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Impact of High Light on Reactive Oxygen Species Production within Photosynthetic Biological Membranes

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Abstract

In this study we describe the mechanisms of reactive oxygen species (ROS) production in the photosynthetic electron transport chain of higher plants chloroplasts under illumination. We implement an improved method for the measurement of hydrogen peroxide (H_2O_2) production in lipid phase of photosynthetic membranes of chloroplasts. Total rate of H_2O_2 production and the production within the thylakoid membrane under operation of photosynthetic electron transport chain is evaluated. Obtained data show that even in the presence of an efficient electron acceptor, methyl viologen, an increase in light intensity leads to an increase in H_2O_2 production mainly within the thylakoid membranes. The role of H_2O_2 produced within the photosynthetic biological membrane is discussed.

Keywords: Higher plants, Photosynthesis, Thylakoid membrane, Light intensity, Hydrogen peroxide

1. Introduction

Biological membranes are the inherent structures of living cells since a lot of biochemical processes proceed with the membrane components involvement. Such vital assemblies as a respiratory electron-transport chain and a photosynthetic electron-transport chain are embedded into membranes of different organelles. In higher plants the primary reactions of photosynthesis occur in the thylakoid membranes of chloroplasts and are catalyzed by pigment-protein complexes of the photosynthetic electron-transport chain: photosystem II (PSII) and photosystem I (PSI). Pigments of photosystems capture and transfer light energy to the primary electron donors of the PSII and PSI reaction centers for subsequent photochemical reactions. PSII and PSI are surrounded by the light-harvesting antenna complexes. The electron flow between PS II and PSI is mediated by the plastoquinone pool, cytochrome b_6/f-complex and plastocyanin.

During photosynthesis molecular oxygen (O_2, dioxygen) is evolved in water oxidizing complex associated with PSII. However, the photosynthetic electron transport chain components are also known to reduce the molecular oxygen. As a result of the reduction of O_2 molecules, reactive oxygen species (ROS) are formed. The primary product of dioxygen reduction is superoxide radical, $O_2^{\bullet -}$, which is reduced to hydrogen peroxide via various pathways. In aqueous part of chloroplasts (stroma) two superoxide radicals produce hydrogen peroxide via reaction of dismutation catalyzed by superoxide dismutase.

Chloroplasts are one of the main sources of ROS in plants when exposed to light (Mubarakshina et al., 2010). It was previously found that an increase in light intensities results in an increase in both superoxide radical and hydrogen peroxide (H_2O_2) production by the photosynthetic electron-transport chain (Ivanov et al., 2012). It has been discovered that the total increase in $O_2^{\bullet -}$ and H_2O_2 production resulted from the increase in the processes within the thylakoid membrane, where the electron-transport chain components are located rather than in stroma (Mubarakshina et al., 2006). In particular, the direct evidence about the hydrogen peroxide production within the thylakoid membrane has been provided in (Borisova-Mubarakshina et al., 2012). Production of superoxide radical within the membrane

has recently been proved in (Kozuleva et al., 2011; Kozuleva et al., 2014). The membrane superoxide radical, as stated, is converted into H_2O_2 via the reaction with plastohydroquinone, reduced plastoquinone (Mubarakshina et al., 2006; Mubarakshina, Ivanov, 2010). This reaction can be considered as a neutralization of superoxide radical, the protonated form of which, perhydroxyl radical, is known to be a strong oxidizing molecule leading to lipid peroxidation of the membrane (Møller et al., 2007).

The production of hydrogen peroxide within the thylakoid membrane was previously shown using isolated thylakoids when oxygen was the only acceptor of electrons from the photosynthetic electron-transport chain. In this context, the aim of the present work is to study the possible production of hydrogen peroxide within the membrane in the presence of an efficient electron acceptor methyl viologen that simulates the physiologically relevant conditions in term of electron transport rate. Moreover, the effect of light intensity on the H_2O_2 formation within the thylakoid lipid bilayer phase has been evaluated.

2. Methodology

2.1 Isolation of Thylakoids

Thylakoids were isolated from the 10–14 old leaves of pea plants grown in a greenhouse under natural light, as described in (Mubarakshina et al., 2006). Chlorophyll content was determined in 95% ethanol extract according to (Lichtenthaler, 1987).

2.2. Measurement of Cytochrome C Reduction Rate and the Rate of Oxygen Concentration Changing in the Thylakoid Suspension

Reduction of cytochrome *c* (cyt *c*) by thylakoids was measured as an increase in absorbance at 550 nm with a reference at 540 nm, using a dual-wavelength spectrophotometer (Hitachi 557, Japan). The photomultiplier was shielded from actinic light with a blue-green filter SZS-22 (Russia). Differential molar extinction coefficient of ferri-/ferro-cyt *c* used was 19 mM^{-1} cm^{-1} (Davis and San Pietro, 1977). Superoxide-dependent rate of reduction of cyt *c* was calculated as the difference between the rate of reduction of cyt *c* in the absence of SOD (superoxide dismutase – a water-soluble enzyme that catalyzes the dismutation reaction of superoxide radicals to H_2O_2 and O_2) and the rate of reduction of cyt *c* in the presence of SOD. The rate of oxygen evolution/uptake was measured in a thermostatic glass cell with a volume of 0.3 mL at 21 ℃ using Clark-type pO_2-electrode connected to the computer via ADC. The reaction mixtures in both the pO_2-electrode vessel and the spectrophotometric cuvette were illuminated with red light. Light intensity was varied using neutral filters and was measured as photon flux density, μmol quanta m^{-2} s^{-1}, using a Li-Cor quantum meter. The source of light for the pO_2-electrode vessel was red LED (Epistar, 660 nm), and light from halogen lamp for the spectrophotometric cuvette was transmitted through a red cut-off filter ($\lambda > 620$ nm). The basic reaction mixture contained 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 50 mM HEPES-KOH (pH 7.6), and thylakoids corresponding to 10 μg Chl $(mL)^{-1}$.

3. Results and Discussion

3.1. The Influence of MV on the Electron Transport Rate at Different Light Intensities

As described in the Introduction, hydrogen peroxide may be produced in aqueous phase, i.e. outside the thylakoid membrane as well as within the thylakoid membrane (Mubarakshina et al., 2006). The aim of this work was to study the hydrogen peroxide production within the thylakoid membrane in the presence of an efficient electron acceptor, which can provide high electron transport rates relatively close to physiological ones. Unfortunately, it was not possible to apply ferredoxin (Fd), the native electron acceptor from PSI, since it reduces with a high efficiency (Asada et al., 1974) cytochrome *c* (cyt *c*), which is used to prevent the hydrogen peroxide production in aqueous phase (see part 3.2). This circumstance does not permit to provide a saturating concentration of cyt *c* for the prevention of hydrogen peroxide production in the presence of Fd. Therefore, another efficient electron acceptor methyl viologen (MV), which accepts electrons from F_A/F_B components of PSI and transfers them to oxygen (k = 8×10^8 M^{-1} s^{-1}), producing superoxide radicals, was used. Km (MV) is equal to 3 μM (Ivanov et al., 1980). In order to reach a saturating concentration of cyt *c* in the presence of MV, lower concentration, 1 μM, was used.

Molecular oxygen is evolved in water-oxidizing complex associated with PSII. When oxygen is the final acceptor, that is a case when either no additional acceptors or MV is used, the rate of oxygen uptake owing to oxygen reduction overcomes the rate of oxygen evolution in PSII. Therefore the oxygen uptake is observed. To investigate the influence of 1 μM MV on the photosynthetic electron transport rate, the oxygen uptake rate was measured using thylakoid suspension. Two light intensities 25 μmol quanta $m^{-2}s^{-1}$ and 180 μmol quanta $m^{-2}s^{-1}$ of red light have been implemented.

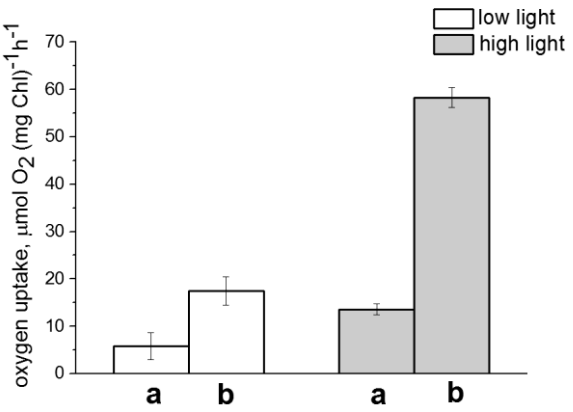

Figure 1. Effect of light intensity on the oxygen uptake rate in the absence (a) and presence (b) of 1 μM MV. White columns – light intensity of red light 25 μmol quanta $m^{-2}s^{-1}$, grey columns – light intensity of red light 180 μmol quanta $m^{-2}s^{-1}$.

Values are presented as mean ± SE of three repetitions. Three independent experiments show the same tendency.

It can be seen that the oxygen uptake rate, which reflects the total electron transport rate,

increases significantly in the presence of 1 μM MV (Fig. 1). Furthermore in the presence of MV the oxygen uptake rate was much higher at light intensity of 180 μmol quanta $m^{-2}s^{-1}$ than at 25 μmol quanta $m^{-2}s^{-1}$.

The rate of electron transport along photosynthetic electron-transport chain can be estimated from the rate of oxygen uptake according to known stoichiometry: $4e^-$ per $1O_2$ consumed (Allen, Hall, 1973). As calculated, the electron transport rate measured in the presence of MV at 180 μmol quanta $m^{-2}s^{-1}$ was appr. 240 μequivalents e^- $(mg\ Chl)^{-1}$ h^{-1}. This is less than the maximal physiological rates, however it is similar to that observed under some stress conditions, e.g. at limited CO_2 supply due to closed stomata (Vico et al., 2013). Therefore it can be concluded that the conditions relatively close to physiological ones in term of electron transport rate can be reached using MV and high light intensity.

3.2. Determination of the Saturating Concentration of Cyt C

Formation of hydrogen peroxide within the thylakoid membrane was studied using cyt *c*, a water-soluble protein, which acts as a scavenger of superoxide radicals oxidizing them to O_2. Unable to penetrate into the thylakoid membrane, cyt *c* interacts with superoxide radicals outside the thylakoid membrane, preventing formation of hydrogen peroxide there. In order to trap all superoxide radicals produced outside the thylakoid membrane cyt *c* should be added in sufficient amount (saturating concentration). Moreover it is important to establish the saturating concentration of cyt *c* for superoxide-dependent reduction rate since it is known that cyt *c* can be reduced not only by superoxide radicals, but also by components of the photosynthetic electron transport chain of thylakoids (Kruk et al., 2003). For that reason the cyt *c* reduction rates in the absence and presence of superoxide dismutase (SOD) have been measured (Fig. 2).

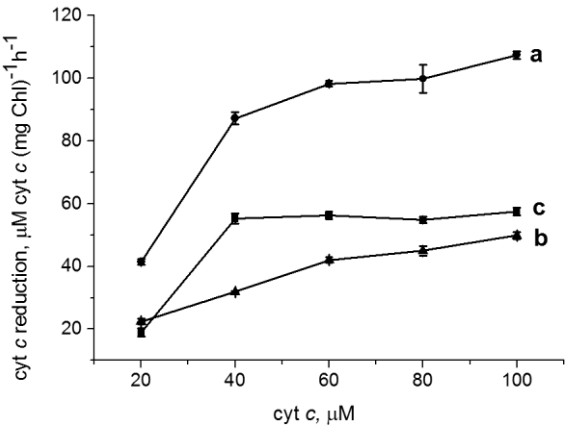

Figure 2. The dependence of cyt c reduction rate in the light on the cyt *c* concentration.
a – cyt *c* reduction rate in the absence of SOD (superoxide dismutase);
b – cyt *c* reduction rate in the presence of SOD;
c – superoxide-dependent cyt *c* reduction rate calculated as a difference between (a) and (b).
Light intensity 180 μmol quanta $m^{-2}s^{-1}$ of red light.

Values are presented as mean ± SE of three repetitions. Four independent experiments show the

same tendency.

Experiments to determine the saturating concentration for the superoxide-dependent cyt *c* reduction rate as a difference between (a) and (b) in Fig. 2 revealed that in the presence of 1 μM MV the saturation of the rate was reached at cyt *c* concentration of 40 μM (Fig. 2c). This means that at this concentration cyt *c* scavenges all superoxide radicals produced outside the thylakoid membrane, inhibiting the production of H_2O_2 in the aqueous part. Similar concentration was found previously when no efficient electron acceptors were used (Mubarakshina et al., 2006).

3.3. The Effect of Cyt C Addition on Oxygen Evolution Rate in the Presence of MV at Different Light Intensities

In the presence of cyt *c*, which is both an additional non-autooxidable acceptor and an oxidant of superoxide radicals, the rate of oxygen uptake owing to oxygen reduction does not overcome the rate of oxygen evolving by the system. Therefore the addition of cyt *c* into thylakoid suspension results in oxygen evolution instead of oxygen uptake (Fig. 3 *vs* Fig. 1).

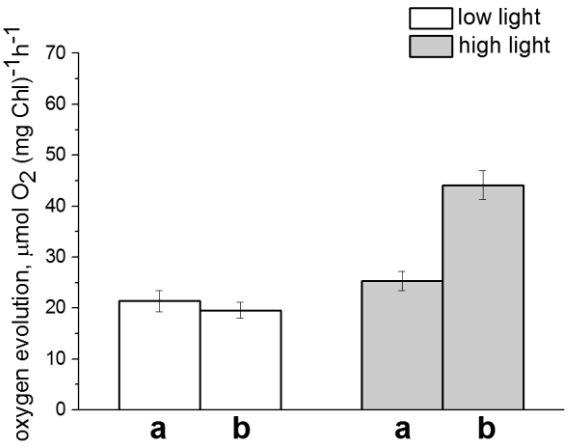

Figure 3. Effect of light intensity on the oxygen evolution rate in the absence (a) and presence (b) of 100 U mL^{-1} catalase. The reaction medium contained 1 μM MV and 60 μM cyt *c*. White columns – light intensity of red light 25 μmol quanta $m^{-2}s^{-1}$, grey columns – light intensity of red light 180 μmol quanta $m^{-2}s^{-1}$.

Values are presented as mean ± SE of three repetitions. Three independent experiments show the same tendency.

To answer the question, whether hydrogen peroxide is formed within the thylakoid membrane even in the presence of MV, the oxygen evolution rate in the thylakoid suspension in the absence and presence of catalase was measured using cyt *c* at saturating concentration (Fig. 3). Catalase – an enzyme decomposing hydrogen peroxide to form molecular oxygen and water. If an additional increase in the oxygen evolution rate can be observed under these conditions when catalase is added, it indicates the formation of H_2O_2 production within the membrane since cyt *c* prevents the formation of H_2O_2 outside the membrane.

Fig. 3 shows that at 25 μmol quanta $m^{-2}s^{-1}$ the addition of catalase had no effect on oxygen

evolution. The addition of catalase at 180 μmol quanta $m^{-2}s^{-1}$ resulted in a noticeable increase in the oxygen evolution rate. Thus under high light conditions even in the presence of MV, H_2O_2 is formed within the thylakoid membrane. The difference between oxygen evolution rate in the presence and absence of catalase, presented in Fig. 3, can be used to estimate the amount of H_2O_2 formed within the thylakoid membrane.

The stoichiometry of H_2O_2 decomposition by catalase gives one molecule of O_2 from two molecules of H_2O_2. In high light the rate of H_2O_2 production within the thylakoid membrane is estimated to be 38±4 μmoles H_2O_2 mg Chl^{-1} h^{-1} (Fig. 3). If the rate of H_2O_2 production in the absence of cyt *c* in Fig. 1 (it is equal to the oxygen uptake rate multiplied by two) would be taken as 100%, then it can be estimated that the fraction of H_2O_2 production within the thylakoid membrane reaches 30%.

3.4. The Pathways of Hydrogen Peroxide Formation in the Absence and Presence of MV

In the absence of either MV or other acceptor, such as Fd, the terminal cofactors of PSI, F_A/F_B, reduce O_2 to superoxide radical. F_A/F_B are situated at PSI subunit PsaC, which is exposed to stroma. Together with PsaD and PsaE subunits they form a docking site for a water-soluble protein Fd. Based on this structure, one may propose that O_2 reduction by F_A/F_B in the absence of MV leads to $O_2^{\bullet-}$ appearance outside the membrane. Further these superoxide radicals are converted to H_2O_2 via dismutation reaction if cyt *c* is absent (pathway 1 of H_2O_2 production; Figure 4A). It cannot be excluded that a small portion of $O_2^{\bullet-}$ may dismutate to H_2O_2 in the close vicinity of the thylakoid membrane surface without being trapped by cyt *c* even if cyt *c* is added (pathway 2 of H_2O_2 production; Figure 4A).

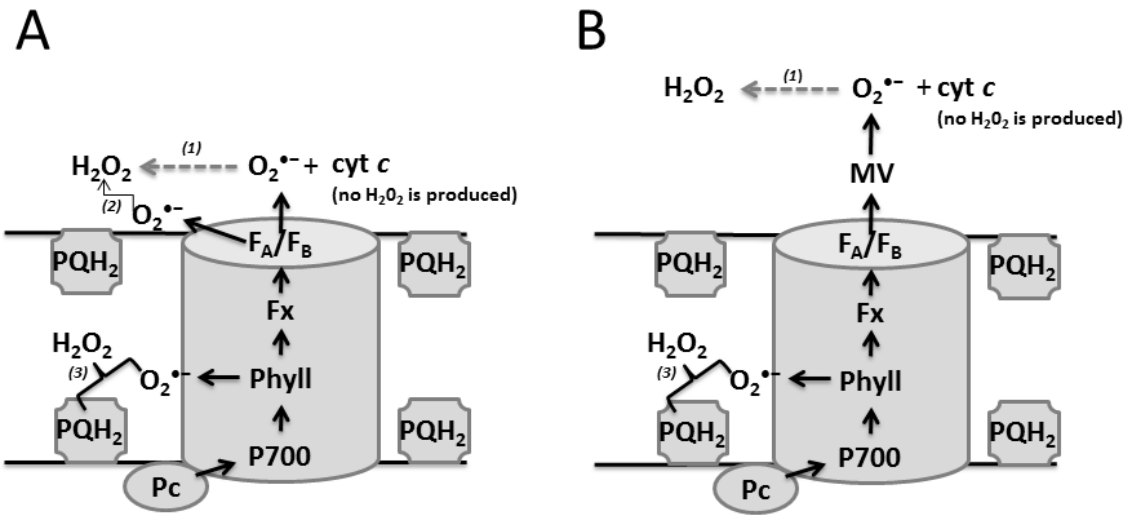

Figure 4. The tentative scheme of the electron transfer and H_2O_2 production pathways in PSI in the absence (A) and presence of MV (B). Black arrows – the electron transfer pathways. Dashed arrows – the pathway of H_2O_2 production in the absence of cyt *c*.

P700 – reaction center of PSI; F_X, [4Fe-4S] cluster, and Phyll, phylloquinone, – intermediate cofactors of electron transfer in PSI; F_A and F_B – the terminal cofactors of electron transfer; PC, plastocyanin, – a donor of electrons for PSI; PQH_2, plastohydroquinone.

Another source of superoxide radicals outside the membrane could be the reaction of O_2 with plastosemiquinone of the plastoquinone pool at the membrane–water interface (not shown) (Mubarakshina, Ivanov, 2010). The source of superoxide radicals detected within the membrane (Kozuleva et al., 2011) is still under debate (Kozuleva et al., 2014). Both pheophytin, the primary electron acceptor in PSII, and phylloquinone, a secondary electron acceptor in PSI, possess negative midpoint potentials that enables O_2 reduction in the membrane where Em ($O_2/O_2^{\bullet-}$) is close to –500 ÷ –600 mV (Afanas'ev, 1989). Phyllosemiquinone in PSI shows a longer life-time than reduced pheophytin in PSII, thus phyllosemiquinone seems to be more probable candidate for superoxide radical production within the membrane (Em values (Phyll/Phyll$^{\bullet-}$) in A_{1A-} and A_{1B-} sites are –671 ÷ –844 mV, respectively (Ptushenko et al., 2008)). The role of phylloquinone in O_2 reduction was shown recently (Kozuleva et al., 2014, see Introduction).

The pathway 3 of H_2O_2 production occurs within the thylakoid membrane via reaction of $O_2^{\bullet-}$ with plastohydroquinone, the fully reduced plastoquinone (PQH_2) (Figure 4A, B; see Introduction). It is worth noting that under continuous light conditions plastohydroquinone spreads along the entire membrane, and the quantity of plastohydroquinone is quite high. Therefore $O_2^{\bullet-}$ produced deeply within the membrane by phyllosemiquinone has a high probability to reach the plastohydroquinone molecule.

The advantage of MV usage, except the providing the higher electron transport rate, is that the addition of MV partly or significantly diminishes the pathway 2 of H_2O_2 production. As a result, hydrogen peroxide is mainly produced within the membrane by the pathway 3 (Figure 4B). In the present study we provide data showing the hydrogen peroxide production within the thylakoid membrane in the presence of an efficient electron acceptor.

4. Conclusion and Perspectives

The main question that arises: what role H_2O_2 formed within the thylakoid lipid bilayer can play? We propose that the H_2O_2 may be a signal molecule, which triggers the acclamatory changes at the level of thylakoid membrane. For example, it is known that under the changes in the spectral composition of light, light-harvesting complexes of thylakoid membranes can be reorganized, this process was named "state transition". The outer part of LHCII of higher plants and green algae can migrate from PSII to PSI and back that would lead to changes in the size of both LHCII and LHCI (Aro and Ohad, 2003). Phosphorylation of LHCII proteins by kinase STN7 (Rochaix et al., 2012) leads to migration of LHCII from PSII to PSI (state 2) when light preferential to PSII is implemented. Dephosphorylation by phosphatase enzyme PPH1/TAP38 (Pribil et al., 2010; Shapiguzov et al., 2010) provides a return of LHCII from PSI to PSII (state 1) under far-red light preferential to PSI. Thus, state transition process is a short-term adaptation of the photosynthetic apparatus to changes in light conditions, which allows redistribution of the excitation energy between two photosystems.

A key role in the initiation of state transition is attributed to the redox state of the plastoquinone pool (Allen et al., 1981). However, the molecular carrier of the signal that mediates the connection of plastoquinone pool and state transition initiation is still unknown. Since hydrogen peroxide is produced within the thylakoid membrane with the plastoquinone pool

components involvement (see introduction), we propose that H_2O_2 can be the signal. Being produced within the membrane, H_2O_2 can diffuse into the lumen (inside thylakoid space) and oxidizes cysteine residues of STN7 kinase converting the enzyme to an active state thus initiating state transition. At present the investigation of H_2O_2 on state transition process as well as on the redox state of STN7 kinase is in progress.

Another open question that still exists is the estimation of input of the H_2O_2 produced within the thylakoid membrane into the total H_2O_2 production by chloroplasts in the presence of native electron acceptors.

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