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Photosynthetic electron flow to oxygen and diffusion of hydrogen peroxide through the chloroplast envelope via aquaporins☆

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ABSTRACT

Light-induced generation of superoxide radicals and hydrogen peroxide in isolated thylakoids has been studied with a lipophilic spin probe, cyclic hydroxylamine 1-hydroxy-4-isobutyramido-2,2,6,6-tetramethylpiperidinium (TMT-H) to detect superoxide radicals, and the spin trap α-(4-pyridyl-1-oxide)-N-tert-butylnitron (4-POBN) to detect hydrogen peroxide-derived hydroxyl radicals. Accumulation of the radical products of the above reactions has been followed using electron paramagnetic resonance. It is found that the increased production of superoxide radicals and hydrogen peroxide in higher light is due to the enhanced production of these species within the thylakoid membrane, rather than outside the membrane. Fluorescent probe Amplex red, which forms fluorescent product, resorufin, in the reaction with hydrogen peroxide, has been used to detect hydrogen peroxide outside isolated chloroplasts using confocal microscopy. Resorufin fluorescence outside the chloroplasts is found to be suppressed by 60% in the presence of the inhibitor of aquaporins, acetazolamide (AZA), indicating that hydrogen peroxide can diffuse through the chloroplast envelope aquaporins. It is demonstrated that AZA also inhibits carbonic anhydrase activity of the isolated envelope. We put forward a hypothesis that carbonic anhydrase presumably can be attached to the envelope aquaporins. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

1. Introduction

Chloroplasts use energy of sunlight to oxidize H_2O and evolve O_2. Electrons are transferred via the photosynthetic electron transport chain to $NADP^+$, which in its reduced form (NADPH) is used for CO_2 assimilation. An excess of light energy beyond the necessary one to fulfill CO_2 assimilation can be achieved not only under strong light but also under moderate and even low light, as well as under other environmental stress conditions. Additionally to thermal dissipation in antenna, the electron flow from the photosynthetic electron transport chain to molecular oxygen (the so-called Mehler reaction) can eliminate the excess of light energy, thus preventing the chain from photoinhibition (for review see Ref. [1]). Besides, the electron flow to O_2 leads to formation of reactive oxygen species (ROS) such as superoxide anion radical, $O_2^{\bullet-}$, and hydrogen peroxide, H_2O_2. Under normal functional conditions the reduction of molecular oxygen may represent about 5–10% of the total photosynthetic electron flow in C3 plants, however it can increase to 30% and even higher under stress conditions [2,3].

The production of $O_2^{\bullet-}$, the primary product of the Mehler reaction, was repeatedly shown in isolated thylakoids, using specific $O_2^{\bullet-}$ detectors such as cytochrome *c* (cyt *c*), epinephrine [4], ascorbate [5], Tiron [6,7], 5,5-dimethyl-pyrrolin n-oxide (DMPO) [7,8] and 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO) [9]. These compounds are hydrophilic, meaning that they detect $O_2^{\bullet-}$ outside the thylakoid membrane. The possibility of $O_2^{\bullet-}$ production within the thylakoid membrane was proposed by Takahashi and Asada [10], who provided some indirect indications of this process. Recently, the first clear demonstration of $O_2^{\bullet-}$ generation within the thylakoid membrane was presented using cyclic hydroxylamines to detect $O_2^{\bullet-}$ [11]. The evidence supporting the formation of H_2O_2, the secondary product of the Mehler reaction, outside and within the thylakoid membrane was also presented [12]. However, potential capacities of the different formation pathways of the two ROS under various light conditions still remain obscure.

ROS are known to be damaging molecules. However, in the early stages of stress, the ROS that are produced also play a major role in cellular signaling pathways and this is particularly the case with H_2O_2 (for review see Ref. [13]). Initiation of the signaling pathways is obligatory for acclimation of plants to environmental stress

Abbreviations: Amplex Red, 10-acetyl-3,7-dihydroxyphenoxazine; AZA, acetazolamide; CA, carbonic anhydrase; CHA, cyclic hydroxylamine; Chl, chlorophyll; cyt *c*, cytochrome *c*; Gr D, gramicidin D; MV, methyl viologen; POBN, α-(4-pyridyl-1-oxide)-N-tert-butylnitron; PQH_2, plastohydroquinone, plastoquinol; PSI, photosystem I; PSII, photosystem II; ROS, reactive oxygen species; SOD, superoxide dismutase; TMT-H, 1-hydroxy-4-isobutyramido-2,2,6,6-tetramethylpiperidinium

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conditions in order to maintain the main chloroplast function, *i.e.*, photosynthesis (for recent reviews see Refs. [14–17]). H_2O_2 leads to vast changes on the level of gene expression in plants [18–22]. For example, it was shown that H_2O_2 might be the redox signal that activates the expression of cytosolic ascorbate peroxidase [23–25]. The retrograde signaling with involvement of H_2O_2 is likely to be implemented by inducing protein phosphorylation of mitogen-activated protein kinases (MAPKs) [26], which are involved in signaling pathways [27]. The reversible redox modulation of Cys residues of the proteins is most likely to be the mechanism of the H_2O_2-mediated activation of MAPK pathways. Previously we demonstrated that a fraction of H_2O_2 produced in chloroplasts can diffuse from the chloroplasts to cytoplasm [28]. This implies that H_2O_2 can initiate signaling pathways both inside and outside the organelle. The mechanism of H_2O_2 diffusion through the chloroplast envelope however is still under debate.

Aquaporins, *i.e.*, channel proteins, facilitate the transport not only of water but also of small neutral solutes (urea, boric acid, silicic acid) and gasses (NH_3 and CO_2) through the plasma membrane and intracellular membranes of plant cells (for review see Ref. [29]). It has been recently found that the tobacco (*Nicotiana tabacum*) inner chloroplast envelope membrane contains the aquaporin Nt AQP1, which enables CO_2 transport through the membrane [30]. The expression of human and plant aquaporins in yeast revealed that aquaporins facilitated the diffusion of hydrogen peroxide across the membranes [31].

The aims of the present work were i) to study the capacities of the pathways of $O_2^{\bullet-}$ and H_2O_2 generation by the photosynthetic electron transport chain in different locations within the chloroplast, namely in the chloroplast stroma (outside the thylakoid membrane) and within the thylakoid membrane, under different light conditions; ii) to study the role of the chloroplast envelope aquaporins in H_2O_2 diffusion. It is shown that the increase of $O_2^{\bullet-}$ and H_2O_2 production by thylakoids with the increase in the light intensity results from the acceleration of these processes within the thylakoid membrane rather than outside. Further, hydrogen peroxide is shown to diffuse out of isolated chloroplasts through the chloroplast envelope mostly via aquaporins. The evidence on the presence of carbonic anhydrase in the chloroplast envelope is presented, and it is suggested that carbonic anhydrase can be attached to aquaporins.

2. Materials and methods

2.1. Plant material

Spinach plants (*Spinacia oleracea* L.) and pea plants (*Pisum sativum* L.) were grown in the greenhouse with 16 hour day-light period (light intensity 150 μmol quanta $m^{-2}\,s^{-1}$) at 22 °C and 8 hour night period at approximately the same temperature. The experiments with the isolated thylakoids were conducted using 10–14 day old pea plants. When the work was performed with intact chloroplasts, 4–5 week old spinach plants were used.

2.2. Thylakoid preparation

Thylakoids were isolated from pea leaves as described by Khorobrykh and Ivanov [32], and resuspended in the medium containing 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$ and 25 mM HEPES (pH 7.6) and stored on ice. Ascorbate was not included in the isolation medium when thylakoids were used for detection of $O_2^{\bullet-}$ by cyclic hydroxylamine.

2.3. Chloroplast preparation

Chloroplasts were isolated from spinach leaves according to Ref. [33] (centrifugation in a Percoll step gradient) with some modifications. The medium for 40% Percoll contained 3.33 mM EDTA, 1.66 mM $MgCl_2$, 83.3 mM HEPES (pH 7.6) and 0.55 M sorbitol. The medium for 80% Percoll contained 10 mM EDTA, 5 mM $MgCl_2$, 250 mM HEPES (pH 7.6) and 1.65 M sorbitol. Two green bands were separated after Percoll gradient centrifugation. The lower band that corresponded to intact chloroplasts was used. The chloroplasts were washed in resuspension buffer without Percoll. The degree of intactness of chloroplasts was higher than 95% as tested by ferricyanide (1 mM $K_3[Fe(CN)_6]$) photoreduction test [34] based on the ratio of light-driven O_2 evolution measured in intact and osmotically shocked chloroplasts.

2.4. Photosynthetic activity of chloroplasts and thylakoids

Photosynthetic activity of chloroplasts and thylakoids was measured in a temperature-controlled (21 °C) vessel with a Clark-type O_2-electrode. The reaction medium for chloroplasts contained 0.33 M sorbitol, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES (pH 7.6) at 50 μg Chl mL^{-1}. Intact chloroplasts were shocked for 45 s in 5 mM $MgCl_2$ and 25 mM HEPES (pH 7.6). Photosynthetic oxygen evolution in the broken chloroplasts in the presence of $K_3[Fe(CN)_6]$ was 240 ± 38 μmol O_2 mg $Chl^{-1}\,h^{-1}$. 1 μM Gramicidin D (GrD) was added in order to suppress proton gradient. The photosynthetic oxygen evolution rate by intact chloroplasts in the presence of 0.5 mM phosphoglycerate and 4 mM $NaHCO_3$ was 58.7 ± 5.9 μmol O_2 mg $Chl^{-1}\,h^{-1}$.

The reaction medium for thylakoids contained 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES (pH 7.6), 20 μg Chl mL^{-1} and 25 μM methyl viologen (MV).

2.5. Purification of the chloroplast envelope

Purification of the chloroplast envelope from spinach chloroplasts was done as described by Douce and Joyard [35] using sucrose gradient. Sucrose solutions contained 20.5 and 31.8% sucrose (w/w) corresponding to 0.6 M and 0.93 M, respectively, and 10 mM tricine, 4 mM $MgCl_2$, pH 7.8 (at 2 °C). Discontinuous sucrose gradient was prepared in the tubes by carefully layering in succession 12 mL each of 31.8% and 20.5% sucrose solutions. 15 mL of swelling chloroplasts were then layered onto the gradient and the tubes were centrifuged at $72{,}000 \times g$ for 1 h at 3 °C. The chloroplast components were clearly separated giving three distinguishable subfractions: a tightly-packed, dark green pellet at the bottom of the tubes (a thylakoid subfraction); a yellow band at the interface of the two sucrose layers (an envelope membrane subfraction) and a brown supernatant (a soluble subfraction).

2.6. Treatment of chloroplast envelope with acetazolamide

The isolated envelope membranes were diluted and then concentrated again by centrifugation at $12{,}000 \times g$ for 15 min using the centrifugal filter tube (0.5 mL) Microcon YM-10 (Millipore) in order to minimize the sucrose concentration. The obtained solution was divided into two tubes, the content of one was incubated with 1 mM AZA during 30 min. Then both tubes were centrifuged at $12{,}000 \times g$ for 15 min using the same filter, and the supernatants were removed.

2.7. Electron paramagnetic resonance measurements

Electron paramagnetic resonance (EPR) measurements were performed with EMX-6 ESR spectrometer (Bruker, Germany). To detect $O_2^{\bullet-}$, cyclic hydroxylamine (CHA) 1-hydroxy-4-isobutyramido-2,2,6,6-tetramethylpiperidinium (TMT-H) was used. After reaction with superoxide radical, TMT-H forms nitroxide radical ($TMT^{\bullet}$) that can be detected by EPR spectroscopy [36]. The advantage is that the rate constant of TMT-H oxidation by $O_2^{\bullet-}$ is $4.9 \times 10^3\,M^{-1}\,s^{-1}$, which is two orders of magnitude higher than the constants of $O_2^{\bullet-}$

trapping by such established spin traps as DMPO and EMPO [37]. Stock solution of TMT-H (20 mM) in 0.9% NaCl, containing 50 μM desferoxamine and purged with argon, was prepared daily and kept under argon on ice. Desferoxamine was used to decrease autooxidation of CHA catalyzed by trace amounts of iron ions. The samples were placed in 1 mm i.d. glass capillaries and studied at room temperature in ER4102ST microwave cavity with 50% transparent irradiation grid. The $TMT^{\bullet}$ spectra were recorded at room temperature, 9.47 GHz microwave frequency, 100 kHz modulation frequency, 4 G modulation amplitude and 20 mW microwave power. The kinetics of $TMT^{\bullet}$ accumulation were recorded by monitoring the amplitude of the low field component of the $TMT^{\bullet}$ spectrum at the same microwave power and modulation amplitude, the time constant was 0.3 s. To calibrate the spectrometer sensitivity, 10^{-5} M solution of Tempol, stable nitroxide radical, was measured under identical conditions. This calibration was used to calculate the nitroxide concentration. The reaction medium contained 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES (pH 7.6), 50 μM desferoxamine, thylakoids at 100 μg Chl mL^{-1}, 3 mM TMT-H and 1 μM GrD.

To detect H_2O_2-derived hydroxyl radicals, the spin trap α-(4-pyridyl-1-oxide)-N-tert-butylnitron (4-POBN) was used. The addition of 50 μM FeEDTA produced $^{\bullet}OH$ from H_2O_2 via the so-called Fenton reaction. 4% ethanol was added to allow the reaction between the spin trap and H_2O_2-derived hydroxyl radical. The reaction medium contained 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES (pH 7.6), thylakoids at 15 μg Chl mL^{-1} and 1 μM GrD. 50 mM POBN, ethanol and FeEDTA were added immediately after illumination. The spectra were recorded after 5 min of incubation in 1 mm i.d. glass capillaries at room temperature, 9.47 GHz microwave frequency, 100 kHz modulation frequency, 1 G modulation amplitude, 63 mW microwave power and were the average of 2 scans.

Reaction mixtures with chloroplasts or thylakoids were illuminated through the red cut-off filter (λ > 600 nm).

2.8. Measurements of carbonic anhydrase activity

The carbonic anhydrase (CA) activity was assayed with a glass electrode at 2 °C in 13.6 mM veronal buffer (pH 8.4). The reaction was initiated by the addition of water saturated with CO_2 at 0 °C. The time of pH change from 8.1 to 7.6 was measured. The CA activity was calculated taking into account the time of spontaneous hydration that was measured under the same conditions by the addition of the corresponding volume of the medium instead of the sample. The CA activity was expressed in μmol H^+ min^{-1} mg $Protein^{-1}$ after reassessment of the buffer capacities both of the medium and the samples measured by titration with 0.1 N HCl.

2.9. Measurements of protein content

The protein content of the chloroplast envelope was determined according to Lowry et al. [37].

2.10. Observation of H_2O_2 using confocal microscope

H_2O_2 was followed by the AmplexRed fluorescence assay as described in [28], using confocal microscope Leica TCS SPE. The Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in the presence of horseradish peroxidase and forms resorufin, the fluorescent product. Resorufin has a fluorescence emission maximum of 587 nm. Where indicated, 1 mM AZA (in dimethyl sulfoxide) was added. The chloroplast suspension was scanned with 488 nm laser for resorufin excitation and 635 nm laser for chlorophyll excitation. Emission was detected from 540 to 600 nm for resorufin fluorescence and from 640 to 750 nm for chlorophyll fluorescence. Laser powers of the microscope were set to 5% and 10%, respectively, to avoid photobleaching and chlorophyll degradation. The lasers acted as the sources of photosynthetically active light, no additional light source was used.

3. Results

3.1. The effect of light intensity on $O_2^{\bullet-}$ and H_2O_2 production in a thylakoid suspension

To investigate generation of $O_2^{\bullet-}$ and H_2O_2 by the photosynthetic electron transport chain in different locations within the chloroplast, namely outside the membrane (hydrophilic area) and within the thylakoid membrane (lipophilic area), and the capacities of these pathways under different light conditions, isolated thylakoids as a model system were used.

Cyclic hydroxylamines (CHAs) are widely accepted for $O_2^{\bullet-}$ detection in animal cells and tissues (see Ref. [36] and references therein). In the present work, $O_2^{\bullet-}$ in a thylakoid suspension was detected using lipophilic CHA, TMT-H. TMT-H was found to have no effect on the photosynthetic electron transport chain of thylakoids and could not be oxidized by components of the chain, including $P700^+$ [11]. All measurements were conducted in the presence of desferoxamine, the chelator of iron ions, in order to exclude the possible reaction of CHA with H_2O_2-derived hydroxyl radicals [36]. Owing to its rather high lipophilicity (partition coefficient of 35 between octanol and phosphate buffer, pH 7.4), TMT-H was able to enter the thylakoid membrane.

Light-induced accumulation of the nitroxide radical, $TMT^{\bullet}$, was measured in the absence and in the presence of superoxide dismutase (SOD) under 250 and 500 μmol quanta m^{-2} s^{-1} of light (Fig. 1). It was previously shown that 3 mM was the saturating concentration of TMT-H to detect all superoxide radicals produced in a thylakoid suspension, competing efficiently with the reaction of spontaneous dismutation of superoxide radicals [11]. SOD, a water-soluble enzyme, was applied to catalyze the dismutation of superoxide radicals to prevent the reaction of TMT-H with $O_2^{\bullet-}$ outside the thylakoid membrane. Since SOD can adhere to the thylakoid membrane surface [38,39], it provides dismutation of superoxide radicals produced not only in the bulk phase but also in the aqueous regions in the vicinity of the thylakoid membrane. Fig. 1 shows that the increase in the light intensity led to an increase of the $TMT^{\bullet}$ accumulation rate both in the absence and in the presence of SOD; the latter indicated clearly an increase of $O_2^{\bullet-}$ generation within the thylakoid membrane. While total $TMT^{\bullet}$ accumulation in a thylakoid suspension increased appr. 2.5-fold under change of the light intensity from 250 to 500 μmol quanta m^{-2} s^{-1}, the $TMT^{\bullet}$ accumulation in the presence of SOD increased appr. 4-fold (the average of the 5 experiments). It is difficult to estimate the exact capacities of $O_2^{\bullet-}$ generation in lipophilic *vs.* hydrophilic areas, since $TMT^{\bullet}$ formed within the membrane can be partly reduced by components of the electron transport chain [11], and moreover, the "intramembrane" $O_2^{\bullet-}$ can react with PQH_2 (for review see Ref. [40]). Both reactions could accelerate in higher light that might result in underestimation of the actual rate of $O_2^{\bullet-}$ generation within the membrane.

The estimation of H_2O_2 generation in lipophilic *vs.* hydrophilic areas does not have the above complications, the more so that the reaction of $O_2^{\bullet-}$ with PQH_2 produces H_2O_2. Generation of H_2O_2-derived hydroxyl radicals was previously measured by EPR using the spin trap 4-POBN [41]. The addition of catalase suppressed the EPR signal in thylakoids completely, and no signal was obtained in the dark and in the absence of FeEDTA as well [28]. The latter showed that $^{\bullet}OH$ radicals were not generated directly in the light but originated from the H_2O_2 molecules that were accumulated during illumination. Thus, the used approach allows H_2O_2 production to be reliably measured in thylakoids.

Fig. 2 shows the EPR signal of the nitroxide radicals produced in the reaction of 4-POBN with H_2O_2-derived hydroxyl radicals obtained using isolated thylakoids after 3 min of illumination in the absence

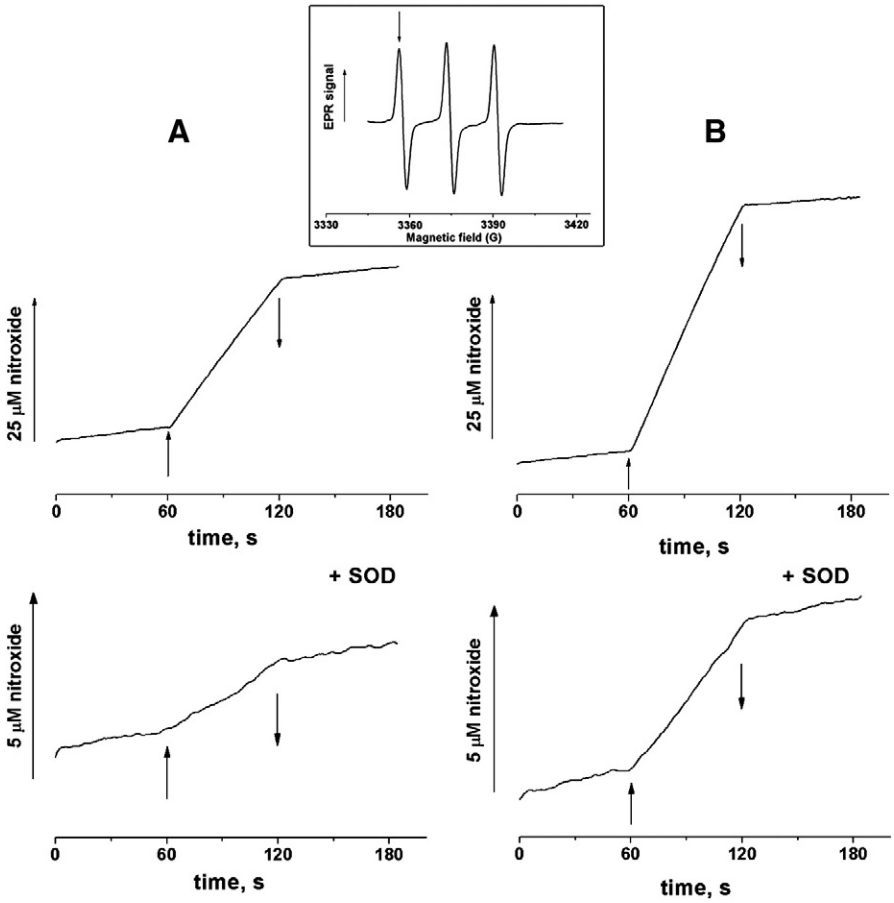

Fig. 1. The effect of light intensity on accumulation of nitroxide radicals produced in the reaction of TMT-H with superoxide radicals in a thylakoid suspension, in the presence and in the absence of superoxide dismutase (SOD). Thylakoids (100 μg Chl mL^{-1}) were illuminated in the presence of 1 μM GrD and 3 mM TMT-H. Where indicated, 100 U mL^{-1} SOD was added. A — 250 μmol quanta m^{-2} s^{-1}, B — 500 μmol quanta m^{-2} s^{-1} of red light (λ > 600 nm). Arrows: on set and off set of light. The inset shows the spectrum of $TMT^{\bullet}$ obtained after illumination of a thylakoid suspension. The arrow indicates the low-field component of the spectrum, which was used for the nitroxide kinetic monitoring.

and in the presence of exogenously added 40 μM cytochrome *c* (cyt *c*). Cyt *c* is an effective trap of $O_2^{\bullet-}$. Being a water-soluble substance, cyt *c* is not able to penetrate inside the membranes. Previously it was found that at pH 7.8 the superoxide-dependent cyt *c* reduction rate (calculated as the difference between cyt *c* reduction rates measured in the absence and in the presence of SOD) saturated at 30 μM concentration of cyt *c* in high light. This demonstrated that 30 μM cyt *c* was enough to trap all superoxide radicals, preventing H_2O_2

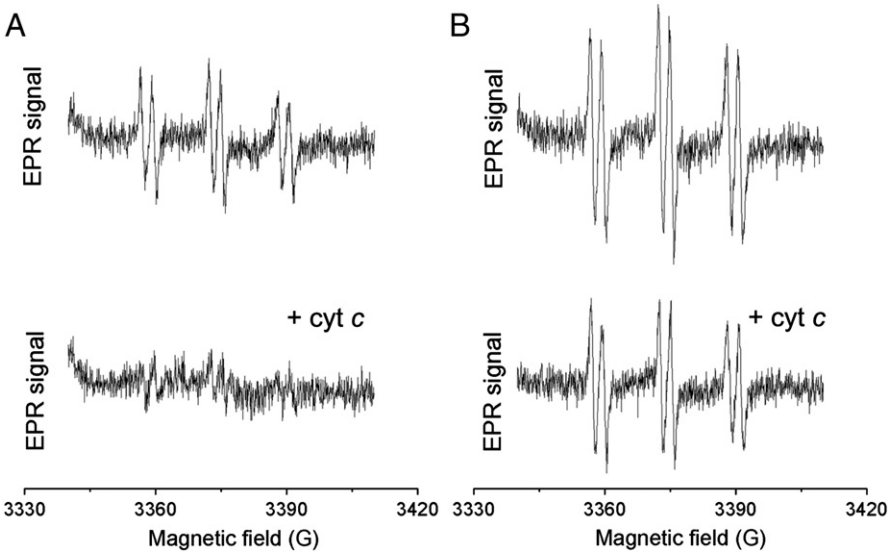

Fig. 2. The effect of light intensity on the signal size of the nitroxide radicals produced in the reaction of 4-POBN with H_2O_2-derived hydroxyl radicals in a thylakoid suspension, in the presence and in the absence of cytochrome *c* (cyt *c*). Thylakoids (15 μg Chl mL^{-1}) were illuminated for 3 min in the presence of 1 μM GrD. Where indicated, 40 μM cyt *c* was added. A — 50 μmol quanta m^{-2} s^{-1}, B — 500 μmol quanta m^{-2} s^{-1} of red light. (λ > 600 nm). The spectra are shown on the same scale.

production outside the membrane [12]. Added cyt *c* can be tightly attached to the membrane [42,43], implying that it can react with $O_2^{\bullet-}$ even in the aqueous regions in the vicinity of the thylakoid membrane.

The EPR signal of the trapped H_2O_2-derived hydroxyl radicals in the absence of cyt *c* (*i.e.*, total H_2O_2 production) after illumination of thylakoids with 500 μmol quanta m^{-2} s^{-1} of light was almost twice as big as that obtained after illumination with 50 μmol quanta m^{-2} s^{-1} of light (Fig. 2). While a very small signal was detected in the presence of cyt *c* after illumination with 50 μmol quanta m^{-2} s^{-1}, a significant signal was obtained after illumination with 500 μmol quanta m^{-2} s^{-1} that means that H_2O_2 production within the thylakoid membrane strongly increases with the increase in the light intensity. It has been estimated that while almost no H_2O_2 was produced within the thylakoid membrane in low light (Fig. 2A), H_2O_2 production in high light within the membrane represented about 50% of total H_2O_2 production by thylakoids (Fig. 2B). It is noteworthy that the signal presented in Fig. 2B in the absence of cyt *c* is almost equal to the sum of the signals presented in Fig. 2A without cyt *c* and Fig. 2B in the presence of cyt *c*. Addition of KCN, the inhibitor of SOD, did not influence the EPR signal amplitude in the presence of cyt *c* (data not shown). This indicated that the H_2O_2 production was not the result of possible dismutation of superoxide radicals, which could be catalyzed by chloroplastic SOD if this SOD attached to the membrane during isolation of thylakoids.

3.2. The role of aquaporins in H_2O_2 appearance outside isolated chloroplasts

Previously we have shown that a fraction of H_2O_2 produced inside chloroplasts can leave the chloroplasts, thus escaping the efficient antioxidant system located in the chloroplast stroma [28]. In the present study the diffusion of H_2O_2 through the chloroplast envelope was investigated using intact spinach chloroplasts in the presence and in the absence of acetazolamide (AZA), which was established to be an efficient inhibitor of aquaporins [44–46]. AZA interacts with guanidyl group of Arg, with the backbone carbonyl of Gly, with the carboxyl of Asp, as well as with Ser, His, Ile and Asn of aquaporins. This leads to blocking H_2O diffusion via aquaporins [44,45]. AZA cannot easily penetrate through the membranes and it has been found that 1 mM AZA did not penetrate through the membranes of isolated protoplasts even after 10 min of incubation [47]. For detection of H_2O_2, the Amplex Red reagent was used (see Materials and methods). It was checked that without any chloroplasts AZA had no effect on the resorufin fluorescence, neither in the presence nor in the absence of added H_2O_2 (not shown). Methyl viologen (MV) was added in all the cases as an efficient acceptor of electrons from Photosystem I (PSI). MV transfers electrons to O_2 leading to an increase of H_2O_2 generation (Table 1, see also Ref. [48]).

As shown in Fig. 3, no resorufin fluorescence was detected without illumination. Strong resorufin fluorescence was observed outside the chloroplasts after 3 min of illumination, showing that a significant amount of H_2O_2 diffused out of the chloroplasts. In the presence of 1 mM AZA the level of resorufin fluorescence was much lower. Fig. 4 shows that the fluorescence of resorufin outside the chloroplasts after 3.5 min of illumination was approximately 60% lower in the presence of AZA than in the absence of AZA. In the presence of MV the Calvin cycle was not able to operate, thus the effect of AZA on the resorufin fluorescence was obviously not a result of the Calvin cycle disruption via inhibition of carbonic anhydrase (CA) by AZA. Addition of AZA had almost no effect on the photosynthetic electron transport rate of isolated thylakoids in the presence of MV and, hence, H_2O_2 production; a small inhibitory effect was detected in the presence of both MV and Gr D (Table 1). In the case of intact chloroplasts even such small inhibitory effect of AZA on H_2O_2 production would be minimal since AZA could not easily penetrate through the membranes and react with the components of the photosynthetic electron transport chain [47]. Moreover, Gr D was excluded from the reaction medium when isolated chloroplasts were used. Therefore data demonstrating the inhibition of the resorufin fluorescence by AZA outside isolated chloroplasts indicated that H_2O_2 diffused out of the chloroplasts through the channels formed by aquaporin proteins in the chloroplast envelope.

Table 1
The influence of AZA on the rate of oxygen uptake in the light (500 μmol quanta m^{-2} s^{-1}) in isolated pea thylakoids in the absence and in the presence of Gr D. MV (25 μM) was used in all the cases. Where indicated, 1 μM Gr D and 1 mM AZA were added.

Additions	O_2 uptake, μmol O_2 mg Chl^{-1} h^{-1}
Thylakoids + MV	37.9 ± 4.1
Thylakoids + MV + Gr D	191.2 ± 8.4
Thylakoids + MV + AZA	35.4 ± 2.7
Thylakoids + MV + Gr D + AZA	131.4 ± 2.2

3.3. Binding of acetazolamide to the chloroplast envelope

In order to study whether AZA can be attached to any of the chloroplast envelope components, the effect of AZA incubation with the envelope isolated from spinach chloroplasts on the activity of the exogenously added bovine CA was examined. AZA is a non-covalently binding inhibitor of CAs, and can easily leave its adhesion site after dilution. Bovine CA is an enzyme, which belongs to the so-called alpha family of CA and has high sensitivity to the inhibitor [49]. A very small decrease of the bovine CA activity was observed in the presence of untreated chloroplast envelope (Table 2), and this was probably the result of recalculation of the CA activity per protein since the quantity of proteins increased in the presence of the envelope preparations. The activity of bovine CA was almost completely inhibited in the presence of the chloroplast envelope treated with AZA (Table 2). This implied that after incubation of the envelope membrane with AZA, the inhibitor evidently combined with the envelope preparations.

We have found that the envelope membrane itself possesses CA activity (Table 2). These data could evidence the presence of CA associated with the chloroplast envelope. Upon incubation with AZA, the CA activity of the envelope was inhibited by 57%. This pointed out that AZA could be attached to the chloroplast envelope CA.

4. Discussion

In this study we have presented evidence that $O_2^{\bullet-}$ and H_2O_2 can be generated by the electron carriers of the photosynthetic electron transport chain in the light not only outside the thylakoid membrane (hydrophilic area, *i.e.*, the stroma *in vivo*) but also within the thylakoid membrane (lipophilic area) (Figs. 1 and 2). The data obtained with lipophilic $O_2^{\bullet-}$ detector, TMT-H, (Fig. 1) indicated that in higher light "intramembrane" $O_2^{\bullet-}$ generation preferentially increased as compared to the "extramembrane" one. Using the spectra presented in Fig. 2 it is possible to estimate that "intramembrane" production of H_2O_2 (measured in the presence of cyt *c*) increases approximately 3 times greater than H_2O_2 production outside thylakoids (calculated as the difference between the signals in the absence and in the presence of cyt *c*) with the increase in the light intensity. Thus, an increase of total H_2O_2 production by the photosynthetic electron transport chain in higher light resulted mostly from the increased production of H_2O_2 within the thylakoid membrane. This conclusion is in agreement with the assumption made in Ref. [12], where the rates of total oxygen reduction in thylakoids and cyt *c* reduction were compared at different light intensities.

Taking into account the position of electron transport chain carriers in the thylakoid membrane, their properties and the literature data, one may consider that F_A and F_B in PSI and plastosemiquinone molecules in the PQ-pool reduce O_2 to $O_2^{\bullet-}$ close to the membrane

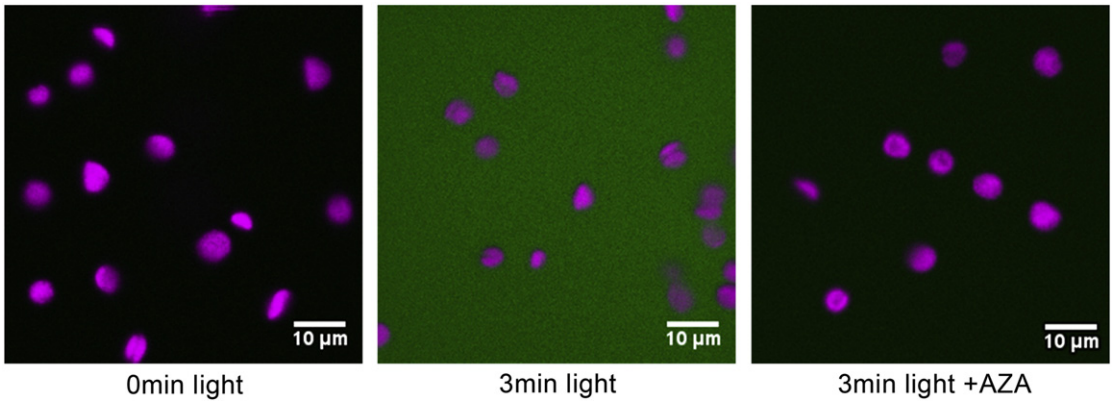

Fig. 3. The effect of acetazolamide (AZA) on the resorufin fluorescence produced in the reaction of Amplex Red with H_2O_2 in a chloroplast suspension. Chloroplasts were illuminated during 3 min in the presence of 25 μM methyl viologen (MV). Magenta color — chlorophyll fluorescence, green color — resorufin fluorescence. Where indicated, 1 mM AZA was added before illumination.

surface (for review see Ref. [40]); while A_1 and F_X in PSI generate $O_2^{\bullet-}$ more deeply in the membrane [50], (for review see also Ref. [40]). In the former case the superoxide radicals leave the membrane quite readily, dismutate and produce hydrogen peroxide outside the thylakoid membrane. The superoxide radicals generated deeply in the membrane can, at least partly, move out of the thylakoid membrane as well. Thus, the measured ratio of the "extramembrane" to the "intramembrane" $O_2^{\bullet-}$ depends on the properties of the used traps, namely their lipophilicity, the rate constant of the reaction with $O_2^{\bullet-}$, the product stability and so on. In both cases superoxide radicals can be reduced by plastoquinol (PQH_2) situated predominantly near the membrane boundary [51], producing H_2O_2 within the thylakoid membrane. Obviously, the superoxide radicals generated deeply within the thylakoid membrane have more chances to be engaged in this reaction. Owing to the higher solubility in water, H_2O_2 molecules formed by this way leave the membrane and are detected using POBN outside the membrane together with those formed by dismutation of superoxides. In order to prevent the latter reaction, cyt *c* was used in the experiments presented in Fig. 2.

It was shown that oxygen reduction in thylakoids was significant even in the presence of both $NADP^+$ and ferredoxin [52]. Ferredoxin-dependent oxygen reduction was saturated at lower light intensities compared to the rate of total oxygen reduction. This reveals that enhanced oxygen reduction in high light is executed by the membrane-bound carriers of the photosynthetic electron transport chain in the presence of the efficient electron acceptor as well. As stated above, in high light both $O_2^{\bullet-}$ and H_2O_2 are generated by these carriers mostly within the thylakoid membrane.

The change of the preferential places of these ROS generation can be essential for plant acclimation to the fluctuations in light intensity, since the ways of ROS production are important for executing the signaling function by ROS [14]. Probably, ROS produced in different locations within the chloroplast can initiate different signaling pathways. The signaling network in the chloroplast stroma is widely studied, and many of its characteristics become clear. H_2O_2 in the chloroplast stroma can interact with certain sensors that trigger the organelle-specific retrograde signaling in order to regulate the expression of the nuclear genes encoding the chloroplast components [14]. Some of signal sensors such as thylakoid protein kinases [53] are associated with the thylakoid membrane, and the signaling pathway initiated by such kinases may involve H_2O_2 produced within the membrane.

We have shown that H_2O_2 produced inside chloroplasts appeared outside the chloroplasts (Fig. 3, see also Ref. [28]); in this case H_2O_2 can interact with the sensors involved in the integrated signaling network of the whole cell. The very efficient system of H_2O_2 scavenging in the chloroplast stroma gives a limited possibility for H_2O_2 to leave chloroplasts, and the fraction of H_2O_2, which diffuses out of chloroplasts is not high [28]. However, the flow of H_2O_2 from the chloroplast stroma to cytoplasm should increase under stress conditions in the case of inefficient operation of the stromal antioxidant system (see Ref. [28] for H_2O_2 diffusion out of chloroplasts when ascorbate–ascorbate peroxidase system does not operate). The result of such increased H_2O_2 outflow should differ from the one provided by another signaling pathway, which could possibly involve H_2O_2 produced within the membrane.

The next question concerns the mechanism of H_2O_2 diffusion through the chloroplast envelope. Mercury, silver and gold compounds are well-known aquaporin inhibitors that work due to covalent modification of cysteine residues within the water pore. However, plant

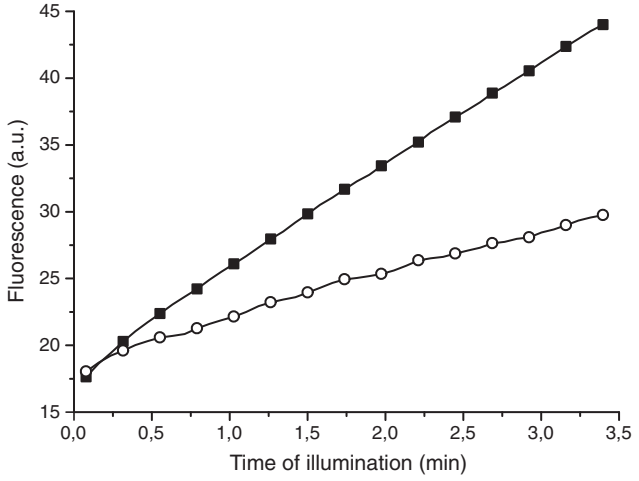

Fig. 4. Light-induced changes of the resorufin fluorescence outside chloroplasts in the absence (squares) and the presence (circles) of 1 mM AZA. The conditions are the same as in Fig. 3. The levels of the resorufin fluorescence outside the chloroplasts were calculated as average fluorescence in the pixels, which did not display chlorophyll fluorescence.

Table 2
The influence of the chloroplast envelope incubation with AZA on the bovine CA activity and the envelope CA activity (see Materials and methods). Where indicated, 0.0011 mg of the chloroplast envelope proteins and 0.004 mg of the bovine CA proteins were used.

Additions	CA activity, μmol H^+ min^{-1} mg $Protein^{-1}$
Bovine CA	7963 ± 294
Bovine CA + envelope	6127 ± 0
Bovine CA + envelope/AZA	248 ± 5
Envelope	1216 ± 252
Envelope/AZA	523 ± 288

aquaporins do not have Cys residues at conserved positions, thus other residues may be involved in inhibition of the plant aquaporins; that is the reason why the above-mentioned inhibitors do not affect the plant aquaporins [54]. Furthermore, they inhibit the Calvin cycle enzymes. We have found that the photosynthetic activity of chloroplasts was completely inhibited by $AgNO_3$ at rather low concentration of 20 μM (data not shown). AZA is established to be a CA inhibitor, which blocks efficiently aquaporin channel, suppressing H_2O diffusion via aquaporins [44,45]. Here using AZA, we have shown that H_2O_2 can diffuse out of chloroplasts through the envelope membrane aquaporins as well (Fig. 5A). In the experiments with the isolated envelope membranes of spinach chloroplasts we demonstrated that AZA was indeed attached to the chloroplast envelope components (Table 2).

It was shown that photoinhibition of PSII and PSI occurred in cyanobacteria cells with blocked aquaporins [55]. It is known that photoinhibition of PSII can be mediated by ROS [56,57]. We suggest that the inhibition of the activities the both photosystems observed in Ref. [55] could be mediated by H_2O_2, which accumulated in high concentration inside the cell as a result of blocking the aquaporins.

In the course of the present work we have demonstrated the presence of the CA activity associated with the chloroplast envelope (Table 2). This is in agreement with the data presented in Ref. [58], where the CA activity was detected in chloroplast envelope membranes isolated from *Chlamydomonas reinhardtii*. CA in animal cells represents a part of the functional protein macro complexes, the so-called metabolones, consisting of the transport and other proteins, and aquaporins can be a part of the metabolones [59]. It has also been shown that CA is bound to transport channels both at the inner and the outer sides facilitating diffusion of CO_2 through the mammal plasma membrane [60]. Moreover, the transcript levels of aquaporin genes and CA genes depend on each other [61], and these genes are expressed in the same tissues [62]. A close relationship of the biological characteristics of CA and aquaporins was proposed in Ref. [63]. It was recently demonstrated that CA operation protects the cells from H_2O_2-induced apoptosis [64]. Taking into account the presence of CA in the chloroplast envelope (Table 2, see also Ref. [58]) and that the chloroplast envelope aquaporins facilitate CO_2 diffusion through the envelope [30], we suggest that CA in the chloroplast envelope can be combined with the aquaporins. In this case the inhibitory effect of AZA on the H_2O_2 diffusion may be a possible result of AZA binding with CA: the changed conformation of CA could induce conformational changes of aquaporin proteins, leading to blocking the aquaporins (Fig. 5B).

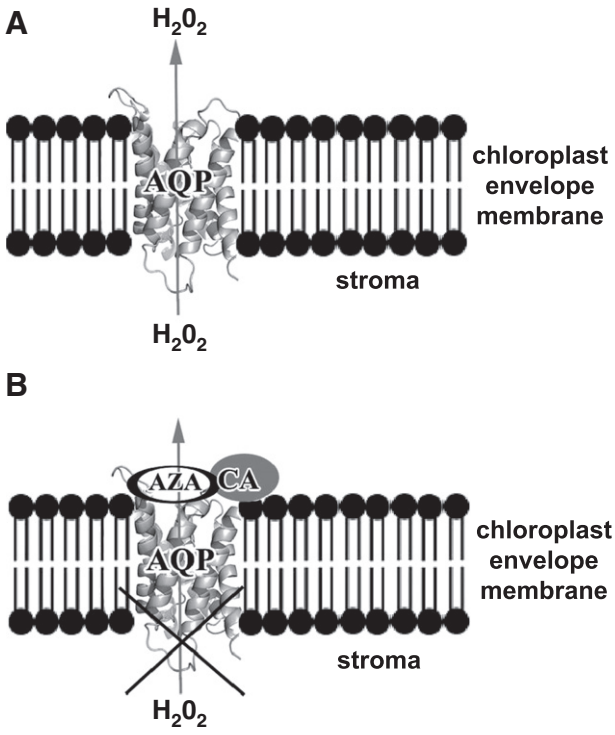

Fig. 5. The scheme of H_2O_2 diffusion through the chloroplast envelope aquaporin (AQP) (A); and the hypothetical scheme of the inhibition of H_2O_2 diffusion via AQP by acetazolamide (AZA) (B).

In this study the obtained results support the assumptions, which were made in our earlier works [11,12] on generation of $O_2^{\cdot-}$ and H_2O_2 by the electron transport chain components within the thylakoid membrane. It is important to note that the "intramembrane" production of the ROS increases more significantly that the "extramembrane" production under strong light. The new findings give the insight into the potential places of signaling action of these ROS. Owing to low partition coefficient of H_2O_2 between octanol and water, and much lower one between benzene and water [65], H_2O_2 molecules cannot readily cross the biological membranes. Thus, it seems logical for the aquaporins in the chloroplast envelope to fulfill the role of the channels for H_2O_2 diffusion. Such mechanism of H_2O_2 diffusion through the chloroplast envelope can obviously permit to control efficiently the H_2O_2 flow. It is possible that CA, which is attached to aquaporins of the chloroplast envelope, may be involved somehow in such control.

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