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Photoconsumption of Oxygen in Photosystem II Preparations under Impairment of the Water-Oxidizing Complex

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Abstract—Oxygen consumption in photosystem II (PSII) preparations in the light was 2 μmol O_2/h per mg Chl at weakly acidic and at neutral pH values. It increased fourfold to fivefold at pH 8.5-9.0. The addition of either artificial electron donors for PSII such as $MnCl_2$ or diphenylcarbazide, or diuron as an inhibitor of electron transfer from Q_A, the primary bound quinone acceptor, to Q_B, the secondary bound quinone acceptor of PSII, resulted in a decrease in oxygen consumption rate at basic pH to value close to ones measured at pH 6.5. Such additions did not affect oxygen consumption at lower pH values. The induction of variable chlorophyll fluorescence yield in the light differed greatly at pH 6.5 and 8.5. While at pH 6.5 the fluorescence yield, after an initial fast rise almost to F_{max}, only slightly decreased, at pH 8.5 after such a rise it dropped promptly to a low value. The additions of the artificial electron donors at pH 8.5 resulted in the induction kinetics close to that observed at pH 6.5. These data indicate impairment of electron donation to $P680^+$ that could be caused by damage to the water oxidation system at basic pH values. In experiments with PSII preparations treated with Tris to destroy the water-oxidizing complex, photoconsumption of oxygen in the entire pH region was close to the values in untreated preparations at basic pH. In untreated preparations the rate of light-induced oxygen consumption decreased in the presence of catalase, which decomposes H_2O_2, as well as in the presence of electron acceptor potassium ferricyanide. From these data it is suggested that the light-induced oxygen consumption in PSII is caused by two processes, by an interaction of O_2 with organic radicals, which were formed due to oxidation of components of the donor side of this photosystem (proteins, lipids, pigments) by cation-radical $P680^+$, as well as by oxygen reduction by still unidentified components of PSII.

Key words: oxygen, hydrogen peroxide, superoxide anion-radical, photosystem II

The formation of compounds with low redox potential takes place in the photosynthetic electron transport chain (PETC) and it is considered as one of the basic sources for the creation of active oxygen species in chloroplasts [1]. The reduction of O_2 by PETC was first shown by Mehler [2]. It is currently thought that the basic site of oxygen reduction in the PETC is the acceptor side of photosystem I (PSI) and that the primary product is superoxide radical ($O_2^{\cdot-}$) [3-5]. The formation of active oxygen species can also occur in other sites of the PETC besides PSI [6]. The reduction of oxygen by pigment–protein complex of photosystem II (PSII) at the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, diuron) and silicomolybdate has been shown [7]. The photoproduction of hydrogen peroxide in PSII preparations suppressed by inhibitors of electron transfer (DCMU and *o*-phenanthroline) [8] was ascribed to reduction of O_2 by plastoquinone Q_B or $Pheo^-$. The reduction of cytochrome *c* by PSII preparations in the presence of NaN_3 or in PSII preparations treated with $CaCl_2$ or tetracyanoethylene was also regarded as an evidence for superoxide formation in the site of $Pheo^-$ and/or Q_A^- [9]. The photoproduction of $O_2^{\cdot-}$ by thylakoid membranes in the presence of 2′,4′-dinitrophenylether of 2-iodo-4-nitrothymol (DNP-INT) (the inhibitor of oxidation of plastoquinone by cytochrome b_6/f complex) or in the presence of low concentrations of DCMU was

Abbreviations: Chl) chlorophyll; DCMU) 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron); DNP-INT) 2′,4′-dinitrophenylether of 2-iodo-4-nitrothymol; DPC) diphenylcarbazide; F_{max}) maximum level of chlorophyll fluorescence; MDA) monodehydroascorbate; PETC) photosynthetic electron transport chain; PSI, PSII) photosystems I and II, respectively; Pheo) pheophytin, the primary electron acceptor of PSII; P680) the primary electron donor of PSII; Q_A and Q_B) primary and secondary plastoquinone electron acceptors of PSII; Tiron) 4,5-dihydroxy-1,3-benzene disulfonic acid; TPB) tetraphenylboron.

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demonstrated using voltametric technique and ascribed to reduction of O_2 by Q_A^- [10].

Foremost, oxygen consumption induced by electron transfer results from the interaction of O_2 with the reduced electron acceptors; this produces superoxide redicals followed by their dismutation to H_2O_2. Taking into account oxygen evolution as a result of water oxidation in PSII, this process can be described by the following reactions:

$$H_2O - 2e^- \rightarrow 1/2\ O_2 + 2H^+ ; \quad (1)$$

$$2\ O_2 + 2\ e^- \rightarrow 2\ O_2^{\cdot-} ; \quad (2)$$

$$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2 . \quad (3)$$

In this case the addition of superoxide radical traps leads to an increase in the oxygen consumption rate (see reaction (4), where D_{red} and D_{ox} are reduced and oxidized forms of trap):

$$2\ O_2^{\cdot-} + 2\ H^+ + 2\ D_{red} \rightarrow 2\ H_2O_2 + 2\ D_{ox} . \quad (4)$$

On the other hand, the addition of catalase results in a decrease in the oxygen consumption rate (see reaction (5)):

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2 . \quad (5)$$

According to the stoichiometry of the above reactions, the resulting change in oxygen content in the medium will be equal to zero. However, if the oxygen evolution taking place when water is oxidized by PSII is excluded (for example, if an artificial electron donor instead of water is used), oxygen consumption will be observed even in the presence of catalase.

Second, oxygen consumption can occur on the interaction of oxygen with radicals of organic molecules ($R^{\cdot}$) with formation of corresponding peroxides (see reaction (6)):

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} . \quad (6)$$

The present work presents new data on the light-induced oxygen consumption in PSII preparations isolated from spinach chloroplasts. It is shown that inhibition of electron flow from water to reaction center of PSII results in the stimulation of the light-induced oxygen consumption. Based on the experimental results, it is suggested that the light-induced oxygen consumption in PSII is caused by two processes: an interaction of O_2 with organic radicals, which are formed due to oxidation of components of the donor side of this photosystem (proteins, lipids, pigments) by cation-radical $P680^+$, as well as an oxygen reduction by a still unidentified components of PSII.

MATERIALS AND METHODS

PSII enriched membrane fragments were isolated from spinach chloroplasts as described earlier [11] with some modifications [12]. The samples resuspended in the medium containing 0.4 M sucrose, 20 mM NaCl, 25 mM Mes-NaOH buffer, pH 6.5, at chlorophyll concentration 2 mg/ml were frozen with 20% glycerol in liquid nitrogen and stored at −196°C. Then they were slowly thawed and washed twice in medium containing 0.4 M sucrose, 20 mM NaCl, 5 mM Mes-NaOH buffer, pH 6.5. The oxygen evolution rate under continuous illumination of the untreated PSII membrane fragments with 100 μM 2,6-dichloro-*p*-benzoquinone/1 mM K_3FeCN_6 added as electron acceptors was 290 μmol O_2/h per mg Chl. Oxygen measurements were made in a temperature-controlled chamber using a Clark-type electrode. The samples were illuminated with red light ($\lambda > 600$ nm, 400 μmol photons/sec per m^2).

Complete (>95%) removal of Mn from the membrane fragments was carried out using 1 M Tris-HCl (pH 8.0) plus $MgCl_2$ treatment [13].

Chlorophyll fluorescence yields were measured by using a modulated fluorescence technique (PAM fluorimeter, model 101, Walz, Germany). The intensity of the modulated measuring light was 12 μmol photons/sec per m^2 and the red actinic light approximately 450 μmol photons/sec per m^2. A saturated light flash was generated by using an FL-103 lamp (Walz).

The assay medium contained 0.4 M sucrose, 20 mM NaCl, and buffer (50 mM Tricine for pH 8.5 and 9.0; 50 mM Hepes for pH 7.0-8.0; 50 mM Mes for pH 5.5-6.5; 50 mM Mes/glycine for pH 4.5 and 5.0.)

Chlorophyll concentration was determined according to [14].

RESULTS

Figures 1 and 2 show the light-induced oxygen consumption in PSII membrane fragments in the absence of electron acceptors. It can be seen (Fig. 2) that from pH 4.5 to pH 7.5 the oxygen consumption rate was closed to 2 μmol O_2/h per mg Chl and independent of the pH. The rate is considerably (by a factor of 4-5) increased upon alkalization of the medium to pH 8.5-9.0. The oxygen consumption rate at alkaline pH is considerably (close to the rate observed at pH 6.5) decreased by the addition of artificial electron donor for PSII (100 μM $MnCl_2$ or 100 μM diphenylcarbazide (DPC)). Addition of DCMU (5 μM) in the reaction medium also led to a decrease in oxygen uptake rate at pH 8.5-9.0, whereas at acidic and neutral pH values the presence of both these electron donors and DCMU had no effect on oxygen consumption (Fig. 2).

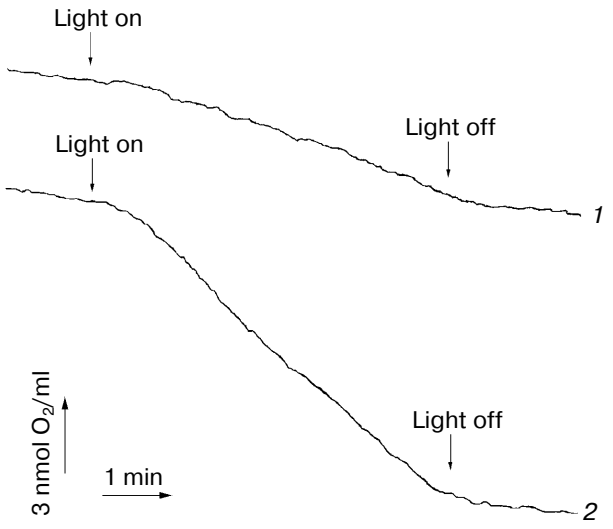

Fig. 1. Kinetics of photoinduced oxygen consumption by PSII membrane fragments at pH 6.5 (*1*) and 8.5 (*2*). The measurement conditions are described in "Materials and Methods". The chlorophyll concentration is 15 μg/ml.

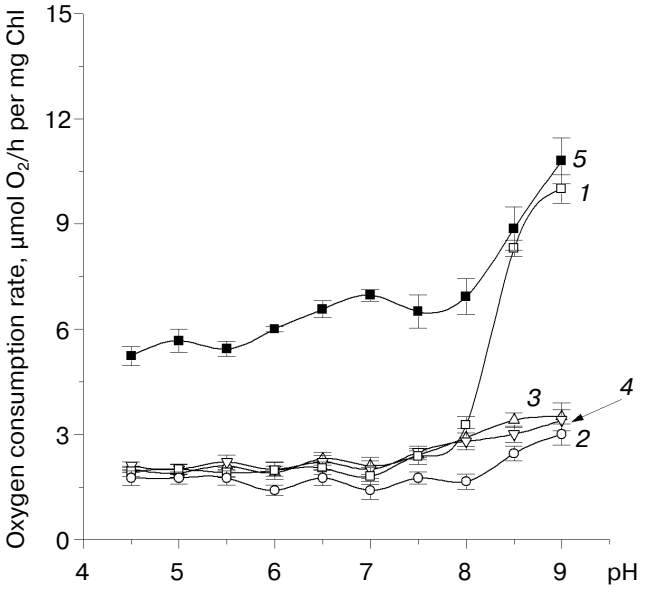

Fig. 2. pH dependence of oxygen consumption rate in photosystem II preparations in the absence (*1*) and presence of 5 μM DCMU (*2*), 100 μM $MnCl_2$ (*3*), and 100 μM DPC (*4*); in PSII preparations treated with 1 M Tris-HCl (*5*). The measurement conditions and the treatment of PSII by 1 M Tris-HCl are described in "Materials and Methods". The chlorophyll concentration is 15 μg/ml.

The measurements of kinetics of chlorophyll fluorescence yield in PSII preparations showed that at pH 6.5 (Fig. 3a) chlorophyll fluorescence yield slightly decreased after an initial fast rise almost to F_{max}, whereas at pH 8.5 (Fig. 3b) after such a rise it dropped promptly to a low value. The addition of artificial electron donors $MnCl_2$ (not shown) or DPC (100 μM) to the samples at pH 8.5 led to complete restoration of kinetics of chlorophyll fluorescence yield (Fig. 3c) close to that observed at pH 6.5 (Fig. 3d).

Thus, the results presented above indicate that damage to electron flow from water to reaction center of PSII at high pH [15] results in the stimulation of the light-induced oxygen consumption. This also is seen by experiments showing that damage to the water oxidizing complex in PSII preparations by 1 M Tris-HCl buffer led to increasing (by a factor of 4) of the light-induced oxygen consumption at low and neutral pH (Fig. 2).

The subsequent research of the light-induced oxygen consumption in PSII membrane fragments at high pH showed that the addition of other artificial electron donor (tetraphenylboron (TPB) [16] or NH_2OH) for PSII also led to the decrease of oxygen consumption rate. However, the decrease of oxygen consumption rate by these electron donors was less (30-35%) (Fig. 4) than that (70%) after the addition of 100 μM $MnCl_2$ or 100 μM DPC (Figs. 2 and 4).

The decrease in the rate of O_2 consumption after the addition of 50 μM K_3FeCN_6 as an electron acceptor indicates that oxygen reduction is one of the reasons for the observed oxygen consumption (Fig. 4). If the observed oxygen consumption is caused by reduction of O_2 with generation of superoxide radical, the presence of superoxide radical traps would result in the increase in photoconsumption oxygen rate (see reaction (4)). However, it is necessary to take into account possible additional effects of superoxide radical traps. Thus, ascorbate as the trap of $O_2^{\cdot-}$ can be used in the context of its ability to act as an electron donor for PSII [17], especially when the donor side of PSII is damaged. As seen in Fig. 4, the rate of oxygen photoconsumption is decreased (by 30%) in the presence of 1 mM ascorbate. Similar effect of the inhibition of oxygen uptake is observed on the addition of 5 mM Tiron (Fig. 4), which also can be used as the trap of $O_2^{\cdot-}$ [18]. This seems to indicate that Tiron can also serve as an electron donor for PSII. This ability of Tiron was confirmed by experiments showing the reactivation of the kinetics of photoinduced changes of chlorophyll fluorescence yield with Tiron in PSII preparations depleted of Mn^{2+} (>95%) (S. Zharmukhamedov, V. Klimov, unpublished results).

The decrease in observed oxygen photoconsumption upon the addition of catalase (Fig. 4) indicates that reduction of O_2 leads to production of superoxide radicals followed by its dismutation to H_2O_2. The effect of the inhibition of oxygen photoconsumption in PSII preparations was observed both in the presence and in the absence of artificial electron donors (Fig. 4). The least

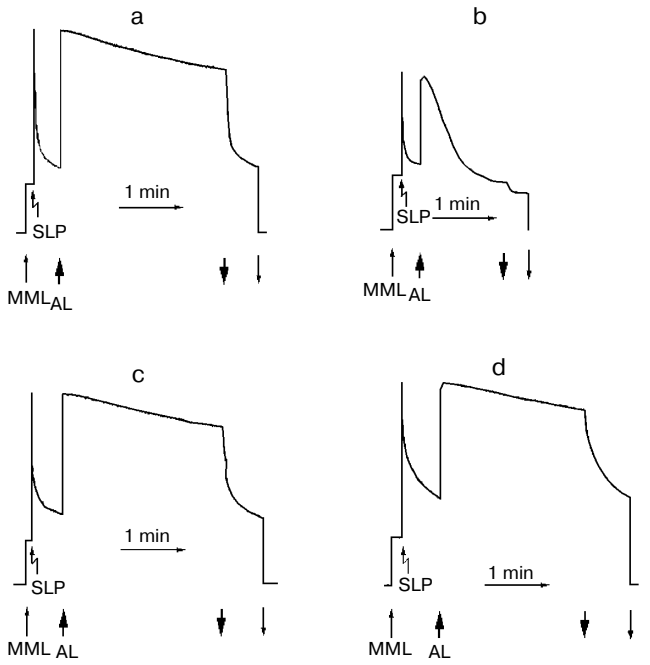

Fig. 3. Kinetics of photoinduced changes in chlorophyll fluorescence yield (ΔF) related to photoreduction of Q_A in PSII membrane fragments at pH 6.5 (a and c) and pH 8.5 (b and d). In the absence (a, b) and presence of 100 μM DPC (c, d). Abbreviations: MML, modulated measurement light; SLP, saturated light pulse; AL, actinic light. The measurement conditions are described in "Materials and Methods". The chlorophyll concentration is 10 μg/ml.

repressible action of catalase was observed in the presence of $MnCl_2$, about 20% relative to the rate of oxygen photoconsumption determined in the presence this electron donor. The catalase effect becomes more accentuated (40-50%) in the presence of other electron donors (ascorbate or Tiron) which act both as artificial electron donor and superoxide radical trap. In the presence of TPB or NH_2OH the oxygen photoconsumption suppressed by catalase was about 40%. The suppression of oxygen photoconsumption rate by catalase (less than 15%) was also observed in the presence of K_3FeCN_6 (a non-autoxidizable artificial electron acceptor).

DISCUSSION

The results of this study show that the increase of the light-induced oxygen consumption in PSII preparations is due to damage of water oxidizing complex and the inhibition of electron flow from water to $P680^+$. Therefore, the inhibition of oxygen photoconsumption observed both upon the addition of artificial electron donors leading to the reactivation of electron donation to $P680^+$ and upon the addition of DCMU blocking electron transfer beyond Q_A and promoting the recombination of separated charges in the $P680^+Pheo^-$ state is related to a decreased lifetime of $P680^+$.

The inhibition of electron donation to $P680^+$ can lead to the oxidation of organic molecules by $P680^+$, the strongest biological oxidant (E_o = 1.12 V) [19], with formation of radicals of organic molecules, $R^{\bullet}$. Oxygen interacts with the radicals of organic molecules with the formation of corresponding hydroperoxide $ROO^{\bullet}$ (see reaction (6)). The activation of carotenoid photobleaching observed upon illumination of PSII membrane fragments completely lacking Mn was eliminated by adding $MnCl_2$ or DCMU [20]. This fact can be considered as the result of carotenoid oxidation by oxygen, which is one of the indications of our finding that the donor side of PSII takes part in oxygen photoconsumption. It is possible that the oxygen photoconsumption is related to the processes of photoinhibition located in the donor side of PSII.

The increase in the lifetime of $P680^+$ requires electron removal from acceptors of PSII. The decrease in the oxygen photoconsumption observed upon the addition of catalase gives evidence of electron withdrawal to oxygen resulting in hydrogen peroxide formation. It is known that under conditions that destabilize the water-oxidizing complex such as high pH, oxygen evolution does not

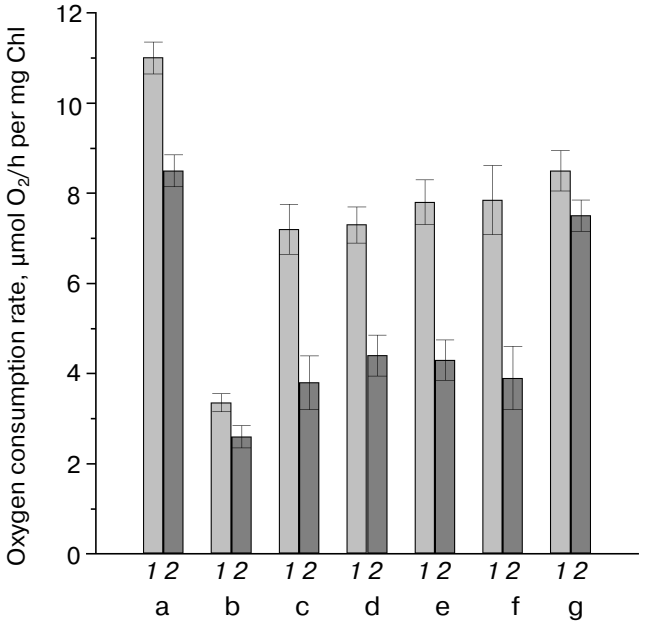

Fig. 4. Influence of catalase on the oxygen photoconsumption by PSII preparations at pH 9.0 in the absence (a) and presence of 100 μM $MnCl_2$ (b), 100 μM NH_2OH (c), 100 μM TPB (d), 5 mM Tiron (e), 1 mM ascorbate (f) and 50 μM $K_3[Fe(CN)_6]$ (g). The measurement was done in the absence (*1*) and presence of catalase (500 units/ml) (*2*). The chlorophyll concentration is 15 μg/ml.

occur. Thus, according to stoichiometry of the reactions (see reactions (2), (3), and (5)), the decrease in the oxygen photoconsumption rate in the presence of catalase by 2.5 μmol O_2/h per mg Chl (Fig. 4) corresponds to the rate of H_2O_2 production equal to 5 μmol H_2O_2/h per mg Chl. This rate is close to that of H_2O_2 production determined using the luminol-peroxidase technique in similar PSII membrane fragments at pH 8.5 [8].

In the presence of ascorbate, NH_2OH, and Tiron the production of H_2O_2 was about 7.9, 6.8, and 7.0 μmol H_2O_2/h per mg Chl, respectively. This may be the result of oxygen reduction taking place by means of one-electron path, and H_2O_2 production occurs not only in reaction of dismutation (see reaction (3)), but also in the interaction of $O_2^{\cdot-}$ with these compounds (see reaction (4)). In our experiments we used ascorbate and Tiron as the superoxide radical traps (see "Results"). In the first place, hydroxylamine is usually used as electron donor for PSII. However, it is known that hydroxylamine is able to interact with superoxide radicals and so it is used in detection of $O_2^{\cdot-}$ production [21]. The effect of catalase in the presence of TPB on the rate of oxygen consumption is quite similar to effects of ascorbate, NH_2OH, and Tiron. This fact indicates that TPB can interact with $O_2^{\cdot-}$ yielding H_2O_2. Taking into account the wide use of TPB, it would be interesting to check this assumption.

Additional evidence of superoxide radical production upon illumination of PSII preparations at high pH is that superoxide radical traps (which are also able to act as electron donors) did not lead to significant decrease in oxygen consumption in comparison with such donors as DPC and $MnCl_2$ (Figs. 2 and 4). It should be noted that the addition of ascorbate at low (100 μM) concentration (in which its ability to act as electron donor might be reduced) resulted in the increasing of oxygen consumption by 15%.

In accordance with stoichiometry of the reactions (see reactions (2) and (3)) at pH 8.5-9.0, the oxygen photoconsumption rate associated with the reduction of O_2 is equal to the rate of H_2O_2 production, which in the absence of electron donors and acceptors is about 5 μmol O_2/h per mg Chl. In this case, the rate of oxygen photoconsumption not associated with the reduction of O_2 and taking place on the donor side of PSII is about 6 μmol O_2/h per mg Chl. Under the circumstances when oxygen evolution was completely absent the oxygen consumption rate 5 μmol O_2/h per mg Chl resulted from the reduction of O_2 corresponds the rate of electron transfer to O_2 to be 10 μmol e^-/h per mg Chl (see reactions (2) and (3)). Therefore, the production of $R^{\cdot}$ with the rate of 10 μmol $R^{\cdot}$/h per mg Chl should result in the same rate of oxygen consumption caused by interaction of these radicals with O_2. However, the obtained oxygen consumption rate in our experiments shows that not all arising radicals seem to react with O_2 since they can undergo dismutation or react with intrinsic "traps".

It can be seen (Fig. 4) that the addition catalase in the presence of 50 μM K_3FeCN_6 leads to the inappreciable (about 1 μmol O_2/h per mg Chl) decreasing of oxygen photoconsumption rate, which corresponds to the rate of H_2O_2 production of 2 μmol H_2O_2/h per mg Chl. The H_2O_2 production in the presence of 50 μM K_3FeCN_6 might be explained by the incomplete inhibition of the electron transfer from components of acceptor side of PSII to O_2 by this hydrophilic electron acceptor. It should be noted, however, that the effective electron donor $MnCl_2$ decreased the H_2O_2 production, which could be calculated from the catalase effect. In the presence of 100 μM $MnCl_2$ the rate of H_2O_2 production was about 2 versus 5 μmol H_2O_2/h per mg Chl detected in its absence. This shows that the inhibition of electron transfer from water to reaction center of PSII results in the stimulation of H_2O_2 production. The assumption of H_2O_2 formation in the donor side of PSII when water oxidizing complex is damaged was put forward earlier [22-25].

Thus, light-induced oxygen consumption in PSII is stimulated by the inhibition of electron transport from water to the reaction center of PSII, P680. In this case oxygen photoconsumption occurs as a result of both oxygen reduction by reduced components of PSII yielding superoxide followed by its dismutation to H_2O_2 and the interaction of O_2 with organic radicals, which were formed due to oxidation of components of the donor side of PSII (proteins, lipids, pigments) by $P680^+$.

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