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The involvement of photosystem II-generated H_2O_2 in photoinhibition

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The involvement of H_2O_2 generated by photosystem II (PSII) in the process of photoinhibition of thylakoids with a functional oxygen-evolving complex (OEC) was investigated. The rate of photoinhibition was decreased to the rate of loss of activity in the dark when bovine Fe-catalase was present during the photoinhibitory illumination. Photoinhibition was accelerated for both Cl^--depleted and Cl^--sufficient thylakoids when KCN was present to inhibit the thylakoid-bound Fe-catalase. We propose that these preparations become photoinhibed by reactions with H_2O_2 produced via oxidation of water by the Cl^--depleted OEC and by reduction of O_2 at the Q_B site when PSII is illuminated without an electron acceptor.

Photosystem II; D1 protein; Photoinhibition; H_2O_2

1. INTRODUCTION

Light drives the electron transfer reactions of photosystem II (PSII) which result in the reduction of plastoquinone and the oxidation of water to molecular oxygen [1,2]. However, high light intensities also lead to the inhibition of electron transfer in PSII by a process known as photoinhibition [3]. While evidence suggests that photoinhibition can originate on the reducing side of PSII [3–5], photoinhibition of the reactions on the oxidizing side have been documented under a variety of conditions [6–10].

The components responsible for light-induced charge separation and electron transfer in the PSII reaction center are bound to the D1 (32 kDa) and D2 (34 kDa) proteins [1,2]. Evidence indicates that the manganese, calcium and chloride required for water oxidation are also bound primarily to D1 and D2 [12,13] but the 47 kDa protein may also contribute ligands [14,15]. Although the D1/D2 heterodimer has an apparent twofold symmetry [16], electron transfer is directed exclusively through tyrosine-161 (Y_Z) and the bound pheophytin of D1 [2]. As a result of photoinhibition, the D1 protein degrades much more rapidly than the other proteins of the reaction center [17]. Degradation of the D1 protein can be induced even when highly purified PSII core preparations are subjected to light intensities that induce photoinhibition [18]. From this observation it has been hypothesized that one of the eight proteins that comprizes the PSII core has protease activity.

The rate of photoinhibition and degradation of D1 is accelerated when the OEC is depleted of Cl^- [7,19]. We have recently shown that Cl^- is required by the OEC to prevent the oxidation of water to H_2O_2 when the enzyme has accumulated only two of the four oxidizing equivalents required to oxidize water to O_2 [20]. When PSII preparations are illuminated in the absence of an electron acceptor, the reaction center has also been shown to produce H_2O_2 that does not originate from water [21]. This peroxide is thought to originate from the reduction of O_2 at the Q_B binding site of D1.

We now present evidence which shows that the H_2O_2 produced by PSII is responsible for photoinhibition of PSII reaction centers that have not been depleted of manganese. Peroxide has been documented to inactivate several enzymes via the protein convalent modification. From these results we propose that the degradation products of D1 initially produced by photoinhibitory illumination result from reactions with H_2O_2.

2. MATERIALS AND METHODS

Thylakoid membranes were prepared from market spinach in a darkened room using the Cl^--depletion procedure of Jegerschöld et al. [19]. The membranes were stored at −80°C at a concentration of 3–5 mg Chl/ml. The initial rate of O_2 evolution was measured at saturating light intensities using a Clark-type electrode with 800 μM dichlorobenzoquinone as an electron acceptor. The assay medium contained 25 mM HEPES-NaOH pH 7.5, 2 mM $MgSO_4$, 200 mM sucrose and 40 mM NaCl, unless noted otherwise. The rates of O_2 evolution in the Cl^--depleted thylakoids prior to photoinhibition

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Abbreviations: PSII, photosystem II; OEC, oxygen-evolving complex; Q_B, second quinone acceptor in PSII; P_{680}, primary electron donor chlorophylls; Y_Z, tyrosine-161 on D1 which is the immediate electron carrier between the OEC and P_{680}.

were typically 180 μmol O_2·mg Chl^{-1}·h^{-1} when assayed in the Cl^--sufficient reaction mixture.

To induce photoinhibition, thylakoids were suspended at a concentration of 100 μg Chl/ml in 10 mM sodium phosphate pH 7.4, 100 mM sucrose, 5 mM $MgSO_4$ and, in experiments performed in the presence of Cl^-, 50 mM NaCl. A quartz-halogen source (Oriel Inc.) filtered through 7 cm of 5% $CuSO_4$ and 3 cm of water was used to illuminate the thylakoids such that the light intensity on the sample was 780 μE·m^{-2}·s^{-1}. The samples were illuminated in a 1.5 cm diameter vessel that was stirred continuously and maintained at 20°C by a thermostatically controlled water bath. Aliquots were removed at various intervals during the illumination, diluted tenfold into the assay reaction mixture and assayed immediately for O_2 evolution activity.

3. RESULTS

Thylakoids depleted of Cl^- retained 90% of the original activity after a 50 min dark incubation at 20°C when assayed for photosynthetic O_2 evolution in the presence of Cl^- (Fig. 1). When illuminated in the presence of Cl^- at intensities sufficient to induce photoinhibition, the rate of loss of activity was significantly greater than the rate in darkness. When the Cl^--depleted thylakoids were photoinhibited in the absence of Cl^- the rate of photoinhibition was about 2-fold faster than in the presence of Cl^- as previously observed [18,19]. Since the illumination of Cl^--depleted PSII core preparations produces H_2O_2 [20], it is possible that the photoinhibition observed in Fig. 1 resulted from peroxide-induced reactions.

Thylakoid membranes contain significant amounts of an endogenous Fe-catalase which can only be removed by detergent-washing [22,23]. This membrane-associated catalase, which decreases the levels of H_2O_2 in thylakoids, can be inhibited by addition of 1 mM KCN [24]. To determine if the H_2O_2 produced by PSII

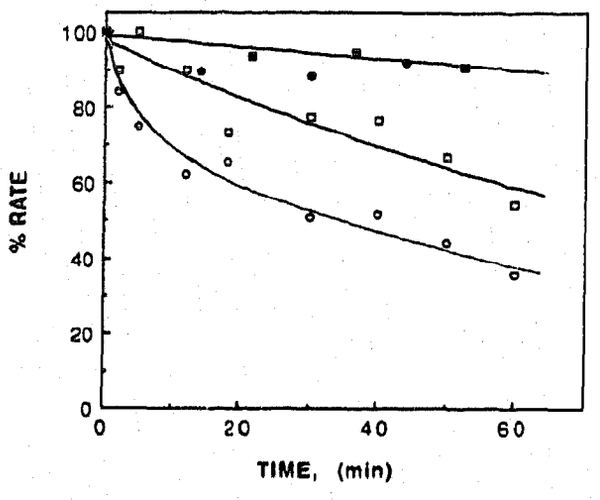

Fig. 1. Inhibition of O_2 evolution in Cl^--depleted thylakoids as a function of the duration of illumination with 780 μE·m^{-2}·s^{-1} in the absence (○) or presence (□) of 60 mM Cl^- or in darkness in the absence of Cl^- (●). The data are plotted as the percent of the rate of O_2 evolved prior to the photoinhibitory illumination using DCBQ as an electron acceptor as described in section 2.

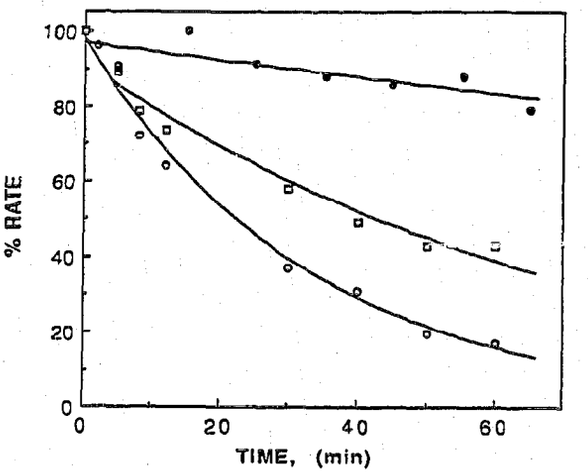

Fig. 2. The time-dependent loss of O_2 evolving activity in Cl^--depleted thylakoids as a function of the duration of illumination with 780 μE·m^{-2}·s^{-1} in the absence (□) or presence (○) of 1 mM KCN or in darkness in the presence of 1 mM KCN (●). The data are expressed as in Fig. 1.

contributed to photoinhibition, the effect of KCN on the time dependence of photoinhibition of Cl^--depleted thylakoids was investigated (Fig. 2). The best fit of the data to a first-order decay process is shown by the solid curves in Figs. 2–4 from which the apparent rate constants for photoinhibition were determined. The magnitude of changes in the rate of photoinhibition was determined from the change in the rate constant derived from this fit. As shown in Fig. 2, inhibition of the endogenous Fe-catalase increased the rate at which water oxidation was inhibited at high intensity light by about 2-fold. The rate of loss of activity in the dark was not affected by the addition of KCN, indicating that the increased rate of loss of activity in the light was due to an effect of KCN on the light-dependent inhibition process. Since the rate of O_2 evolution activity was unaffected when assayed in the presence of 1 mM KCN (data not shown), this reagent does not inhibit electron transfer in PSII directly at 1 mM concentrations. Thus, the increased rate of the photoinhibition observed in the presence of KCN resulted from the higher concentrations of H_2O_2 present when the thylakoid-bound Fe-catalase was inhibited.

The presence of KCN increased the rate of photoinhibition not only in thylakoids depleted of Cl^- but also when Cl^- was present during the illumination to induce photoinhibition as shown in Fig. 3. In all of the experiments described here, photoinhibition was induced by illumination of the thylakoids in the absence of an exogenous electron acceptor. Illumination of PSII preparations in the absence of an electron acceptor has been found to produce H_2O_2 from a source other than the oxidation of water by the OEC [21]. It has been hypothesized that this H_2O_2 originates from the reduction of O_2 on the reducing side of PSII. It seems likely that this second source of H_2O_2 is responsible for the

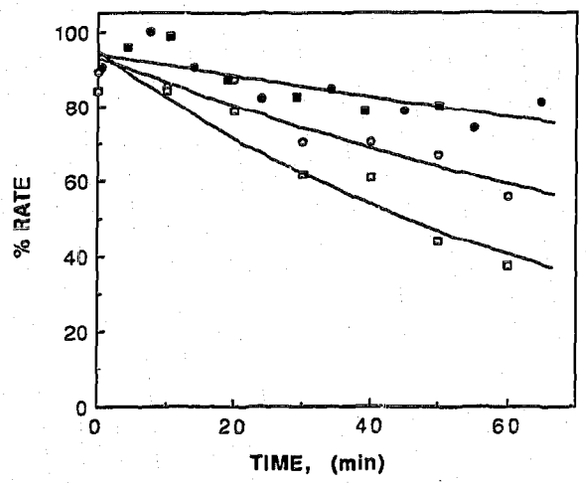

Fig. 3. Inhibition of O_2 evolving activity in Cl^--depleted thylakoids as a function of the time illuminated with 780 $\mu E \cdot m^{-2} \cdot s^{-1}$ with 60 mM Cl^- in the absence (○) or presence (□) of 1 mM KCN or in darkness in the absence (●) or presence (■) of 1 mM KCN. The data are expressed as in Fig. 1.

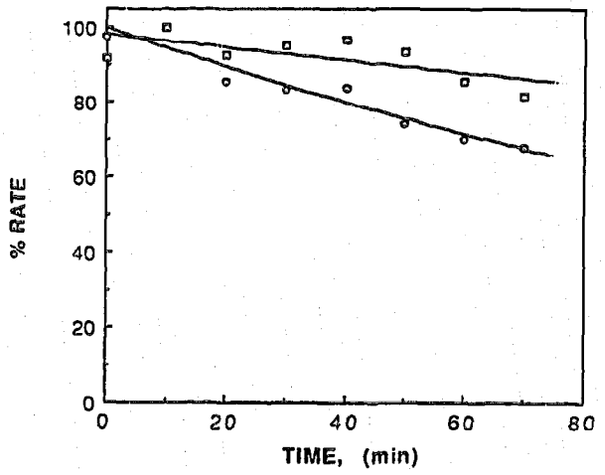

Fig. 4. The time-dependence of inhibition of O_2 evolution in thylakoids as a function of the duration of illumination with 780 $\mu E \cdot m^{-2} \cdot s^{-1}$ in the absence (○) or presence (□) of 5000 units/ml of bovine Fe-catalase. The data are plotted as in Fig. 1.

KCN-dependent loss of activity that results from the illumination of Cl^- sufficient thylakoids in Fig. 3.

If photoinhibition in Cl^--sufficient thylakoids also results from the production of peroxide by PSII, the inhibition should be decreased by the addition of exogenous Fe-catalase. As shown in Fig. 4, the addition of bovine Fe-catalase to the media during the photoinhibition treatment decreased the rate of loss of O_2 evolution activity induced by the light to a rate comparable to the loss of activity observed in darkness.

4. DISCUSSION

The results presented here indicate that when the manganese of the OEC has not been removed by NH_2OH or Tris-washing, photoinhibition is induced by H_2O_2 generated by PSII. This photosystem has been shown to produce H_2O_2 by two mechanisms. First, we have previously obtained evidence that suggests that the OEC oxidizes water to H_2O_2 when depleted of the Cl^- required to form a normal S_2-state [20]. Second, H_2O_2 can also originate from a source other than water if Cl^--sufficient PSII preparations are illuminated in the absence of a quinone that can serve as an electron acceptor at Q_B [21]. Presumably, this latter production of H_2O_2 results from the reduction of O_2 at the Q_B site. In the experiments presented here, photoinhibition was induced by illumination in the absence of a PSII electron acceptor. Inhibition of the endogenous Fe-catalase by KCN increased the rate of photoinhibition whether or not the membranes were depleted of Cl^-. This indicates that there is a site for H_2O_2 production that leads to photoinhibition in addition to the Cl^--dependent site on the OEC. In support of this second site originating from the reduction of O_2, Jegerschöld and Styring [25] have shown that under anaerobic conditions, photoinhibition occurs only when the OEC is depleted of Cl^-.

Peroxide is particularly destructive to the function of a number of proteins which bind and/or catalyze the formation or consumption of O_2 including hemoglobin, myoglobin, cytochrome *c* peroxidase and the Cu/Zn-superoxide dismutase [26-28]. With the exception of superoxide dismutase, these proteins contain an iron bound to a heme that undergoes reactions with H_2O_2 similar to the reactions that are part of the normal catalytic mechanism of horseradish peroxidase, cytochrome *c* peroxidase and prostaglandin H synthase. These reactions involve the H_2O_2-induced oxidation of the heme-iron by a single electron to form a ferryl group [29,30]. Formation of this group also results in the oxidation of a nearby tyrosine to form a cation radical [31,32]. In the peroxidases, the tyrosine radical and the ferryl group serve to catalyze the peroxidation of the substrates. These peroxidase reactions can also inactivate proteins by covalently modifying the proteins to contain adducts of styrene, 17β-estradiol, morphine or diethylstilbesterol [33,34]. In PSII, the Q_A and Q_B sites bind plastoquinones which closely resemble morphine and diethylstilbesterol and, thus, might be susceptible to inactivation by covalent modification via peroxide-induced reactions. This mode of inactivation is less likely since most data indicate that the site of photoinhibition is on the oxidizing side of PSII [6-11].

Hemoglobin, myoglobin and cytochrome *c* can also be oxidized by H_2O_2 to form tyrosine-radicals [33]. The inactivation of hemoglobin, myoglobin and cytochrome *c* by H_2O_2 has been found to proceed when the tyrosine-radicals produced by H_2O_2 induce the formation of dityrosine crosslinks within the protein or to a nearby protein subunit [26]. The formation of dityrosine crosslinks within the D1 protein induced by

H_2O_2 is also a possible means of inactivation, particularly if tyrosine-161 on D1 (Y_Z), which is responsible for electron transfer from the OEC to P680 [35–37], becomes crosslinked.

The Cu/Zn-superoxide dismutase normally catalyzes the dismutation of superoxide to form O_2 and H_2O_2. However, at alkaline pH, excess H_2O_2 reduces the copper from Cu^{2+} to Cu^{+} which is followed by an irreversible inactivation [28]. In a manner similar to the heme proteins, the inactivation has been correlated to the formation of a free radical on an amino acid followed by the covalent modification of an amino acid (possibly histidine) near the metal. Evidence now supports the formation of a histidine radical as an intermediate in the catalytic cycle of the OEC [38]. This radical has been observed in preparations depleted of Cl^- and has been implicated in the process of photoinhibition [39]. It seems likely that peroxide could also inactivate PSII via reactions with histidine as observed for superoxide dismutase.

The involvement of H_2O_2 in the process of photoinhibition has been confined in this study to the reactions that lead to photoinhibition when the manganese of the OEC has not been removed by NH_2OH or Tris-washing. When depleted of manganese. PSII reaction centers become sensitive to photoinhibition at much lower light intensities than required for the studies presented here [7,11]. Increasing evidence indicates that the photoinduced lesion in the Mn-depleted preparations results from the inability of Y_Z to become oxidized [11]. Due to the much longer lifetime of the $Y_Z^{\cdot +}$ radical when the OEC is destroyed [40,41], this loss of Y_Z may result from the formation of dityrosine crosslinks that occur without the help of H_2O_2. It is also possible that some peroxide might be generated on the reducing side of PSII upon illumination.

High light intensities also induce the degradation of the D1 protein at rates which are rapid compared to the degradation of the other PSII proteins. Evidence suggests that the photodegradation of D1 occurs as a result of photoinhibition. Recently, Virgin et al. [18] reported that highly purified PSII core preparations are subject to photoinhibition and photo-induced degradation of D1. The major fragments of D1 observed as initial degradation products had molecular masses of 23 kDa and 13–16 kDa. To account for the appearance of these fragments, it was proposed that one of the seven polypeptides that composes the core preparation is a protease [18].

Alternatively, we suggest that the proteolysis of the D1 protein may result from reactions induced by H_2O_2. A possible means by which this might occur is by a reaction similar to that known to be catalyzed by NH_2OH [42]. Hydroxylamine is capable of breaking the peptide bond of a protein when asparagine precedes glycine in the sequence by a nucleophilic attack of the carbonyl on the succinimidyl group that these amino acids form. Although no N-G pairs are found in D2, two are found in D1 from spinach at positions 104 and 284. Proteolysis of D1 at these positions leads to the formation of three peptides with molecular weights of about 24 300, 13 800 and 5800. The first two peptides are close in size to the major initial breakdown products of D1. The lack of detection of the smallest peptide by SDS-PAGE previously may have resulted from the percent acrylamide in the gel.

A wide variety of reactions caused by H_2O_2 may in fact lead to photoinhibition and degradation of D1 which will ultimately be resolved by sequence analysis of the D1-degradation products. Experiments are currently underway to resolve this question.

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