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Review

Molecular mechanisms of production and scavenging of reactive oxygen species by photosystem II ☆

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ABSTRACT

Photosystem II (PSII) is a multisubunit protein complex in cyanobacteria, algae and plants that use light energy for oxidation of water and reduction of plastoquinone. The conversion of excitation energy absorbed by chlorophylls into the energy of separated charges and subsequent water–plastoquinone oxidoreductase activity are inadvertently coupled with the formation of reactive oxygen species (ROS). Singlet oxygen is generated by the excitation energy transfer from triplet chlorophyll formed by the intersystem crossing from singlet chlorophyll and the charge recombination of separated charges in the PSII antenna complex and reaction center of PSII, respectively. Apart to the energy transfer, the electron transport associated with the reduction of plastoquinone and the oxidation of water is linked to the formation of superoxide anion radical, hydrogen peroxide and hydroxyl radical. To protect PSII pigments, proteins and lipids against the oxidative damage, PSII evolved a highly efficient antioxidant defense system comprising either a non-enzymatic (prenyllipids such as carotenoids and prenylquinols) or an enzymatic (superoxide dismutase and catalase) scavengers. It is pointed out here that both the formation and the scavenging of ROS are controlled by the energy level and the redox potential of the excitation energy transfer and the electron transport carries, respectively. The review is focused on the mechanistic aspects of ROS production and scavenging by PSII. This article is part of a Special Issue entitled: Photosystem II.

1. Introduction

Photosystem II (PSII) is a pigment–protein complex embedded in the thylakoid membrane of higher plants, algae and cyanobacteria. The capture of solar energy by the pigments in the PSII antenna complex and the charge separation in the PSII reaction center play a crucial role in the conversion of solar energy into the biologically useful chemical energy essential for the production of the overall biomass on the Earth. As a by-product of such energy conversion, molecular oxygen is released into the atmosphere, which is essential for respiration, a process of crucial importance for all aerobic life on the Earth. Apart of the oxygen-based respiration, molecular oxygen serves as a precursor of reactive oxygen species (ROS), which at low concentration play an important role in the defense against infection, the cell signaling and the apoptosis [1]. However, when ROS are formed in the excess, the oxidation of cellular biomolecules by ROS initiates the oxidative damage of proteins, lipids and nucleic acids.

In the thylakoid membrane, ROS are formed when the absorption of light by chlorophylls exceeds the capacity for the energy utilization by photosynthetic apparatus [2–4]. It is well established that another pigment–protein complex, photosystem I (PSI), is the main source of ROS produced in the thylakoid membrane mainly due to the fact that the electron transport across the thylakoid membrane is terminated on the stromal side of PSI. On the other hand, ROS production by PSII is of importance when the excitation energy delivery to the PSII reaction center is limited or the electron transport chain between the photosystems is inhibited. Under the limitation of excitation energy delivery from the PSII antenna complex to the PSII reaction center, chlorophyll serves as an effective photosynthesizer that transfers the

Abbreviations: AsA, ascorbate; Car, carotenoid; Chl_{D1}, Chl_{D2}, monomeric chlorophylls bound to the D1 and D2 subunits of PSII, respectively; CP29, CP26, and CP24, minor chlorophyll a/*b* binding protein complex; CP43 and CP47, the core antenna complex of PSII; Cyt b_{559}, cytochrome b_{559}; DCMU, 3–3,4-dichlorophenyl-1,1-dimethylurea; E_m, midpoint redox potential; EPR, electron paramagnetic spectroscopy; Fe^{3+}–O^-, ferric-oxo intermediate; Fe^{3+}–OO, ferric-peroxo intermediate; Fe^{3+}–OOH, ferric-hydroperoxo intermediate; HP, high potential; IP, intermediate potential form; LP, low potential; H_2O_2, hydrogen peroxide; $HO^{\bullet}$, hydroxyl radical; LHCII, light-harvesting complex of PSII; MDA, monodehydroascorbate radical; P_{D1} and P_{D2}, weakly-coupled chlorophyll dimer bound to the D1 and D2 subunits of PSII; Pheo, phephytin *a*—primary electron acceptor of PSII; $Pheo_{D1}$ and $Pheo_{D2}$, pheophytins *a* bound to the D1 and D2 subunits of PSII, respectively; PQ, plastoquinone; $PQ^{\bullet-}$, plastosemiquinone radical; PQH_2, plastoquinol; PSI, photosystem I; PSII, photosystem II; P680, primary electron donor of PSII; Q_A, primary quinone electron acceptor of PSII; Q_B, secondary quinone electron acceptor of PSII; RC, reaction center; ROS, reactive oxygen species; SCP, small chlorophyll-binding protein; $^1O_2$, singlet oxygen; $O_2^{\bullet-}$, superoxide anion radical; SOD, superoxide dismutase; SOO, superoxide oxidase; SOR, superoxide reductase; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl, α-Toc, α-tocopherol; α-TOOH, 8-hydroperoxy-tocopherone; α-TQ, α-tocopherolquinone; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Tyr_Z, redox active tyrosine residue Tyr161of the D1 protein; WSCP, water-soluble chlorophyll-binding protein

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excitation energy to molecular oxygen. As the redox potentials of the redox couples in PSII covers a broad range, starting at a highly negative value (Em(Pheo/Pheo$^-$) = −610 mV, pH 7) and terminating by a highly positive value (Em(P680$^+$/P680) = +1250 mV, pH 7) [4], PSII has a capability of either reducing molecular oxygen or oxidizing water on the electron acceptor (stromal) and the electron donor (lumenal) side of the membrane, respectively.

The transfer of excitation energy is accompanied with the formation of singlet oxygen ($^1O_2$). Singlet oxygen is generated by the triplet–singlet excitation energy transfer from the triplet excited state of chlorophyll (triplet chlorophyll, $^3Chl^*$) to the triplet ground state of molecular oxygen (triplet molecular oxygen, $^3O_2$). The triplet chlorophyll is formed either by the intersystem crossing from the singlet excited state of chlorophyll (singlet chlorophyll, $^1Chl^*$) in the PSII antenna complex or by the charge recombination of primary radical pair ^{1}[P680$^+$Pheo$^-$] in the PSII reaction center [6–11].

The electron transport on the electron acceptor side of PSII is linked to the leakage of electrons to molecular oxygen [12]. The non-enzymatic and enzymatic one-electron reductions of molecular oxygen result in the formation of superoxide anion radical ($O_2^{\bullet-}$). Superoxide anion radical is either reduced to free hydrogen peroxide (H_2O_2) or forms bound peroxide by the interaction with the non-heme iron [13]. The reduction of H_2O_2 by free metals (iron or manganese ions) or the reduction of bound peroxide by the non-heme iron results in the formation of hydroxyl radical (HO$^\bullet$). On the electron donor side of PSII, an incomplete oxidation of water is associated with the formation of H_2O_2[14,15]. Hydrogen peroxide is either oxidized to $O_2^{\bullet-}$ by highly oxidizing species such as the redox active tyrosine residue Tyr_Z or reduced to HO$^\bullet$ by manganese released from the manganese complex [16–18].

To prevent deleterious effect of ROS on pigments, proteins and lipids, the non-enzymatic and the enzymatic scavenging of ROS is engaged. Whereas the non-enzymatic antioxidants are mainly effective in the scavenging of ROS formed by the excitation energy transfer ($^1O_2$), the enzymatic antioxidants are crucial in the prevention of the deleterious effect of ROS formed by electron transport ($O_2^{\bullet-}$, H_2O_2, HO$^\bullet$). Superoxide anion radical is scavenged mainly on the electron acceptor side of PSII by cytochrome b_{559} (cyt b_{559}) [19,20] and superoxide dismutase (SOD) attached to the stromal side of the thylakoid membrane at the vicinity of PSII [21,22]. Hydrogen peroxide is scavenged predominantly on the electron donor side of PSII by the manganese complex [14,16,23,24] and the heme catalase attached to the lumenal side of the thylakoid membrane closed to PSII [25]. This review is focused on the production and scavenging of ROS formed by both the excitation energy transfer and the electron transport processes.

2. ROS production by excitation energy transfer

Singlet oxygen is generated by the triplet–singlet excitation energy transfer from the triplet chlorophyll to molecular oxygen. The triplet chlorophyll is formed either by the intersystem crossing from the singlet chlorophyll in the PSII antenna complex or by the charge recombination of primary radical pair ^{1}[P680$^+$Pheo$^-$] in the PSII reaction center (Fig. 1) [6,8,10,11]. In spite of the fact that chlorophylls are located mainly in the PSII antenna complex, the formation of $^1O_2$ in the PSII antenna complex by the intersystem crossing is rather neglectable. On the other hand, in the PSII reaction center, which contains only 6 chlorophylls, the charge recombination is considered as the main reaction pathway for $^1O_2$ generation in PSII.

2.1. $^1O_2$ generation in the PSII antenna complex

The chlorophylls coordinated to the protein matrix of the major chlorophyll a/*b* binding protein complex (light harvesting complex LHCII), the minor chlorophyll a/*b* binding protein complexes (CP29, CP26, and CP24) and the core antenna complexes (CP43 and CP47) of PSII are known to absorb the excitation energy that is transferred from the PSII antenna complex to the PSII reaction center. Under certain circumstances, when the singlet–singlet excitation energy transfer between chlorophylls is limited, the lifetime of the singlet excited state of chlorophyll raises and the alternative pathways become involved in the de-excitation of singlet chlorophyll. If the singlet excited state is not deactivated to the ground state such as in the absence of quenching by zeaxanthin or chlorophyll itself, the probability of the intersystem crossing and the formation of triplet chlorophyll is increased. When the triplet–triplet energy transfer from the triplet chlorophyll to carotenoid is ineffective, the triplet excitation energy from the triplet chlorophyll is transferred to molecular oxygen. The triplet–singlet energy transfer from the triplet chlorophyll to molecular oxygen results in $^1O_2$ formation (Fig. 1).

2.1.1. Triplet chlorophyll formation in the PSII antenna complex

The chlorophylls bound to the proteins in the PSII antenna complex are distanced and oriented in such a way that the efficient energy transfer to the PSII reaction center is maintained. Under such circumstances, very fast excitation energy transfer to the neighborhood chlorophyll is expected to prevent the formation of triplet chlorophyll. However, when the singlet–singlet energy transfer to the neighborhood chlorophyll is limited, the quantum yield of intersystem crossing is enhanced (Fig. 1, reaction 1). The intersystem crossing involves a change in the orientation of the spin of excited electron i.e. the conversion of the singlet excited state with antiparallel spin to the triplet excited state with parallel spin. To prevent the formation of triplet chlorophylls, the chlorophylls are coupled with carotenoids. Whereas xanthophylls prevent the formation of triplet chlorophyll in LHCII, carotenoids quench the triplet chlorophylls in the PSII core antenna complexes CP43 and CP47. The formation of triplet excited state is of importance mainly in the weakly-coupled, uncoupled and unbound chlorophylls in which the triplet excitation energy is not effectively quenched.

2.1.1.1. Weakly-coupled and uncoupled chlorophylls. The strong coupling between chlorophylls and carotenoids requires that both molecules are within van der Waals distance i.e. the distance between π-systems is less than 4 Å. However, it has been demonstrated that not all chlorophylls in the PSII antenna complex are in the close distance with the carotenoids. The crystal structures of LHCII [26,27] and PSII core complex [28,29] show that some carotenoids are located at the distance large than van der Waals distance. The large distance between the weakly-coupled chlorophyll and carotenoid results in the decrease in the efficiency of triplet energy quenching.

In the partially damaged or incorrectly assembled PSII antenna complex, some of the chlorophylls are bound to their chlorophyll binding sites, however, the chlorophyll–carotenoid coupling is lost. It has been shown that both the LHCII and the PSII core antenna complexes CP43 and CP47 contain uncoupled chlorophylls [30]. Using fluorescence-detected magnetic resonance spectroscopy, Santabarbara et al. [31] demonstrated that two triplet chlorophylls are formed in the PSII antenna complex—one triplet chlorophyll in the LHCII and one in the PSII core antenna complex CP43 and CP47.

2.1.1.2. Unbound chlorophyll. In the damaged PSII antenna complex, some of the chlorophylls are released from their binding sites. The formation of unbound chlorophylls is of importance mainly during the re-synthesis of damaged proteins, when chlorophylls are temporarily unbound from the damaged proteins. Under such circumstances, the probability of triplet chlorophyll formation significantly increases. To prevent the formation of deleterious triplet chlorophylls, the small chlorophyll-binding protein (SCP) [32] and the water-soluble chlorophyll-binding protein (WSCP) [33] were proposed to temporarily bind unbound chlorophylls. It has been demonstrated that SCP is involved in

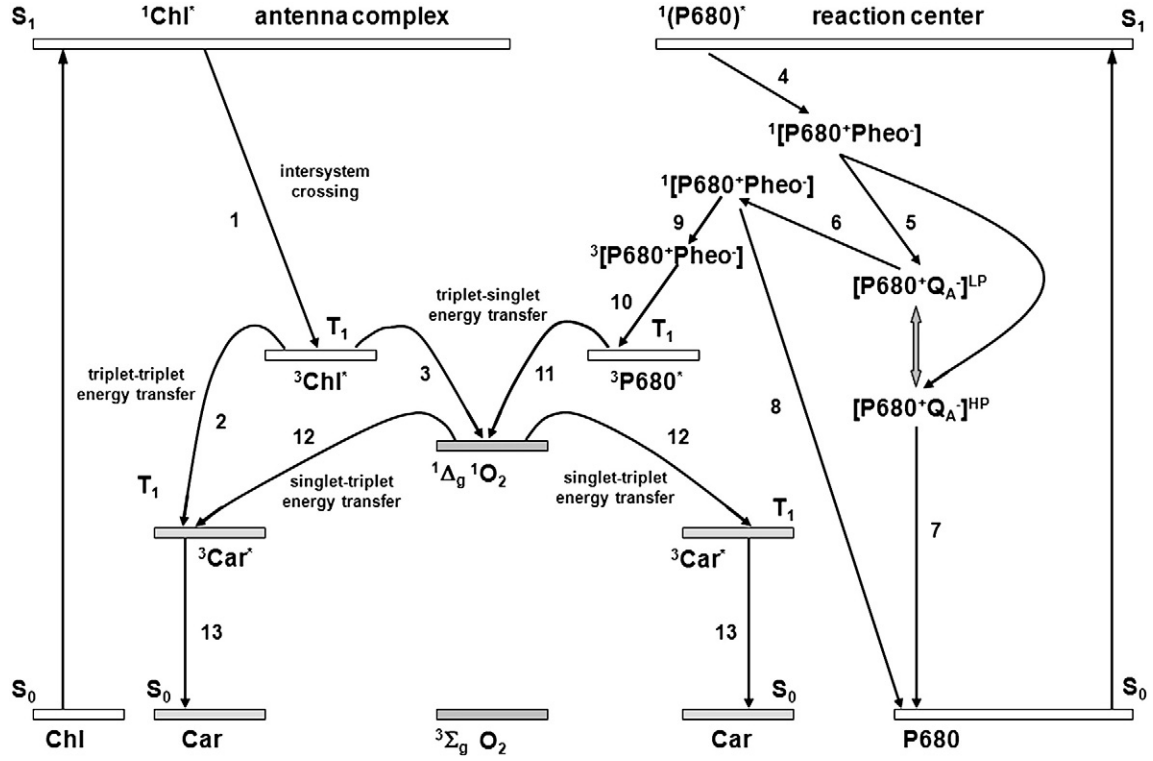

Fig. 1. Singlet oxygen generation in the antenna complex and the reaction center of PSII. In the PSII antenna complex, the absorption of visible light by ground state chlorophyll (Chl) (S_0 electronic state) forms the singlet chlorophyll ($^1Chl^*$) (S_1 electronic state). When the singlet–singlet energy transfer to the neighborhood chlorophyll is limited, the triplet chlorophyll ($^3Chl^*$) is formed by intersystem crossing (reaction 1). The strong coupling between chlorophyll and carotenoid results in the excitation energy transfer from the triplet chlorophyll to carotenoid forming the triplet carotenoid ($^3Car^*$) (reaction 2). The triplet–triplet energy transfer from the triplet chlorophyll to carotenoid is possible as the triplet energy level of carotenoid is below the triplet energy level of chlorophyll. When chlorophyll is weakly-coupled, uncoupled or unbound, the triplet excitation energy is transferred to molecular oxygen forming singlet oxygen ($^1O_2$) (reaction 3). As the energy level of $^1O_2$ ($^1\Delta_g$) is below the triplet energy level of chlorophyll, the triplet–singlet energy transfer from the triplet chlorophyll to molecular oxygen is possible. In the PSII reaction center, charge separation results in the formation of primary radical pair $^1[P680^+Pheo^-]$ (reaction 4). The electron transfer from $Pheo^-$ to Q_A forms the secondary radical pair $[P680^+Q_A^-]$ (reaction 5). The reverse electron transport from Q_A^- to Pheo results in the formation of $[P680^+Pheo^-]$ radical pair with lower energy compared to the primary radical pair $^1[P680^+Pheo^-]$ formed by charge separation (reaction 6). When the midpoint redox potential of $Pheo/Pheo^-$ and Q_A/Q_A^- redox couples is more positive, the charge recombination of $[P680^+ Q_A^-]$ (reaction 7) and $^1[P680^+Pheo^-]$ (reaction 8) radical pairs to the singlet ground state P680 is preferred. The radical pair $^1[P680^+Pheo^-]$ can be converted to the triplet radical pair $^3[P680^+Pheo^-]$ by change in the spin orientation (reaction 9). When the midpoint redox potential of Q_A/Q_A^- is more negative, the charge recombination of the triplet radical pair $^3[P680^+Pheo^-]$ results in the formation of triplet excited state $^3P680^*$ (reaction 10). The triplet excitation energy $^3P680^*$ is transferred to molecular oxygen forming $^1O_2$ (reaction 11). In the both antenna complex and reaction center of PSII, the interaction of $^1O_2$ and carotenoid results in the formation of triplet carotenoid ($^3Car^*$) and molecular oxygen (reaction 12). The singlet–triplet energy transfer from $^1O_2$ to carotenoid is possible, as the triplet energy level of carotenoid is below the energy level of $^1O_2$ ($^1\Delta_g$). The triplet excitation energy of carotenoid is converted into the heat (reaction 13). The singlet–singlet, triplet–triplet, triplet–singlet and singlet–triplet excitation energy transfer refer to the final states of the donor and acceptor of the excitation energy.

the PS II repair cycle as a temporary reservoir of chlorophylls, while PS II components are replaced [34–36]. Using EPR spin-trapping spectroscopy, Schmidt et al. [37] demonstrated that the light-induced $^1O_2$ formation in the reconstituted WSCP-chlorophyll complex was significantly prevented compared to free chlorophylls. The authors proposed that the tight enclosure of chlorophylls by the tetrameric protein structure of WSCP avoids the interaction of triplet chlorophylls with molecular oxygen and thus prevents $^1O_2$ formation. More recently, the crystal structure of WSCP-chlorophyll complex purified from leaves of *Lepidium virginicum* has shown that a homotetramer comprising four protein chains forms the hydrophobic cavity in which chlorophylls are tightly packed and isolated from molecular oxygen [38]. The authors assumed that WSCP prevents the physical contact between chlorophylls and molecular oxygen.

2.1.2. Quenching of triplet chlorophylls by carotenoids in the PSII antenna complex

The excitation energy transfer from the triplet chlorophyll to carotenoid results in the formation of the ground state of chlorophyll and the triplet excited state of carotenoids ($^3Car^*$) (Fig. 1, reaction 2). The triplet–triplet energy transfer from the triplet chlorophyll to carotenoid is possible, as the triplet energy level of carotenoid is below the triplet energy level of chlorophyll [39,40]. The triplet–triplet energy transfer takes place via Dexter mechanism, which requires the exchange of electrons between chlorophylls and carotenoids. As the electron exchange occurs only on the short-range distance, the Dexter mechanism requires the orbital overlap between chlorophylls and carotenoids. Since the triplet energy level of carotenoid is lower than the singlet energy level of $^1O_2$ ($^1\Delta_g$) the triplet carotenoid is unable to transfer the triplet energy to molecular oxygen (Fig. 1).

Each monomer of the trimeric LHCII and the monomeric CP29, CP26, and CP24 protein complexes coordinates four xanthophylls—two luteins, neoxanthin and violaxanthin [26]. It has been demonstrated that the α-branch (lutein) and the β-branch (neoxanthin, violaxanthin/zeaxanthin) xanthophylls have a distinct and complementary role in the photoprotection of PSII [41]. Using recombinant LHCII with the modified carotenoid composition, Croce et al. [42] showed that two luteins are required for the quenching of triplet chlorophylls, whereas neoxanthin is rather an effective quencher of $^1O_2$. The crystal structures of LHCII obtained by electron diffraction at 3.4 Å [26] and X-ray crystallography at 2.7 Å [27] showed that two luteins located in the center of the monomer are bound to chlorophylls within van der Waals distance. The triplet chlorophylls are quenched mainly by the lutein bound at the L1 site, whereas the lutein at the L2 site is less effective in the quenching of triplet chlorophylls [43]. The authors demonstrated that chlorophylls (Chls 610, 612, 613) and (Chls 602, 603) are strongly

coupled to the luteins bound at the L1 and L2 sites, respectively. Contrary, chlorophylls (Chls 611 and 614) are distanced far away from the lutein bound at the L1 and L2 sites. The nearest carotenoid to Chl 611 distanced at 7.7 Å is violaxanthin at V1 site, which is unable to quench the triplet chlorophyll [44]. Due to the lack of coupling between Chl 611 and lutein, Chl 611 is not protected and it possibly contributes significantly to the overall triplet chlorophyll formation in LHCII. As Chl 614 is energetically coupled with Chl 613 distanced at 9.3 Å, the singlet–singlet energy transfer from Chl 614 to Chl 613 at least partially protects Chl 614 [43] (Fig. 2A).

The three-dimensional crystal structure of PSII from thermophilic cyanobacteria *Thermosynechococcus elongatus* showed that nine

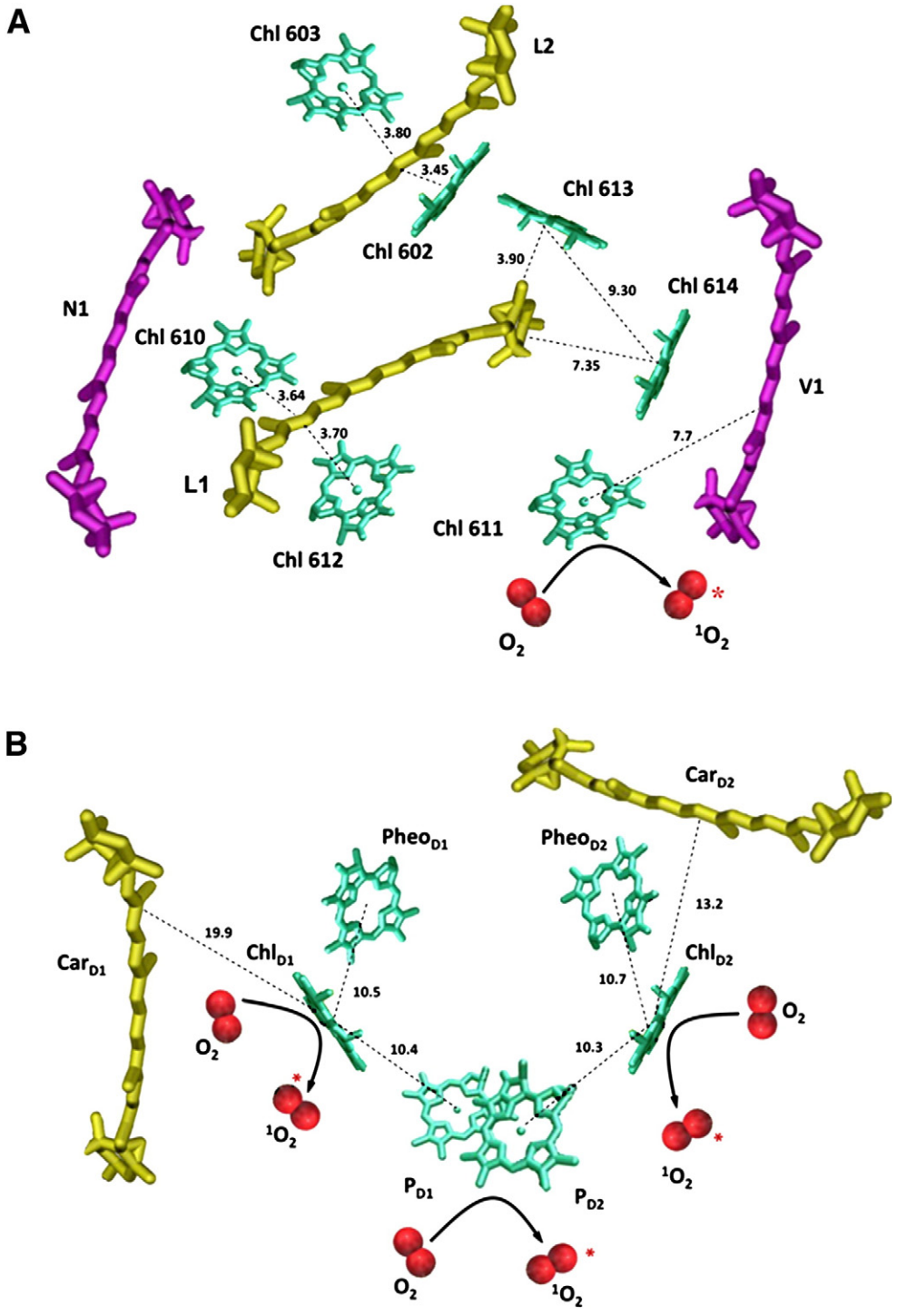

Fig. 2. Singlet oxygen generation in the light-harvesting complex and the reaction center of PSII. A. As the conjugated π-systems of chlorophylls (Chls 602, 603, 610, 612, 613) and luteins (L1 and L2) are at van der Waals distance i.e. less than 4 Å, the triplet excitation energy of these chlorophylls is efficiently quenched by luteins. Contrary, the triplet excitation energy of chlorophyll 611 (Chl 611) is not sufficiently quenched by luteins due to the large distance to luteins (L1 and L2) and in the presence of molecular oxygen is transferred to molecular oxygen forming $^1O_2$. The structural model is based on the three-dimensional crystal structure of the spinach major light-harvesting complex [27]. The pigment–pigment distances are taken from [43]. B. The triplet excitation energy delocalized on the weakly-coupled chlorophyll dimer P_{D1} and P_{D2} is transferred to molecular oxygen forming $^1O_2$. Alternatively, the triplet–triplet energy transfer from chlorophyll monomers Chl_{D1} and Chl_{D2} to molecular oxygen results in $^1O_2$ formation. The arrangement of cofactors is based on the three-dimensional crystal structure of PSII from the thermophilic cyanobacteria *Thermosynechococcus elongatus* [29].

β-carotenes are associated with the PSII core antenna complex—five β-carotenes with CP47 and four β-carotenes with CP43 [45,46]. The PSII core antenna CP47 contains chlorophylls strongly coupled with a cluster of four β-carotenes located at the monomer-monomer interface. Contrary, chlorophylls in CP43 are more distant from β-carotenes without any van der Waals contact indicating a lower efficiency of the triplet chlorophyll quenching. Müh et al. [46] showed that only three chlorophylls (Chls 17, 22, 27) in CP47 and two chlorophylls (Chls 17, 22) in CP43 are coupled with β-carotenes.

2.1.3. Triplet–singlet energy transfer to molecular oxygen in the PSII antenna complex

Due to the fact that the reverse conversion from the triplet chlorophyll to the singlet chlorophyll is forbidden, the triplet chlorophyll survives in the excited state for a longer period compared to the singlet chlorophyll. Under such circumstances, the probability of interaction of triplet chlorophyll with the surrounding molecules is enhanced. When molecular oxygen is present in the close proximity of the triplet chlorophyll, the excitation energy is transferred from the triplet chlorophyll to molecular oxygen (Fig. 1, reaction 3). The triplet–singlet energy transfer from the triplet chlorophyll to molecular oxygen results in the formation of $^1O_2$ and the ground state of chlorophyll. The triplet–singlet energy transfer from the triplet chlorophyll to molecular oxygen is possible, as the energy level of $^1O_2$ ($^1\Delta_g$) is below the triplet energy level of chlorophyll. The excitation energy transfer from the triplet chlorophyll to molecular oxygen proceeds via the formation of $^1(T_1 \cdot {}^3\Sigma)$ encounter complexes [47].

$$O_2(^3\Sigma_g^-) + {}^3Chl^* \rightarrow {}^1(T_1 \cdot {}^3\Sigma) \rightarrow O_2(^1\Delta_g) + Chl$$

Singlet oxygen formation in the PSII antenna complex has been demonstrated using $^1O_2$ phosphorescence at 1270 nm [48,49] and EPR spin-trapping spectroscopy [50,51]. Zolla et al. [50,51] demonstrated that the exposure of the isolated LHCII to high-intensity light results in $^1O_2$ generation as confirmed by the formation of TEMPO EPR signal. The observation that illumination of the apoproteins brings about no formation of $^1O_2$ revealed that $^1O_2$ is formed by photosensitization reaction by chlorophylls. The finding that $^1O_2$ formation was lowered in the monomeric antenna complex indicates that the monomerization represents protection against $^1O_2$.

2.2. $^1O_2$ generation in the PSII reaction center

In addition to the formation of triplet chlorophyll in the PSII antenna complex, the triplet chlorophyll is formed by the charge recombination in the PSII reaction center. As the recombination of the primary radical pair $^1[P680^+Pheo^-]$ is fast (i.e. occurs before spin conversion), the charge recombination of the primary radical pair $^1[P680^+Pheo^-]$ does not lead to the formation of triplet chlorophyll. However, when the lifetime of the primary radical pair $^1[P680^+Pheo^-]$ is prolonged due to the reduction, the double protonation or the complete loss of the primary quinone electron acceptor Q_A, the charge recombination of $^1[P680^+Pheo^-]$ radical pair might produce the triplet chlorophyll [5,8,52]. In contrast to the PSII reaction center, $^1O_2$ production in the PSI reaction center is neglectable as the charge recombination between the long-lived $P700^+$ and the reducing species on the PSI electron acceptor side is unlikely mainly due to the efficient electron transport to molecular oxygen.

2.2.1. Triplet chlorophyll formation in the PSII reaction center

Primary charge separation results in the formation of primary radical pair $^1[P680^+Pheo^-]$ (Fig. 1, reaction 4) which is stabilized by the electron transfer to Q_A forming the secondary radical pair $[P680^+Q_A^-]$ (Fig. 1, reaction 5). When the forward electron transport from Q_A to the secondary quinone electron acceptor Q_B is inhibited, the reverse electron transport from Q_A^- to Pheo brings about the formation of radical pair $^1[P680^+Pheo^-]$ (Fig. 1, reaction 6). Due to the energy loss during the relaxation process, the radical pair $^1[P680^+Pheo^-]$ formed by the reverse electron transport has been proposed to have a lower energy compared to the primary radical pair $^1[P680^+Pheo^-]$ formed by charge separation [6,53]. The charge recombination of $[P680^+Q_A^-]$ radical pair results in the formation of the ground state P680, whereas the radical pair $^1[P680^+Pheo^-]$ recombines either in the ground state P680 or the triplet excited state $^3P680^*$. Whereas the charge recombination of $[P680^+Q_A^-]$ radical pair to the ground state P680 accounts for about 20% of the overall recombination, the charge recombination of $^1[P680^+Pheo^-]$ radical pair in the ground state P680 or the triplet excited state $^3P680^*$ is the main reaction pathway with the yield of about 80% [5,9,54]. The reaction pathway of charge recombination is determined by the midpoint redox potential of Q_A/Q_A^-, Pheo/$Pheo^-$ and $P680^+$/P680 redox couples [7,55].

2.2.1.1. $[P680^+Q_A^-]$ recombination to ground state P680. The shift in the midpoint redox potential of Q_A/Q_A^- redox couple ($Em = -80$ mV, pH 7) [56] to a more positive value increases the energy gap between $[P680^+Q_A^-]$ and $^1[P680^+Pheo^-]$ radical pairs. Under such circumstances, the charge recombination of $[P680^+Q_A^-]$ radical pair to the ground state P680 is preferred (Fig. 1, reaction 7). It has been demonstrated that the midpoint redox potential of Q_A/Q_A^- redox couple is increased by the binding of urea-type of herbicide (DCMU) to the Q_B site [56].

2.2.1.2. $^1[P680^+Pheo^-]$ recombination to ground state P680. When the midpoint redox potential of Pheo/$Pheo^-$ redox couple ($Em = -610$ mV, pH 7) [58,59] is more positive, the free energy gap between $^1[P680^+Pheo^-]$ and $P680^*$ is enhanced and the charge recombination of $^1[P680^+Pheo^-]$ radical pair occurs mainly to the ground state P680 (Fig. 1, reaction 8). It has been demonstrated that the modification of protein environment around Pheo [55,60] and P680 [61] affects the midpoint redox potential of Pheo/$Pheo^-$ and $P680^+$/P680 redox couples.

2.2.1.3. $^1[P680^+Pheo^-]$ recombination to triplet excited state $^3P680^*$. The shift in the midpoint redox potential of Q_A/Q_A^- redox couple to a more negative value decreases the free energy gap between $[P680^+Q_A^-]$ and $^1[P680^+Pheo^-]$. Under such conditons, the reverse electron transport from Q_A^- to Pheo and the formation of radical pair $^1[P680^+Pheo^-]$ is promoted. The radical pair $^1[P680^+Pheo^-]$ can be converted to the triplet radical pair $^3[P680^+Pheo^-]$ by the change in spin orientation (Fig. 1, reaction 9). The charge recombination of the triplet radical pair $^3[P680^+Pheo^-]$ results in the formation of triplet excited state $^3P680^*$ (Fig. 1, reaction 10). The midpoint redox potential of Q_A/Q_A^- redox couple is decreased either by the binding of phenolic-type of herbicide (bromoxynil) to the Q_B site [53] or the site-directed modification of the protein environment around Q_A site [57].

It is generally considered that the triplet excited state formed by the charge recombination is located on the weakly-coupled chlorophyll dimer P_{D1} and P_{D2} (P680) [62–66]. However, several lines of evidence have been provided that the triplet excited state might reside on the chlorophyll different from P_{D1} and P_{D2} dimer [67–71]. Based on the low-temperature EPR spectroscopy, the triplet excited state was proposed to reside on the accessory chlorophyll that is a structural analogue to the accessory bacteriochlorophyll in the bacterial reaction center [67]. In agreement with this proposal, the data obtained from the time-resolved FTIR [68,69] and the time-resolved EPR [70] spectroscopy support that the triplet excited state is located on the chlorophyll monomer of D1 protein Chl_{D1} located between P_{D1} and $Pheo_{D1}$ (Fig. 2B). The temperature dependence of the FTIR spectra showed that the triplet energy is distributed in a thermal

equilibrium between Chl_{D1} and P_{D1} and P_{D2} dimer. Whereas at the low temperature, the triplet energy is localized on the accessory chlorophyll Chl_{D1}, at room temperature, the triplet energy is delocalized between Chl_{D1} and P_{D1} and P_{D2} dimer [69]. The authors proposed that the triplet–triplet energy transfer from P_{D1} and P_{D2} dimer to Chl_{D1} is possible, as the triplet energy level of Chl_{D1} is below the triplet energy level of P680. More recently, the data obtained from the transient absorption spectroscopy has shown that the triplet excited state is located on the chlorophyll monomer of D2 protein Chl_{D2} located between P_{D2} and $Pheo_{D2}$ (Fig. 2B) [71].

2.2.2. Inability of carotenoids to quench triplet chlorophyll in the PSII reaction center

Several lines of evidence have been given that two β-carotenes are coordinated to the D1 and D2 proteins [72,73]. The three-dimensional crystal structure data showed that β-carotenes on D1 (Car_{D1}) and D2 (Car_{D2}) proteins are distanced 19.9 Å and 13.2 Å from Chl_{D1} and Chl_{D2}, respectively [29]. Due to the fact that chlorophylls are not bound within van der Waals distance (<4 Å), the triplet chlorophyll is not sufficiently quenched by β-carotene [73,74]. It has been proposed that the primary function of β-carotene in the PSII reaction center is quenching of $^1O_2$ [74,75]. Using nanosecond transient absorption spectroscopy, it has been demonstrated that the triplet carotenoid assigned to Car_{D2} is formed by the interaction with the triplet chlorophyll located on Chl_{D2} [71]. Based on the analogy with triplet chlorophyll quenching in the bacterial reaction center, the authors proposed that Car_{D2} serves as an efficient quencher of the triplet chlorophyll. The authors suggested that the triplet chlorophyll is formed on the D1 branch, when Q_A is oxidized or doubly reduced. Due to the large distance to Car_{D1}, the triplet chlorophyll formed on the D1 branch is not quenched by Car_{D1}. However, when Q_A is reduced, the triplet chlorophyll is formed on Chl_{D2}, which can be quenched by Car_{D2}.

2.2.3. Triplet–singlet energy transfer to molecular oxygen in the PSII reaction center

The triplet excitation energy $^3P680^*$ is transferred to molecular oxygen forming $^1O_2$ (reaction 11). Direct evidence on $^1O_2$ formation was provided by the measurements of $^1O_2$ phosphorescence at 1270 nm [76], chemical trapping [65] and EPR spin-trapping spectroscopy [77–79]. Durrant et al. [62] demonstrated that in the absence of molecular oxygen, the lifetime of $^3P_{680}^*$ is approximately 1 ms, whereas it is significantly shortened in the presence of molecular oxygen to approximately 30 μs. Based on this observation, the authors suggested that $^1O_2$ is formed by the excitation energy transfer from $^3P680^*$ to molecular oxygen. Evidence on the excitation energy transfer from $^3P680^*$ to molecular oxygen has been provided by a direct correlation between the loss of the triplet chlorophyll $^3P680^*$ and the formation of $^1O_2$ as detected by phosphorescence at 1270 nm in the PSII reaction center [80]. Using EPR spin-trapping spectroscopy, Hideg et al. [78] showed that the illumination of thylakoid membranes results in $^1O_2$ formation accompanied by the formation of carbon-centered radicals [77,81].

3. ROS production by electron transport

When electron transport from the manganese complex to plastoquinone is limited, ROS are formed both on the electron acceptor and the electron donor side of PSII [12]. The leakage of electrons to molecular oxygen on the electron acceptor side of PSII forms $O_2^{\bullet-}$ known to initiate a cascade reaction leading to the formation of H_2O_2 and highly oxidizing $HO^{\bullet}$. The incomplete oxidation of water on the electron donor side of PSII brings about the formation of H_2O_2 which is either oxidized to $O_2^{\bullet-}$ by Tyr_Z or reduced to $HO^{\bullet}$ by manganese released from the manganese complex.

3.1. $O_2^{\bullet-}$ production on the electron acceptor side of PSII

Reduction of molecular oxygen on the electron acceptor side of PSII results in the formation of $O_2^{\bullet-}$ (Fig. 3). As the standard redox potential of $O_2/O_2^{\bullet-}$ redox couple is negative ($E'_0 = -160$ mV, pH 7) [82] (Table 1), the only reducing species with a high reducing power are able to provide an electron to molecular oxygen. It has been recently pointed out that E'_0 ($O_2/O_2^{\bullet-}$) = −160 mV (pH 7) is relevant when the concentration of molecular oxygen is of the same order as the concentration of $O_2^{\bullet-}$ [12]. However, the concentration of molecular oxygen (hundreds μM) differs at least by three orders from the concentration of $O_2^{\bullet-}$ (hundreds nM) formed by PSII. According to the Nernst equation $E = -0.16 + 0{,}06 \log [O_2/O_2^{\bullet-}]$, the difference in the concentration shifts the redox potential of $O_2/O_2^{\bullet-}$ redox couple to positive values. In agreement with these considerations, it has been shown that the xanthine oxidase ($Em = -60$ to $+60$ mV, pH 7) and the FeEDTA complex ($Em = +117$ mV, pH 7) have a capability to reduce molecular oxygen [82].

Reduction of molecular oxygen proceeds either by the non-enzymatic or the enzymatic reaction pathways. The non-enzymatic reduction of molecular oxygen to $O_2^{\bullet-}$ is maintained by Pheo [13,19], tightly bound (Q_A) [13,83], loosely bound (Q_B) [84] and free (PQ) [85] plastosemiquinone. In the enzymatic reaction pathway, molecular oxygen is reduced by the heme iron of low-potential (LP) form of cyt b_{559} (Fig. 3).

3.1.1. Non-enzymatic reduction of molecular oxygen to $O_2^{\bullet-}$

3.1.1.1. Reduction of molecular oxygen by pheophytin. In spite of the fact that Pheo has enough redox power to reduce molecular oxygen (Em ($Pheo/Pheo^-$) = −610 mV, pH 7) [58,59], $Pheo^-$ lifetime is short to effectively reduce molecular oxygen (the electron transfer from $Pheo^-$ to Q_A is in several hundreds picoseconds). However, when the forward electron transport from $Pheo^-$ to Q_A is slowdown due to the overreduction of the electron acceptor side of PSII, the lifetime of $Pheo^-$ is enhanced. Under such circumstances, the lifetime of $Pheo^-$ is determined mainly by the charge recombination between $Pheo^-$ and the oxidizing species on the electron donor side of PSII. As the recombination of the primary radical pair $^1[P680^+Pheo^-]$ is fast (~tens nanoseconds) [5], the probability of electron transport from $Pheo^-$ to molecular oxygen is low. However, the recombination of negative charge on Pheo with positive charge on Tyr_Z and higher oxidizing states of the manganese complex is considerably slower, allowing thus the leakage of electrons from Pheo to molecular oxygen (Fig. 3, reaction 1). It is proposed here that the leakage of electrons from $Pheo^-$ to molecular oxygen prevents double reduction of Q_A and thus protects Q_A from the sequential protonation and release from its binding site.

3.1.1.2. Reduction of molecular oxygen by tightly bound plastosemiquinone Q_A^-. As the midpoint redox potential of Q_A/Q_A^- redox couple is less negative ($Em = -80$ mV, pH 7) compared to the midpoint redox potential of $Pheo/Pheo^-$ redox couple, the reduction of molecular oxygen by the tightly bound plastosemiquinone Q_A^- is thermodynamically less likely. However, contrary to $Pheo^-$, the pronouncedly longer lifetime of Q_A^- (several hundreds microseconds) [5,87] significantly enhances the probability of electron donation to molecular oxygen (Fig. 3, reaction 2). When the electron transport to Q_B is limited, the lifetime of Q_A^- is determined by the recombination of $[P680^+Q_A^-]$ (several milliseconds) and $S_2Q_A^-$ (several seconds) radical pairs [54].

3.1.1.3. Reduction of molecular oxygen by loosely bound plastosemiquinone Q_B^-. The reduction of molecular oxygen by the loosely bound plastosemiquinone at the Q_B site (Q_B^-) (Fig. 3, reaction 3) is less favorable from the thermodynamic point of view ($Em(Q_B/Q_B^-) = -45$ mV, pH 7) [86]. In the light, the lifetime of Q_B^- is determined by

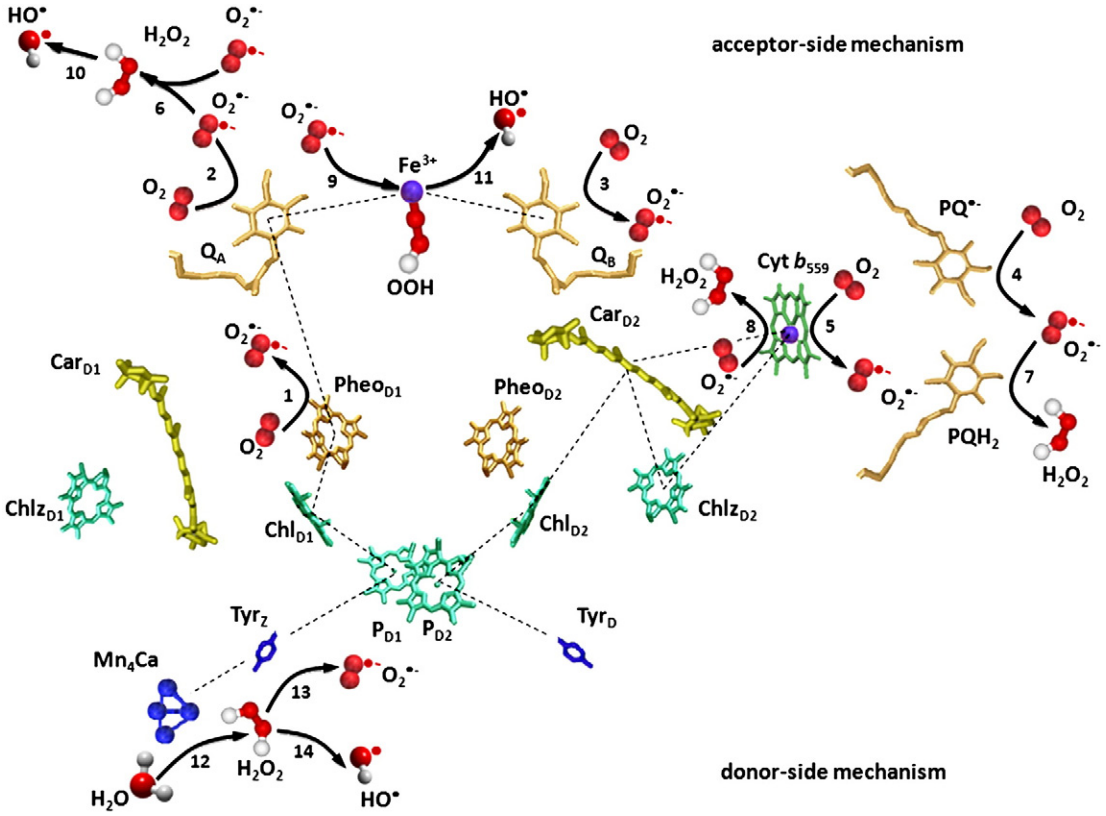

Fig. 3. ROS production on the electron acceptor and the electron donor side of PSII. On the electron acceptor side of PSII, the one-electron reduction of molecular oxygen by pheophytion (Pheo) (reaction 1), tightly bound plastosemiquinone (Q_A) (reaction 2), loosely bound plastosemiquinone (Q_B) (reaction 3), free plastosemiquinone (PQ) (reaction 4) and cytochrome b_{559} (cyt b_{559}) (reaction 5) results in the formation of superoxide anion radical ($O_2^{\bullet-}$). Superoxide anion radical is reduced either by another $O_2^{\bullet-}$ (reaction 6) or plastoquinol (PQH_2) (reaction 7) to free hydrogen peroxide (H_2O_2). The interaction of $O_2^{\bullet-}$ with the heme iron of cyt b_{559} (reaction 8) or the non-heme iron (reaction 9) forms the ferric-hydroperoxo intermediate. The protonation of the proximal oxygen of the ferric-hydroperoxo intermediate by the proton released from the heme-binding histidine of cyt b_{559} causes the formation of H_2O_2. The reduction of H_2O_2 by free metals such as Fe^{2+} or Mn^{2+} (reaction 10) or the reduction of the ferric-hydroperoxo intermediate by the non-heme iron (reaction 11) form hydroxyl radical ($HO^{\bullet}$). On the electron donor side of PSII, the two-electron oxidation of water results in the formation of H_2O_2 (reaction 12). The one-electron oxidation of H_2O_2 by tyrosine residue (Tyr_Z) results in the formation of $O_2^{\bullet-}$ (reaction 13). One-electron reduction of H_2O_2 by free Mn^{2+} released from the manganese complex forms $HO^{\bullet}$ (reaction 14). The arrangement of cofactors is based on three-dimensional crystal structure of PSII from thermophilic cyanobacteria *Thermosynechococcus elongatus* [29].

Table 1
One-electron standard (E'_0) and midpoint (Em) redox potentials at pH = 7 for the selected redox couples involved in the formation of ROS in PSII.

A) Standard redox potential E'_0 (pH 7).

Redox couple	E'_0 (mV)	Ref.
$O_2/O_2^{\bullet-}$	−160	[82]
$H_2O_2/HO^{\bullet}$	+460	[98]
$O_2^{\bullet-}/H_2O_2$	+890	[82]
Fe^{3+}/Fe^{2+}	+110	[97]
Mn^{3+}/Mn^{2+}	+1200	[109]

B) Midpoint redox potential Em (pH 7) of redox couples in PSII.

Redox couple	Em (mV)	Ref.
$Pheo/Pheo^-$	−610	[58]
$PQ/PQ^{\bullet-}$	−170	[86]
Q_A/Q_A^-	−80	[56]
Q_B/Q_B^-	−45	[86]
Q_B^-/Q_BH_2	+290	[86]
$PQ^{\bullet-}/PQH_2$	+370	[86]
Fe^{3+}/Fe^{2+} (LP cyt b_{559})	−40 to +80	[20]
Fe^{3+}/Fe^{2+} (IP cyt b_{559})	+125	[20]
Fe^{3+}/Fe^{2+} (HP cyt b_{559})	+310 to +400	[20]
Fe^{3+}/Fe^{2+} (non-heme)	+400	[94]
$Tyr_Z^{\bullet}/Tyr_Z$	+1100 to +1200	[5]
$P680^+/P680$	1250	[5]

Q_A^- to Q_B^- electron transport (several hundreds microseconds) [5,87], whereas in the dark, the lifetime of Q_B^- is determined by $S_2Q_B^-$ recombination (the recombination of $S_2Q_B^-$ is several tens seconds i.e. 20-fold slower compared to $S_2Q_A^-$ [54]). Apart of the loosely bound plastosemiquinone at the Q_B site, the fully reduced plastoquinol dianion (PQ^{2-}) loosely bound to the Q_B site was proposed to serve as a reductant to molecular oxygen [88]. As the midpoint redox potential of Q_B^-/Q_BH_2 redox couple is highly positive ($Em = +290$ mV, pH 7) [86], the reduction of molecular oxygen by plastoquinol is thermodynamically unfavorable.

3.1.1.4. Reduction of molecular oxygen by free plastosemiquinone $PQ^{\bullet-}$. Based on the analogy to the reduction of molecular oxygen by ubisemiquinone in the inner mitochondrial membrane, Ivanov et al. [85] proposed that $O_2^{\bullet-}$ is formed by the reduction of molecular oxygen by plastosemiquinone radical ($PQ^{\bullet-}$) in the PQ-pool (Fig. 3, reaction 4).

$$O_2 + PQ^{\bullet-} \rightarrow O_2^{\bullet-} + PQ$$

As the midpoint redox potential of $PQ/PQ^{\bullet-}$ redox couple ($Em = -170$ mV, pH 7) is more negative than the midpoint redox potential of the other quinones [86], the reduction of molecular oxygen by $PQ^{\bullet-}$ seems to be highly favorable from the thermodynamic point of view. However, the probability of $PQ^{\bullet-}$ formation by the interaction of plastoquinone and plastoquinol in the PQ-pool is low.

$$PQ + PQH_2 \rightarrow 2PQ^{\bullet-} + 2H^+$$

3.1.2. Enzymatic reduction of molecular oxygen to $O_2^{\bullet-}$

The enzymatic reduction of molecular oxygen with a subsequent production of $O_2^{\bullet-}$ has been proposed by the heme iron of LP form of cyt b_{559} [89] (Fig. 3, reaction 5). As the midpoint redox potential of Fe^{3+}/Fe^{2+} redox couple in LP form of cyt b_{559} ranges from −40 to +80 mV (pH 7), the reduction of molecular oxygen by the ferrous heme iron of LP form of cyt b_{559} is feasible. Based on the observation that the exogenous plastoquinones with a different side-chain length enhance $O_2^{\bullet-}$ production in the following order PQ-1>PQ-2>PQ-9 (PQ-n, *n* isoprenoid units in the side-chain), it has been proposed that plastoquinone is involved in $O_2^{\bullet-}$ production. Due to the higher polarity and the smaller molecular mass of short-chain plastoquinones, the short-chain plastoquinones (PQ-1, PQ-2) are able to bind more effectively in the vicinity of the heme iron of LP form of cyt b_{559} at the relatively polar membrane region. The observation that the inhibition of Q_A to Q_B electron transport by DCMU decreased $O_2^{\bullet-}$ production to about half reveals that the ferric heme iron of LP form of cyt b_{559} is reduced by $Pheo^-$. The three-dimensional crystal structure of PSII from thermophilic cyanobacteria showed that both pheophytins on D1 ($Pheo_{D1}$) and D2 ($Pheo_{D2}$) proteins are far away from the heme iron of cyt b_{559} to sufficiently maintain a direct electron transport (the heme iron is located approximately 50 Å and 30 Å from $Pheo_{D1}$ and $Pheo_{D2}$, respectively) [29]. Based on these considerations, it has been proposed that plastoquinone bound in the vicinity of cyt b_{559} mediates electron transport from $Pheo_{D2}$ to the heme iron of cyt b_{559} [89]. More recent crystal structure of PSII showed that the head group of plastoquinone bound at Q_C site is located 10.1 Å and 17.1 Å from $Pheo_{D2}$ and the heme group of cyt b_{559}, respectively [90,91]. It seems likely that electron transport from $Pheo_{D2}$ to the heme iron occurs via plastoquinone bound at Q_C site, which fills the gap between the normally inactive $Pheo_{D2}$ and the heme iron of cyt b_{559}. As the midpoint redox potential of $Pheo_{D2}/Pheo_{D2}^-$ redox couple is more negative by about 80–210 mV than the midpoint redox potential of $Pheo_{D1}/Pheo_{D1}^-$ redox couple, the formation of $Pheo_{D2}^-$ is less favorable from the thermodynamic point of view [92]. It is proposed here that the binding of plastoquinone at Q_C site might modulate the midpoint redox potential of $Pheo_{D2}/Pheo_{D2}^-$ and thus maintains the reduction of $Pheo_{D2}$. The binding of plastoquinone to Q_C site might serve as a protective valve for the side electron transport to molecular oxygen when forward electron transport is limited due to Q_A overreduction.

3.2. H_2O_2 production on the electron acceptor side of PSII

Hydrogen peroxide is formed by the one-electron reduction of $O_2^{\bullet-}$ via the non-enzymatic and the enzymatic reaction pathways (Fig. 3). In the non-enzymatic reaction pathway, the spontaneous dismutation [13,93] and the one-electron reduction of $O_2^{\bullet-}$ by plastoquinol [85] result in the formation of free peroxide. In the enzymatic reaction pathway, the interaction of $O_2^{\bullet-}$ with the heme and non-heme iron result in the formation of free and bound peroxide, respectively [13,20].

3.2.1. Non-enzymatic reduction of $O_2^{\bullet-}$ to H_2O_2

Several lines of evidence have been provided that $O_2^{\bullet-}$ formed on the electron acceptor side of PSII spontaneously dismutates to H_2O_2 [13,93]. Superoxide anion radical spontaneously dismutates to H_2O_2 and molecular oxygen in a reaction that requires the direct interaction of two negatively charged $O_2^{\bullet-}$ (Fig. 3, reaction 6).

$$O_2^{\bullet-} + O_2^{\bullet-} \rightarrow H_2O_2 + O_2$$

As two molecules with the same charge are repulsed, the interaction of two negatively charged $O_2^{\bullet-}$ is rather limited. Contrary, due to the lack of charge on the protonated form of superoxide $HO_2^{\bullet}$ (hydroperoxyl radical), $HO_2^{\bullet}$ easily interacts either with the negatively charged $O_2^{\bullet-}$ or the neutral $HO_2^{\bullet}$. As the pK_a value of $HO_2^{\bullet}$ is 4.8 [1], $HO_2^{\bullet}$ is formed at the acid microenvironment, whereas $O_2^{\bullet-}$ is preferred at the neutral pH. When protons are available e.g. at the membrane edge, the spontaneous dismutation is favored, whereas in the interior of the membrane, the dismutation reaction is preferably catalyzed by PSII metal centers.

Superoxide anion radical formed in PQ pool is reduced by plastoquinol (PQH_2) to H_2O_2 (Fig. 3, reaction 7) [85].

$$O_2^{\bullet-} + PQH_2 \rightarrow H_2O_2 + PQ^{\bullet-}$$

Due to the large difference between the midpoint redox potential of $PQ^{\bullet-}/PQH_2$ redox couple ($Em = +370$ mV, pH 7) [86] and the standard redox potential of $O_2^{\bullet-}/H_2O_2$ redox couple ($E'_0 = +890$ mV, pH 7) [82] (Table 1), the reduction of $O_2^{\bullet-}$ by PQH_2 is highly favorable.

3.2.2. Enzymatic reduction of $O_2^{\bullet-}$ to H_2O_2

Recently, it has been demonstrated that the light-induced oxidation of the ferrous heme iron in Tris-treated PSII membranes with the restored high-potential (HP) form of cyt b_{559} is prevented by exogenous SOD [20]. Based on this observation, it has been suggested that the reduction of $HO_2^{\bullet}$ by the ferrous heme iron of HP form of cyt b_{559} results in the formation of free peroxide and ferric heme iron of the intermediate-potential (IP) form of cyt b_{559} (Fig. 3, reaction 8). As the midpoint redox potential of Fe^{3+}/Fe^{2+} redox couple in HP form of cyt b_{559} is less positive ($Em \sim +310$ to $+400$ mV, pH 7) than the standard redox potential of $HO_2^{\bullet}/H_2O_2$ redox couple ($E'_0(O_2^{\bullet-}/H_2O_2) = +890$ mV, pH 7) [82] (Table 1), the reduction of $HO_2^{\bullet}$ by the ferrous heme iron of HP form of cyt b_{559} is favored. In the first step, the interaction of $HO_2^{\bullet}$ with the ferrous heme iron of HP form of cyt b_{559} results in the oxidation of the ferrous heme iron to the ferric heme iron and the formation of the ferric-hydroperoxo intermediate (Fe^{3+}–OOH) (Fig. 4A, reaction 3). In the second step, the deprotonation of the heme-binding histidine residue results in the conversion of HP to IP form of cyt b_{559} and the formation of free peroxide. The deprotonation of the heme-binding histidine residue results in the cleavage of the heme-oxygen bridge, whereas the released proton is provided to the proximal oxygen of the ferric-hydroperoxo intermediate during the formation of free peroxide (Fig. 4A, reaction 4). The cleavage of the heme-oxygen bridge is followed by the restoration of the sixth coordination of the ferric iron with nitrogen of the imidazole ring.

Apart of the interaction of $HO_2^{\bullet}$ with the heme iron of cyt b_{559}, the interaction of $O_2^{\bullet-}$ with the non-heme iron has been proposed to form the bound peroxide (Fig. 3, reaction 9) [13]. As the midpoint redox potential of Fe^{3+}/Fe^{2+} redox couple of the non-heme iron is +400 mV (pH 7) [94], the reduction of $O_2^{\bullet-}$ to H_2O_2 ($E'_0(O_2^{\bullet-}/H_2O_2) = +890$ mV, pH 7) by the ferrous non-heme iron is favorable. In the first step, the interaction of $O_2^{\bullet-}$ with the ferrous non-heme iron results in the oxidation of the ferrous non-heme iron to the ferric non-heme iron and the formation of the ferric-peroxo intermediate (Fe^{3+}–OO) (Fig. 4B, reaction 1). In the second step, the protonation of the ferric-peroxo intermediate forms the ferric-hydroperoxo species (Fe^{3+}–OOH) (Fig. 4B, reaction 2).

3.3. $HO^{\bullet}$ production on the electron acceptor side of PSII

Hydroxyl radical is formed by the one-electron reduction of either the free or the bound peroxide [95,96]. In the classical Fenton mechanism, free peroxide is reduced by free metals. Apart of the classical Fenton mechanism, $HO^{\bullet}$ is formed by the reduction of peroxide coordinated to the PSII metal center via the ferric-oxo species as an intermediate product.

3.3.1. Reduction of free peroxide to $HO^{\bullet}$

In the Fenton reaction pathway, the free peroxide is reduced to $HO^{\bullet}$ and OH^- by free metals such as iron released from the damaged PSII (Fig. 3, reaction 10) [13].

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^- + Fe^{3+}$$

As the standard redox potential of free iron ($E'_0(Fe^{3+}/Fe^{2+}) = +110$ mV, pH 7) [97] is lower than the standard redox potential of

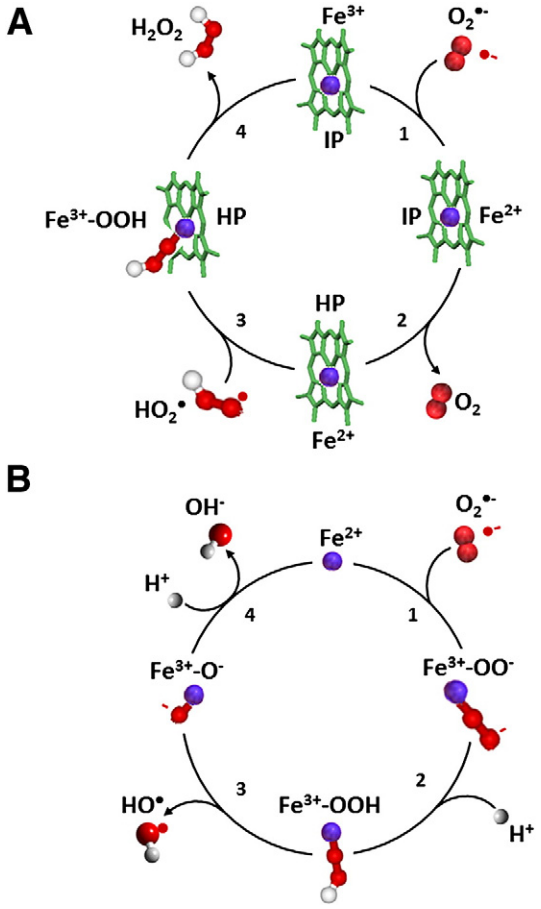

Fig. 4. Interaction of superoxide anion radical with the heme (A) and the non-heme (B) iron in PSII. A. The reduction of the ferric heme iron of IP form of cyt b_{559} by superoxide anion radical ($O_2^{\bullet-}$) results in the formation of the ferrous heme iron of IP form of cyt b_{559} (reaction 1) and the conversion of the IP to HP form of cyt b_{559}, while molecular oxygen is formed (superoxide oxidase activity of cyt b_{559}) (reaction 2). The interaction of hydroperoxyl radical ($HO_2^{\bullet}$) with the ferrous heme iron of HP form of cyt b_{559} forms the ferric-hydroperoxo intermediate (Fe^{3+}–OOH) (reaction 3) followed by the conversion of HP to IP form of cyt b_{559} and the formation of free peroxide (reaction 4). B. The interaction of $O_2^{\bullet-}$ with the ferrous non-heme iron results in the formation of the ferric-peroxo intermediate (Fe^{3+}–OO) (reaction 1). The protonation of the ferric-peroxo intermediate forms the ferric-hydroperoxo intermediate (Fe^{3+}–OOH) (reaction 2). The reduction of ferric-hydroperoxo intermediate brings about the formation of the ferric-oxo intermediate (Fe^{3+}–O^-), while $HO^{\bullet}$ is formed (reaction 3). The protonation of the ferric-oxo intermediate results in the liberation of hydroxo anion (OH^-) and the fromation of ferrous non-heme iron (reaction 4).

$H_2O_2/HO^{\bullet}$ redox couple ($E'_0(H_2O_2/HO^{\bullet}) = +460$ mV, pH 7) [98] (Table 1), the reduction of H_2O_2 by free ferrous iron is thermodynamically favored.

3.3.2. Reduction of bound peroxide to $HO^{\bullet}$

The reduction of ferric-hydroperoxo intermediate (Fe^{3+}–OOH) formed by the interaction of $O_2^{\bullet-}$ with the ferrous non-heme iron results in $HO^{\bullet}$ and HO^- (Fig. 3, reaction 11) [13]. As the midpoint redox potential of Fe^{3+}/Fe^{2+} redox couple of the non-heme iron is +400 mV (pH 7) [94] is lower than the standard redox potential of $H_2O_2/HO^{\bullet}$ redox couple is +460 mV (pH 7) [98] (Table 1), the reduction of bound peroxide by the ferrous non-heme iron is favorable. In the first step, the reduction of ferric-hydroperoxo intermediate (Fe^{3+}–OOH) results in the formation of ferric-oxo intermediate (Fe^{3+}–O^-) and $HO^{\bullet}$ (Fig. 4B, reaction 3). In this reaction, the ferric non-heme iron is reduced by Q_A^-, whereas the formed ferrous iron reduces the hydroperoxo ligand. In the seconds step, the protonation of the ferric-oxo intermediate brings about the release of HO^-, while the ferrous non-heme iron is formed (Fig. 4B, reaction 4).

3.4. H_2O_2 production on the electron donor side of PSII

The incomplete oxidation of water by the manganese complex results in the formation of H_2O_2 (Fig. 3, reaction 12) [23]. The two-electron oxidation of water has been proposed to involve the transition from either S_2 to S_0 state [14,99] or S_1 to S_{-1} state [100].

$$2H_2O + Mn^n\text{–}Mn^n \rightarrow H_2O_2 + Mn^{n-1}\text{–}Mn^{n-1}$$

It has been proposed that peroxide is formed by the interaction of either two hydroxo group coordinated to the different manganese ions of the manganese complex [14] or the hydroxo group with the oxo group coordinated to the same manganese ion of the manganese complex [15]. Fine and Frasch [14] suggested that the peroxide is formed by the interaction of the hydroxo group formed by the coordination of unprotonated water to the manganese ion and the hydroxo group formed by the coordination of unprotonated water to the proximal manganese ion at the chloride-binding site. Wydrzynski et al. [15] proposed that the peroxide is formed by the interaction between the hydroxo group formed by the coordination of unprotonated water to the manganese ion and the terminal oxo group normally coordinated to the manganese ion. Recently, Antal et al. [101] could not detect a significant amount of H_2O_2 associated to the two-electron oxidation of water during S_3 to S_1 state transition. These observations indicate that S_3 to S_1 state transition is unlikely involved in the formation of H_2O_2 on the electron donor side of PSII.

3.5. $O_2^{\bullet-}$ production on the electron donor side of PSII

The one-electron oxidation of H_2O_2 by the oxidizing species on the electron donor side of PSII has been postulated to form $O_2^{\bullet-}$ [16,102]. It has been proposed that H_2O_2 is oxidized by the redox active tyrosine residue Tyr_Z (Fig. 3, reaction 13).

$$H_2O_2 + Tyr_Z^{\bullet} \rightarrow O_2^{\bullet-} + Tyr_Z$$

The one-electron oxidation of H_2O_2 to $O_2^{\bullet-}$ can be favored only by the strong oxidant with the midpoint redox potential more positive than the standard redox potential of the $O_2^{\bullet-}/H_2O_2$ redox couple ($E'_0(O_2^{\bullet-}/H_2O_2) = +890$ mV, pH 7) (Table 1). As the midpoint redox potential of $Tyr_Z^{\bullet}/Tyr_Z$ redox couple is ~1100–1200 mV [5,103], the oxidation of H_2O_2 by $Tyr_Z^{\bullet}$ is favorable. In PSII capable of water oxidation, the reduction of $Tyr_Z^{\bullet}$ by the manganese complex occurs in the μs–ms range, depending on the oxidation state of manganese ions [104]. However, in the absence of the manganese complex, the lifetime of $Tyr_Z^{\bullet}$ is determined by the reduction maintained by an exogenous reductant such as ascorbate. It has been demonstrated that ascorbate (AsA) in the lumen of thylakoid membrane serves as an efficient electron donor to $Tyr_Z^{\bullet}$ forming monodehydroascorbate radical (MDA) [105,106]. As the standard redox potential of AsA/MDA redox couple is +320 mV (pH 7) [107], the reduction of $Tyr_Z^{\bullet}$ by ascorbate is more favored than the reduction of $Tyr_Z^{\bullet}$ by H_2O_2.

3.6. $HO^{\bullet}$ production on the electron donor side of PSII

Hydrogen peroxide is reduced to $HO^{\bullet}$ and OH^- by free metals such as manganese ion released from the damaged manganese complex (Fig. 3, reaction 14) [17,18,108].

$$H_2O_2 + Mn^{2+} \rightarrow HO^{\bullet} + OH^- + Mn^{3+}$$

As the standard redox potential of free manganese is highly positive ($E'_0(Mn^{3+}/Mn^{2+}) = +1200$ mV, pH 7) [109] (Table 1), the free manganese ion has no redox power to reduce H_2O_2. However, the redox potential of manganese can be significantly modulated by the coordination environment of manganese. It seems likely that the redox potential of manganese released from the damaged

manganese complex is decreased by the temporal coordination of manganese to the extrinsic protein bound on the lumenal side of PSII. It has been demonstrated that the 23 kDa extrinsic protein may play a role in manganese binding during the turnover of the D1 protein [110,111]. Furthermore, the standard redox potential of $H_2O_2/HO^{\bullet}$ redox couple increases when pH is decreased. As the proton transfer across the thylakoid membrane and the proton release from water decrease the pH in the lumen below 4, the modulation of redox potential of $H_2O_2/HO^{\bullet}$ redox couple by pH is mainly relevant on the electron donor side of PSII. Based on these considerations, it is proposed that the decrease in the redox potential of manganese by the coordination to the protein and the increase in the standard redox potential of $H_2O_2/HO^{\bullet}$ redox couple with the pH decrease make the reduction of H_2O_2 by manganese thermodynamically more favorable.

4. Non-enzymatic ROS scavenging

Reactive oxygen species are known to induce the oxidative damage to pigments, proteins and lipids in the thylakoid membrane. In the prevention of deleterious effect of ROS on the membrane components, the non-enzymatic and the enzymatic ROS scavenging are engaged. The non-enzymatic ROS scavengers involve the hydrophobic antioxidants such as prenyllipids comprising of carotenoids (neoxanthin, zeaxantin and β-carotene) and prenylquinols (α-tocopherol, plastoquinol) known to eliminate the deleterious effect of $^1O_2$. Singlet oxygen scavenging occurs either by the excitation energy transfer (physical scavenging) or by the electron transport (chemical scavenging) [112,113]. The physical scavenging of $^1O_2$, denoted as $^1O_2$ quenching, is maintained by carotenoids, whereas the chemical scavenging of $^1O_2$ is proceeded predominantly by prenylquinols. In the physical type of $^1O_2$ scavenging, the excitation energy from $^1O_2$ is transferred to the quencher forming the triplet excited state of the quencher, the energy of which is lost as heat [114]. Typically, one molecule of quencher can deactivate several hundreds of $^1O_2$ molecules. In the chemical type of $^1O_2$ scavenging, the interaction of $^1O_2$ with the scavenger modifies the structure of scavenger irreversibly in such a way that typically one molecule of scavenger can eliminate the deleterious effect of one $^1O_2$ molecule. Under such circumstances, the continuous re-synthesis of scavengers is required to keep its level sufficient for the photoprotection of PSII.

4.1. $^1O_2$ scavenging by carotenoids

The excitation energy transfer from $^1O_2$ to carotenoid results in the formation of the ground triplet state of molecular oxygen ($^3O_2$) and the triplet excited state of carotenoid ($^3Car^*$) (Fig. 1, reaction 12). The triplet carotenoid decays radiationless into the ground state (Car), while the triplet excitation energy is converted effectively into heat (Fig. 1, reaction 13) [39].

$$^1O_2 + Car \rightarrow O_2 + {}^3Car^*$$

$$^3Car^* \rightarrow Car + heat$$

The singlet–triplet energy transfer from $^1O_2$ to carotenoid is possible, as the triplet energy level of carotenoid is below the energy level of $^1O_2$ [115]. The singlet–triplet energy transfer from singlet oxygen ($O_2(^1\Delta_g)$) to carotenoid proceeds via the formation of $^1(^1\Delta \cdot S_0)$ encounter complex. The later deactivates irreversibly by the internal conversion to $^1(T_1 \cdot {}^3\Sigma)$ encounter complexes leading to the formation of molecular oxygen and triplet carotenoid [116].

$$O_2(^1\Delta_g) + Car \rightarrow {}^1(^1\Delta \cdot S_0) \rightarrow {}^1(T_1 \cdot {}^3\Sigma) \rightarrow O_2(^3\Sigma_g) + {}^3Car^*$$

4.1.1. $^1O_2$ scavenging by xanthophylls

Singlet oxygen formed in LHCII is effectively quenched by xanthophylls. Whereas α-xanthophyll (lutein) is an efficient quencher of the triplet chlorophyll, β-xanthophyll (zeaxanthin and neoxanthin) serves as a quencher of $^1O_2$ [41]. Under high light, violaxanthin is converted to zeaxanthin via the enzymatic removal of the epoxy groups from violaxanthin [117]. The conversion of violaxanthin to zeaxanthin is catalyzed by the enzyme violaxanthin de-epoxidase, while the reverse conversion of zeaxanthin to violaxanthin is performed by zeaxanthin epoxidase.

4.1.2. $^1O_2$ scavenging by β-carotene

Singlet oxygen formed in the PSII core antenna complex and the PSII reaction center is quenched by β-carotene [73,75]. Singlet oxygen quenching is mainly of importance in the PSII reaction center, where β-carotenes are not bound in the proximity of triplet chlorophylls. It was demonstrated that $^1O_2$ formation measured by time-resolved luminescence at 1270 nm was pronouncedly enhanced when one β-carotene was removed from the PSII reaction center [118].

4.2. $^1O_2$ scavenging by prenylquinols

Apart of physical scavenging of $^1O_2$ by carotenoids, the chemical scavenging of $^1O_2$ by prenylquinols such as α-tocopherol (α-Toc) [75,119–122] and plastoquinol (PQH_2) [123–125] plays an important role in the elimination of deleterious effect of $^1O_2$ on pigments, proteins and lipids.

4.2.1. $^1O_2$ scavenging by α-tocopherol

The oxidation of α-Toc by $^1O_2$ was shown to form 8-hydroperoxy-tocopherone (α-TOOH) intermediate, which hydrolyzes to α-tocopherolquinone (α-TQ) [126].

$$^1O_2 + \alpha - Toc \rightarrow \alpha - TOOH \rightarrow \alpha - TQ$$

The authors assumed that the part of α-TOOH, which is not converted to α-TQ, remains in the thylakoid membrane. As the chemical properties of α-TOOH are similar to those of lipid peroxides, it is likely that α-TOOH might promote lipid peroxidation and consequently contribute to the overall $^1O_2$ production by Russell-type mechanism. In this reaction, the self-reaction of two lipid peroxyl radicals forms $^1O_2$ via a putative intermediary tetraoxide [1].

As the oxidation of α-Toc by $^1O_2$ is an irreversible reaction, a continuous re-synthesis of α-Toc is required to keep its level sufficient for the photoprotection [120]. Due to the fact that the chromanol ring of α-Toc is likely located close to the membrane edge [127,128], α-Toc is effective in the scavenging of those $^1O_2$ which escape the antioxidant barrier formed by carotenoid in the interior of the thylakoid membrane.

4.2.2. $^1O_2$ scavenging by plastoquinol

Several lines of evidence have been recently provided on $^1O_2$ scavenging by plastoquinol (PQH_2) [123,124]. The oxidation of PQH_2 by $^1O_2$ was shown to results in the formation of plastoquinone (PQ) which is further oxidized by $^1O_2$ to the plastoquinone derivate with one (PQ(OH)) and consequently three ($PQ(OH)_3$) hydroxyl groups in the side chain [126].

$$PQH_2 \xrightarrow{^1O_2} PQ \xrightarrow{^1O_2} PQ(OH) \xrightarrow{^1O_2} PQ(OH)_3$$

In spite of the fact that the quenching rate constant of PQH_2 is several times lower compared to that of α-Toc, PQH_2 has a large capability to scavenge $^1O_2$. In comparison to α-Toc, PQH_2 is formed by the reduction of PQ directly in the thylakoid membrane. The plastoquinol ring is located deeper in the membrane within the highly hydrophobic interior of the thylakoid membrane, where $^1O_2$ is formed.

5. Enzymatic ROS scavenging

The enzymatic scavenging of ROS, which involves enzymes such as SOD and catalase, represents an efficient way for the elimination of ROS formed by the electron transport. Evidence has been provided that either the intrinsic metalloproteins or small protein subunits of PSII exhibit the antioxidant activity [20–23,25].

5.1. $O_2^{\bullet-}$ scavenging by superoxide dismutase

Superoxide dismutase catalyzes the dismutation of $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen. It has been demonstrated that $O_2^{\bullet-}$ is scavenged by cyt b_{559} [19,20] and SOD attached to the stromal side of the thylakoid membrane at the vicinity of PSII [21,22]. The intrinsic SOD activity of cyt b_{559} provides the first line of defense against $O_2^{\bullet-}$ in the membrane interior, where $O_2^{\bullet-}$ is formed. On the other hand, SOD attached to the thylakoid membrane is proposed to play a role as the second defense against those $O_2^{\bullet-}$ which diffuse from the membrane interior to the stroma.

5.1.1. $O_2^{\bullet-}$ scavenging by cyt b_{559}

It is well known that the redox active metal in SOD is able to both oxidize and reduce $O_2^{\bullet-}$ depending on the oxidation state of the metal and the protonation state of the nearby residues [129,130]. The redox active heme iron of cyt b_{559} has been shown to be both oxidized and reduced by $O_2^{\bullet-}$ depending on the oxidation state of the heme iron and the protonation state of histidine residues [20,131,132]. Based on this analogy, Tiwari and Pospíšil [20] proposed that the uprotonated IP form of cyt b_{559} serves as superoxide oxidase (SOO) that catalyzes the one-electron oxidation of $O_2^{\bullet-}$ to molecular oxygen, whereas the protonated HP^{red} form of cyt b_{559} serves as superoxide reductase (SOR) that catalyzes the one-electron reduction of $O_2^{\bullet-}$ to H_2O_2. The reduction of the ferric heme iron by $O_2^{\bullet-}$ results in the formation of the ferrous heme iron, whereas O_2 is released as a by-product (Fig. 4A, reactions 1 and 2). As the standard redox potential of the $O_2/O_2^{\bullet-}$ redox couple ($E'_0 = -160$ mV, pH 7) [82] is lower that the midpoint redox potential of Fe^{3+}/Fe^{2+} redox couple in IP form of cyt b_{559} ($Em = +125$ mV, pH 7) [20,131] (Table 1), the reduction of the ferric heme iron by $O_2^{\bullet-}$ is favorable from the thermodynamic point of view. The oxidation of the ferrous heme iron by $HO_2^{\bullet}$ results in the formation of the ferric heme iron and H_2O_2 (Fig. 4A, reactions 3 and 4). From the thermodynamic point of view, the oxidation of the ferrous heme iron by $HO_2^{\bullet}$ is favorable, as the midpoint redox potential of Fe^{3+}/Fe^{2+} redox couple in HP form of cyt b_{559} ($Em = +310$ mV, pH 7) [20,131] is less positive than the standard redox potential of the $O_2^{\bullet-}/H_2O_2$ redox couple ($E'_0 = +890$ mV, pH 7) [82] (Table 1).

5.1.2. $O_2^{\bullet-}$ scavenging by FeSOD

In the chloroplasts, copper-zinc SOD (Cu/ZnSOD) and iron SOD (FeSOD) were described [133]. Whereas the Cu/ZnSOD is attached to the thylakoid membrane at the vicinity of PSI, the FeSOD was demonstrated to be attached to the stromal side of the thylakoid membrane closed to PSII [21,22,134]. Based on the immunoblot analysis of the protein extracted from PSII membranes, Navari-Izzo et al. [21] showed that the 29 kDa protein was recognized as FeSOD. More recently, Zhang et al. [22] have demonstrated increased level of $O_2^{\bullet-}$ production and D1 degradation in the transgenic tobacco plants with severely decreased chloroplastic FeSOD.

5.2. H_2O_2 scavenging by catalase

Catalase is a peroxidase type of enzyme that catalyzes the disproportion of H_2O_2 in water and molecular oxygen. Several lines of evidence have been provided that H_2O_2 is scavenged by the manganese complexes [23,135] and the heme catalase attached to the lumenal side of the thylakoid membrane at the vicinity of PSII [25,136]. The intrinsic catalase activity of the manganese complex plays a role in the first line of defense against H_2O_2 formed by the incomplete oxidation of water by the manganese complex. Contrary, the heme catalase attached to the lumenal side of the thylakoid membrane serves as the second defense against H_2O_2 released into the lumen.

5.2.1. H_2O_2 scavenging by the manganese complex

The two-electron reduction and oxidation of H_2O_2 was shown to occur both in the dark and light [16,137,138]. The dark reaction of H_2O_2 has been assumed to involve either the S_1/S_{-1} cycle or flash-triggered S_2/S_0 cycle, whereas the light reaction was proposed to be mediated by free Mn^{2+}.

5.2.1.1. Dark reaction. The two-electron reduction and oxidation of H_2O_2 catalyzed by the manganese complex were proposed from the measurements of the yield of molecular oxygen evolved after a single flash in the presence of H_2O_2 [137]. The author demonstrated that the manganese complex catalyzes the disproportion of H_2O_2 in water and molecular oxygen. Based on the steady-state production of molecular oxygen from H_2O_2, it has been proposed that the manganese complex completes many catalytic cycles. The disproportion of H_2O_2 was demonstrated to proceed via two sequential reactions [14,16,24,102,139,140]. In the initial reaction, H_2O_2 is oxidized by the manganese complex to molecular oxygen (S_2 to S_0 or S_1 to S_{-1} state transitions), whereas in the sequential reaction, H_2O_2 is reduced to water by the manganese complex (S_0 to S_2 or S_{-1} to S_1 state transitions).

5.2.1.2. Light reaction. In addition to the dark reaction, the oxidation of H_2O_2 to O_2 was examined in the light [138]. Hydrogen peroxide was shown to be an efficient electron donor to PSII after the removal of manganese from the manganese complex. Due to the observation that the two-electron oxidation of H_2O_2 to molecular oxygen was abolished after the addition of EDTA, it has been proposed that H_2O_2 oxidation is mediated by free manganese [138]. In the absence of free manganese, H_2O_2 was inefficient to donate electron to PSII, whereas the presence of free manganese supports the oxidation of H_2O_2 and the electron donation to PSII. Based on this, it was proposed that manganese plays a role of the redox mediator in the electron donation to PSII. Due to the observation that the addition of H_2O_2 to the intact thylakoid in light causes the release of manganese from the manganese complex, Saudsky and Yocum [140] proposed that manganese released from the manganese complex causes two-electron oxidation of H_2O_2. The light-induced H_2O_2 oxidation depends on the presence of free or loosely bound manganese that can originate from the damage manganese complex.

5.2.2. H_2O_2 scavenging by the heme catalase

Frasch and Mei [141] demonstrated that the solubilization of PSII membranes with octyl glucoside significantly increases H_2O_2 production. The author assumed that the enhancement in H_2O_2 production is due to detergent-induced removal of intrinsic catalase. However, as the manganese complex was shown to be insensitive to octyl glucoside treatment, the authors suggested that rather than the manganese complex it is another component associated with PSII that is responsible for catalase-like activity. In accordance with this suggestion, Quensel and Åkerlund [142] demonstrated that the removal of manganese by hydroxylamine-treatment results only in the partial inhibition of catalase activity. Based on these observations, the heme-catalase was suggested to be associated with PSII [135]. Later, it was shown that it is mainly PSII membrane associated heme-catalase which is responsible for the disproportion of H_2O_2 to water and molecular oxygen in PSII [25]. The heme-catalase was identified as the 63 kDa protein located on the lumenal side of thylakoid membrane closed to PSII [136].

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