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Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy

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Abstract

Exposure of isolated spinach thylakoids to high intensity illumination (photoinhibition) results in the well-characterized impairment of Photosystem II electron transport, followed by degradation of the D1 reaction centre protein. In the present study we demonstrate that this process is accompanied by singlet oxygen production. Singlet oxygen was detected by EPR spectroscopy, following the formation of stable nitroxide radicals from the trapping of singlet oxygen with a sterically hindered amine TEMP (2,2,6,6-tetramethylpiperidine). There was no detectable singlet oxygen production during anaerob photoinhibition or in the presence of sodium-azide. Comparing the kinetics of the loss of PS II function and D1 protein with that of singlet oxygen trapping suggests that singlet oxygen itself or its radical product initiates the degradation of D1.

Abbreviations: HEPES – 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; PS – Photosystem; TEMP – 2,2,6,6-tetramethylpiperidine; TEMPO – 2,2,6,6-tetramethylpiperidine-1-oxyl

Introduction

High intensity illumination of plants invokes a complex series of stress reactions, known as photoinhibition (Powles 1984). This process includes the functional impairment of Photosystem (PS) II electron transport and the structural damage of one of the PS II reaction centre proteins (D1) (recent reviews: Andersson and Styring 1991, Barber and Andersson 1992, Prasil et al. 1992, Aro et al. 1993). Although there is a lack of complete agreement on the mechanism responsible for D1 degradation, it is generally described as a two-step process. First, the protein is triggered (marked for degradation) by a photophysical process, which is followed by the proteolytic degradation of the triggered protein (Aro et al. 1990, Richter et al. 1990a). This assumes that the PS II reaction centre undergoes light-induced modifications, which expose the otherwise protected cleavage region(s) of the D1 protein to a putative protease (Aro et al. 1990, Richter et al. 1990a).

There is a general consensus that the primary target of photoinhibition is the reaction centre of PS II. The protein backbone of the reaction centre complex is consisted of the heterodimer of the D1 and D2 proteins (and cytochrome *b*-559) (Namba and Satoh 1987). The D1/D2 heterodimer binds or contains the reaction centre chlorophyll (P_{680}), the pheophytin (Pheo) and quinone electron acceptors (Q_A and Q_B) as well as the electron donor tyrosine residues (Tyr-Z and Tyr-D). It most likely provides the binding

site for the water splitting enzyme, too (see Anderson and Styring 1991 and references therein). Energy absorption in the reaction centre chlorophyll results in charge separation between P_{680} and Pheo, followed by a sequence of charge stabilizing reactions at the acceptor- and donor sides of PS II.

Recent studies have demonstrated that continuous strong illumination can damage electron transport both at the donor and acceptor sides of PS II (reviewed by Prasil et al. 1992). Donor side photoinhibition most likely affects the functioning of Tyr-Z or P_{680}. In contrast, the impairment of the acceptor side function is due to an unusual, double reduction of Q_A (Setlik et al. 1990, Vass et al. 1992). Double-reduced Q_A is an abnormal state, which is inactive in mediating electron transfer toward Q_B. Illumination of these modified PS II centers facilitates the recombination of the $P_{680}{}^{+}Pheo^{-}$ primary charge pair leading to the formation of $^3P_{680}$ with a high yield (Van Mieghem et al. 1989, Vass et al. 1992, Vass and Styring 1993). Triplet chlorophyll is known to react with the triplet ground state of oxygen, producing the highly reactive singlet oxygen (reviewed by Elstner 1987, Asada and Takahashi 1990). Singlet oxygen was suggested to play an important role in damaging the D1 protein (Durrant et al. 1990, Vass et al. 1992, Macpherson et al. 1993), but the mechanism of this process has not been fully characterized yet.

The involvement of oxygen radicals in photoinhibition has also been concluded on the basis of experiments in which externally added free radical scavengers or defense enzymes slowed down photoinactivation (Barényi and Krause 1985, Setlik et al. 1990, Richter et al. 1990b, Sopory et al. 1990). However, the chemical identification of active oxygen species meets several experimental problems. Although most of the applied scavengers and enzymes are more reactive to certain types of active oxygen than to others, they are able to react with a variety of radicals. This way, the identification of the involved radicals cannot be based solely on the effect of these reagents. On the other hand, due to the limited access of water soluble chemicals to the proposed site of radical production (in the membrane), the failure of a scavenger to affect photodamage does not necessarily exclude the participation of its putative target radical from the process.

Other methods, such as fluorescence or electron paramagnetic resonance (EPR), which are based on the observation of certain physical characteristics of active oxygen provide more solid basis for identification. Free radicals can be spin-trapped with nitroso and nitron reagents converting them to more stable nitroxide radicals (reviewed by Janzen 1971, Evans 1979, Halliwell and Gutteridge 1989). Singlet oxygen, which is a strong electrophile but not a radical, can oxidize TEMP (a sterically hindered amine) to a stable N-oxyl radical, TEMPO (Aurich 1982), which can be detected by EPR spectroscopy (Lion et al. 1976, 1980). Also, in the absence of reactions $^1O_2$ returns to the ground state with light emission around 1270 nm as spontaneous monomolar decay (Kanofsky 1983, 1984). This infrared chemiluminescence was recently applied for the detection of $^1O_2$ from photodamaged PS II reaction centre preparations (Telfer et al. 1992, Macpherson et al. 1993). Unfortunately, the emission spectrum utilized in the above method is composite (the 1268 nm peak is sitting atop a declining spectral component of chlorophyll phosphorescence), making it difficult to apply as quantitative analysis.

Here, we report data on the detection of singlet oxygen via its reaction with a sterically hindered secondary amine (TEMP) yielding a nitroxide free radical (TEMPO) which is detectable by EPR spectroscopy. This sensitive technique enabled us to determine the kinetics of singlet oxygen production at various stages of photoinhibition and discuss its role in the process.

Materials and methods

Thylakoid membranes were isolated from market spinach as described by Takahashi and Asada (1982) and suspended in a HEPES buffer (40 mM, pH 7.5) containing 0.4 M sucrose, 15 mM NaCl and 5 mM $MgCl_2$.

Two ml alliquots of thylakoids, diluted with the same buffer to 100 μg chlorophyll/ml, were exposed to high intensity (1000 $\mu E/m^2/s$) illumination from a Tungsten lamp while stirred in a

temperature controlled glass cuvette at 20 °C for the time indicated in the figures.

Anaerob conditions were established by flushing the samples with argon, followed by the addition of 10 mM glucose, 10 μg/ml glucose oxidase and 10 μg/ml catalase, according to the method described by Durrant et al. (1990).

Steady-state oxygen evolution was measured with a Clark-type oxygen electrode at room temperature. The 3 ml assay contained thylakoids (50 μg chlorophyll) in the above buffer and 1 mM dimethyl-benzoquinone as an electron acceptor.

There is a general consensus that all PS II proteins other than D1 are stable (except for a limited loss of D2) (Andersson et al. 1992, Aro et al. 1993 and references therein), therefore we followed the structural damage of PS II by measuring the decrease in the amount of the D1. Degradation of the D1 protein was followed by immunoblotting as described by Barbato et al. (1991). Before electrophoresis, samples of photoinhibited thylakoids were kept in the dark for 15 min at room temperature. SDS–PAGE was performed on a 12–17% linear acrylamide gradient gel containing 6 M urea. The resolved proteins were electroblotted and identified by using an antibody raised against the D1 protein (kindly provided by Dr R. Barbato). Densitometric analysis of the immunodecorated blots was performed using a Biorad densitometer (Biorad 1650) in reflectance mode, attached to a Hewlett Packard integrator (HP 33941).

Singlet oxygen was determined in samples containing 10 mM TEMP, by measuring the EPR absorption of the stable nitroxide radical (TEMPO) which is produced from the reaction between $^1O_2$ and TEMP (Lion et al. 1976). In order to measure the rate of $^1O_2$ trapping during a certain period of photoinhibition, untreated thylakoids were pre-illuminated with high intensity light, then TEMP was added to the illuminated suspension and photoinhibition was continued for the period of time required.

Nitroxides, specially 6-membered heterocyclic radicals such as TEMPO are very sensitive to reducing agents (Couet et al. 1985) which are produced in illuminated thylakoids, too. These can reduce the radical to a labile diamagnetic N-hydroxylamine (TEMP-OH). However, these N-hydroxylamines are oxidized back to the EPR active form almost spontaneously, which can be accelerated by airating the sample in the presence of catalytic amounts of PbO_2. Therefore, photoinhibited thylakoids were mixed with ethylacetate (1:2 V/V) and allowed to separate into two phases for a few minutes. The upper, organic phase (containing the reduced amine, but no photosynthetically active thylakoids) was removed and re-oxidized with a catalytic amount (10–30 μg) of PbO_2 before EPR spectroscopy.

The $^1O_2$ yielding photodynamic reaction of toluidine blue (Pottier et al. 1975) was performed in ethylacetate. One mM toluidine blue was illuminated for 20 min in the presence of 10 mM TEMP. This reaction leads to nitroxide radical production, as described by Lion et al. (1976, 1980).

For reference EPR spectrum, the stable nitroxide radical (TEMPO) was measured in ethylacetate solution at 10 μM concentration.

EPR spectra of the nitroxide radical were measured with a Brucker ECS-106 spectrometer utilizing the ECS-106 data acquisition program. X-band spectra were recorded at room temperature with 9.43 GHz microwave frequency, 16 mW microwave power and 100 kHz modulation frequency, as described earlier (Hideg and Vass 1993).

Results

Figure 1 demonstrates free radical production during photoinhibition. In the upper part of the figure, the EPR spectrum of TEMP is compared after its reaction with untreated and photoinhibited thylakoids. Figure 1a shows that no significant radical formation was detected when the amine (TEMP) was added to untreated samples. The appearance of the EPR signal in photoinhibited thylakoids in the presence of TEMP (Fig. 1b) shows that stable paramagnetic radicals are formed in the reaction between a product of photoinhibition and the diamagnetic amine. The symmetrical triplet is characteristic to the EPR spectrum of the paramagnetic N-oxyl radical (TEMPO), which is formed when singlet oxygen is trapped by TEMP. This is evidenced by the EPR spectrum of the reaction between the

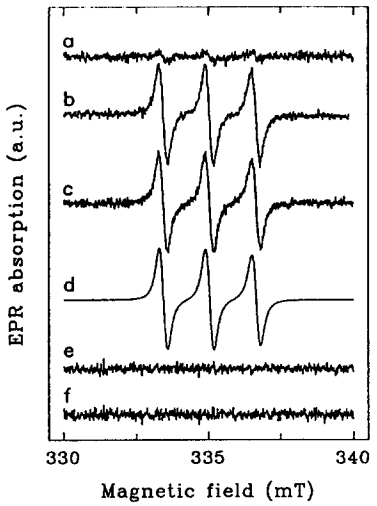

Fig. 1. EPR detection of trapping singlet oxygen by TEMP in isolated spinach thylakoids. Thylakoid membranes containing 10 mM TEMP were kept (a) for 15 min in the dark or were photoinhibited for 15 min (b) without addition, (e) in the presence of 10 mM NaN_3 or (f) in the absence of oxygen. Trace (c) represents the EPR spectrum from the photodynamic reaction of 1 mM toluidine blue and oxygen in the presence of 10 mM in buffer solution. Trace (d) is the EPR signal from 10 μM TEMPO. All EPR spectra were recorded in ethylacetate (for details see 'Materials and methods'). Microwave frequency 9.43 GHz, microwave power 16 mW, modulation frequency 100 kHz. Amplification parameter: (a, b, c, e, f) 1×10^5 and (d) 1×10^3.

amine and $^1O_2$ from an in vitro photodynamic reaction of toluidine blue with oxygen (Fig. 1c), as reported by Lion et al. (1976, 1980). No EPR signal emerged after illuminating TEMP in a buffer only (data not shown).

The identity of the EPR spectrum of the radical produced in photoinhibited thylakoids (Fig. 1b) and of the EPR spectrum of TEMPO (Fig. 1d) supports the assumption that $^1O_2$ is produced during photoinhibition.

The EPR signal was not observed in thylakoids photoinhibited in the presence of both TEMP and NaN_3 (Fig. 1e). Sodium-azide is known as a singlet oxygen scavenger (Hasty et al. 1972, Denke and Krinsky 1977) and, although we are aware of its ability to influence many enzymes and of its reactivity to other radicals (reviewed by Halliwell and Gutteridge 1989), we regard its inhibitory effect on the formation of the characteristic EPR signal as corroboration of $^1O_2$ production. As it is shown in Fig. 1f, anaerob photoinhibition did not result in $^1O_2$ production either, demonstrating that the radical was yielded from a reaction requiring molecular oxygen.

Comparing the EPR signal of the nitroxide radical, produced by trapping of $^1O_2$ by TEMP in photodamaged thylakoids with that from a known concentration of TEMPO gives approximately 10^{-7} moles for the concentration of $^1O_2$ in the membrane after 15 min of photoinhibition. This, however, can only provide a rough estimate, since neither the probability that $^1O_2$ reaches the amine nor the efficiency of trapping are known.

Figure 2A illustrates the increase of $^1O_2$ concentration during photoinhibition. Curve *a* in Fig. 2A is the EPR signal from thylakoids illuminated with high intensity light for 15 min but allowed to react with the trap (TEMP) during the last minute only. This signal is much smaller than the one from samples which were illuminated for the same time interval but in the presence of the trap from the beginning of photoinhibition (compare curves *b* in Figs. 1 and 2A), because $^1O_2$ itself, unlike TEMPO, is not accumulated during the process. If no trapping reagent is present, $^1O_2$ reacts with other compounds in the sample, while in the presence of TEMP some of the produced singlet oxygen will

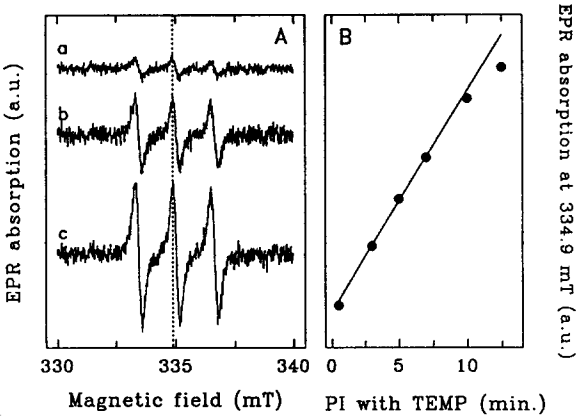

Fig. 2. The kinetics of singlet oxygen trapping by TEMP in isolated thylakoids exposed to photoinhibition. (A) Samples were illuminated for (a) 15, (b) 20 and (c) 25 min and were mixed with 10 mM TEMP at the end of the fourteenth minute of illumination. The EPR spectra were recorded as in Fig. 1. (B) EPR absorption at a magnetic field indicated by dashed line in Fig. 2A is shown as a function of illumination time. Dots represent EPR data, the solid line shows the linear fit determining the trapping rate.

be trapped and detected as signal from the stable nitroxide radical. When pre-illuminated thylakoids were exposed to high light for longer time (5, 10 min) following the addition of TEMP, the induced EPR signal was larger (Fig. 2A, curves *b* and *c*).

The trapping rate of $^1O_2$ can be determined as the rate of nitroxide accumulation. Plotting the EPR signal measured at a frequency characteristic to the nitroxide radical versus the time of photoinhibitory illumination required for the accumulation of this amount of nitroxide, the slope of the plot gives the $^1O_2$ trapping rate (Fig. 2B). Declination from this line indicates that the amine substrate (TEMP) was used up, therefore these data were not considered. Following this method, the concentration of the substrate was not limiting at any time of the experiment. On the other hand, it was low enough not to perturb the experimental conditions, since the kinetics of the loss of electron transport activity was not influenced by the presence of 10 mM TEMP during photoinhibition (data not shown).

Singlet oxygen was measured for several short, 10–15 min periods of the whole time course of photoinhibition, and changes in its trapping rate are compared to changes in photosynthetic activity and D1 protein degradation in Fig. 3. Oxygen evolution was 70% inhibited after 25 min of photoinhibition and completely impaired after 40 min. Steady-state oxygen evolution was not affected in samples kept in the dark for 30 min (data not shown), therefore, the decrease shown in Fig. 3 is attributed to photodamage only. Protein degradation occurred much slower, about 50% of D1 was lost after 2.5 h of photoinhibition. The inset in Fig. 3 shows the immunoblot of D1 protein which served as the basis of the data presented in the figure. The data of D1 damage and PS II impairment are in agreement with the time course of protein degradation and loss of PS II function

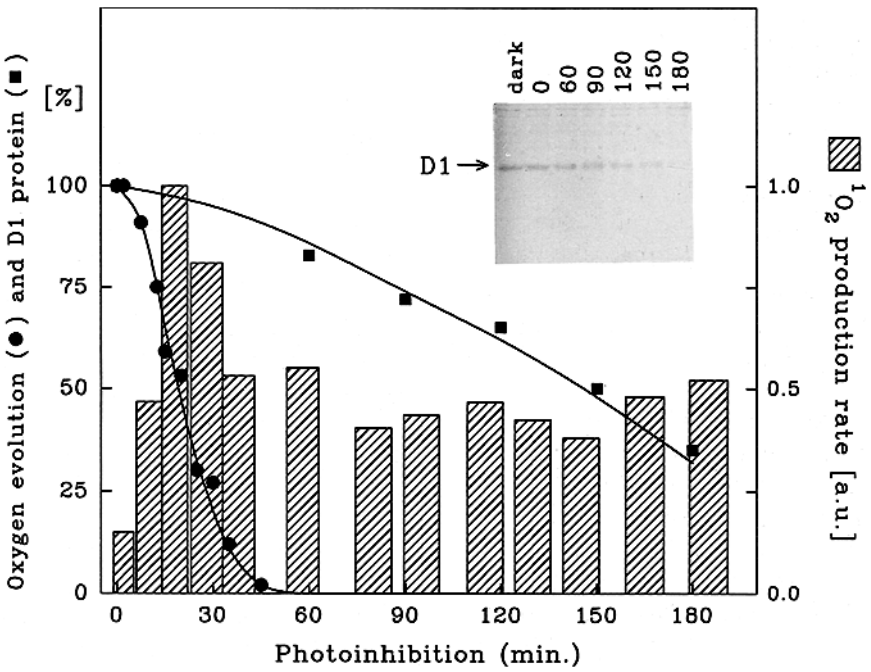

Fig. 3. The kinetics of oxygen evolution, D1 protein degradation and singlet oxygen production during photoinhibition of isolated thylakoids. Samples were photoinhibited for various periods of time, and the rate of oxygen evolution (●) and the amount of D1 protein (■) were detected. The 100% for oxygen evolution rate is 193 μM O_2/mg chlorophyll/h. The histograms show singlet oxygen trapping rate determined from the slope of EPR absorption vs. photoinhibition time as illustrated in Fig. 2B. The width of the histogram columns correspond to the time interval in which the trapping rate was determined. One unit in the y-scale represent approximately 10 nM $^1O_2$ trapped per minute. *Inset*: Immunoblot showing D1 degradation after various times of photoinhibition. The numbers refer to the duration of photoinhibition in minutes. The first lane is a dark control: D1 in a sample which was kept in the dark for 3 h at room temperature.

reported by Hundal (1992) for thylakoids photoinhibited under similar experimental conditions.

We found that the trapping rate of singlet oxygen was increasing quickly during the first minutes of photoinhibition reaching a maximum 20–25 min after the onset of high intensity light (histogram in Fig. 3). After longer exposure to high light, a smaller rate of $^1O_2$ production (approximately half of the maximal rate) was detected which showed a slow decline with increasing time of photoinhibition.

Discussion

Active oxygen species have long been considered as damaging agents in the process of light-induced destruction of the D1 reaction centre protein of PS II (Barényi and Krause 1985, Setlik et al. 1990, Richter et al. 1990b, Sopory et al. 1990). The possibility that singlet oxygen is produced during photoinhibition has also been raised (see Asada and Takahashi 1987). However, strong indication of this process was provided only recently by demonstrating in anaerobically photoinhibited PS II membranes the light-induced formation of $^3P_{680}$ (Vass et al. 1992), a sensitizer of $^1O_2$ formation, and its effective quenching by oxygen (Vass and Styring 1992). $^1O_2$ production in PS II was further corroborated by detection of infrared chemiluminescence in isolated PS II reaction centre complexes (Macpherson et al. 1993), but direct evidence for $^1O_2$ formation during photoinhibition of thylakoid membranes was still lacking.

The trapping experiments presented here utilizing a sterically hindered amine (TEMP) provide the evidence that singlet oxygen is produced in photodamaged thylakoids. Our results partly contradict an earlier publication of Sopory et al. (1990), who pointed out the importance of oxyradicals in photodamage but excluded the participation of $^1O_2$. However, their latter conclusion was based on the failure of a scavenger (selenomethionine) to inhibit the process in *Spirodela*, which could be explained by a limited access of the chemical to the site of $^1O_2$ production; and to the inefficiency of D_2O to affect the time course of damage, which could only be expected if $^1O_2$ decayed in a monomolecular reaction (Halliwell and Gutteridge 1989, Gorman and Rodgers 1992).

In order to explain the observed changes in the trapping rate of $^1O_2$, one should consider that the ratio of trapped and free (not trapped) singlet oxygen (i.e. the probability that $^1O_2$ will be trapped instead of following its natural pathway) is governed by the reactivity of $^1O_2$ to TEMP compared to its reactivity to other compounds. This depends on concentrations of the trap (TEMP), the $^1O_2$ and that of the competing reaction partners. In our experiments the concentration of TEMP was not limiting, so the observed changes in $^1O_2$ trapping rate could reflect changes in either the production rate or in the kinetics of the reactions competing with the TEMP substrate for $^1O_2$.

In the initial phase of photoinhibition (5–15 min) an increasing concentration of $^1O_2$ is induced in parallel with an increase in the number of PS II centers which are incapable of secondary electron transport (shown by decreasing photosynthetic oxygen evolution) but able to perform primary charge separation (as expected in the absence of D1 degradation). This supports the assumption that $^1O_2$ is formed in the interaction of $^3P_{680}$ with the triplet ground-state of oxygen, in PS II centers which can still form $P_{680}^+Pheo^-$, the precursor state of $^3P_{680}$ (Durrant et al. 1990, Vass et al. 1992). Between 20–45 min of photoinhibition the trapping rate of $^1O_2$ was decreasing faster than the amount of the D1 protein, i.e. than the number of centers with functional primary charge separation and $^3P_{680}$ formation. This way, the fast decrease in $^1O_2$ trapping rate is unlikely to report a decrease in the production rate, which is proportional to the amount of centers with intact $^3P_{680}$ formation (and D1 protein). A more likely explanation is an increased reactivity of other, natural substrates (protein, lipid or pigment components of PS II, see further discussion below) to $^1O_2$, the onset of reactions competing with TEMP for $^1O_2$. Such an effect could be caused by a structural change of the PS II reaction centre, which was shown to occur in the early phase of photoinhibition (Vass et al. 1992).

It is very important to note that detectable D1 protein degradation begins when high levels of singlet oxygen production is reached, and the

slow decline in the amount of the D1 protein is accompanied with the slow decrease of the $^1O_2$ trapping rate after 60 min photoinhibition. This supports earlier proposals about the involvement of $^1O_2$ in damaging (triggering for degradation) the D1 protein (Durrant et al. 1990, Vass et al. 1992, Macpherson et al. 1993): i.e. the singlet oxygen produced in $^3P_{680}$ forming reaction centers is consumed in a reaction which modifies the D1 protein and triggers it for proteolytic degradation. In the following we consider possible mechanisms of the $^1O_2$ induced D1 protein damage.

Several amino acids were found to be vulnerable to modification by oxidative stress (for review see Rice-Evans et al. 1991), specially histidine, tryptophan and methionone (Nilsson et al. 1972, Matheson et al. 1975). Histidine is frequently used as a $^1O_2$ trap in spectrophotometric assays (Kraljic and Mohsni 1978, Chakraborty and Tripathy 1992), and it was reported as one of the common sites for oxidative covalent modifications of proteins (Levine 1983). Some of the histidine residues, which were suggested to provide the binding site of P_{680} in fourth and fifth transmembrane helix of D1 (Trebst 1986, Michel and Deisenhofer 1988), could be oxidized by the $^1O_2$ produced in the reaction of triplet chlorophyll with oxygen.

On the other hand, because the above amino acid residues are susceptible to oxidation by other oxyradicals, too (Elastner 1987, Halliwell and Gutteridge 1989), it cannot be excluded that products of a $^1O_2$ initiated free radical cascade are also involved in the process. It has been suggested, that free radicals can propagate in proteins quite far from the site where they were produced via a chain reaction involving several amino acid residues (Rice-Evans et al. 1991). Beside amino acids, singlet oxygen could also attack the carbon-carbon double bonds of carotenoids or chlorophyll whose breakdown would promote structural changes (Barber and Andersson 1992, De Las Rivas et al. 1992).

It was shown that after the attack of active oxygen, proteins are more sensitive to proteolytic hydrolysis (Wolff et al. 1986, Davies et al. 1987), as a result of structural modifications (Davies et al. 1987, Davies and Delsignore 1987). Similarly to these examples, the reaction of $^1O_2$ (or its products) with D1 does not necessarily marks the cleavage site itself. The oxidative damage may only alter a few amino acid residues resulting in changes in the secondary and tertiary protein structure in such a way that the proposed sites of scission become exposed to the protease.

In summary, EPR spectroscopy provides direct experimental evidence that $^1O_2$ is produced in chloroplast thylakoids exposed to high light in the presence of oxygen. We suggest that singlet oxygen itself or a (yet unidentified) product of a reaction using $^1O_2$ as a substrate triggers the D1 protein for degradation. This targeting process is not prompt, the degradation of the triggered protein starts only after several minutes of exposure to oxidative stress. It is of note that initiation of lipid peroxidation by $^1O_2$ (Heath and Packer 1968, Takahama and Nishimura 1975) is not likely involved (neither as the reaction decreasing the affinity of $^1O_2$ to trapping, nor as a radical source for D1 targeting), since it was observed only in the late phase of photodamage when the amount of the D1 protein decreased by more than 50% (Hundal 1992).

In order to investigate the possible indirect role of $^1O_2$ and the involvement of a radical cascade, experiments with spin traps, as well as with sterically hindered amines other than TEMP are in progress in our laboratory.

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