Journal of Photoscience (2000), Vol. 7(3), pp. 87 – 95

Photodynamic Action by Endogenous Non-Chlorophyll Sensitizer As a Cause of Photoinhibition

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As sunlight is not always optimized for every terrestrial plant in terms of light quality, quantity and duration, some plants suffer detrimental effects of sunlight exposure under certain conditions. Photoinhibition of photosynthesis is a typical phenomenon representing harmful light effects, commonly observed in many photosynthetic organisms. It is generally accepted that functional, structural loss of photosystem II complex (PS II) is the primary event of photoinhibition. Accumulating data also suggest that singlet oxygen ($^1O_2$) is the main toxic species directly involved in it. There are two different views on the specific site and mechanism of $^1O_2$ production in the photosynthetic membrane. One of them favors the PS II reaction center, where the primary charge pairs recombination occurs as a prerequisite for the generation of $^1O_2$, and the other inclines to photosensitized $^1O_2$ formation by a substance located outside PS II. This article describes how we, as the advocators of the latter concept, have arrived at the conclusion that $^1O_2$ immediately involved in PS II photodamage is largely generated from the Rieske center of the cytochrome b_6/f complex and diffuses into PS II, attacking the reaction center subunits

key words: Photoinhibition, Photosystem II, photosensitization, Rieske center, Singlet oxygen

Introduction

Photon taken up by photosynthetic cells is efficiently converted into chemical energy that is subsequently transduced into all forms of biological energy through a variety of cellular processes. Light is therefore an absolute prerequisite for autotrophic growth of photosynthetic organisms. Nevertheless, light is not always beneficial to plants but potentially harmful under certain conditions, as is frequently observed, for instance, in low light-adapted plants upon exposure to bright sunlight. The detrimental effects of sunlight in organisms are largely blamed on shorter wavelength UV radiation, i.e. UV-B and UV-C. Yet, there is abundant evidence that high light-induced loss of cellular functions and structures has also been associated with the interaction between organisms and longer wavelength radiation involving UV-A and visible light.

In plant cells, deleterious light effect is most evident in chloroplasts, as manifested by a sharp decrease in the capacity of photosynthesis, which is generally termed as photoinhibiton of photosynthesis. Photoinhibition is an extremely complex phenomenon, comprising various phases, some of which overlap and thus difficult to resolve. Numbers of hypotheses and schemes have naturally been proposed to describe the underlying mechanisms, reflecting many different observations in detail [1-3 and Refs. therein]. Despite the diversity of view as to which process predominantly takes place in the photosynthetic apparatus leading to photoinhibition, the only consensus is that the photosystem II complex (PS II) is the primary target of photoinhibition damage to chloroplasts. Various experimental data suggest that photoinhibition is caused by changes on either the donor side or the acceptor side of PS II [3-5] and that some subunits of PS II reaction center complex (RC II) suffer degradation under photo-inhibitory conditions [1, 3, 6].

A copious body of evidence indicates that singlet oxygen ($^1O_2$), a very reactive oxygen species, is a key intermediate initiating or mediating photodamage to PS II [7-13]. Due to its high electrophilic nature, $^1O_2$ can readily attack proteins with some amino acid residues possessing electron-rich functional groups. In plant cells, the conditions that favor photoformation of $^1O_2$ are found in the thylakoid that is the site of high oxygen concentration and abundant in chlorophyll, a putative photosensitizing agent. Therefore, it is very likely that $^1O_2$ involved in photoinhibition originates in the photosynthetic membrane. However, one may note from the literature that there are two different views on the major site and mechanism of $^1O_2$ formation, which have yet to be reconciled. A view is that $^1O_2$ generates from the reaction center chlorophyll (P_{680}) in PS II itself, which might be intuitive because PS II is the primary site of photoinhibi-

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Received 30 July 2000; accepted 30 August 2000

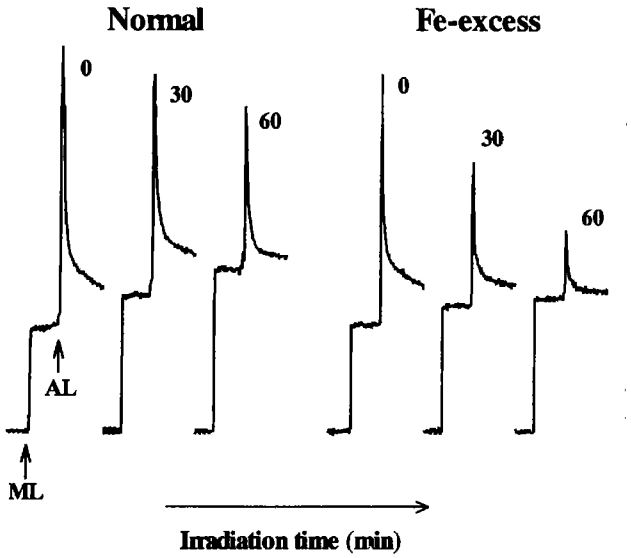

Figure 1. Fluorescence induction of pea leaf chlorophyll. The leaves supplied with excessive iron during growth show higher rate of decrease in photochemical efficiency (Fv/Fm) under high irradiance conditions (800 W/m^2), as compared with those grown with an adequate iron supply

tion. Conforming to this, $^1O_2$ production has been detected in PS II samples, such as BBY-type PS II and PS II core complex preparations, under photoinhibitory conditions [10, 11, 13-15]. The postulation is that $^1O_2$ is formed by the reaction of molecular oxygen with the triplet state P680 which arises when the acceptor side fails to properly function [3, 13, 16]. Another view is that $^1O_2$ is largely generated from the outside of PS II by certain Fe-S clusters and diffuses into the PS II core, which appears supported by a number of evidence provided from this laboratory [7, 9, 12, 17, 18].

In this article, it has by no means been intended to write a comprehensive review on various aspects of photoinhibition of photosynthesis that have been dealt in a vast number of papers in the last two decades. It will rather be focused on how we have approached to photoinhibition problems at the molecular levels, showing relevant results obtained, and what the results implicate with respect to a novel mechanistic aspect of photoinhibition in plant cells.

PS II DAMAGE AS A DETERMINANT OF PHOTOINHIBITION

Evidence for PS II as the primary target of photoinhibition has been provided by varieties of biophysical and biochemical measurements. Let us start from chlorophyll fluorescence. Since the fluorescence quantum yield of chlorophyll in isolated chloroplasts, protoplasts, or intact leaves is dependent on the microenvironment in the thylakoid, it can be used to probe the organizational, functional states of the membrane [19-21]. Although the fluorescence emission aris-

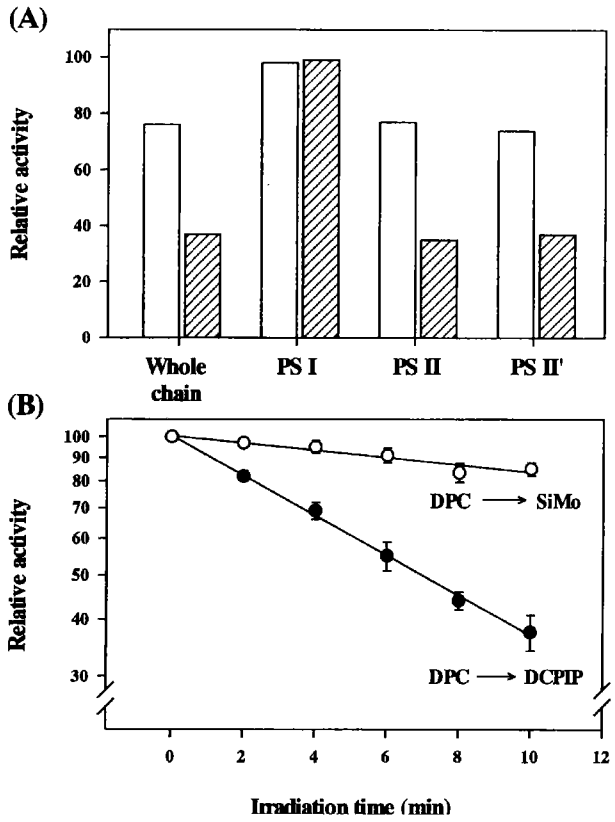

Figure 2. Photosynthetic electron transport activity of isolated thylakoids subjected to irradiation (500 W/m^2). Panel A shows the effects of light alone (open bars) and light plus rose bengal (slashed bars) on whole chain activity, PS I activity, and the oxygen evolving complex-independent PS II (PS II′) activity. Panel B shows the effect of light plus rose bengal on the QB-dependent (DPC R DCPIP) and -independent (DPC R SiMo) electron transfer through PS II.

es largely from chlorophylls of PS II, the fluorescence emitted from PS I can also be readily quantified at liquid nitrogen temperature. Analysis of the fluorescence emitted from PS I and PS II has revealed that the pigment bed of PS II is markedly disrupted by photoinhibitory treatment while only a slight perturbation occurs in the pigment bed of PS I. However, the fluorescence induction that is dominated by a change in the state of PS II is more informative than the steady state fluorescence with regard to photochemical efficiency of the photosynthetic apparatus. For instance, the ratio of the variable fluorescence (Fv) to the maximum fluorescence (Fm) is proportional to the quantum yield of PS II photochemistry, i.e. water splitting and plastoquinone reduction. A decrease in Fv/Fm is symptomatic of photoinhibitory stress in plants, indicating a loss of the photochemical efficiency.

Kinetic study on photosynthetic electron transport provides a powerful tool to determine the photoinhibition target in the thylakoid. Measuring the rates of whole chain (PS II R PS I) electron transport, partial transport through PS II and partial transfer through PS I, respectively, has produced results

[1, 22-25] that conform to what can be inferred from the fluorescence data. That is, the electron transport activity of PS II in strongly irradiated thylakoids, chloroplasts and leaves is markedly reduced while the activity of PS I remains almost uninhibited, indicating that only PS II is damaged rather specifically. PS II electron transport activity can be further fractionated by various techniques; one of these is the use of artificial electron donors and acceptors targeting specific carrier components within the complex. Kinetically analyzing PSII-catalyzed redox reactions of several artificial electron donor-acceptor pairs has led to a conclusion that the Q_B binding site on the D1 protein is most likely the initial, specific site of photodamage within the PS II complex [1, 12, 26].

There is abundant evidence that among chloroplast proteins the D1 protein of RC II is predominantly degraded *in vivo* as well as *in vitro* upon exposure to bright light [1, 27], although the D2 protein is also known to be susceptible to photodegradation [28]. The degradation of the D1 protein may therefore be regarded as the 'real' phtoinhibition and the earlier events leading to D1 degradation would be reversible processes that disappear immediately when over-energized state of PS II is dissipated upon the cessation of irradiation. Many kinetic data obtained with both *in vivo* and *in vitro* systems indicate, however, that photoinactivation of PS II electron transport does not appear readily reversible and always precedes D1 degradation, implying that functional loss of PS II *per se* prior to the structural loss is indeed photoinhibition. In this respect, the degradation of RC II subunits could be the initial step of the repair process of the already-impaired PS II complex. Circumstantially conforming to such notion, rapid turnover and synthesis of the D1 protein have often been observed whenever the degree of photoinhibition is high [2]. Whatever physiological implication of D1 degradation is, assay of D1 degradation can be a reliable method for quantitative assess of PS II damage if experiments are carefully designed.

PUTATIVE MEDIATOR OF PHOTOINHIBITION PROCESS

According to information available from the literature, it seems that there are two classes of reactive chemical species associated with the early stage events of photoinhibition, which have been either implicated by experimental results or simply hypothesized. One involves the radicals of some redox intermediates of water-plastoquinone oxido-reductive reac-

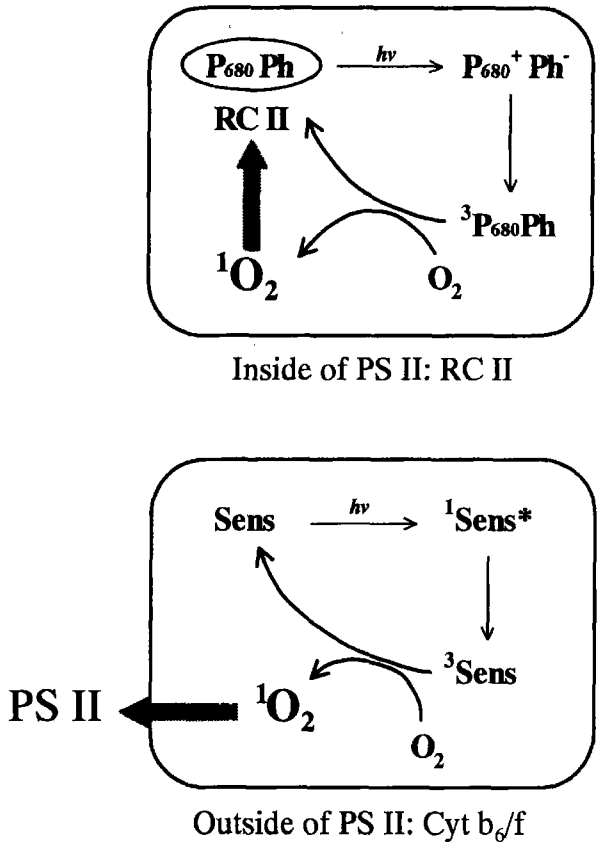

Figure 3. Possible sites and mechanisms of $^1O_2$ production in the thylakoid membrane associated with photoinhibition damage to PS II.

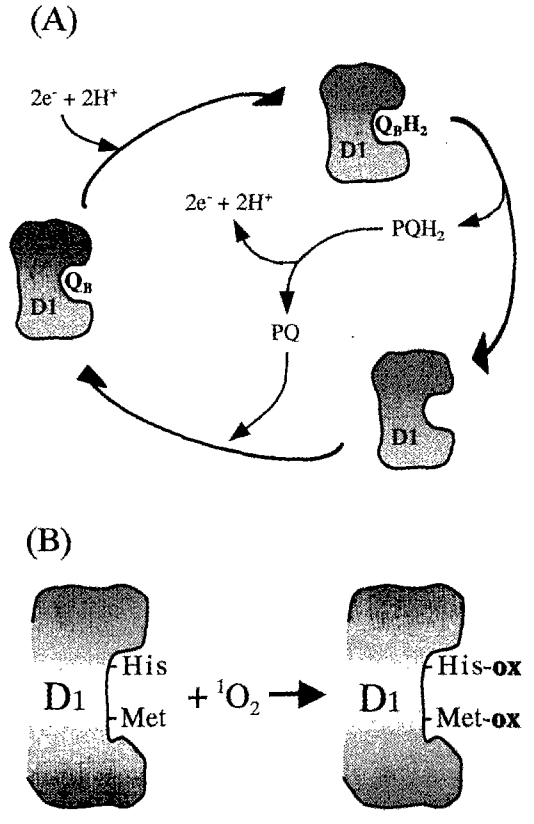

Figure 4. The role of the D1 protein in the redox-cycling plastoquinone (A) and the existence of $^1O_2$- sensitive residues, histidine and methionine, at the QB site (B)

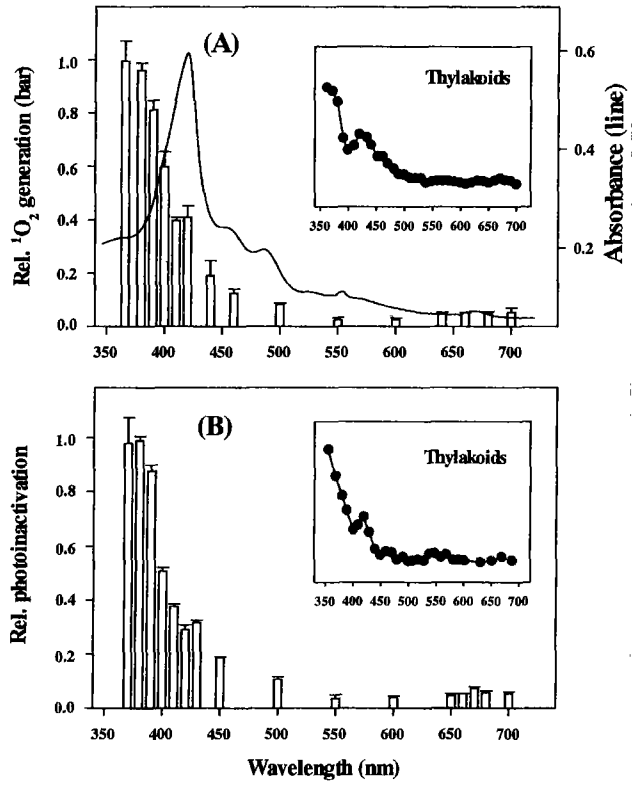

Figure 5. Action spectra for the formation of $^1O_2$ (A) and the inactivation of PS II core complex (B) by Cyt b_6/f. Action spectra measured with thylakoids are shown in insets for comparison. Absorption spectrum of isolated Cyt b_6/f is also shown in panel A.

tions, such as Yz^+, P_{680}^+ and Q_B^- [1, 6, 29]. The other comprises activated oxygen species formed by the excited pigments in the presence of molecular oxygen or as side-products of redox reactions in thylakoid membranes [1, 30, 31]. Nevertheless, there can now be little doubt that $^1O_2$ is the principal mediator of PSII photodamage although the possibility of other potentially reactive species involved in photoinhibition process may not be ruled out. $^1O_2$ is produced from various sources. In biological systems the major mechanism of formation is by energy transfer from triplet excited sensitizers to molecular oxygen at its ground triplet state [30, 32]. Triplet state of a molecule is formed usually via intersystem crossing from singlet excited state. In the case of the reaction center chlorophyll P_{680}, however, its triplet state is presumed to be formed by recombination of the primary charge pairs, $P_{680}^+ + Pheo^-$ R $^3P_{680} + Pheo$ [3, 13, 33] when the charge propagation is somehow blocked. Then, the transfer of excitation energy from long-lived $^3P_{680}$ to molecular oxygen can take place, producing $^1O_2$. This mechanism may be plausible because, under photoinhibitory conditions, nonphysiological over-reduction of the first quinone acceptor (Q_A) occurs which accompanies an increase in formation of the chlorophyll triplet [16].

The term 'photoinhibition' was originally defined by

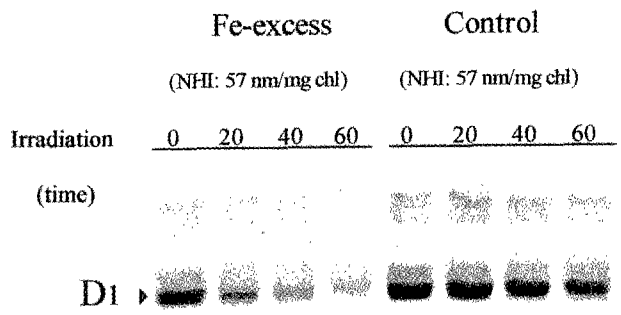

Figure 6. Degradation of the D1 protein in thylakoids irradiated at 500 W/m^2. SDS-PAGE reveals that thylakoids with a high content of NHI are more susceptible to D1 degradation than those with a lower NHI content.

Osmond [34] about two decades ago as a phenomenon resulting from overexcitation of light harvesting pigment assemblies in excess of the potential of photosynthetic electron transport system to orderly dissipate the excitation energy. Since then, numbers of molecular mechanisms have been proposed on the basis of the Osmond's definition. In fact, the concept of the primary charge recombination and the resulting $^1O_2$ formation with respect to photoinhibition was also formulated to explain how overexcitation of the pigment assemblies can lead to the damage-initiating process in PS II. If such elaborate mechanism of $^1O_2$ production holds true, the quantum efficiency for $^1O_2$-mediated PS II photodamage should be higher in the region of photosynthetically active radiation (PAR) than in the rather short wavelength region of the sunlight spectrum. This is because excitation or overexcitation of the pigment assemblies is achieved most efficiently by absorbing PAR. However, such is likely not the case, as has been indicated by the action spectra for inactivation of photosynthetic electron transport in isolated chloroplasts [7] and for D1 degradation in *Spirodela* plants [35]. Both spectra clearly demonstrate that photoinhibition is induced much more efficiently by UV to blue (250 - 450 nm) light than by photosynthetically more active red (600 - 700 nm) light. Then, there must be some photosensitizing system(s) involved in PSII damage other than the reaction center chlorophyll (P_{680}) and/or light harvesting chlorophyll.

HIGH SENSITIVITY OF THE D1 PROTEIN TO $^1O_2$ ATTACK

The primary structure of the D1 protein carries two unique sequences containing His-Met pairs, which are found only in other proteins interacting with quinones [36]. Cross-linking experiment has revealed that a herbicide azidoatrazine, which competes with plastoquinone for the secondary quinone (Q_B) binding site, is linked to methionine-214 of one of the

His-Met pairs [37], indicating that the Q_B site on the D1 protein involves at least one of these pairs. The existence of histidine and methionine residues at this active site is suggestive of a high susceptibility of the D1 protein to oxidative modification by $^1O_2$; for these residues are well known to readily react with $^1O_2$ [32]. The loss of PS II activity as the initial event of photoinhibition may thus be thought to result from the $^1O_2$-mediated Q_B site modification that, in turn, triggers degradation of the D1 protein itself in some manner. Supporting this concept, a PS II-directed herbicide DCMU, which binds to the Q_B site and can thus limit the accessibility of $^1O_2$ to the possible target, effectively suppresses D1 degradation under photoinhibitory conditions [12]. However, some workers regards P_{680} as the main target of $^1O_2$ attack [13, 38].

Biochemical nature of D1 degradation has yet to be fully elucidated. It is not very conceivable that $^1O_2$ or other reactive species formed in irradiated thylakoids can cause a direct cleavage of peptide bonds in the D1 protein, fragmenting the whole protein into so many polypeptides and even amino acids. Such photochemical cleavage, if any, could be responsible only for the initial breakdown of the protein, even though there is no evidence supporting this notion. For the present, abundant data speak in favor of proteolytic breakdown. In line with this concept, D1 degradation is significantly mitigated by the presence of protease inhibitors [39-41]; furthermore, the degradation occurs in a complete darkness at room temperature once the samples have been subjected to photoinhibitory treatment in the cold [42]. Then, the relevant question is how the chemical modification turns the D1 protein into a substrate for preteolysis. The most popular model at present is that a conformational change of the modified protein brings it into contact with neighboring proteolytic site [4, 33].

There is evidence that the protease involved, at least, in the initial step of D1 degradation is an integral part of PSII itself, functioning as a serine-type enzyme [40, 43, 44] which suffers irreversible inhibition by diisopropyl fluorophosphate (DPF), a serine-targeting covalent inhibitor. Therefore, isotope-labeled DPF has been used to identify a PS II polypeptide possessing protease activity, producing results that shows the possibility of CP43, a light-harvesting chlorophyll protein directly attached to the D1/D2 duplex in RC II, to have a preoteolytic activity [33]. A contradictory result to this is, however, that D1 degradation occurs also in isolated reaction center particles consisting only of D1, D2, cytochrome b_{559} and the *psbI* gene product when exposed to bright light, indicating that the degradation is autoproteolytic rather than CP43-catalyzed [40, 45]. Although the differing views on the PS II component catalyzing D1 cleavage remain to be reconciled, this may be resolved by accepting that more than one polypeptides of PS II have proteinase activity.

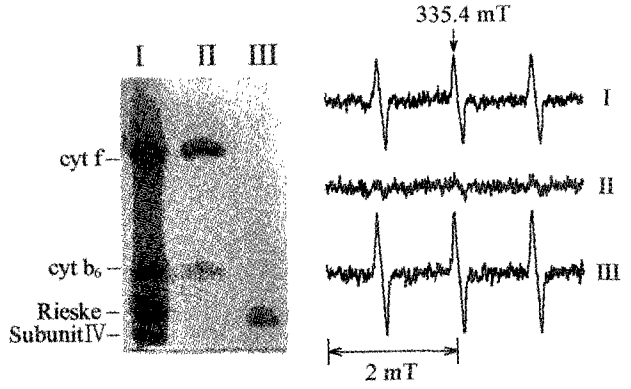

Figure 7. ESR spectra of TMPD-N-oxyl radical formed by the reaction of TMPD with $^1O_2$ generated from Cyt b_6/f (I), the Rieske-depleted complex (II) and the Rieske protein (III).

NON-CHLOROPHYLL PHOTOSENSITIZER IN THYLAKOIDS

Action spectra for certain biological processes induced by light have often provided a clue to the chromophores immediately responsible for the light-driven processes. We therefore measured several action spectra related to photoinhibition in higher plants, such as those for $^1O_2$ generation from thylakoid membrane, for D1 degradation in thylakoids, and for inactivation of whole chain electron transport of chloroplasts [7, 46]. As it turned out, while all the action spectra show a remarkable resemblance to each other, they bear only a little, if any, spectroscopic characteristics of chlorophyll, which may allow one to exclude the possibility of any chlorophyll in PS II as the major photosensitizing agent producing $^1O_2$. Besides the main photosynthetic pigments, there exist several types of chromophores in thylakoids, such as hemes and Fe-S clusters that absorb UV/visible light but do not function as photon collectors. Because of low concentrations and low extinction coefficients, the contribution of these minor pigments to the total absorption of the thylakoid is so small that their detection *in situ* by molecular spectroscopic techniques is extremely difficult. Nevertheless, their absorption properties can be deduced from the spectra of several cytochromes and Fe-S proteins isolated from other sources. Interestingly enough, those action spectra related to photoinhibition appeared similar to the deduced absorption spectra of Fe-S clusters in terms of general shape and the peak position, which tempted us to assume that thylakoid Fe-S clusters act as the major photosensitizer generating $^1O_2$ in the photosynthetic apparatus. Conforming to this assumption, mersalyl acid treatment of thylakoids resulted in a drastic decrease in $^1O_2$ production as well as in D1 degradation: note that mersalyl acid destroys rather specifically Fe-S clusters [47].

If the Fe-S centers are indeed photosensitizing chromophores in thylakoid membranes, it would rather be natural to expect that the larger the content of the nonheme iron moieties, the more vulnerable the chloroplast is to harmful effect of high light. To scrutinize this point, a set of thylakoid samples with different Fe-S content were prepared from mung bean and pea leaves grown in the presence of soluble iron at varied concentrations. We then measured the rates of $^1O_2$ production, electron transport inactivation, and D1 degradation in thylakoids under high irradiance conditions [46, 48]. Every parameter determined clearly indicated, as it turned out, that the susceptibility of the thylakoid to photoinhibition increases with increasing content of its Fe-S clusters. In consistent with this, *In vivo* photoinhibition also is exacerbated as the Fe-S content of thylakoids increases, as has been assessed by leaf fluorescence characteristics [46] as well as by CO2 -uptake rate of attached leaves [48].

In fact, we have evidence good enough for hypothesizing that the nonheme iron moiety with acid labile sulfide in general is an endogenous photogenerator of $^1O_2$ in cells. The mitochondron, which is abundant in Fe-S clusters as is the thylakoid, shows a significant susceptibility to light-induced loss of its biochemical function: this mitochondrial photosensitivity also appears closely associated with photodynamic action of certain Fe-S clusters localized to the inner membrane [49-52]. Further, it has been demonstrated that even an artificial nonheme iron moiety, synthesized by the reaction of bovine serum albumin with inorganic sulfide and ferrous iron, acts as a photosensitizer *via* $^1O_2$ mechanism [53]. Despite the sensitizing ability of Fe-S clusters, however, the quantum yield for photogenerating 1O2 may depend on their types and, more importantly, on the microenvironments of their location [54]. For instance, if a certain Fe-S cluster is located in immediate proximity to some substances or chemical groups that quench effectively either the Fe-S triplet state or $^1O_2$, the quantum yield and photosensitization efficiency of the nonheme iron would be low.

THE RIESKE FE-S CENTER, THE MAJOR SITE OF $^1O_2$ GENERATION

Photosensitization by thylakoid Fe-S clusters via $^1O_2$ has been questioned on the basis that, while PS II samples, such as BBY type PS II and PS II core complex preparations, produce $^1O_2$ under photoinhibitory conditions, PS I particles does not to any significant extent [15]. Since most of thylakoid Fe-S clusters are localized to PS I, the failure of $^1O_2$ detection in irradiated PS I particles has been taken as evidence against our proposition on the role of Fe-S clusters in photoinhibition. Nevertheless, this does not totally exclude the possibility that a certain Fe-S group takes part in the process of PS II damage through $^1O_2$-mediated photosensitization; for there is another Fe-S moiety in the thylakoid membrane that is the Rieske Fe-S center of the cytochrome b_6/f complex (Cyt b_6/f). The most recent data clearly indicate that Cyt b_6/f in light produces 1^1O_2 far more efficiently than PS II and causes photoinactivation of PS II core complex, which are effectively suppressed by azide and enhanced by mediun deuteration [18].

Cyt b_6/f comprises two f-type cytochromes, a cytochrome b_6, a Rieske protein and a subunit IV protein, and its brown color arises from blue-violet absorption largely by the heme groups but only marginally by the nonheme iron. Therefore, the photodynamic action of Cyt b_6/f may not in itself be convincing evidence for accepting the Rieske center as the responsible sensitizer. Notwithstanding, the heme groups can safely be removed from the candidates because the action spectra for $^1O_2$ production and PS II inactivation by Cyt b_6/f under illumination have no resemblance to the absorption spectra of cytochromes in any form. Instead, both spectra appear to carry the spectroscopic property of the Rieske protein [55]. However, it is thought that rather direct evidence supporting the Rieske center is provided by the observation that the Rieske protein in the isolated state produces $^1O_2$ in light whereas the Rieske-depleted complex consisting of cytochromes b6 and f and subunit IV does not [18].

CONCLUDING REMARKS

The problems regarding $^1O_2$ formation in chloroplasts, such as the mechanism and the major site, have been the subjects of extensive investigation, for this activated oxygen species is regarded in many respects as the main toxic intermediate that gives rise to damage to PS II during photoinhibition. We now arrive at the conclusion that $^1O_2$ immediately involved in functional loss of PS II is not formed within PS II but rather generated from the outside, specifically from Cyt b_6/f with the Rieske center being the generator.

The thylakoid membrane contains various antioxidant substances that scavenge $^1O_2$, including α-tocopherol and β-carotene. Unsaturated fatty acid moieties of polar membrane lipids also contribute a share to $^1O_2$ removal from the membrane. $^1O_2$ could therefore be eliminated before it reaches the substrates. However, the scavengers themselves are consumed during the scavenging reactions. Thus, the rate of $^1O_2$ production may exceed the rate of $^1O_2$ scavenging at a certain point, particularly in thylakoids exposed to bright light if the scavengers are not regenerated fast enough to counter a continuous attack of the active oxygen. In this situation, even a biological apparatus located at a distance could not be effectively protected anymore. With respect to this conjecture it would be worth mentioning that rose bengal, eosin Y and hematoporphyrin, which are efficient photodynamic

generators of $^1O_2$, promote severe inactivation of PS II and D1 degradation without affecting other components of the electron transport system of thylakoids under low irradiance conditions [9]. Such effect mimics apparently high light-induced effect in thylakoids. As all those chemicals are not believed to directly bind PS II core in which PS II electron transfer takes place, their effect may more likely result from oxidative reaction(s) of certain PS II component(s) with $^1O_2$ that is generated from the outside and diffuses into PS II. Furthermore, the fact that the lifetime of $^1O_2$ is substantially extended in apolar environments of bilayered membranes compared with that in aqueous media should also be taken into account in assessing the dimension of the *in vivo* effects of $^1O_2$ production by Cyt b_6/f in thylakoids.

Acknowledgement – Jin Jung thanks his former graduate students who have done their best during their stay in the laboratory to produce such significant results, some of which are presented herein. Financial support from Korea Ministry of Agriculture-Special Grants Research Program (Grant 295097-5) is also acknowledged.

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