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Review

Production of reactive oxygen species by photosystem II

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ABSTRACT

Photosysthetic cleavage of water molecules to molecular oxygen is a crucial process for all aerobic life on the Earth. Light-driven oxidation of water occurs in photosystem II (PSII) — a pigment–protein complex embedded in the thylakoid membrane of plants, algae and cyanobacteria. Electron transport across the thylakoid membrane terminated by NADPH and ATP formation is inadvertently coupled with the formation of reactive oxygen species (ROS). Reactive oxygen species are mainly produced by photosystem I; however, under certain circumstances, PSII contributes to the overall formation of ROS in the thylakoid membrane. Under limitation of electron transport reaction between both photosystems, photoreduction of molecular oxygen by the reducing side of PSII generates a superoxide anion radical, its dismutation to hydrogen peroxide and the subsequent formation of a hydroxyl radical terminates the overall process of ROS formation on the PSII electron acceptor side. On the PSII electron donor side, partial or complete inhibition of enzymatic activity of the water-splitting manganese complex is coupled with incomplete oxidation of water to hydrogen peroxide. The review points out the mechanistic aspects in the production of ROS on both the electron acceptor and electron donor side of PSII.

1. Introduction

Molecular oxygen released as a by-product of the photosynthetic oxidation of water has allowed oxygen-based respiration, a process essential for most life on the planet. However, an oxygen-rich environment is something of a mixed blessing since life under such circumstances is confronted with the formation of dangerous reactive oxygen species (ROS). While the production of ROS can be important in several cellular processes (e.g. defense against infection, cellular signaling), the presence of ROS is more often associated with damage to cellular components such as proteins, lipids and nucleic acids. Protein complexes involved in the electron transport processes are thermodynamically capable of inadvertently reacting with molecular oxygen and thus producing ROS. Such reactions are not only wasteful of energy but also potentially dangerous to the proteins themselves. Evolution has kept such inefficient processes to a minimum level. When the formation of ROS is not wholly eliminated, a broad range of non-enzymatic and enzymatic scavengers has been evolved in order to minimize the damage to cellular components.

In the plant cell, electron transport processes in chloroplasts and mitochondria are the potential source of ROS [1]. The major generation site of ROS in the thylakoid membrane of chloroplast is photosystem I (PSI) and photosystem II (PSII) [2]. Although ROS are mainly produced by PSI, the light-induced production of various types of ROS was demonstrated in PSII. In this respect, PSII itself is unique: 1) it is the source of molecular oxygen and 2) it performs redox chemistry which spans the redox range from lower than −600 mV to higher than 1.25 V i.e. with extremely reducing and extremely oxidizing potentials [3,4]. Its reducing components are capable of reducing molecular oxygen, whereas the oxidizing components are by definition capable of oxidizing water. Furthermore, PSII contains a number of chlorophyll molecules that can act as a photosensitizer for the production of singlet oxygen ($^1O_2$). It has been demonstrated that electron-hole recombination chemistry can produce a triplet state of chlorophyll that converts triplet molecular oxygen into its reactive singlet form [5–7] (for a recent review see [8–10]). In this review, a focus is given on the formation of ROS occurring under both the reducing and oxidizing conditions on the electron acceptor and donor side of PSII, respectively: superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$).

Abbreviations: ADRY, agents *a*ccelerating the *d*eactivation *r*eactions of water-splitting enzyme *Y*; Chl_Z, redox active chlorophyll in PSII; Cyt b_{559}, cytochrome b_{559}; DCMU, 3-3,4-dichlorophenyl-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide; DPC, diphenylcarbazid; *E*m, midpoint redox potential; EDTA, ethylenediaminetetraacetic acid; EMPO, 5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide; EPR, electron paramagnetic spectroscopy; LCC, lauroylcholine chloride; PSII, photosystem II; Pheo, phephytin — primary electron acceptor of PSII; PPBQ, phenyl-p-benzoquinone; PQ, plastoquinone; $PQH^{\cdot}$, plastosemiquinone radical; P680, primary electron donor of PSII; Q_A, primary plastoquinone electron acceptor of PSII; S_i, redox state of water-splitting Mn complex; SOD, superoxide dismutase; Tiron, 1,2-dihydroxybenzene-3,5-disulphonate; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Tyr_Z, redox active tyrosine residue of D1 protein

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2. PSII and ROS

2.1. PSII photochemistry

Photosynthetic splitting of water molecules into molecular oxygen occurs in PSII, which acts as a water:plastoquinone oxidoreductase (Fig. 1) (for a recent review on PSII see e.g. [11–13], for the X-ray structure of PSII, see [14,15]). Charge separation takes place among the central pigments upon excitation either directly or through excitation energy transfer from the large array of light collecting pigments (not shown). The first detectable radical pair involves a chlorophyll cation $P680^+$ (localized on the P_{D1} and P_{D2} dimer) and pheophytin anion $Pheo^-$ (localized on the $Pheo_{D1}$). The electron from $Pheo^-$ is transferred to a tightly bound plastoquinone molecule, Q_A, which acts as a one-electron carrier [16–18]. The $P680^+$ cation is oxidizing sufficiently to remove an electron from a tyrosine residue, Tyr 161 of D1, forming the neutral tyrosyl radical, $Tyr_Z^{\cdot}$ [19,20]. The electron from Q_A^- is transferred to a firmly bound plastoquinone acceptor, Q_B, forming the relatively stable semiquinone, Q_B^- [21]. Charge separation and charge stabilization are completed within a millisecond time range, with the early steps occurring in the picosecond time scale. The overall process is highly efficient with a quantum yield close to 100% i.e. about 1 eV of the 1.8 eV of the red photon's energy is stored in the final stable radical pair.

The subsequent excitations of the PSII reaction center produce further change separation and charge stabilization reactions. The water-splitting Mn complex undergoes a consecutive series of five intermediates (S_0, S_1, S_2, S_3 and S_4) [22–26]. Whereas the $S_1 \rightarrow S_2$ transition solely involves the oxidation of manganese, the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4 \rightarrow S_0$ transitions are accompanied by a proton release from the water-splitting Mn complex. The arginine residue Agr357 of the CP43 protein (CP43-Arg357) has been recently proposed to serve as the catalytic base in the water-splitting Mn complex [27,28]. On the PSII electron acceptor side, the double reduction and protonation of plastoquinone at the Q_B-binding site leads to the formation of plastoquinol which is released into the membrane and replaced by an additional plastoquinone molecule [21].

A secondary electron transfer pathway exists in PSII, with electrons donated to $P680^+$ by β-carotene (Car_{D2}). It has been demonstrated that the β-carotene cation is reduced by cytochrome b_{559} (cyt b_{559}) or by monomeric chlorophyll Chlz ($Chlz_{D2}$) when the cyt b_{559} is already oxidized [29–32]. The oxidized cyt b_{559} can be reduced by plastoquinone from the pool or from the Q_B-binding site. This is usually considered as a protective pathway aimed at preventing the accumulation of the highly oxidizing $P680^+$. It has been recently suggested, however, that the pathway exists in order to prevent accumulation of the β-carotene cation, which is itself highly oxidizing but importantly cannot act as a quencher of singlet oxygen or chlorophyll triplet [33].

2.2. Two reaction pathways for ROS generation by PSII

The redox components of PSII are spread over a wide scale of redox potentials ranging from the highly negative redox potential of the Pheo/$Pheo^-$ redox couple ($Em = -610$ mV, pH 7) to the highly positive redox potential of the $P680^+$/P680 redox couple ($Em = 1.25$ V, pH 7) or higher [4,13,34]. Thus pathways for ROS formation are expected to occur from the reducing species on the PSII

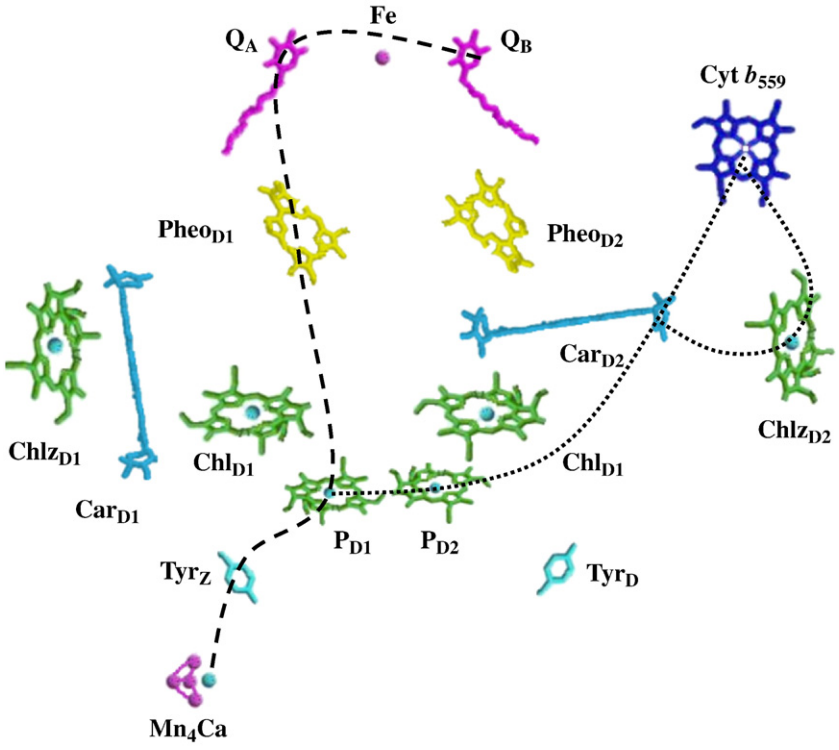

Fig. 1. Photosystem II reaction center structure model. Side view of the redox cofactors involved in the forward electron transfer reaction (dashed line) and the secondary electron transfer pathway (dotted line). Arrangement of cofactors in PSII on the central subunits of D1 and D2 protein: P_{D1} and P_{D2}, chlorophyll *a* dimer; Chl_{D1} and Chl_{D2}, chlorophyll monomers of D1 and D2 protein, respectively; $Pheo_{D1}$ and $Pheo_{D2}$, pheophytin of D1 and D2 protein, respectively; Q_A, primary quinone electron acceptor; Q_B, secondary quinone electron acceptor; Fe, non-heme iron; Tyr_Z, redox active tyrosine residue of D1 protein; Tyr_D, redox active tyrosine residue of D2 protein; Mn_4Ca, water-splitting Mn complex; $Chlz_{D1}$ and $Chlz_{D2}$, peripheral chlorophyll of D1 and D2 protein, respectively; Car_{D1} and Car_{D2}, β-carotene of D1 and D2 protein, respectively. Heme iron of cytochrome b_{559} (cyt b_{559}) is coordinated to α and β subunits of cyt b_{559}. The forward electron transfer reactions involve the primary charge separation on the P_{D1}/P_{D2} dimer and $Pheo_{D1}$, the subsequent reduction of Q_A and Q_B and the oxidation of Tyr_Z and Mn_4Ca complex. The secondary electron transfer reactions include the reduction of the P_{D1}/P_{D2} dimer by Car_{D2} in a linear (cyt $b_{559} \Rightarrow Chlz_{D2} \Rightarrow Car_{D2}$) or a branched (cyt $b_{559} \Rightarrow Car_{D2} \Leftarrow Chlz_{D2}$) pathway.

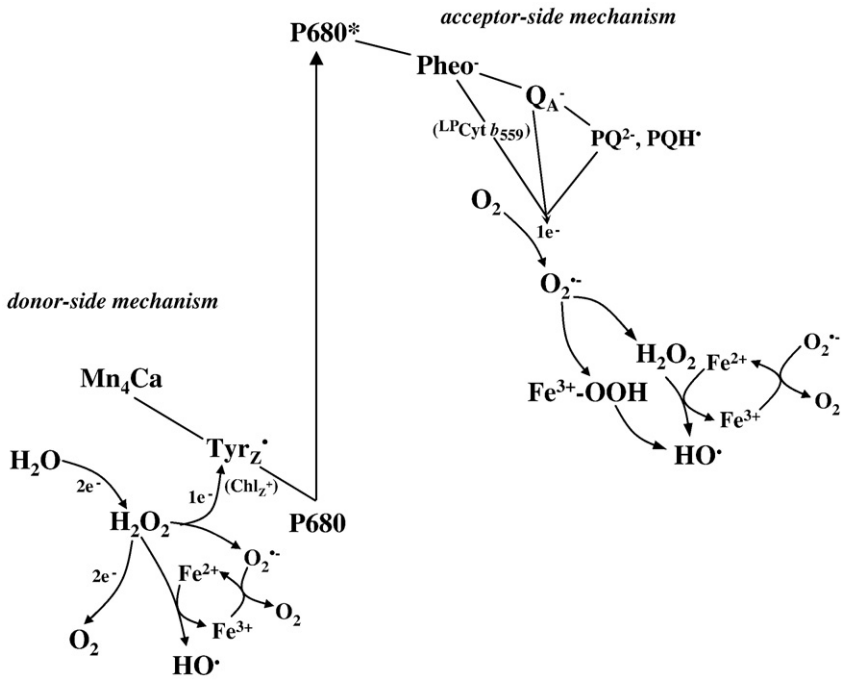

Fig. 2. Production of ROS by acceptor- and donor-side mechanism in PSII. On the electron acceptor side of PSII, one-electron reduction of molecular oxygen by the reducing side of PSII results in the formation of $O_2^{\cdot-}$ which subsequently either dismutates to free H_2O_2 or interact with ferrous non-heme iron forming a ferric-hydroperoxo intermediate. In the presence of free metals (Fe^{2+}, Mn^{2+}), the reduction of H_2O_2 results in the formation of $HO^{\cdot}$ via Fenton-type reaction. The reduction of ferric-hydroperoxo intermediate by an electron presumably from Q_A^- has been suggested to form $HO^{\cdot}$. On the electron donor side of PSII, two-electron oxidation of H_2O by the modified water-splitting Mn complex results in the formation of H_2O_2. Hydrogen peroxide has been proposed to be either oxidized to $O_2^{\cdot-}$ by tyrosine radical $Tyr_Z^{\cdot}$ or reduced to $HO^{\cdot}$ by free metals the most likely being Fe^{2+}, Mn^{2+} released from damaged PSII.

electron acceptor side and the highly oxidizing components on the PSII electron donor side (Fig. 2).

3. ROS generation on the PSII electron acceptor side

Due to the relatively low redox potential required for reduction of molecular oxygen (Em ($O_2/O_2^{\cdot-}$) = −160 mV, pH 7) [35], the number of potential reductants within PSII is limited. Several lines of evidence have been given indicating that under certain circumstances (e.g. absence or full reductions of the PQ pool) molecular oxygen is reduced by the PSII electron acceptor species [36–38]. There is still debate as to which of the reduced acceptors is the actual reductant of molecular oxygen. From the thermodynamic point of view, $Pheo^-$ and Q_A^- are able to reduce molecular oxygen: $Pheo^-$ (Em = −610 mV, pH 7) with a favorable driving force and Q_A^- (Em = −80 mV, pH 7) with only a small thermodynamic barrier to be overcome. These anions have short lifetimes under normal circumstances; however, under certain conditions (e.g. under strong light, during the assembly of PSII etc.) they may have lifetimes long enough to allow them reducing molecular oxygen. Plastoquinone molecules firmly bound at the Q_B-binding site such as Q_B^-, Q_BH_2 are less reducing species: Em (Q_B/Q_B-) = −45 mV, Em (Q_B-/Q_BH_2) = 290 mV and Em (Q_B/Q_BH_2) = 120 mV (pH 7) [39–41]. Thus the reduction of molecular oxygen by plastoquinones is less thermodynamically favorable; however, their particularly long lifetimes may nevertheless make them important as reductants of molecular oxygen. The reduction of molecular oxygen is the starting point for a series of reactions leading to the generation of ROS on the electron acceptor side of PSII. The reaction pathway involves a classical cascade of reactions which is comprised of the formation of $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ (Scheme 1).

$$O_2 \xrightarrow{(1)} O_2^{\bullet-} \xrightarrow{(2)} H_2O_2 \xrightarrow{(3)} HO^{\bullet}$$

Scheme 1. Successive univalent reduction of molecular oxygen to hydroxyl radicals. The reaction pathway involves 1) one-electron reduction of O_2 to $O_2^{\cdot-}$, 2) dismutation of $O_2^{\cdot-}$ to H_2O_2 and 3) one-electron reduction of H_2O_2 to $HO^{\cdot}$.

3.1. $O_2^{\cdot-}$ production

The one-electron reduction of molecular oxygen by the reducing side of PSII has been pondered over for many years [36,42,43]. The light-induced formation of $O_2^{\cdot-}$ in PSII membranes has been demonstrated using an assay consisting of cytochrome *c* reduction in the presence of xanthine/xanthine oxidase [37] or voltametric methods [38]. The development of a spin-trap compound in recent years has enabled the application of a spin-trapping EPR technique for monitoring $O_2^{\cdot-}$ production by PSII involving either DEPMPO [44,45] or the EMPO spin trap compound [46,47].

The primary electron acceptor $Pheo^-$ [37] and the primary quinone electron acceptor Q_A^- [38] were proposed to serve as the reductant of molecular oxygen. Furthermore, evidence has been provided that plastoquinol and cyt b_{559} can donate an electron to molecular oxygen [48–53] (Fig. 2).

3.1.1. $Pheo^-$ as a reductant to O_2

Based on the fact that $Pheo^-$ is a strong reductant (Em = −610 mV, pH 7), the reduction of molecular oxygen by $Pheo^-$ is highly thermodynamically favorable. Ananyev et al. [37] have demonstrated that D1/D2/cyt b_{559} complexes, which lack Q_A, exhibit a significant rate of cytochrome (III) reduction in the light, indicating that $Pheo^-$ reduces molecular oxygen. The authors revealed that in the absence of the electron donor DPC, no reduction of cytochrome (III) was obtained, whereas in the presence of DPC a significant rate of cytochrome (III) reduction was observed. The rate of $O_2^{\cdot-}$ production was estimated to be higher than 21 μmol $O_2^{\cdot-}$ mg^{-1} Chl h^{-1}. However, in fully intact PSII, the reduction of molecular oxygen by $Pheo^-$ is much less likely due to the fast electron transfer from $Pheo^-$ to Q_A^- (300–500 ps) [13,16]. When Q_A is reduced, the lifetime of $Pheo^-$ is prolonged, in all probability being mainly determined by

charge recombination (the $P680^+Pheo^-$ radical pair lifetime is 20 ns [54]). Under these conditions, the formation of a $Tyr_Z^{\cdot}(H^+)Pheo^-$ radical pair is probable in certain PSII centers. Such a state, which has never been detected, is predicted to have a significantly longer lifetime than the $P680^+Pheo^-$ radical pair and thus in consequence more chance to donate an electron to molecular oxygen. In an even smaller fraction of PSII centers, the oxidation of the water-splitting Mn complex may occur providing an even longer-lived $Pheo^-$. In the most extreme case, if S_3 (and possibly S_0) were present prior to illumination, $Pheo^-$ would be formed in the absence of an electron hole for the charge recombination. Under these conditions, the lifetime of $Pheo^-$ would be only limited by its electron donation route to another component. When molecular oxygen is absent, $Pheo^-$ donates an electron to Q_A^- forcing it to undergo a second reduction process, which probably involves protonation and damage of the Q_A site. When molecular oxygen is present, it seems likely that it picks up the electron from $Pheo^-$ forming $O_2^{\cdot -}$.

Based on these considerations, it is proposed that the illumination of PSII centers with Q_A^- already present will provide a distribution of radical pairs involving $Pheo^-$, with the long-lived species being present in the smallest proportions. In addition in a very small fraction of PSII centers, $Pheo^-$ could be trapped in a reduced form through irreversible electron donation from water. Under these conditions, the yield of $O_2^{\cdot -}$ formation would be related to the lifetime of $Pheo^-$ and thus in a small fraction of PSII centers the yield could be significant. All of this could occur in an extremely small fraction of PSII centers as the presence of Q_A^- is known to "close" PSII. This would indicate that the electrostatic influence of Q_A^- is such that the normally high quantum yield of charge separation drops to about 15% in plant PSII with a full complement of light harvesting chlorophylls [55,56]. When Q_A^- is reduced to the fully reduced form (chemically or by $Pheo^-$ using strong light), the centers are "reopened" and the high yield of charge separation is restored. Further illumination of such centers in the presence of molecular oxygen could result in high yields of $O_2^{\cdot -}$ formation providing the PSII electron donor side remains functional. However, such conditions are unlikely to occur in vivo.

3.1.2. Q_A^- as a reductant to O_2

The reduction of molecular oxygen by Q_A^- is less favorable from the thermodynamic point of view (redox potential of the Q_A/Q_A^- redox couple $Em = -80$ mV, pH 7); however, it better fits the kinetic criteria. The redox potential of $O_2/O_2^{\cdot -}$ redox couple ($Em = -160$ mV, pH 7) is only relevant if the concentration of O_2 is equal to the concentration of $O_2^{\cdot -}$. However, in PSII membranes the actual concentration of O_2 (250 μM) highly exceeds the concentration of $O_2^{\cdot -}$ (hundreds nM) formed by PSII. Therefore, the redox power required for reduction of O_2 becomes from Nernst equation $E = -0.16 + 0.06 \log [O_2/O_2^{\cdot -}]$ close to 0 mV or even higher. In redox terms, the reduction of molecular oxygen by Q_A^- becomes more favorable from the thermodynamic point of view.

Due to the slow electron transfer from Q_A^- to a PQ molecule bound at the Q_B-binding site (200–400 μs) [40,56], the Q_A^- lifetime is high enough to sufficiently interact with molecular oxygen. When the Q_B-binding site is unoccupied or a herbicide is present, the Q_A^- lifetime is even prolonged. Under these conditions, the Q_A^- lifetime is mainly determined by a charge recombination. When reversible states of the water-splitting Mn complex are present (i.e. the so-called S_2 and S_3 states), the Q_A^- lifetime is around 1 s. However, when irreversible redox states of the water-splitting Mn complex are present (S_0 or S_1), no charge recombination can take place. The lifetime of Q_A^- may then be determined by reduction of molecular oxygen, a rate that is expected to be second order depending on the concentration of molecular oxygen.

Based on the analogy to the mitochondrial electron transfer chain, the involvement of Q_A^- in $O_2^{\cdot -}$ production seems to be likely. In mitochondria, ubisemiquinone is known to provide an electron to molecular oxygen forming $O_2^{\cdot -}$. Liu et al. [57] have demonstrated that ubiquinone-reconstructed D1/D2/cyt b_{559} complexes produce $O_2^{\cdot -}$, whereas in the absence of ubiquinone $O_2^{\cdot -}$ production was completely diminished. Since ubiquinone mimics the native quinone at the Q_A-binding site, these observations indicate that it is likely Q_A^- that donates an electron to molecular oxygen.

Based on these considerations, it seems probable that both $Pheo^-$ and Q_A^- donate an electron to molecular oxygen; however, under very different conditions. It seems much more likely that Q_A^- is a more relevant reductant of molecular oxygen under physiological conditions, whereas the reduction of molecular oxygen by $Pheo^-$ dominates, when Q_A is damaged. It has recently been proposed that the dominant reductant of molecular oxygen changes over the time course of photoinhibition in vitro [47]. The authors have suggested that Q_A^- serves as a reductant to molecular oxygen in the early phase, whereas at later times it is $Pheo^-$ that donates an electron to molecular oxygen. This suggestion is consistent with the observation that Q_A^- is lost in the later phases of photoinhibition as monitored by the loss in the $Q_A^-Fe^{2+}$ ($g = 1.84$) EPR signal [47,58].

3.1.3. PQ as a reductant to O_2

From the thermodynamic point of view, the reduction of molecular oxygen by plastosemiquinone and plastoquinol is less favorable; however, due to their extremely long lifetimes, one can consider these molecules as reliable components providing electrons to molecular oxygen. Indeed, the dianionic plastoquinol (PQ^{2-}) firmly bound at the Q_B-binding side was proposed in order to reduce molecular oxygen (Fig. 2) [59]. The authors suggested that under certain conditions, when the fully reduced PQ^{2-} is bound to the Q_B-binding side (i.e. in the presence of a strong reductant), molecular oxygen diffuses to the Q_B-binding side and oxidizes PQ^{2-}, while $O_2^{\cdot -}$ is formed. However, considering the redox potential of Q_B^-/Q_BH_2 ($Em = 290$ mV, pH 7), the reduction of molecular oxygen by PQ^{2-} is highly unfavorable from the thermodynamic point of view and therefore the formation of $O_2^{\cdot -}$ by PQ^{2-} at the Q_B-binding side is unlikely to occur. A more likely candidate would seem to be the firmly bound plastosemiquinone at the Q_B-binding site (PQ^-) whose redox potential (Em (Q_B/Q_B^-) $= -45$ mV, pH 7) fits the thermodynamic criteria better.

Evidence was provided that the plastosemiquinone radical ($PQH^{\cdot}$) in the PQ pool is involved in $O_2^{\cdot -}$ production [50–52]. It was demonstrated that a reasonable rate of light-induced consumption of O_2 was observed in the thylakoid membrane in the presence of DNP-INT, the inhibitor of plastoquinol oxidation by the cyt b_6/f complex [50]. The authors proposed that prior to diffusion of $O_2^{\cdot -}$ into the hydrophilic exterior of the thylakoid membrane, $O_2^{\cdot -}$ was reduced by plastoquinone to H_2O_2. The rate of the $O_2^{\cdot -}$ production was estimated at about 5–6 μmol mg Chl^{-1} h^{-1}. From the thermodynamic point of view, the reaction is feasible (Em ($PQ/PQH^{\cdot}$) $= -170$ mV, pH 7); however, the probability of $PQH^{\cdot}$ formation is very low (the equilibrium constant of the reaction $PQH_2 + PQ \Leftrightarrow PQH^{\cdot}$ is around 10^{-10}) [39].

3.1.4. Cyt b_{559} as a reductant to O_2

Several authors have proposed that cyt b_{559} is involved in O_2 reduction (Fig. 2) [48,49,60]. From the thermodynamic point of view, the reduction of molecular oxygen by the low-potential (LP) form of cyt b_{559} ($Em = 40$–80 mV, pH 7) is feasible, whereas the high-potential form (HP) ($Em = 400$ mV, pH 7) has no power to reduce molecular oxygen. In agreement with the thermodynamic assumption, it has been shown that the LP form of cyt b_{559} is reoxidized by molecular oxygen, whereas its HP form tends to be unaffected by molecular oxygen [48,49,53].

The three-dimensional structure of PSII from thermophilic cyanobacteria has revealed that Pheo is far enough away from the heme iron of cyt b_{559} to sufficiently maintain a direct electron transfer reaction. It has been demonstrated that heme iron is located approximately 50 Å

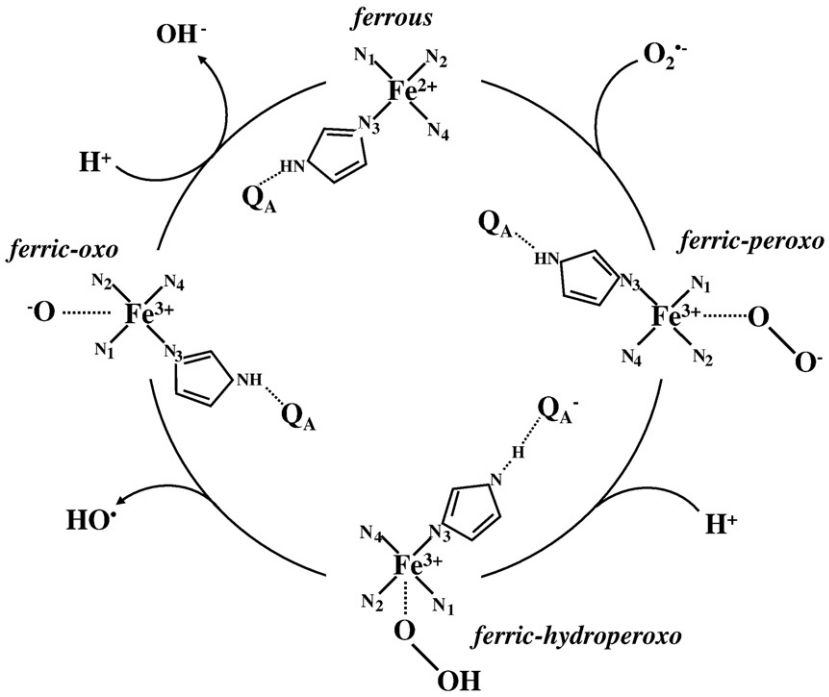

Fig. 3. Involvement of the non-heme iron in the formation of $HO^{\cdot}$ by the reduction of bound peroxide. The interaction of $O_2^{\cdot -}$ and ferrous heme iron results in the oxidation of ferrous iron and the formation of ferric-peroxo species. When protons are available, the protonation of peroxo group results in the formation of ferric-hydroperoxo species. The reduction of the ferric-hydroperoxo species leads to $HO^{\cdot}$ production, whereas the ferric-oxo species is formed. In this reaction, the ferric heme iron is reduced by the electron form the nearby Q_A, whereas the hydropero ligand is reduced by the ferrous heme iron. Protonation of oxo ligand leads to the formation of the hydroxo group and the liberation of hydroxo anion.

from $Pheo_{D1}$ (pheophytin on D1 protein) and 30 Å from $Pheo_{D2}$ (pheophytin on D2), respectively [14,15]. Kruk and Strzalka [49] have demonstrated that this distance gap might be fulfilled by plastoquinones. The authors have shown that synthetic short-chain plastoquinones (PQ-1 and PQ-2) facilitate electron transport from Pheo to LP cyt b_{559}. More recently, it has been demonstrated that the presence of short-chain plastoquinones enhanced $O_2^{\cdot -}$ production in PSII membranes [53]. Using the EPR spin-trapping spectroscopy, the author has demonstrated that exogenous plastoquinones with a different side-chain length enhanced $O_2^{\cdot -}$ production in the following order: PQ-1>PQ-2>PQ-9. Due to their increased polarity and smaller molecular size, short-chain plastoquinones penetrate more easily into the interior of the membrane and are bound in the vicinity of the heme of LP cyt b_{559} at the relatively polar membrane region. Despite the fact that short-chain plastoquinones are not natural components of the thylakoid membrane, the involvement of native, long-chain plastoquinones in $O_2^{\cdot -}$ production may have a relevance under physiological conditions.

A similar mechanism for electron donation to molecular oxygen was proposed in phagocytic cells such as neutrophils and macrophages [61,62]. In phagocytic cells, cyt b_{559} as a part of the NADPH oxidase, was demonstrated to reduce molecular oxygen. Based on the analogy to NADPH oxidase, it would seem probable that LP cyt b_{559} contributes to overall production of $O_2^{\cdot -}$ by PSII.

3.2. H_2O_2 production

Measurements of H_2O_2 production in PSII membranes in the presence of labeled water ($H_2^{18}O$) have demonstrated that little if any of the H_2O_2 produced by PSII arises from H_2O and thus the likely origin of H_2O_2 production is from O_2 reduction [42]. An increase in the concentration of molecular oxygen was shown to stimulate H_2O_2 production, whereas removal of molecular oxygen by flushing with nitrogen greatly reduced H_2O_2 production. Based on these observations, the authors suggested that H_2O_2 is produced by a dismutation of $O_2^{\cdot -}$ formed by the reduction of molecular oxygen on the PSII acceptor side (Fig. 2). The latter proposal was supported by an observation that H_2O_2 production is completely suppressed by the artificial electron acceptor DCPIP [36].

3.2.1. Formation of free peroxide

Using a luminol-peroxidase assay, Klimov and his co-workers have shown that illumination of PSII membranes results in the production of H_2O_2 which is gradually accumulated in the medium (the amount of H_2O_2 formed under saturating flashes was about 0.01 H_2O_2 molecule per PSII and flash) [36]. Based on the observation that H_2O_2 production was completely suppressed by exogenous catalase, the authors proposed that the illumination of PSII membranes results in the formation of free H_2O_2. It has been suggested that free H_2O_2 is formed by a dismutation of $O_2^{\cdot -}$ known to occur either spontaneously or catalyzed by endogenous SOD [63,64]. It is well established that the anionic form $O_2^{\cdot -}$ is in pH-dependent equilibrium with its protonated form perhydroxyl radical ($HO_2^{\cdot}$) which has a p*K*a of approximately 4.8 [63,65]. At neutral pH the anionic form is the major species, whereas at low pH the equilibrium is shifted toward the protonated form. Due to electrostatic interactions, $HO_2^{\cdot}$ dismutates spontaneously at several orders of magnitude faster than $O_2^{\cdot -}$ does. When the protons are available within the medium, the spontaneous dismutation is favored, whereas at physiological pH the dismutation reaction is preferably catalyzed by SOD.

3.2.2. Formation of bound peroxide

Recently, new evidence has been provided indicating that illumination of PSII membranes results in the formation of bound peroxide [47]. Based on the observation that $HO^{\cdot}$ formation is insensitive to the presence of exogenous catalase, it has been proposed that the interaction of $O_2^{\cdot -}$ with a PSII metal center results in the formation of bound peroxide. Three metal centers were proposed as potential candidates for the site of bound peroxide: the water-splitting Mn complex, cyt b_{559} and the non-heme iron. Due to the observation that the water-splitting Mn complex itself is not required for the formation of $HO^{\cdot}$, it has been proposed that the water-splitting Mn

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \quad (1)$$

$$Fe^{2+}\text{–}OOH \longrightarrow Fe^{3+}\text{–}O^{-} + HO^{\bullet} \longrightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \quad (2)$$

$$X^{ox} + H_2O_2 \longrightarrow X + O_2^{\bullet -} \quad (3)$$

Scheme 2. Decomposition of H_2O_2: (1) one-electron reduction of free H_2O_2, (2) one-electron reduction of bound peroxide and (3) one-electron oxidation of H_2O_2.

complex is unlikely to be involved in the binding of peroxide. The effect of pH and formate on $HO^{\bullet}$ production has been taken as support for the involvement of the non-heme iron in the formation of bound peroxide; however, the role of cyt b_{559} has not been completely excluded.

The authors proposed that the interaction of $O_2^{\bullet -}$ with non-heme iron results in the oxidation of ferrous iron and the formation of ferric-peroxo species (Fig. 3). An electron transfer from ferrous iron to coordinated $O_2^{\bullet -}$ was suggested to occur as an inner-sphere electron transfer reaction. When protons are available in the vicinity of the non-heme iron, the ferric-peroxo species is protonated forming a ferric-hydroperoxo species. Based on the analogy to superoxide reductase (SOR), the mechanism for the formation of ferric-hydroperoxo species by PSII is likely. Superoxide reductase, an enzyme catalyzing the reduction of $O_2^{\bullet -}$ to H_2O_2, contains a non-heme iron liganded by four histidines and a cysteine [66,67]. In analogy to SOR, the non-heme iron in PSII is coordinated by four histidines and bicarbonate. Several lines of evidence were provided demonstrating that CN^- and NO can replace the cysteine ligand in SOR [68,69], whereas bicarbonate is exchangeable by these exogenous ligands in PSII [70,71].

3.3. $HO^{\bullet}$ production

3.3.1. Reduction of free peroxide

It is well established that free H_2O_2 is reduced by metals forming $HO^{\bullet}$ via the Fenton reaction, well known in inorganic chemistry (Scheme 2, Eq. 1) [72,73]. In many biological systems, H_2O_2 is reduced by several low-valent metals such as Fe^{2+}, Mn^{2+} or Cu^{+}, among which Fe^{2+} is the strongest reductant. Making use of the spin-trapping EPR technique, it has been demonstrated that illumination of PSII membranes results in the formation of $HO^{\bullet}$ [44–47,74]. Based on the observation that $HO^{\bullet}$ production is enhanced in the presence of Cu^{+}, it was proposed that $HO^{\bullet}$ is formed by one-electron reduction of H_2O_2 via the Fenton reaction [74]. The production of $HO^{\bullet}$ has been recently demonstrated in the presence of either pheonolic- or urea-type herbicides [75]. Using the EMPO spin-trap, the authors have demonstrated that in the presence of DCMU, the production of $HO^{\bullet}$ was diminished by exogenous catalase, confirming the involvement of H_2O_2. Furthermore, it was shown that the addition of an artificial electron acceptor, DCPIP, resulted in the suppression of $HO^{\bullet}$ production [46]. Based on the measurements of the six-line hexaquo-Mn^{2+} EPR signal, it was demonstrated that Mn^{2+} released after high-light intensity illumination might be involved in the reduction of H_2O_2 to $HO^{\bullet}$ [47]. In agreement with this observation, the 33 kDa protein released from the water-splitting Mn complex has been proposed to serve as a temporary reservoir of Mn^{2+} [76].

3.3.2. Reduction of bound peroxide

In addition to free peroxide, $HO^{\bullet}$ might also be produced by the reduction of peroxide bound to a metal center (Scheme 2, Eq. 2) [64,77–81]. Using the EPR spin-trapping technique, it has been demonstrated that $HO^{\bullet}$ is produced by the reduction of peroxide bound to non-heme iron [47]. By simultaneous measurements of $HO^{\bullet}$ production and Fe^{3+} ($g=8$) EPR signal in PSII membranes, it has been shown that $HO^{\bullet}$ production exhibits a similar pH-dependence as the ability to oxidize the non-heme iron: both processes gradually decreased as the pH value was lowered below pH 6.5. The author has proposed that the reduction of the ferric-hydroperoxo species results in the formation of the ferric-oxo species, whereas $HO^{\bullet}$ is produced (Fig. 3). The ferric iron was proposed to be reduced by an endogenous reductant, the most likely being Q_A^-, whereas the ferrous iron produced was suggested to reduce the hydroperoxo ligand. As the midpoint redox potential of non-heme iron and the $H_2O_2/HO^{\bullet}$ redox couple is 400 mV and 460 mV (pH 7), respectively, the reduction of peroxide by non-heme iron is feasible from thermodynamic point of view. Whereas the Fenton reaction involves the outer-sphere electron transfer with no direct binding of peroxide to iron, the reduction of bound peroxide proceeds as an inner-sphere electron transfer reaction that strictly requires the direct binding of peroxide to iron.

In agreement with this proposal, other authors have demonstrated that non-heme iron appeared to be oxidized by H_2O_2 [82,83]. It has been shown that incubation of PSII membranes with glucose/glucose oxidase system under aerobic conditions has induced significant oxidation of the non-heme iron, in all probability due to the formation of H_2O_2. Other authors have demonstrated that in addition to the catalase-mediated disproportionation of H_2O_2, the non-heme iron in PSII causes a disproportionation of H_2O_2 probably by its reduction to $HO^{\bullet}$ [84]. Miyao et al. [85] have demonstrated that the degradation of the D1 protein by treatment with H_2O_2 was not affected by chelators (EDTA) and was more pronounced in PSII preparations containing non-heme iron (PSII core complexes) than PSII preparations lacking non-heme iron (isolated PSII reaction centers). It was shown in support of these observations that peroxide treatment of thylakoid membranes from the site-directed mutant, in which a histidine residue liganded to the non-heme iron was replaced by leucine, did not show peroxide-induced protein fragmentation [86].

4. ROS generation on the PSII electron donor side

The abstraction of electrons from H_2O molecules is driven by the primary electron donor $P680^+/P680$ redox couple which has the highest midpoint redox potential in PSII ($Em=1.25$ V, pH 7). As the midpoint redox potential of the O_2/H_2O redox couple ($Em=0.81$ V, pH 7) is lower than the midpoint redox potential of the $P680^+/P680$ redox couple, water oxidation to molecular oxygen is thermodynamically favorable. In principle, the water oxidation to molecular oxygen might occur either 1) in a concerted multi-electron reaction, in which two H_2O molecules are oxidized simultaneously during the last S-state transition or 2) in a consecutive step-electron reaction, in which two H_2O molecules are partially oxidized at an earlier S-state. The concerted multi-electron oxidation of water during the last S-state transition results in the direct evolvement of O_2, whereas the stepwise oxidation of water was proposed to involve the formation of non-radical (peroxo, hydroxo, oxo) and a radical (peroxyl, hydroxyl, oxyl) intermediate species.

The involvement of an intermediate species in the water oxidation cycle was suggested more than 30 years ago. From that time, several models involving an intermediate species in the water oxidation cycle have been proposed (for a recent review see [22,24,26,27]). Despite the extensive progress in this field over the last decade, the formation of an intermediate species in the water oxidation cycle remains unresolved mainly due to two reasons 1) the powerful spectroscopic methods are insensitive to the intermediate species and 2) the intermediate species are too short lived to be detected. Recent evidence has shown, however, that the intermediate species are likely involved in the water-splitting enzymatic cycle [87,88]. Using a high pressure technique, the authors demonstrated that water oxidation to molecular oxygen proceeds through an endergonic electron transfer reaction from the highest oxidation state to the intermediate and an exergonic electron transfer reaction from the intermediate to molecular oxygen. The authors have proposed that the intermediate state in all probability involves the formation of a peroxo species.

4.1. H_2O_2 production

In addition to the production of peroxide at the PSII electron acceptor side, peroxide can also be produced at the PSII electron donor side. Several lines of evidence have been given in order to indicate the production of H_2O_2 on the PSII electron donor side. The formation of H_2O_2 produced on the PSII donor side was demonstrated either in the thylakoid membranes or PSII membranes exposed to various treatments including salt-washing [36,89,90], chloride-depletion [91–93], high pH [94], low pH [36], heat [95], ADRY reagent [96] and lauroylcholine [43,97]. It has been demonstrated that the modification of the PSII donor side enhanced H_2O_2 production several times when compared to untreated PSII. Whereas in the untreated PSII, H_2O_2 is produced in the range of several units of μmol mg Chl^{-1} h^{-1}, in the treated PSII, H_2O_2 production reaches several tens of μmol mg Chl^{-1} h^{-1} (Table 1). The rate of H_2O_2 production in the PSII membranes is affected by the presence of the PSII membrane associated heme catalase which catalyzes decomposition of H_2O_2 into water and molecular oxygen. Due to this intrinsic catalase activity, the use of either PSII core complexes [94] or catalase-depleted PSII membranes [98] was shown to be advantageous in these studies.

At present, it is not apparent whether the light-induced formation of H_2O_2 is a result of a side reaction or whether H_2O_2 is formed as an intermediate in the normal pathway of water oxidation. Based on the evidence that H_2O_2 is produced mainly in PSII modified on the donor side (depleted by the extrinsic protein or chloride), it is more likely that H_2O_2 production has been raised from the side reactions in the perturbed water-splitting Mn complex.

4.1.1. H_2O_2 production in extrinsic protein-depleted PSII

The illumination of salt-washed PSII depleted by the 17, 23 and/or 33 kDa extrinsic proteins results in H_2O_2 production [36,43,89,90]. It has been observed that the release of 17 and 23 kDa extrinsic proteins significantly stimulates the H_2O_2 production in the inside-out thylakoid (Table 1) [89]. Based on this, the authors have suggested that H_2O_2 is produced on the PSII electron donor side, most likely at the catalytic site of the water-splitting Mn complex. It has been proposed that due to the absence of 17 and 23 kDa proteins, H_2O_2 is dissociated from the water-splitting Mn complex more easily. Wydrzynski et al. [91] have shown that the production of H_2O_2 is insensitive to the presence of the artificial electron acceptors such as DCPIP or PPBQ confirming the H_2O_2 production on the PSII electron donor side. The partial removal of extrinsic proteins caused by 1 M NaCl, LCC or 70% ethylene glycol treatment has resulted in the formation of about 40–50 μmol H_2O_2 mg Chl^{-1} h^{-1}, whereas the complete removal of extrinsic proteins followed by 1 M $CaCl_2$ has caused an almost double production of about 76 μmol H_2O_2 mg Chl^{-1} h^{-1} (Table 1).

4.1.2. H_2O_2 production in chloride-depleted PSII

Several authors have demonstrated that chloride-depleted PSII produced more H_2O_2 than active PSII (Table 1) [46,92–94]. When chloride-depleted PSII membranes were illuminated in the presence of an artificial electron acceptor (silicomolybdate), the production of H_2O_2 was unchanged indicating a significant contribution of the PSII electron donor side to the overall H_2O_2 production [46,93]. Making use of PSII core complexes, which lack the 17 and 23 kDa extrinsic proteins, Fine and Frasch [94] have demonstrated that H_2O_2 production requires low chloride concentration (1.5–3 mM Cl^-), whereas higher chloride concentration (above 8 mM Cl^-) prevents H_2O_2 production. In low-chloride conditions, a pH increase stimulates H_2O_2 production inversely to the decrease in O_2 evolution and reaches a maximum at pH 7.2. These observations indicate that the water-splitting Mn complex requires Cl^- in order to prevent H_2O_2 production. Based on the correlation between the pH dependence for H_2O_2 production and the inhibition of O_2 evolution in chloride-depleted PSII, the authors have proposed that the hydroxo anion (OH^-) is a substrate for H_2O_2 production. Using a coupled assay with Fe-catalase, Wydrzynski et al. [91] have demonstrated that illuminations of PSII membranes at 8 mM sucrose (low sucrose) caused H_2O_2 production, whereas in the presence of 400 mM sucrose (high sucrose) H_2O_2 production was significantly suppressed. Two possible explanations for the effect of sucrose on H_2O_2 production were proposed: (1) sucrose alters the chloride-binding side or (2) sucrose blocks the access of other molecules to the chloride-binding side and thus prevents H_2O_2 production.

Table 1
The rate of H_2O_2 production by PSII.

Modification	Treatment	H_2O_2 formation (μmol H_2O_2 mg Chl^{-1} h^{-1})	Reference
None		1	[42]
		2	[46]
		9	[47]
		17	[90]
17, 23 kDa	1 M NaCl	39	[90]
	LCC	37	[90]
	70% EG	53	[90]
17, 23, 33 kDa	1 M $CaCl_2$	76	[90]
Chloride-depleted		55	[94]
		75	[93]

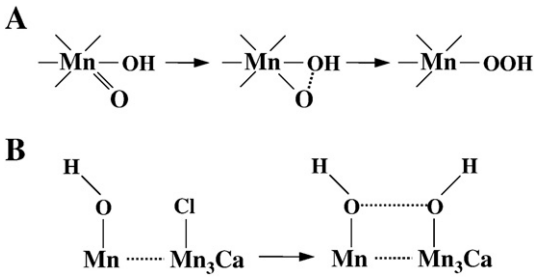

Fig. 4. Production of peroxide by (A) the interaction of the hydroxo group with the terminal the oxo group in 17, 23 and/or 33 kDa extrinsic protein-depleted PSII and (B) the interaction of hydroxo groups in chloride-depleted PSII. In A, the attack of the bound water substrate onto a highly electrophilic terminal oxo ligand coordinated to the same manganese ion results in the formation of bound peroxide. In B, the replacement of Cl^- by the water substrate results in the interaction of the two hydroxo groups and the formation of peroxide.

4.1.3. The water accessibility mechanism

The removal of the 17 and 23 kDa extrinsic proteins was proposed to modify the structure of the water-splitting Mn complex in such a way as to expose the catalytic site to the solvent phase and make it possible for H_2O_2 production [89]. Wydrzynski et al. [99] has proposed that the removal of extrinsic proteins leads to the enhancement of water accessibility to the water-splitting Mn complex and the creation of an alternate peroxide state which is not normally involved in the water oxidation cycle. In the native PSII, the accessibility of the H_2O substrate to the water-splitting Mn complex is controlled by a hydrophobic domain in the surrounding protein matrix, whereas the perturbation of the hydrophobic domain exposes the water-splitting Mn complex to an uncontrolled access of H_2O molecules from the external solvent phase. It has been proposed that a deprotonated water molecule from the external solvent phase is bound to manganese forming a hydroxo ligand. The interaction of the hydroxo ligand with the terminal oxo group, which is coordinated to the water-splitting Mn complex and normally involved in water oxidation, leads to the formation of peroxide (Fig. 4A).

4.1.4. The short-circuiting mechanism

The chloride-binding binding site in chloride-depleted PSII was suggested to be occupied by OH^- which interacts with the second OH^- forming H_2O_2 in the short-circuiting of the S-state cycle (Fig. 4B). The

formation of H_2O_2 was proposed to involve the S_2–S_0 states [94,100], whereas other authors have proposed the involvement of the more reduced S_1–S_{-1} states [95,101]. Two-electron oxidation of H_2O to H_2O_2 requires a redox potential higher than the driving force of PSII. Dismukes et al. [102] have suggested that the replacement of Cl^- by OH^- increases the redox potential of manganese and makes the H_2O_2 formation thermodynamically feasible.

4.2. $O_2^{\cdot -}$ production

Superoxide production on the PSII donor side was proposed based on measurements of oxygen evolution from H_2O_2 in Tris-treated PSII membranes [103,104]. The authors have demonstrated that the light-induced oxygen evolution from H_2O_2 in Tris-treated PSII was prevented by Tiron, a scavenger of $O_2^{\cdot -}$. Based on these observations, one-electron oxidation of H_2O_2 was proposed to generate $O_2^{\cdot -}$ (Scheme 2, Eq. 3). As the midpoint redox potential of the $O_2^{\cdot -}$/H_2O_2 redox couple is high ($Em = 0.89$ V, pH 7) [105], the one-electron oxidation of H_2O_2 to $O_2^{\cdot -}$ can only be achieved by a strong oxidant. Two redox active components of PSII, tyrosine $Tyr_Z^{\cdot}$ [84,106] and chlorophyll cation $Chlz^+$ [103], were proposed in order to serve as an oxidant X^{ox} for H_2O_2 (Fig. 2). The author has proposed that the oxidation of H_2O_2 by $Tyr_Z^{\cdot}$ can occur in Mn-depleted PSII, where $Tyr_Z^{\cdot}$ is long-lived and easily accessible to H_2O_2. In spite of the fact that direct evidence for $O_2^{\cdot -}$ formation on the PSII donor side has not been yet provided, the mechanism of $O_2^{\cdot -}$ formation by a one-electron oxidation of H_2O_2 is likely. In a similar manner, one-electron oxidation of hydroxylamine was demonstrated to result in the formation of a nitroxide radical [107]. Based on the fact that the later reaction only occurs in PSII deprived of the water-splitting Mn complex, the authors proposed that $Tyr_Z^{\cdot}$ is involved in the one-electron oxidation of hydroxylamine.

4.3. $HO^{\cdot}$ production

Making use of the EPR spin-trapping technique, it has been shown that photoinhibition on the PSII electron donor side is dominated by $HO^{\cdot}$, its production was unaffected by removal of molecular oxygen [108,109]. More recently, other authors have demonstrated that $HO^{\cdot}$ production in Ca^{2+}-depleted PSII membranes is completely suppressed in the presence of exogenous SOD, whereas in chloride-depleted PSII membranes removal of $O_2^{\cdot -}$ partially decreased $HO^{\cdot}$ production [46]. Based on this observation, the authors proposed that $HO^{\cdot}$ is produced from H_2O_2 generated on the PSII electron donor side. Recently, $HO^{\cdot}$ production has been demonstrated in PSII membranes under moderate heat stress [110,111]. Based on the observation that exogenous Ca^{2+} and Cl^- ions have partially prevented the formation $HO^{\cdot}$, the authors proposed that $HO^{\cdot}$ is produced by the water-splitting Mn complex. This proposal has been confirmed by the finding that $HO^{\cdot}$ formation was completely abolished in Tris-treated PSII membranes lacking the water-splitting Mn complex. It has been proposed that the heat-induced destabilization of the water-splitting Mn complex modified the environment around the enzyme thus promoting the generation of H_2O_2. Based on the evidence that exogenous catalase completely prevented $HO^{\cdot}$ formation, it has been suggested that $HO^{\cdot}$ is formed by univalent reduction of free H_2O_2 by manganese ions released from the water-splitting Mn complex.

5. Concluding remarks

This review has attempted to point out the mechanistic aspect on ROS production by PSII. The focus has been given on the production of radical ($O_2^{\cdot -}$, $HO^{\cdot}$) and non-radical (H_2O_2) forms of ROS on both the electron acceptor and the electron donor side of PSII. Whereas on the PSII electron acceptor side, ROS are produced by successive univalent reduction of molecular oxygen involving formation of $O_2^{\cdot -}$, H_2O_2 and $HO^{\cdot}$, the partial oxidation of water on the PSII electron donor side forms H_2O_2 that is further either oxidized to $O_2^{\cdot -}$ or reduced to $HO^{\cdot}$.

The production of ROS by PSII has a physiological relevance to the oxidative stress that occurs as a consequence of environmental stress (high light, heat, draught, heavy metals). The focus has been mainly placed on ROS-induced oxidative damage that might contribute to photoinhibition — the process occurring when the absorption of light energy by chlorophylls exceeds the capacity for its utilization.

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