Plant Physiol. (1992) 98, 7–11
0032-0889/92/98/0007/05/$01.00/0

Received for publication May 20, 1991
Accepted July 24, 1991

Involvement of Singlet Oxygen in 5-Aminolevulinic Acid-Induced Photodynamic Damage of Cucumber (*Cucumis sativus* L.) Chloroplasts[1]

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ABSTRACT

Cucumber (*Cucumis sativus* L., cv Poinsette) plants were sprayed with 20 millimolar 5-aminolevulinic acid and then incubated in the dark for 14 hours. The intact chloroplasts were isolated from the above plants in the dark and were exposed to weak light (250 micromoles per square meter per second). Within 30 minutes, photosystem II activity was reduced by 50%. The singlet oxygen ($^1O_2$) scavengers, histidine and sodium azide (NaN_3) significantly protected against the damage caused to photosystem II. The hydroxyl radical scavenger formate failed to protect the thylakoid membranes. The production of $^1O_2$ monitored as *N,N*-dimethyl *p*-nitrosoaniline bleaching increased as a function of light exposure time of treated chloroplasts and was abolished by the $^1O_2$ quencher, NaN_3. Membrane lipid peroxidation monitored as malondialdehyde production was also significantly reduced when chloroplasts were illuminated in the presence of NaN_3 and histidine. Protochlorophyllide was the most abundant pigment accumulated in intact chloroplasts isolated from 5-aminolevulinic acid-treated plants and was probably acting as type II photosensitizer.

Biological tissues that accumulate porphyrins are sensitive to photodynamic reaction and can be destroyed by it (3, 18–20). Porphyric patients suffer from sun burns (14). Photosensitizers are used for photodynamic therapy of cancerous cells (5, 6).

Plants synthesize Chl from ALA[2] via monovinyl and divinyl monocarboxylic routes (23, 24). It is well known that plants treated with ALA accumulate Mg-tetrapyrroles in the dark. Upon exposure of ALA-treated plants to sunlight, these excess tetrapyrroles are photosensitized and consequent photodynamic reactions kill the plants within a few hours (3, 18–20). Photodynamic damage to plants is accompanied by destruction of photosynthetic reactions, and this damage appears to be irreversible (20). $^1O_2$ is produced due to the type II photosensitization reaction of porphyrins (11). It was proposed that $^1O_2$ could be involved in porphyrin-sensitized photodynamic damage of ALA-treated plants (3, 18–20). However, it is difficult to demonstrate the production of $^1O_2$ in intact plants. All Mg-tetrapyrroles that accumulate in response to ALA treatment are localized in the chloroplasts. Therefore, chloroplasts should be the site of photodynamic reactions. $^1O_2$ produced due to a photodynamic reaction could be monitored in chloroplast suspensions. PSII is highly susceptible to slight perturbation of the thylakoid membrane and is known to be affected by photodynamic reactions (20). If $^1O_2$ is the active O_2 species involved in photodynamic reactions, the photodynamic damage would be protected by $^1O_2$ scavengers. The present investigation aimed to determine the nature of active O_2 species involved in photodynamic reactions in intact chloroplasts containing excess Mg-tetrapyrroles.

MATERIALS AND METHODS

Plant Material

Cucumber (*Cucumis sativus* L., cv Poinsette) was grown on Petri plates (14.5 cm diameter) on moist filter paper under continuous white fluorescent light as described previously (21). The temperature was maintained at 25°C. The 6-d-old plants were used throughout the experiments.

ALA Treatment

Each Petri plate, having 15 seedlings, was sprayed with 5 mL of aqueous ALA solution (20 mM, pH 4.8) as described previously (20). Control seedlings were sprayed with distilled water (pH 4.8). After ALA/distilled water treatment, the plants were kept in the dark for 14 h.

Isolation of Intact Chloroplasts

Chloroplast extractions were made under a dim, green light in an isolation medium consisting of 0.4 M sucrose, 10 mM NaCl, and 25 mM Hepes/NaOH buffer (pH 7.6) (21). Intact chloroplasts were obtained through a 40% (v/v) Percoll gradient (4) and were pelleted and resuspended in the same isolation buffer. The chloroplasts were 95% intact. The intactness was determined by the $K_3Fe(CN)_6$ reduction assay method (25). Chl was extracted in 80% acetone. ALA-treated, dark-incubated plants accumulated an excess amount of protochlorophyllide that could interfere with Chl estimation, and thus the latter was corrected for protochlorophyllide absorbance at 626 nm (1).

[1] This work was supported by Department of Science and Technology, Government of India grant No. DST/SP/SO/A44-88 to B.C.T.

[2] Abbreviations: ALA, 5-aminolevulinic acid; $^1O_2$, singlet oxygen; MDA, malondialdehyde; TBA, thiobarbituric acid; RNO, *N,N*-dimethyl, *p*-nitrosoaniline.

Light Treatment

Chloroplast suspensions were placed in a glass tube surrounded by a water jacket connected to a circulating water bath (maintained at 25°C). Illumination was with white light provided by a projection lamp at a flux intensity of 250 μmol m^{-2} s^{-1} obtained via neutral density filters (Blazers).

Electron Transport Activity

Chloroplasts isolated from both control and ALA-treated and dark-incubated plants were illuminated (250 μmol m^{-2} s^{-1}) at a concentration of 1 mg Chl mL^{-1}. The electron transport activity through PSII was determined polarographically using a YSI model 53 Clark-type O_2 electrode connected to a recorder (21). The reaction mixture (3 mL) for phenylenediamine-supported PSII-mediated O_2 evolution consisted of 50 mM Hepes/NaOH (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM NH_4Cl, 1 mM $K_3Fe(CN)_6$, and 1 mM phenylenediamine. Chloroplasts were added to the above reaction mixture at a concentration of 40 μg Chl mL^{-1}. In the above reaction medium, chloroplasts lysed due to osmotic shock.

MDA Production

TBA-reactive MDA production from isolated chloroplasts was determined as described by Heath and Packer (9). The amount of MDA produced was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} at 532 nm and was corrected for nonspecific turbidity at 600 nm. As sucrose interferes with the MDA assay, chloroplasts were suspended in a buffer containing 0.3 M NaCl, 25 mM Hepes/NaOH buffer (pH 7.6) at a concentration of 100 μg Chl mL^{-1}.

RNO Bleaching

The production of $^1O_2$ was determined monitoring RNO bleaching reactions using a spectrophotometric method as described by Joshi and Pathak (12). Histidine was used as a trap for $^1O_2$. Chloroplasts (100 μg Chl mL^{-1}) were incubated with RNO (300 μg mL^{-1}) and 10 mM histidine. Samples were taken at various time intervals. After centrifugation (3000*g* for 10 min), the supernatant was decanted and the absorbance was read at 440 nm. When the $^1O_2$ quencher NaN_3 was used, it was added to the incubation mixture before illumination.

Estimation of Tetrapyrroles

Intact chloroplasts isolated from control and ALA-treated, dark-incubated plants were illuminated at a concentration of 100 μg Chl mL^{-1} as described above. Aliquots were taken after various periods of light treatment and the pigments were extracted in 80% acetone. Fully esterified tetrapyrroles were extracted into hexane, whereas the mono- and dicarboxylic tetrapyrroles remained in the hexane-extracted acetone residue fraction. Quantitative estimation of protoporphyrin IX, Mg-protoporphyrin monoester, and protochlorophyllide from their mixture was carried out spectrofluorometrically (17, 22). The protein content of the extracts was estimated according to Lowry *et al.* (15).

Fluorescence spectra from hexane-extracted acetone residue fraction were recorded in the ratio mode using a computer-driven SLM AMINCO 8000 C spectrofluorometer and corrected for photomultiplier tube sensitivity. Rhodamine B was used in the reference channel as a quantum counter. A tetraphenylbutadiene block was used to adjust the voltage in both the channels (*i.e.* sample as well as reference channels) to 20,000 counts/s at excitation and emission wavelengths of 348 and 422 nm, respectively. Spectra were recorded at excitation and emission bandwidths of 4 nm. The emission spectra were recorded from 580 to 700 nm.

Chemicals

ALA, $K_3Fe(CN)_6$, phenylenediamine, RNO, NaN_3, and TBA were obtained from Sigma Chemical Co.

RESULTS

Protection of Thylakoid Membrane Functions by $^1O_2$ Quenchers

Intact chloroplasts from dark-incubated control and ALA-treated plants were illuminated (250 μmol m^{-2} s^{-1}) for 30 min. This specific light intensity and time were chosen as these caused substantial (50%) damage to the PSII activity of treated chloroplasts and only marginal damage (8%) to control chloroplasts (Fig. 1). Longer exposure (2 h) of chloroplasts to high light intensities (1000 μmol m^{-2} s^{-1}) impaired PSII function in control chloroplasts by 60% and caused complete inhibition in treated chloroplasts (data not shown). The rates of O_2 evolution in control and treated chloroplasts before illumination were 90 μmol mg^{-1} Chl h^{-1}.

To test if the photodynamic damage to the thylakoid membrane function is mediated by active O_2 species, both control and treated chloroplasts were illuminated in the presence of various scavengers of active O_2. In control chloroplasts, none of the scavengers enhanced or inhibited phenylenediamine-supported O_2 evolution (data not shown). However, in treated chloroplasts, the $^1O_2$ scavenger, 10 mM histidine (16), protected PSII-mediated O_2 evolution by 85% and 2 mM NaN_3 (2, 8) by 65% (Fig. 1). Higher concentrations of NaN_3 (10 mM), when added to the control chloroplasts, inactivated PSII function and thus were not used. The hydroxyl radical scavenger, formate (10 mM) (7), did not protect against damage to PSII. The above results suggest that the $^1O_2$ is the active O_2 species involved in photodynamic damage.

Detection of $^1O_2$ Production in Light-Treated Chloroplasts

To demonstrate whether $^1O_2$ is produced during photodynamic reactions, its level was monitored by the RNO bleaching reaction (12). Upon illumination, $^1O_2$-mediated RNO bleaching increased as a function of time in chloroplasts isolated from ALA-treated plants (Fig. 2). The $^1O_2$ induced RNO bleaching was only marginal in control chloroplasts. The $^1O_2$ quencher, NaN_3 (10 mM), reduced light-induced $^1O_2$ production in treated chloroplasts by 90%. The above results demonstrated the production of $^1O_2$ in the illuminated chloroplasts isolated from ALA-treated plants.

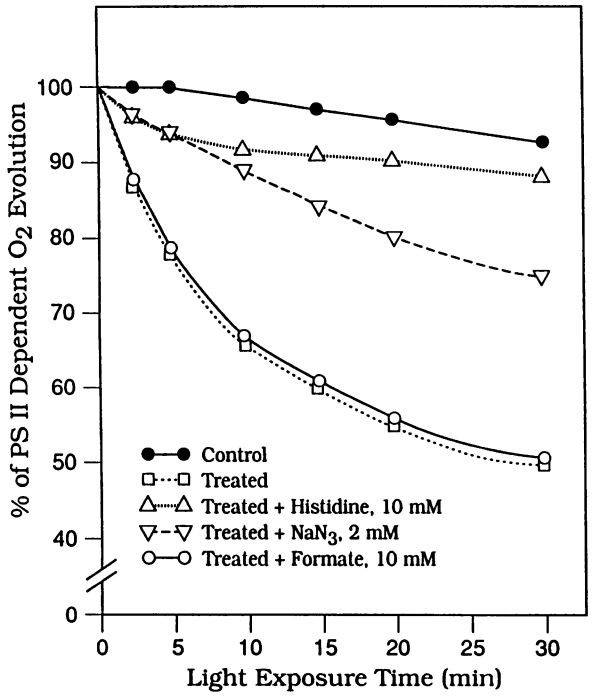

Figure 1. Effect of scavengers of active O_2 species on light (250 μmol m^{-2} s^{-1})-induced damage to the thylakoid membrane-linked PSII photochemical reactions in intact chloroplasts isolated from control and ALA-treated cucumber plants. The scavengers did not have any inhibitory or protective role on phenylenediamine-mediated PSII activity of control chloroplasts (data not shown). The rates of O_2 evolution in control and treated chloroplasts before illumination were 90 μmol/mg chl·h. Other experimental details are as in "Materials and Methods."

Light-Induced Membrane Lipid Peroxidiaton

MDA production is an index of peroxidation of unsaturated membrane lipids. Figure 3 demonstrates that illumination of treated chloroplasts increased production of MDA. As expected, there was also a marginal increase in MDA production in control chloroplasts. $^1O_2$ scavengers such as histidine and NaN_3 protected the chloroplast membranes from photodynamic damage and the MDA production was reduced by 80% in the presence of these compounds. Another free radical scavenger, formate (7), failed to decrease MDA production in treated chloroplasts (data not shown). These data confirm that $^1O_2$ is the active O_2 species involved in photodynamic damage.

Involvement of Tetrapyrroles in Photodynamic Damage

To determine the nature of the pigments involved in photodynamic damage, intact chloroplasts were isolated from ALA-treated plants and exposed to light. As shown in Figure 4, the concentrations of protoporphyrin IX, Mg-protoporphyrin monoester, and protochlorophyllide were very low, *i.e.* less than 0.3 nmol mg^{-1} protein in chloroplasts isolated from control plants incubated in dark for 14 h. After illumination, the concentrations of protoporphyrin IX and Mg-protoporphyrin monoester remained constant up to 1 h.

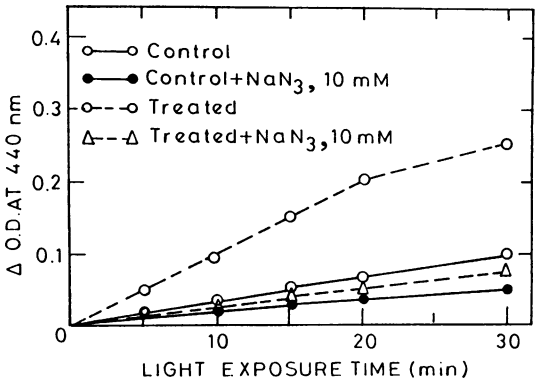

Figure 2. Effect of $^1O_2$ scavenger, NaN_3, on the light-mediated RNO bleaching reaction of intact chloroplasts isolated from control and ALA-treated cucumber plants. Other experimental details are as in "Materials and Methods."

However, the concentration of protochlorophyllide decreased slightly due to its phototransformation to chlorophyllide. Chloroplasts isolated from ALA-treated plants also contained small amounts of protoporphyrin IX and Mg-protoporphyrin monoester. However, the concentration of protochlorophyllide was 6.5-fold higher (1.62 nmol mg^{-1} protein) than that of control chloroplasts. The concentration of protochlorophyllide decreased by 40% after 15 min of light exposure due to its phototransformation to chlorophyllide and did not decrease further even up to 1 h, suggesting that the rest of the protochlorophyllide was nonphototransformable. The protein content of chloroplasts also remained constant throughout the light treatment. The chlorophyllide synthesized from photoconversion of protochlorophyllide could be metabolized to Chl by the action of Chl synthetase, and get integrated into the thylakoid membrane and participate in the photosynthetic reactions. The energy absorbed by nonphototransformable protochlorophyllide would not transfer energy to the photosynthetic reaction centers. Consequently, the absorbed energy

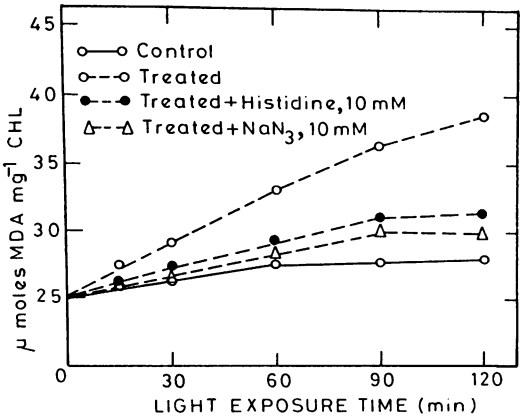

Figure 3. MDA production as a function of duration of the light treatment in chloroplasts incubated in the absence or presence of $^1O_2$ scavengers, NaN_3 and histidine. Other experimental details are as in "Materials and Methods."

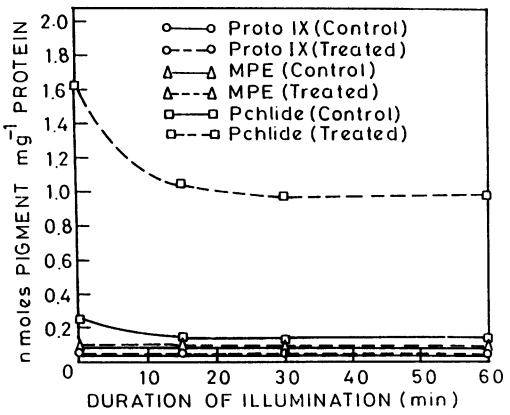

Figure 4. Metabolism of Mg-tetrapyrroles as a function of light exposure time in chloroplasts isolated from control and ALA-treated plants. Other experimental details are as in "Materials and Methods."

could be transferred to molecular O_2, leading to the formation of highly reactive $^1O_2$. Therefore, the nontransformable protochlorophyllide appears to be the most likely candidate responsible for photodynamic damage.

DISCUSSION

We have already shown that in light-exposed ALA-treated plants, the photosynthetic electron transport chain is impaired (20). However, the loss of photosynthetic function in photodynamically damaged plants could be due to secondary effects. As most of the plant tetrapyrroles are localized in chloroplasts, photodynamic reactions photosensitized by ALA-induced overaccumulated tetrapyrroles should start in the chloroplasts, making them the primary site of photodynamic damage. As shown in Figure 1, chloroplasts isolated from ALA-treated plants exposed to light suffer from photodynamic damage. This demonstrates that the photodynamic reactions originate in chloroplasts. The scavengers of $^1O_2$, histidine and NaN_3, protect the thylakoid membrane-linked PSII activity, whereas scavengers of the hydroxyl radical fail to protect the same from photodynamic damage. Similar observations were made in animal tissues in porphyrin-induced photodynamic therapy of cancerous cells, in which only $^1O_2$ scavengers protected the cells from photodynamic damage (5, 6, 10).

The production of $^1O_2$ in ALA-induced photodynamic damage to chloroplasts is shown by bleaching RNO (Fig. 2). The latter is abolished by the $^1O_2$ quencher NaN_3, which suggests a type II photosensitization reaction of excess photoexcited tetrapyrroles with oxygen.

MDA production is an indication of peroxidation of unsaturated membrane lipids. MDA production in chloroplasts isolated from ALA-treated plants is substantially reduced in the presence of $^1O_2$ scavengers such as NaN_3 and histidine (Fig. 3). These confirm that photodynamic damage and consequent membrane lipid peroxidation are mediated by $^1O_2$. Our data suggest that ALA-induced accumulation of nonphototransformable protochlorophyllide is a reasonable candidate for type II photosensitization reactions, consequent production of $^1O_2$, and photodynamic damage. However, this remains to be confirmed by studying the action spectrum of photodynamic damage. If the action spectrum of photodynamic reaction matches the absorption spectrum of protochlorophyllide, it could be concluded that the latter pigment is responsible for photodynamic reactions. In a recent study of the action spectra of photoinhibition of isolated thylakoid membranes exposed to very bright light, the endogenous photosensitizers of $^1O_2$ production were found to be Cyts and iron-sulfur centers of the electron transport chain (13). However, in the present investigation the chromophores responsible for the weak light-induced photodynamic damage, sensitized by accumulated Mg-tetrapyrroles, should be different from Cyts and iron-sulfur centers.

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