (iv) some herbicides such as DCMU and azidoatrazine protect the D_1 protein from photoinduced degradation and removal.

Our results show that the pattern of a damage to thylakoids incurred by the condition identified with the production of $^1O_2$ in membranes at presumably high levels carries all these features. Further, the effects of the presence of a $^1O_2$ scavenger (histidine) and the lifetime extender of $^1O_2$ (D_2O) on PS II damage caused by high concentrations of the intramembrane $^1O_2$ are obviously reproduced in thylakoids exposed to high light intensities. Based on these observations, it appears evident that the events associated with the early phases of photoinhibition damage to thylakoid membranes under aerobic conditions are much the same with the early stage-events occurring in thylakoids upon exposure to high levels of $^1O_2$ production. Particularly worthy of note is that the PS II complex in thylakoids whose Fe-S centers are destroyed by a chemical manipulation shows a markedly reduced

susceptibility to photoinhibition, as determined by the electron transport activity as well as by the extent of D_1 degradation.

Taking the results presented in this paper all together, it may be concluded (1) that, in isolated intact thylakoids and chloroplasts, and also probably in leaves under aerobic conditions, photoinhibition damage to PS II occurs primarily in the acceptor side, the Q_B-binding site most likely being the specific site for initiating PS II damage, (2) that the primary processes of the damage are mainly mediated by $^1O_2$, (3) that this active oxygen is largely produced by the electronically excited Fe-S centers of thylakoids and diffuses into the PS II complex, attacking the functional targets there including the D_1 protein of course, and (4) that the structural and functional modification of the D_1 protein by $^1O_2$ assumably makes it readily vulnerable to the proteolytic degradation through certain biochemical processes which are not yet elucidated.

V. Acknowledgements

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