Photoinhibition of Photosynthesis in Vivo Results in Singlet Oxygen Production Detection via Nitroxide-Induced Fluorescence Quenching in Broad Bean Leaves[†]

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ABSTRACT: In plants experiencing environmental stress, the formation of reactive oxygen is often presumed. In this study, singlet oxygen was detected in broad bean (*Vicia faba*) leaves that were photoinhibited in vivo. Detection was based on the reaction of singlet oxygen with DanePy (dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole) yielding a nitroxide radical (DanePyO) which is EPR active and also features lower fluorescence compared to DanePy. The two (fluorescent and spin) sensor fuctions of DanePy are commensurate, which makes detecting singlet oxygen possible with a spectrofluorimeter in samples hard to measure with EPR spectroscopy [Kálai, T., Hideg, É., Vass, I., and Hideg, K. (1998) *Free Radical Biol. Med. 24*, 649−652]. We found that in leaves saturated with DanePy, the fluorescence of this double sensor was decreased when the leaves were photoinhibited by 1500 μmol m^{-2} s^{-1} photosynthetically active radiation. This fluorescence quenching is the first direct experimental evidence that photoinhibition of photosynthesis in vivo is accompanied by $^1O_2$ production and is, at least partly, governed by the process characterized as acceptor side-induced photoinhibition in vitro.

Illumination with excess photosynthetically active radiation (PAR)[1] is a stress factor for plants (*1*, *2*), known as photoinhibition (*3*). It is widely investigated in combination with other (e.g., low temperatures) stress (*4*), but its occurrence is also recognized in the absence of other factors (*2*, *5*). Photoinhibition has been extensively studied in in vitro systems: isolated, photosynthetically active thylakoid membranes and subthylakoid preparations. It is generally accepted that the primary target of photoinhibition is the reaction center of the photosystem (PS) II subunit of the photosynthetic apparatus. In higher plants, PS II is embedded in the thylakoid membrane of chloroplasts. Its reaction center complex consists of cytochrome b_{559} and the heterodimer of the D1 and D2 proteins (*6*). The D1−D2 heterodimer binds or contains the reaction center chlorophyll (P680), the pheophytin (Pheo), and the 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 (*7*, and references therein). Energy absorption in the reaction center chlorophyll results in charge separation between P680 and Pheo, followed by a sequence of charge-stabilizing reactions at the acceptor and donor sides of PSII.

In vitro studies agree that there is a definitive sequence of events in photoinhibition: excess PAR leads to impairment of PS II electron transport which is followed by selective degradation of the D1 PS II reaction center protein and by more general membrane protein and lipid damage (*8*−*10*). There are both indirect and direct pieces of evidence that reactive oxygen species are produced during photoinhibition. Direct observation of reactive oxygen species by spin trapping EPR spectroscopy demonstrated that photoinhibition by excess PAR is indeed an oxidative stress (*11*−*14*).

In vitro, two types of photoinhibition are recognized, acceptor side-induced and donor side-induced (for reviews, see refs *8* and *10*), although other mechanisms were also suggested to occur under special light conditions (*15*). The two main pathways are distinguished by their light requirement, the primary site of PS II electron transport damage, the reactive oxygen chemistry, the degradation pattern of the D1 protein, and the requirement for oxygen (for reviews, see refs *8* and *10*).

Acceptor side-induced PI occurs when photosynthetically active, oxygen-evolving preparations are illuminated with excess PAR in the presence of oxygen. In this process, double reduction of the first PS II quinone acceptor Q_A results in increased reaction center chlorophyll triplet formation (*16*) and, consequently, in singlet oxygen production. The latter has been confirmed by infrared chemiluminescence (*17*), chemical trapping (*18*), and EPR spectroscopy in oxygen-evolving preparations (*12*, *19*). Damage to the D1 reaction center protein is very likely initiated by singlet oxygen (*16*) which is followed by fragmentation into a 23 kDa N-terminal (*20*) and a corresponding 10 kDa C-terminal (*21*) product.

Photoinhibition follows the donor side-induced route when preparations with the inactive oxygen-evolving complex are

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[1] Abbreviations: DanePy, dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole; DanePyO, dansyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole-1-oxyl; PAR, photosynthetically active radiation (400−700 nm); PI, photoinhibition; PS, photosystem.

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illuminated. In these samples, lower PAR corresponds to more excess energy than in photosynthetically active preparations. It is generally accepted that donor side-induced PI develops if the reduction of chlorophyll and tyrosine cation radicals at the donor side of PS II reaction center is retarded due to impaired electron donation from water (*22*−*24*). Accordingly, it was found that the process does not require the presence of oxygen (*25*, *26*) and is not accompanied by singlet oxygen production in Tris-pretreated thylakoids (*11*). In the PS II reaction center preparation with an inactive donor, donor side-induced PI results in the appearance of 9 kDa N-terminal and 24 kDa C-terminal fragments of the D1 protein (*21*, *27*).

There are strong indications that the above reactive oxygen species are involved in the specific cleavage of the D1 protein; the addition of hydrogen peroxide (*28*) or singlet oxygen-generating substances (*29*−*31*) cause D1 protein fragmentation to specific fragments as does acceptor side-induced photoinhibition by excess PAR. Okada et al. (*31*) recently suggested that the central role of the D1 protein in photoinhibition may be due to its unique amino acid sequence and folding characteristics resulting in higher sensitivity to cleavage by singlet oxygen as compared to the sensitivities of other PS II proteins. In this way, understanding reactive oxygen chemistry and identification of singlet oxygen and free radical production during photoinhibition are important for understanding the mechanism of damage.

An alternative pathway of photoinhibition has been suggested to occur under low, nonsaturating irradiation provided by consecutive flashes of light. This process also leads to degradation of the D1 protein in isolated thylakoid membranes (*32*). According to the model established by Ohad's group, this process is a result of the generation of triplet PS II reaction centers in the dark intervals between flashes, via charge recombination of the primary radical pair (*15*, *33*). Singlet oxygen is also very likely formed in this process, but the amount is below the detection level of presently available methods (*15*).

Various energy-dissipating pathways (*34*), the operation of a D1 protein repair cycle, including degradation, de novo synthesis, and replacement of D1 (*10*, *35*, *36*), lessen damage by photoinhibition in vivo (*37*). However, the question of which mechanism of photoinhibition dominates in vivo is still open. Recent studies aimed at the identification of the pathway on the basis of differences between the fragmentation pattern of the D1 protein in acceptor side-induced PI and donor side-induced PI are ambiguous. Detection of a 10 kDa C-terminal fragment in wheat leaves suggested the acceptor side route (*38*, *39*). On the other hand, an 18 kDa N-terminal D1 protein breakdown product was recently identified in photoinhibited intact pumpkin leaves, supporting the existence of the donor side mechanism in vivo (*40*). Methods other than immunodetection of the D1 protein fragments also suggest donor side-induced PI (*41*, *42*), but neither of these studies excluded the contribution of acceptor side-induced damage.

Identification of reactive oxygen species would provide evidence of whether in vivo photoinhibition follows the acceptor side-induced or the donor side-induced mechanism, but it also meets several obstacles. Conventional techniques, such as the addition of antioxidants or radical scavengers, resulted in only partial protection against photoinhibition

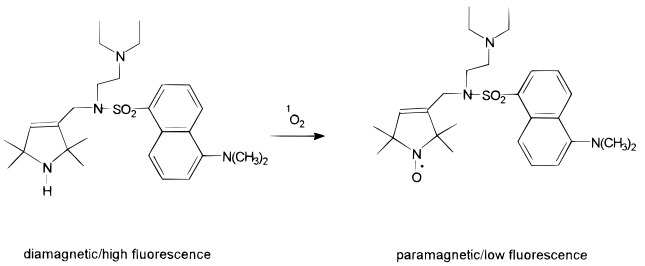

FIGURE 1: Conversion of DanePy into DanePyO by singlet oxygen.

even in in vitro experiments (*28*, *43*−*45*) and are thus not likely to give clear evidence in vivo. Because singlet oxygen production appears to be a unique characteristic of acceptor side-induced PI of PS II (*11*, *19*), its observation during in vivo photoinhibition would provide evidence for the acceptor side mechanism. Unfortunately, the singlet oxygen trapping EPR spectroscopy technique applied in vitro cannot be adapted directly to in vivo experiments, due to the general sensitivity of six-membered nitroxide radicals to reducing agents (*46*), especially to ones produced in illuminated thylakoids (*12*). Also, utilization of any EPR method in vivo, in leaves with a relatively high water content, is limited by a high level of absorption of microwave energy in these samples.

The above difficulties may be overcome by the application of fluorescent probes. Singlet oxygen is known to bleach the fluorescence of a variety of aromatic compounds which may serve as indicators of reactive oxygen production (*47*). Extending these experiments, Blough and Simpson showed that the fluorescence of such compounds closely linked to an EPR active center can be substantially influenced by reactions influencing the paramagnetism of the center (*48*). Utilizing this principle of double (fluorescent and spin) sensors, we were recently able to demonstrate for the first time that the reaction of singlet oxygen with DanePy yields a nitroxide radical DanePyO according to Figure 1. This product is EPR active and also features lower fluorescence compared to DanePy (*49*), which can be readily detected by a spectrofluorimeter in intact leaves. Our in vitro studies showed that the fluorescence quenching of DanePy is selective for singlet oxygen; chemically generated free radicals, such as superoxide or hydroxyl radicals, and lipid peroxidation products resulted in <10% quenching and equal amounts of $^1O_2$ (*49*).

Our experiments with singlet oxygen either generated from chemical substrates or produced in thylakoid membranes undergoing acceptor side-induced photoinhibition proved that monitoring DanePy fluorescence quenching is as informative as EPR spectroscopy (*49*). In this study, we utilized the fluorescent function of this double sensor to study singlet oxygen production in leaves in which direct EPR measurements are ambiguous.

MATERIALS AND METHODS

Broad bean (*Vicia faba* L.) plants were grown under 80−100 μmol m^{-2} s^{-1} PAR at 20−22 °C. For photoinhibition, detached leaves were placed on wet tissue paper and their adaxial sides were exposed to high-intensity (1000, 1500, or 2000 μmol m^{-2} s^{-1}) PAR through an optical fiber guide

from a KL-1500 (DMP) lamp. This method assured that leaves were neither dried out nor heated during photoinhibition. Higher PAR levels caused visible signs of pigment bleaching even after 5−10 min and were not used in this study.

Photosynthetic oxygen evolution was measured with a leaf disk oxygen electrode system (Hansatech) under saturating (5%) CO_2. Data points represent the average of five or six measurements. The typical standard deviation was ±10−12%.

The net D1 protein content was determined using Western blotting after separation of thylakoid membrane proteins by SDS−polyacrylamide gel electrophoresis, according to the method of Barbato et al. (*21*). Thylakoid membranes were prepared from leaves as described previously (*12*). Samples of 1 μg of chlorophyll were found to be optimal for electrophoresis and the following blotting. The D1 protein was identified immunologically by incubating it with an antibody raised against a synthetic peptide corresponding to the C terminus of the pea D1 protein, a kind gift from P. Nixon (Imperial College, London, England). The density of the stains on the immunoblot was determined with a Stratgene Eagle Eye II Video System (Stratgene) densitometer. The integrated density of the bands was used as a relative measure of the D1 protein content. Data shown represent the average of two or three experiments with a ±8−10% standard deviation.

Singlet oxygen was detected in leaves saturated with DanePy before photoinhibition, by monitoring the fluorescence quenching of DanePy. Saturation was performed in a plastic syringe with 10 mL of a 50 mM solution of DanePy (ethanol concentration < 1%) and a leaf inside, by rapidly pulling the plunger while keeping the nozzle closed. This saturation reduced the oxygen-evolving ability of leaves to 10% of that of untreated ones but had no effect on the amount of D1 protein (data not shown). Saturation experiments with water only showed that this decrease in oxygen evolution was caused by the saturation procedure and not by the presence of DanePy (data not shown). The first fluorescence measurement (registration of the DanePy emission spectrum) started 3 min after saturation and was followed by subsequent periods of leaf illumination and fluorescence measurements. Fluorescence emission spectra were recorded with Quanta Master QM-1 (Photon Technology International Inc.) using a 345 nm excitation wavelength and 3 nm excitation and emission slits. Singlet oxygen trapping is characterized as relative fluorescence quenching at the 532 nm emission maximum of DanePy (*49*). Since fluorescence emission maxima are broad, the signal-to-noise ratio of fluorescence quenching data was improved by using the average of six data points around the emission maximum instead of a single value. Fluorescence quenching data represent average of five or six measurements of 532 nm fluorescence from different leaves with a ±7% standard deviation.

When indicated, chloroplast protein synthesis was inhibited with lincomycin. In these experiments, leaf petioles were cut under water and transferred immediately to a 1 g/L aqueous lincomycin solution. Feeding of lincomycin through the petioles proceeded for 4 h in the dark, at room temperature. Chlorophyll concentrations were determined in 80% acetone according to Arnon (*50*).

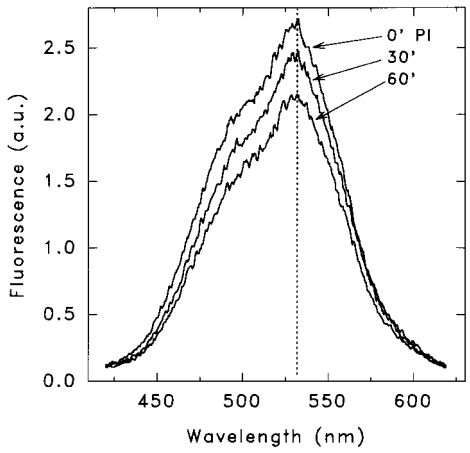

FIGURE 2: Fluorescence emission spectra of DanePy in a broad bean leaves before (0 min) and after exposure to photoinhibition by 1500 μmol m^{-2} s^{-1} PAR for 30 and 60 min.

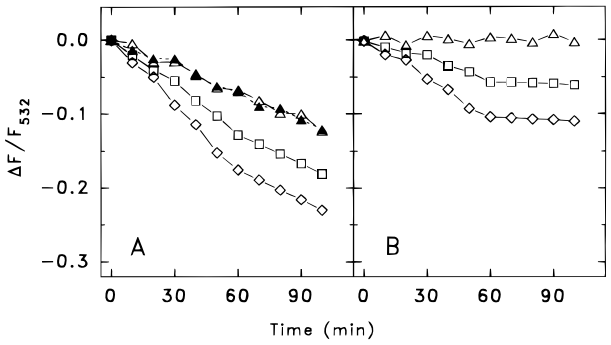

FIGURE 3: Time dependence of DanePy fluorescence quenching in broad bean leaves. After saturation with DanePy, leaves were kept in darkness (▲) or illuminated with 200 (△), 1000 (□), or 2000 (◇) μmol m^{-2} s^{-1} PAR: (A) raw data and (B) data corrected for light-independent fluorescence quenching.

RESULTS

Figure 2 shows fluorescence emission spectra of DanePy in a broad bean leaf before and after exposure to photoinhibition. The emission spectrum is heterogeneous, consisting of a main peak at 532 nm and of a shoulder at 490 nm. The former corresponds to the emission maximum of DanePy in a thylakoid membrane suspension (*49*); the latter is probably emitted from a modified form of the compound. Both peaks were equally affected by photoinhibition-induced processes in the leaf; therefore, only changes in the main peak were considered.

DanePy fluorescence is irreversibly quenched when the leaf is exposed to photoinhibition by PAR. This quenching is stronger after longer photoinhibition (Figure 2) and could be explained as (i) interaction of DanePy with a chemical produced in the photoinhibited leaf or (ii) decay of DanePy. In the following paragraphs, we show that both processes take place.

Figure 3 shows the time dependence of DanePy fluorescence quenching in leaves under various illumination conditions. As demonstrated in Figure 3A, DanePy fluorescence is quenched in leaves even in the dark. However, the extent of this quenching is smaller than the one observed in photoinhibited leaves. Because the dark quenching is not affected by illuminating the leaf with low, non-photoinhibitory PAR (Figure 3A), it is considered a result of DanePy

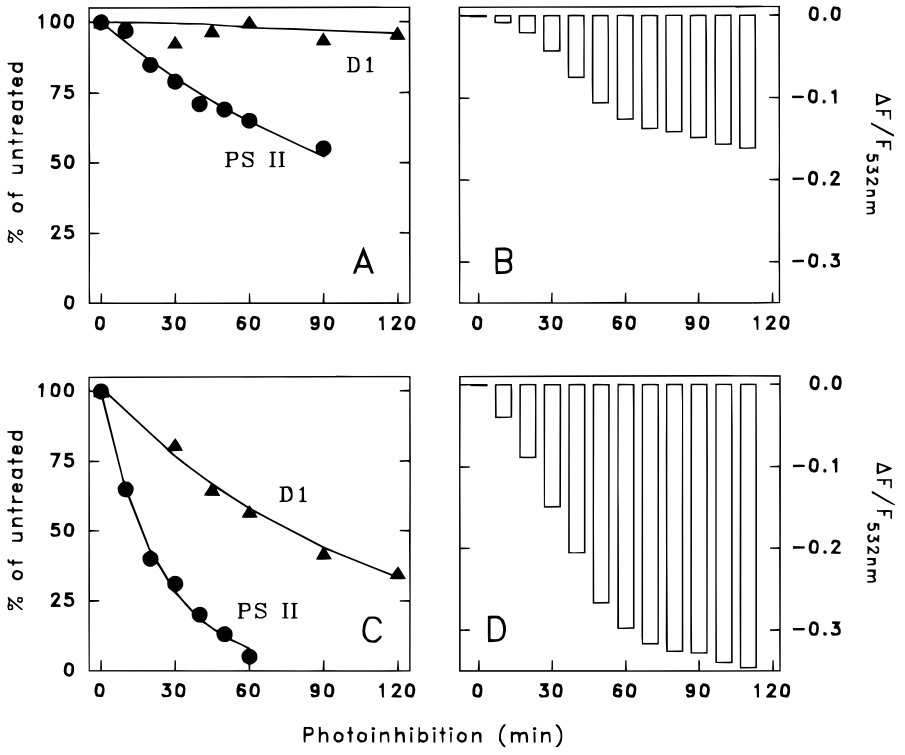

FIGURE 4: Photosynthetic oxygen evolution (●), net D1 protein content (▲), and photoinduced DanePy fluorescence quenching (bars) in broad bean leaves illuminated with 1500 μmol m^{-2} s^{-1} PAR in the absence (A and B) or in the presence (C and D) of lincomycin.

decomposition unrelated to photosynthetic activity. This dark decomposition of the fluorophore is linear; data measured either in the dark or in low light can be fitted well by the same linear function (fitting line not shown). In this way, we assumed that DanePy fluorescence quenching data obtained in photoinhibited leaves are composed of two components: one caused by photoinhibition and another which is also present in the dark. Data measured in illuminated leaves, with or without photoinhibitory conditions, were corrected for this dark decomposition and are shown in Figure 3B.

Figure 4 is a comparison of time courses of DanePy fluorescence quenching, the loss of the D1 protein, and the impairment of photosynthetic oxygen evolution in leaves exposed to photoinhibition by 1500 μmol m^{-2} s^{-1} PAR. These experiments were carried out either in the absence (Figure 4A,B) or in the presence (Figure 4C,D) of lincomycin, an inhibitor of protein synthesis in chloroplasts.

Figure 4A shows that although photosynthetic activity decreased in leaves exposed to photoinhibition by PAR, there was no change in the net amount of D1 protein. In these samples, DanePy fluorescence was gradually quenched, an indication of singlet oxygen production (Figure 4B). This quenching was relatively small in the first 5−10 min and strongest between 30 and 60 min of photoinhibition.

Inhibition of chloroplast protein synthesis in the samples by lincomycin resulted in marked differences in the response of these parameters to photoinhibition compared to the response of samples with active protein synthesis. Exposure to the same photoinhibitory conditions as those in panels A and B of Figure 4 caused net D1 loss and a faster loss of PS II function (Figure 4C). DanePy fluorescence was also quenched in these leaves, demonstrating singlet oxygen production, but it started earlier and was more excessive than in samples photoinhibited without lincomycin (Figure 4D).

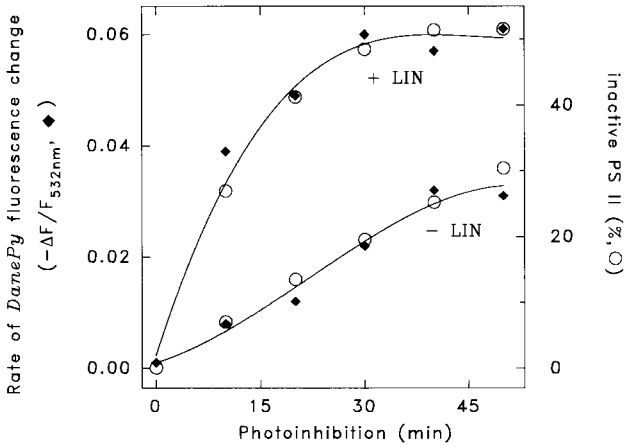

FIGURE 5: Rate of DanePy fluorescence change (◆) and the relative amount of inactive PS II centers (○) in broad bean leaves illuminated with 1500 μmol m^{-2} s^{-1} PAR in the absence (−LIN) or in the presence (+LIN) of lincomycin.

DanePy fluorescence quenching is an accumulative process; i.e., the extent of the quench at a given time point during photoinhibition is characteristic of total singlet oxygen production until that time. In this way, the time course of the production is better characterized as a rate of DanePy fluorescence change, i.e., increase of quenching during a relatively short (5−10 min) time interval. Figure 5 shows such rates in leaves photoinhibited in the absence (−LIN) or in the presence (+LIN) of lincomycin in comparison with the relative amount of inactive PS II centers. The latter were defined as centers structurally intact but without PS II activity. The increase in the number of such centers is consistent with that of singlet oxygen production in both types of samples up to ca. 50 min of photoinhibition. After further photoinhibition, this initial increase in the fluorescence rate change was followed by a decline, to less than

20% of maximal production (data not shown). The relatively lower quenching after 70−80 min was not caused by exhaustion of the sensor in either sample. Experiments with leaves photoinhibited first for 70 min without, and then, after saturation, with DanePy, also resulted in relatively little fluorescence quenching (data not shown).

DISCUSSION

Photoinhibition of photosynthesis occurs under a variety of conditions in nature in many plant species (for review, see ref *1* and references therein). In vivo, photoinhibition includes reversible inhibition of PS II electron transport, a process with a protective role, as well as damaging processes in PS II which are not reversible (*34*). In this latter case, PS II function can only be restored by the operation of a complex repair cycle, including de novo protein synthesis and reassembly of the PS II reaction center (for review, see ref *10* and references therein). Details of damaging processes in PS II are not fully understood. A central issue is whether information from in vitro studies is relevant also to in vivo studies. Part of this problem is whether either of the mechanisms described as in vitro acceptor side-induced PI or donor side-induced PI occurs in leaves exposed to photoinhibition.

Indirect evidence from comparing changes in the amount of D1 protein with that of DCMU binding sites in photoinhibited *Arabidopsis* (*41*) and from the constancy of the quantum yield of photoinhibition under different photon flux densities in pumpkin leaves (*42*) suggested the occurrence of donor side-induced PI in vivo. Supporting this as more direct evidence, fragments typical of donor side-induced PI D1 protein degradation have been identified under in vivo conditions in pumpkin leaves (*40*). However, such fragments were not found either in photoinhibited *Spirodela* (*51*) or in wheat (*38*, *39*), but these authors observed D1 protein breakdown products typical of acceptor side-induced PI.

These results show that both processes characterized as acceptor side-induced PI and donor side-induced PI in vitro are feasible in vivo. Unfortunately, most likely, proteolytic breakdown of the primary D1 protein fragments (*10*) and technical difficulties of predicting accurate fragment sizes (*40*) make the study of the D1 protein degradation pattern difficult in vivo.

Instead of characterizing D1 protein breakdown products, we utilized differences in dominant reactive oxygen species observed under conditions of acceptor side-induced PI and donor side-induced PI in vitro. As we reported previously, in isolated photosynthetic membranes, singlet oxygen production is characteristic of acceptor side-induced PI but not of donor side-induced PI (*11*). These results are based on fluorescence quenching of DanePy (*49*), in which a fluorescent segment is attached to the singlet oxygen trap utilized in our earlier EPR studies (*11*, *12*, *19*). In this way, singlet oxygen production can be detected not only as the appearance of an EPR signal (which meets technical difficulties in leaves) but also as the decrease of fluorescence intensity (*49*).

We found that fluorescence of DanePy decreased in photoinhibited broad bean leaves (Figure 2). This is a result of two processes. The first one is not related to illumination, and the second one is light-dependent (Figure 3A). The origin of the first, dark component is unknown. Because we did not observe such a decay in experiments with isolated thylakoid membranes (data not shown), this component is tentatively assigned to conversion of the sensor into a nonfluorescent, inactive form during leaf metabolism. The second, light-dependent component of the quenching is caused by photoinhibition; low, non-photoinhibitory PAR did not cause photoquenching (Figure 3B). The extent of photoquenching was higher when photoinhibition was carried out under higher PAR.

Our earlier, comparative EPR and fluorescence spectroscopy experiments with DanePy in isolated thylakoid membranes with photoinhibition (*19*) demonstrated that light-induced DanePy fluorescence quenching is an indicator of singlet oxygen production.

This observation of light-induced DanePy fluorescence quenching indicates that singlet oxygen is formed in broad bean leaves exposed to photoinhibition by excess PAR (Figure 4B). Under these conditions, PS II activity decreased to approximately 50% after 90 min, but there was no net D1 protein loss. Further illumination did not increase PS II activity loss and resulted in only a minor decrease of the net amount of D1 (data not shown). Consistent with other studies on photoinhibited leaves (*40*, *52*), this stable steady-state D1 protein content reflects the operation of an efficient repair cycle (*10*). Nevertheless, the loss of photosynthetic activity shows that PS II centers which contain D1 protein but are functionally inactive accumulate during photoinhibition (Figure 5). These centers are potential sites of high-yield chlorophyll triplet and, consequently, singlet oxygen production (*16*). Our experiments show for the first time that such singlet oxygen production occurs in vivo and that it is proportional to the amount of inactive PS II centers (Figure 5).

Lincomycin prevents protein synthesis and photoinhibition in its presence results in a decrease in the steady-state D1 protein concentration (Figure 4C), consistent with earlier results (*52*, *53*). In these leaves, the accumulation of inactive PS II centers is faster under the same photoinhibitory conditions as in untreated leaves with D1 protein repair (Figure 5). Our results support the model postulating functionally inactive centers as sources of singlet oxygen; faster accumulation and a higher concentration of inactive centers in lincomycin-treated leaves result in a higher rate of $^1O_2$ production as in untreated ones (Figure 5).

It is an interesting, common characteristic of all samples that less singlet oxygen was trapped after the accumulation of about 50% inactive PS II centers. A similar effect was observed earlier in EPR spectroscopy detection of singlet oxygen in photoinhibited thylakoid membrane preparations (*11*, *12*), and it does not necessarily reflect decreased singlet oxygen production. It may also be explained as an increased affinity of singlet oxygen for targets other than the sensor (DanePy in this study) and/or its conversion into other type of active oxygen species or free radicals before being trapped by the sensor.

The above results are based on the first application of a double (fluorescent and spin) sensor in plant physiology. With this technique, we demonstrated that singlet oxygen is produced in photoinhibited leaves. This observation shows that PS II in vivo may be damaged by formation of singlet oxygen via interaction of ground-state (triplet) oxygen with triplet chlorophyll formed during charge recombination in

functionally inactive PS II reaction centers; i.e., the reaction route described as acceptor side-induced PI in in vitro samples is operational in vivo. It should also be noted that the contribution of donor side-induced PI to in vivo damage cannot be excluded either, especially when photoinhibition is combined with other stress factors primarily affecting the PS II donor side.

The fates of singlet oxygen in leaf PS II reaction centers are manifold. Some may be scavenged at or close to its production site, e.g., quenched by the reaction center β-carotenes (*18*, *54*). Other singlet oxygen molecules may damage reaction center chlorophylls, lipids, or amino acids of the D1 protein. Possible active oxygen products of these reactions are also detrimental, since neither PS II nor its proximity is sufficiently protected by antioxidant enzymes (for reviews, see refs *55* and *56*). Finally, some singlet oxygens apparently leave the reaction center and reach the fluorescent sensor.

It is generally agreed that the absence of net D1 protein loss during in vivo photoinhibition reflects the balance between D1 protein degradation and repair (*10*, *40*, *52*). Our fluorescence quenching data show that the level of singlet oxygen production is proportional to the concentration of reaction centers to be repaired. When the functioning of the D1 protein repair cycle was inhibited by lincomycin treatment, the number of these centers increased faster and reached a higher level, resulting in increased singlet oxygen production. This supports the notion that rapid degradation and replacement of D1 upon photoinhibitory damage seems to be the main pathway for preventing further membrane and pigment damage in vivo.

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