Abstract: Oxygen is a natural acceptor of electrons in the respiratory pathway of aerobic organisms and in many other biochemical reactions. Aerobic metabolism is always associated with the formation of reactive oxygen species (ROS). ROS may damage biomolecules but are also involved in regulatory functions of photosynthetic organisms. This review presents the main properties of ROS, the formation of ROS in the photosynthetic electron transport chain and in the stroma of chloroplasts, and ROS scavenging systems of thylakoid membrane and stroma. Effects of ROS on the photosynthetic apparatus and their roles in redox signaling are discussed.

Keywords: reactive oxygen species; chloroplasts; photosynthetic electron transport chain; photodamage; redox signaling

1. Introduction

Molecular oxygen (O₂) is a natural acceptor of electrons in the respiratory pathway of aerobic organisms and in many other biochemical reactions. In its ground state, O₂ has two unpaired electrons with parallel spins in two separate π antibonding orbitals. Thus, ground-state O₂ is a triplet diradical. According to Pauli’s principle, O₂ reacts slowly with many biomolecules because spin restriction causes a kinetic barrier, as almost all biomolecules are in the singlet state, having paired electrons with opposite spins. The kinetic barrier of O₂ is removed either by spin inversion of one unpaired electron or by a partial reduction in O₂. Spin inversion requires the absorption of energy and converts the triplet state of O₂ to the singlet state of molecular oxygen (¹O₂). All other forms of active oxygen are produced via an electron transfer mechanism. ¹O₂ and partially reduced forms of oxygen have a higher reactivity towards many organic molecules than the ground state of oxygen and they are collectively called reactive oxygen species (ROS).

ROS can be classified as radical and non-radical species. In addition, ROS can be roughly divided into free ROS, small molecules composed of oxygen and hydrogen only, and incorporated ROS, in which oxygen is bound to other molecules to form reactive oxygen derivatives. Furthermore, a family of reactive species containing nitrogen moieties associated with oxygen are classified as reactive nitrogen species, and reactive oxygen derivatives like lipid peroxyl radicals (LOO∗) can be classified as both ROS and reactive lipid species. The free ROS are ¹O₂, superoxide anion radical (O₂•−), hydroperoxyl radical (HO₂•), hydroxyl radical (HO•) and ozone (O₃). Other molecules containing active oxygen are ROS derivatives. Table 1 presents the most important reactive species containing active oxygen according to this classification.
| Radicals | Non-Radicals |
|----------|--------------|
| Superoxide anion radical, O$_2^{•−}$ | Singlet oxygen, $^1$O$_2$ |
| Hydroperoxyl radical, HO$_2^{•}$ | Hydrogen peroxide, H$_2$O$_2$ |
| Hydroxyl radical, HO$^*$ | Ozone, O$_3$ |
| ROS derivatives and reactive nitrogen species | |
| Peroxyl radical, ROO$^*$ | Organic peroxides, ROOH |
| Alkoxyl radical, RO$^*$ | Peroxynitrite ion, ONOO$^−$ |
| Nitric Oxide, NO$^*$ | Alkyl peroxynitrite, ROONO |
| Nitrogen dioxide, NO$_2^{•}$ | |

Chloroplasts convert light energy to energy for chemical bonds. Light absorption by the chlorophyll (Chl) molecules of Photosystems I and II (PSI and PSII) triggers a sequence of redox reactions along the thylakoid membrane. These reactions result in the oxidation of water, reduction of NADP$^+$ to NADPH and formation of a proton gradient across the thylakoid membrane. In chloroplasts, O$_2$ appears due to light-dependent water-splitting catalyzed by PSII. The steady-state concentration of O$_2$ inside intact chloroplasts in the light depends on the external concentration of O$_2$. At low external O$_2$ concentrations (30 µM), the ratio of the internal to the external is about five, whereas at concentrations corresponding to those in air-saturated water ($≈ 258$ µM), the O$_2$ concentration of isolated chloroplasts is similar to that of the medium [1].

$^1$O$_2$ is mainly formed via the interaction of O$_2$ with the triplet excited state of chlorophyll (3Chl). The reduction in O$_2$ by reduced forms of electron carriers in the photosynthetic electron transfer chain (PETC) can produce O$_2^{•−}$ and H$_2$O$_2$. The reaction of O$_2$ with PETC is considered as the main source of ROS in chloroplasts, and light is essential for this ROS production. ROS can be interconverted by interaction with redox active compounds of the chloroplast, and ROS are produced both via O$_2^{−}$ mediated primary ROS generation mechanisms and ROS-mediated interconversion reactions. ROS are unfavorable for chloroplast functions because ROS cause oxidative damage by reacting with biomolecules. Chloroplasts have ROS scavenging systems to prevent damage [2–5]. In addition to ROS-scavenging systems, chloroplasts have pathways, like non-photochemical quenching of excitation energy (NPQ), cyclic electron flow and plastid terminal oxidase (PTOX)-mediated chlororespiration, that diminish the appearance of long-lived redox active compounds [6–8]. All aerobic organisms have ROS-scavenging mechanisms to prevent ROS damage. Environmental stressors (high light, high or low temperature and others) enhance ROS production in chloroplasts and change the balance between ROS scavenging and ROS production. Imbalances between ROS production and scavenging cause changes in the redox state of the cell through a change in the levels of reduced and oxidized forms of antioxidants like ascorbate (AscH$_2$), glutathione and thiol-containing compounds. Changes in the redox levels of the antioxidants trigger reactions where antioxidants act as ROS processing and signaling mediators that cause changes in gene expression [9,10]. In particular, the formation of ROS in Mehler’s reaction initiates light-signaling that depends on AscH$_2$ and reduced glutathione (GSH) [11].

ROS production quenches excitation energy photochemically via the so-called water–water cycle that consists of the production of O$_2$ in PSII, reduction in O$_2$ to O$_2^{•−}$ and H$_2$O$_2$, scavenging of ROS, and regeneration of the scavenger [12]. The primary function of the water–water cycle is to scavenge ROS. Besides ROS scavenging and the photochemical quenching of excitation energy, the water–water cycle generates a proton gradient across the thylakoid membrane for both ATP production and the enhancement of NPQ [13].

Aerobic metabolism inevitably leads to the formation of ROS, and chloroplasts have to spend metabolic energy to build and maintain the ROS metabolism. The chloroplast provides light sensor functions for the whole plant cell, and the chloroplast ROS network participate in this signaling.
In the present review, we will mainly discuss the roles of $^1\text{O}_2$, $\text{O}_2^{*-}$, $\text{HO}^*$, $\text{H}_2\text{O}_2$, $\text{HO}^*$ and lipid peroxides in chloroplast metabolism. $\text{O}_3$, although it is biologically important, is only shortly discussed because this ROS is not found within cells. The review presents the main properties of ROS and their typical reactions, the formation of ROS in the photosynthetic electron transport chain and in the stroma of chloroplasts, and the ROS-scavenging systems of thylakoid membrane and stroma.

2. ROS Properties and Basic Reactions

2.1. Singlet Oxygen, $^1\text{O}_2$

Ground-state $\text{O}_2$ is a triplet ($^3\Sigma^+_\text{g}\text{O}_2$) and can be converted to the singlet form via the absorption of energy that leads to spin inversion of one unpaired electron. Molecular oxygen has two singlet forms because two electrons with antiparallel spins may reside either in two different orbitals ($^1\Sigma^+_\text{g}\text{O}_2$) or both in one orbital ($^1\Delta\text{gO}_2$). The energy above the ground-state of $\text{O}_2$ is 155 and 92 kJ/mol for $^1\Sigma^+_\text{g}\text{O}_2$ and $^1\Delta\text{gO}_2$, respectively [14]. $^1\Sigma^+_\text{g}\text{O}_2$ is rapidly converted to $^1\Delta\text{gO}_2$ or $^3\Sigma^+_\text{g}\text{O}_2$ and, in the liquid phase, the lifetime of $^1\Sigma^+_\text{g}\text{O}_2$ is only $10^{-11}$ s [14], which is too short for $^1\Sigma^+_\text{g}\text{O}_2$ to take part in biochemical reactions. Therefore, $^1\text{O}_2$ will be used here to designate $^1\Delta\text{gO}_2$. $\text{O}_2$ will be used to designate $^3\Sigma^+_\text{g}\text{O}_2$.

2.1.1. Formation of $^1\text{O}_2$

The most common mechanism of $^1\text{O}_2$ generation is photosensitization, i.e., the reaction of $\text{O}_2$ with a photoexcited sensitizer dye ($S^*$). Both forms of $^1\text{O}_2$, $^1\Sigma^+_\text{g}\text{O}_2$ and $^1\Delta\text{gO}_2$, can be produced via the spin-conserved Reactions (1) and (2).

$$^1\text{S}^* + \text{O}_2 \rightarrow ^3\text{S} + ^1\text{O}_2$$  \hspace{1cm} (1)

$$^3\text{S} + \text{O}_2 \rightarrow ^1\text{S} + ^1\text{O}_2$$  \hspace{1cm} (2)

The second reaction is more common because singlet excited states ($^1\text{S}^*$) are usually short-lived and because only a few dye molecules have a large enough energy gap between the $^1\text{S}^*$ and triplet states ($^3\text{S}$) to convert $\text{O}_2$ to $^1\text{O}_2$ [15]. $\text{Chl}$ reacts rapidly with $\text{O}_2$ with a second-order rate constant close to $10^9 \text{M}^{-1} \text{s}^{-1}$, and the relative quantum yield of $^1\text{O}_2$ generation by chlorophyll $a$ ($\text{Chl} a$) was around 80% when meso-tetraphenylporphyrin and tetra($p$-sulfophenyl) porphyrin were used as standards [16]. The spin transition $\text{O}_2 \rightarrow ^1\Sigma^+_\text{g}\text{O}_2$ is associated with the absorption band of gaseous $\text{O}_2$ at 762 nm. Absorption at 1268 nm, in turn, was found for the transition $\text{O}_2 \rightarrow ^1\Delta\text{gO}_2$ in liquids and in the atmosphere [17].

In addition to photosensitized generation, $^1\text{O}_2$ can be produced by several chemical reactions that usually involve reduced forms of oxygen like $\text{H}_2\text{O}_2$, $\text{O}_2^{*-}$ and reactive oxygen derivatives like organic peroxides (ROOH) and peroxy radicals (ROO*) [14,18].

$^1\text{O}_2$ is produced by decomposition of $\text{H}_2\text{O}_2$ via the Haber–Weiss mechanism (Reactions (3) and (4)) [19,20].

$$\text{H}_2\text{O}_2 + \text{O}_2^{*-} \rightarrow \text{HO}^* + \text{HO}^- + (\text{O}_2 \text{ or } ^1\text{O}_2)$$ \hspace{1cm} (3)

$$\text{H}_2\text{O}_2 + \text{HO}_2^{*-} \rightarrow \text{HO}^* + \text{H}_2\text{O} + (\text{O}_2 \text{ or } ^1\text{O}_2)$$ \hspace{1cm} (4)

The rate constants of Reactions (3) and (4) in aqueous medium are 0.13 to 0.23 M$^{-1}$ s$^{-1}$ and 0.5 M$^{-1}$ s$^{-1}$, respectively [21–23]. It has been suggested that the biologically important oxidant produced by Reaction (3) is $\text{HO}^*$ rather than $^1\text{O}_2$ [24]. $^1\text{O}_2$ can also be produced by $\text{O}_2^{*-}$ dismutation (Reaction (5)) or by electron transfer from $\text{O}_2^{*-}$ to radical ($A^*$) or non-radical ($A$) electron acceptors (Reactions (6) and (7)) [25–27].

$$\text{O}_2^{*-} + \text{O}_2^{*-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + (\text{O}_2 \text{ or } ^1\text{O}_2)$$ \hspace{1cm} (5)

$$\text{O}_2^{*-} + A^+ \rightarrow (\text{O}_2 \text{ or } ^1\text{O}_2) + A^* \text{ or } \text{O}_2^{*-} + A^{*-} \rightarrow (\text{O}_2 \text{ or } ^1\text{O}_2) + A$$ \hspace{1cm} (6)
O₂− + HO• → HO− + 1⁄₂O₂ \( (7) \)

Efficient production of \(^1\)O₂ was found in a reaction of O₂•− with benzoyl peroxide or lauroyl peroxide (Reaction (8)) [28].

\[
2O₂•− + RCOOCR → 2RCO₂− + 2\(^1\)O₂ \( (8) \)
\]

\(^1\)O₂ can be generated via the Russel mechanism (Reaction (9)), in which two ROO• radicals react to form a linear tetraoxide (ROOOOR) intermediate that rapidly decomposes to the corresponding ketone (R=O), alcohol (R–OH) and \(^1\)O₂ [29]. In fact, ROOOOR decomposition releases either \(^1\)O₂ or excited triplet carbonyl (R=\(\text{^3}\text{O}^*\)). However, the relative yield of \(^1\)O₂ is 10%, while the relative yield of R=\(\text{^3}\text{O}^*\) is only 0.01% [30–32].

\[
\text{ROO}^• + \text{ROO}^• \xrightarrow{\Delta} \text{ROO}^• + \text{ROO}^• \rightarrow \text{R-OH} + \text{R=O} + \text{^1O}_2 \( (9) \)
\]

\(^1\)O₂ can be produced in the reaction of O₂ with an R=\(\text{^3}\text{O}^*\) (Reaction (10)), which can be formed by the decomposition of ROOOOR (Reaction (9)) [33].

\[
\text{R=^3O}^* + \text{O}_2 \rightarrow \text{R=O} + \text{^1O}_2 \( (10) \)
\]

Other specific ions, like the hypohalite ion OCl\(^−\) and the molybdate ion MoO\(_4^{2−}\), can react with H₂O₂, forming \(^1\)O₂ (Reactions (11)–(13)) [34–36]; these reactions are probably not of biological importance.

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{H}^+ + \text{OCl}^- & \rightarrow \text{H}_2\text{O} + \text{HCl} + \text{^1O}_2 \( (11) \) \\
\text{MoO}_4^{2−} + \text{H}_2\text{O}_2 & \Delta \text{MoO}_6^{2−} + 2\text{H}_2\text{O} \( (12) \) \\
\text{MoO}_6^{2−} & \rightarrow \text{MoO}_4^{2−} + \text{^1O}_2 \( (13) \)
\end{align*}
\]

2.1.2. Physical Deactivation of \(^1\)O₂

Both excited singlet states of oxygen are metastable and can lose excitation energy via radiative and non-radiative pathways. The latter is physical quenching of \(^1\)O₂. The radiative deactivation is the transition of \(^1\Delta\text{gO}_2\) to O₂ associated with light emission (\(h\nu\); Reaction (14)).

\[
\text{^1O}_2 \rightarrow \text{O}_2 + h\nu \( (14) \)
\]

The phosphorescence spectrum has a major maximum at 1268 nm [37]. The phosphorescence is extremely weak, as the deactivation of \(^1\)O₂ mostly proceeds non-radiatively due to the collision of \(^1\)O₂ with another molecule. The quantum yield of luminescence is from \(10^{-6}\) to \(10^{-5}\) [32]. Non-radiative deactivation mechanisms include electronical-to-vibrational energy transfer, charge-transfer-induced quenching and electronic energy transfer. In the deactivation of \(^1\Sigma^+\text{gO}_2\) and \(^1\Delta\text{gO}_2\) by an electronic-to-vibrational process, the excitation energy of \(^1\)O₂ is converted into vibration of the O₂ molecule and a quencher molecule Qr (Reaction (15)).

\[
\text{^1O}_2 (v = 0) + \text{Qr} (v = 0) \rightarrow \text{O}_2 (v = m) + \text{Qr} (v = n) + E_{mn} \( (15) \)
\]

where \(E_{mn}\) is the energy difference between the reactants and the products and \(v\) is the vibrational energy level of a molecule; \(m\) and \(n\) are vibrational modes.

The deactivation of \(^1\)O₂ by collisions of \(^1\)O₂ with other molecules limits the lifetime of \(^1\)O₂ in many solvents. The lifetime of \(^1\)O₂ for many organic solvents is within 8–100 \(\mu\)s. The substitution of hydrogen with deuterium in the solvent molecule leads to a significant increase in the lifetime of \(^1\)O₂, usually by a factor of ten or more [17,38–40]. The second order rate constant for the deactivation of \(^1\)O₂ via an electronic-to-vibrational process varies widely, from \(10^{-2}\) to \(10^6\) \(\text{M}^{-1} \text{s}^{-1}\) [17].
In addition to the electronic–vibrational non-radiative deactivation, $^1\text{O}_2$ can be deactivated via charge-transfer-induced quenching (Reaction (16)) and an electronic energy transfer mechanism (Reaction (17)).

$$^1\text{O}_2 + ^1\text{A} \rightarrow ^3\text{O}_2\text{A} \rightarrow ^3\text{O}_2\text{A} \rightarrow ^3\text{A} \rightarrow ^3\text{A}_\beta$$  \hspace{1cm} (16)

$$^1\text{O}_2 + \text{A} \rightarrow ^3\text{O}_2\text{A} \rightarrow ^3\text{O}_2\text{A} \rightarrow ^3\text{A} \rightarrow ^3\text{A}_\beta$$  \hspace{1cm} (17)

where A is an acceptor.

Molecules with high triplet energies (more than 94 kJ mol$^{-1}$) and low oxidation potential (midpoint redox potential ($E_m$) around 1.9 V vs. Normal Hydrogen Electrode (NHE)) can deactivate $^1\text{O}_2$ with the charge-transfer mechanism. Second-order rate constants for deactivation of $^1\text{O}_2$ via the charge-transfer mechanism are within $10^3$ to $10^9$ M$^{-1}$ s$^{-1}$ [17]. In the charge-transfer mechanism, the $^1\text{(O}_2\text{A)}$ complex finally dissociates to A and $^2\text{O}_2$ without charge separation. Electronic energy-transfer quenching of $^1\text{O}_2$ occurs via the interaction of molecules with a lower triplet state energy than the energy of $^1\text{O}_2$. The deactivation of $^1\text{O}_2$ via the electronic energy-transfer mechanism is very efficient and its second-order rate constant is close to the diffusion-controlled limit. Carotenoids including $\beta$-carotene and lutein are the most efficient quenchers of $^1\text{O}_2$, and the second order rate constant for many carotenoids is about $10^{10}$ M$^{-1}$ s$^{-1}$ [17,41].

2.1.3. Chemical Reactions of $^1\text{O}_2$

The term “chemical deactivation” of $^1\text{O}_2$ can be applied to reactions in which the products have less reactivity and toxicity towards cell metabolism than $^1\text{O}_2$. The redox potential relative to NHE for the pair $^1\text{O}_2/^2\text{O}_2^+$ is 0.65 V [42]. $^1\text{O}_2$ is an electrophilic agent and reacts with electron-rich organic molecules via three well-known mechanisms.

The ene reaction (Alder-ene reaction) is associated with the formation of a hydroperoxide (Reaction (18)).

$$^1\text{O}_2 + \text{H}_3\text{C}-\text{C}=\text{C}-\text{CH}_3 \rightarrow \text{H}_3\text{C}-\text{C}=\text{C}-\text{CH}_2\text{OOH}$$  \hspace{1cm} (18)

Cycloaddition is associated with dioxetane formation (Reaction (19)).

$$^1\text{O}_2 + \text{C}=\text{C} \rightarrow \text{C}=\text{C}\text{O}=\text{O}$$  \hspace{1cm} (19)

Cycloaddition is associated with aromatic compounds and formation of an endoperoxide via the Diels–Alder mechanism (Reaction (20)).

$$^1\text{O}_2 + \text{C}=\text{C} \rightarrow \text{C}=\text{C}\text{O}=\text{O}$$  \hspace{1cm} (20)

$^1\text{O}_2$ can react with the unsaturated fatty acids of membrane lipids to form both conjugated and non-conjugated diene hydroperoxides (Reaction (21)) with a second order rate constant of about $10^4$ M$^{-1}$ s$^{-1}$ [4,43].
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(22)

\[ \text{O}_2 + \text{AH} \rightarrow \text{HO}_2 + \text{A}^- \] (27)

where DHA and PQ are dehydroascorbate and plastoquinone, respectively.

The lifetimes of \(1\text{O}_2\) in a pure lipid membrane and in the thylakoid membrane have been estimated to be 7 µs and 70 ns, respectively [51]. Thus, the respective diffusion distances, approximated using the diffusion coefficient of \(\text{O}_2\), are 220 and 5.5 nm. The very short lifetime in the thylakoid membrane may be caused by a high concentration of compounds deactivating \(1\text{O}_2\). In a non-aqueous solution like organic solvents, \(\text{O}_2^-\) is a strong deprotonation agent and can be protonated to \(\text{HO}_2^-\) (Reaction (26)).

\[ \text{HO}_2^- + \text{AH} \rightarrow \text{HO}_2 + \text{A}^- \] (26)

The term “chemical deactivation” of \(1\text{O}_2\) can be applied to reactions in which the products have properties and biological roles of \(\text{O}_2^-\). Detailed analyses of the formation of the pair \(1\text{O}_2/\text{O}_2^-\) is 0.65 V [42]. \(1\text{O}_2\) is an electrophilic agent and reacts with electron-rich organic substances. The lifetimes of \(1\text{O}_2\) in a pure lipid membrane and in the thylakoid membrane may be caused by a high concentration of compounds deactivating \(1\text{O}_2\). In a nerve cell, the lifetime of \(1\text{O}_2\) is about 200 ns, which leads to a diffusion distance of about 270 nm [52]. The second-order rate constant for the reaction of \(1\text{O}_2\) with \(\text{AscH}_2\) depends on pH and varies from \(10^7 \text{ M}^{-1} \text{ s}^{-1}\) to \(10^9 \text{ M}^{-1} \text{ s}^{-1}\) [44]. Prenyllipids like PQH\(_2\)-9 and \(\alpha\)-tocopherol react with \(1\text{O}_2\) with second-order rate constants of about \(10^8 \text{ M}^{-1} \text{ s}^{-1}\) [50].

2.1.4. Lifetime and Diffusion Distance of \(1\text{O}_2\)

Solvents and other deactivating compounds play significant roles in controlling the lifetime of \(1\text{O}_2\) and the lifetime, in turn, determines both the diffusion distance and the ability of \(1\text{O}_2\) to react with other substances. The lifetimes of \(1\text{O}_2\) in a pure lipid membrane and in the thylakoid membrane have been estimated to be 7 µs and 70 ns, respectively [51]. Thus, the respective diffusion distances, approximated using the diffusion coefficient of \(\text{O}_2\), are 220 and 5.5 nm. The very short lifetime in the thylakoid membrane may be caused by a high concentration of compounds deactivating \(1\text{O}_2\). In a
nerve cell, the lifetime of $^1$O$_2$ is about 200 ns, which leads to a diffusion distance of about 270 nm [52]. The lifetimes and diffusion distances of $^1$O$_2$ in different tissues have been recently reviewed [23].

2.2. Superoxide Anion Radical, O$_2$$^•$−

The O$_2$$^•$− is the one-electron reduced form of molecular oxygen. Detailed analyses of the properties and biological roles of O$_2$$^•$− are available in several comprehensive reviews [53–56]. O$_2$$^•$− is a deprotonation agent and can be protonated to HO$_2$• (Reaction (26)).

$$O_2$$^•$− + H$^+$ → HO$_2$  

(26)

The deprotonation ability of O$_2$$^•$− depends strongly on the solvent. In aqueous solutions, O$_2$$^•$− is a weak deprotonation agent with pKa of 4.8 due to strong solvation of O$_2$$^•$−. The free energy of hydration for O$_2$$^•$− was estimated to be around 355 kJ mol$^{-1}$ [57]. Therefore, only 0.25% of O$_2$$^•$− is protonated at physiological pH values. In non-aqueous solutions like organic solvents, O$_2$$^•$− is a strong deprotonation agent. For example, in dimethylformamide (DMF), the pKa value of HO$_2$• is around 12 [54]. However, in reality the protonation of O$_2$$^•$− in the presence of a protonated compound AH can accompany the reduction of HO$_2$• to hydroperoxyl anion (HO$_2$−) and occur via a two-step mechanism (Reactions (27) and (28)), which makes O$_2$$^•$− a much stronger deprotonation agent than would follow from its basicity.

$$O_2$$^•$− + AH $\rightarrow$ HO$_2$• + A$^-$  

(27)

$$O_2$$^•$− + HO$_2$• $\rightarrow$ O$_2$ + HO$_2$−  

(28)

which sum up to

$$2O_2$$^•$− + AH $\rightarrow$ O$_2$ + HO$_2$− + A$^-$  

(29)

The equilibrium constant of Reaction (29) is about 10$^9$ [54]. Therefore, in the deprotonation process the pKa value of O$_2$$^•$− should be considered equivalent to a base with pKa 24 [53,54]. The $E_m$ of the pair O$_2$/O$_2$$^•$− is pH-dependent, due to protonation of O$_2$$^•$− and formation of HO$_2$•. In an aqueous solution at pH 7, the $E_m$ of the pair O$_2$/O$_2$$^•$− is −160−−180 mV vs. NHE, and the $E_m$ value becomes more positive under a low pH, around 100 mV [12,58]. In aprotic media offering only a weak solvation of O$_2$$^•$−, O$_2$$^•$− acts as a strong reductant and the redox potential of the pair O$_2$/O$_2$$^•$− is estimated to range between −550 and −600 mV vs. NHE [54] in DMF and around −640 mV in acetonitrile [59,60].

2.2.1. Formation of O$_2$$^•$−

O$_2$$^•$− is mainly formed via the interaction of O$_2$ with reduced compounds having a low redox potential (A) (Reaction (30)).

$$A^-$ + O$_2$ $\rightarrow$ A + O$_2$$^•$−  

(30)

O$_2$$^•$− can be formed in a potentially important equilibrium reaction with semiquinone anion radicals (Q$^•$−) with the formation of the respective quinone Q (Reaction (31)).

$$Q$$^•$− + O$_2$ $\rightarrow$ Q + O$_2$$^•$−  

(31)

The equilibrium constant of Reaction (31) can be determined from the redox potentials of Q/Q$^•$− and O$_2$/O$_2$$^•$−. In aqueous solutions at pH 7, the equilibrium constant for Reaction (31) is estimated as 2 × 10$^{-5}$ for benzosemiquinone with a redox potential around 100 mV, and 26 for durosemiquinone with redox potential around −260 mV [61]. The formation of O$_2$$^•$− via Reaction (31) is favorable for Q$^•$−, with the redox potential of the Q/Q$^•$− pair lower than 160−−180 mV because, with this redox potential, the forward rate constant (k$_{forward}$) of Reaction (31) is larger than the reverse rate constant (k$_{reverse}$). Reaction (31) when Q$^•$− is benzosemiquinone proceeds with k$_{forward}$ of 5 × 10$^5$ M$^{-1}$ s$^{-1}$ and with k$_{reverse}$ of 10$^6$ M$^{-1}$ s$^{-1}$. For durosemiquinone, k$_{forward}$ and k$_{reverse}$ were estimated to be 2.2 × 10$^8$ M$^{-1}$ s$^{-1}$ and
10⁷ M⁻¹ s⁻¹, respectively [61]. However, if O₂•− is efficiently removed after Reaction (31), then the rate of formation depends only on $k_{\text{forward}}$.

2.2.2. Reactions of O₂•−

Reactions of O₂•− with organic and inorganic molecules can proceed in five ways: deprotonation reaction (protonation of O₂•− by H⁺ or the attraction of a proton from a proton donor), attraction of hydrogen, electron transfer reaction, nucleophilic substitution or addition, and addition to a metal or metal complex.

In electron transfer reactions, O₂•− can act as both an oxidant and a reductant. In many cases, the electron transfer reaction involves a deprotonation reaction (deprotonation–oxidation mechanism). O₂•− can reduce or oxidize organic and inorganic molecules like quinones and cytochromes (cyt) or transition metal ions in equilibrium reactions via a one-electron transfer mechanism, Reactions (31)–(33).

$$\text{O}_2^{•−} + \text{cyt (Fe}^{3+}) \rightarrow \text{O}_2 + \text{cyt (Fe}^{2+})$$  \hspace{1cm} (32)

$$\text{O}_2^{•−} + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+}$$ \hspace{1cm} (33)

In many cases, O₂•− oxidizes organic and inorganic molecules by hydrogen attraction via the deprotonation–oxidation mechanism. Oxidation of AscH₂ by O₂•− in aqueous solution (Reaction (34)) proceeds as a two-step reaction (Reactions (35) and (36)) with a second-order rate constant of $3.3 \times 10^5$ M⁻¹ s⁻¹ at pH 7.8 [62–64].

$$\text{O}_2^{•−} + \text{AscH}_2 \rightarrow \text{H}_2\text{O}_2 + \text{Asc}^{•−}$$ \hspace{1cm} (34)

$$\text{HO}_2^{•} + \text{AscH}^{−} \rightarrow \text{H}_2\text{O}_2 + \text{Asc}^{•−}$$ \hspace{1cm} (35)

The same mechanism is suggested for oxidation by O₂•− of thiols as in reduced GSH and lipophilic compounds as in α-tocopherol, Reactions (37)–(40), respectively [64,65].

$$\text{O}_2^{•−} + \text{GSH} \rightarrow \text{H}_2\text{O}_2 + \text{GSH}^{−}$$ \hspace{1cm} (37)

$$\text{HO}_2^{•} + \text{GSH}^{−} \rightarrow \text{H}_2\text{O}_2 + \text{GSH}^{•}$$ \hspace{1cm} (38)

$$\text{O}_2^{•−} + \alpha\text{-Tocopherol-H} \rightarrow \text{H}_2\text{O}_2 + \alpha\text{-Tocopherol}^{−}$$ \hspace{1cm} (39)

$$\text{HO}_2^{•} + \alpha\text{-Tocopherol}^{−} \rightarrow \text{H}_2\text{O}_2 + \alpha\text{-Tocopherol}$$ \hspace{1cm} (40)

In Reactions (37) and (38), GS⁻ and GS⁺ stand for deprotonated reduced glutathione and oxidized glutathione, respectively.

The second-order rate constant of the reaction of O₂•− with α-tocopherol incorporated into soybean or dimyristoyl phosphatidylcholine liposomal membranes was estimated to be $4.9 \times 10^3$ M⁻¹ s⁻¹ [62]. The second-order rate constant of the reaction of O₂•− with GSH was estimated to be $10^3$ M⁻¹ s⁻¹ [66,67].

The main mechanism of deactivation of O₂•− is spontaneous or enzymatic dismutation (Reaction (5)). Non-enzymatic dismutation of O₂•− usually proceeds in aqueous solution and very strongly depends on pH because the protonation of O₂•− determines the rate. Dismutation can be considered as a two-step reaction: protonation of O₂•−, Reaction (41) and a radical–radical reaction between O₂•− and HO₂• or between two molecules of HO₂•—Reactions (42) and (43), respectively.

$$\text{O}_2^{•−} + \text{H}^+ \rightarrow \text{HO}_2^{•}, \text{pKa} = 4.8$$ \hspace{1cm} (41)

$$\text{O}_2^{•−} + \text{HO}_2^{•} + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$ \hspace{1cm} (42)

$$\text{HO}_2^{•} + \text{HO}_2^{•} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$ \hspace{1cm} (43)
The second-order rate constant of the dismutation of \( \text{O}_2^{-}\) has a maximum \((10^8 \text{ M}^{-1} \text{ s}^{-1})\) at pH 4.8, equal to the pKa value of \( \text{HO}_2\). The rate constant decreases with increasing pH and becomes very low, around \(0.3 \text{ M}^{-1} \text{ s}^{-1}\), at alkaline pH. At physiological pH, the rate constant is about \(10^5 \text{ M}^{-1} \text{ s}^{-1}\) [68]. The enzymatic dismutation of \( \text{O}_2^{-}\) is catalyzed by superoxide dismutase (SOD, EC 1.15.1.1). The SOD-catalyzed reaction proceeds as a sequence of oxidation and reduction of \( \text{O}_2^{-}\) by a metal ion (M) of the SOD enzyme, Reactions (44) and (45).

\[
\begin{align*}
\text{M}^{(n+1)+}\text{-SOD} + \text{O}_2^{-} &\rightarrow \text{M}^{n+}\text{-SOD} + \text{O}_2 \quad \text{(44)} \\
\text{M}^{n+}\text{-SOD} + \text{O}_2^{-} + 2\text{H}^+ &\rightarrow \text{M}^{(n+1)+}\text{-SOD} + \text{H}_2\text{O}_2 \quad \text{(45)}
\end{align*}
\]

The rate constant of \( \text{O}_2^{-}\) dismutation catalyzed by SOD is about \(6.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}\) [69].

\( \text{O}_2^{-}\) is a powerful nucleophile in aprotic medium and can be involved in nucleophilic reactions with various organic compounds. \( \text{O}_2^{-}\) reacts with alkyl halides (RX), acyl halides and acyl anhydrides to form ROO* intermediates through nucleophilic substitution reactions [54]. \( \text{O}_2^{-}\) can add to positively charged carbon–carbon double bonds [70] and carbon–nitrogen double bonds [71]. For example, Reactions (46)–(48) illustrate the reaction of \( \text{O}_2^{-}\) with RX, acyl halide and anhydride, respectively. The peroxy and alkoxy radicals are more reactive than \( \text{O}_2^{-}\) itself.

\[
\text{O}_2^{-} + \text{RX} \rightarrow \text{ROO}^* + \text{X}^- \quad \text{(46)}
\]

\[
\begin{align*}
\text{O}_2^- + \text{R} &\rightarrow \text{ROO}^- + \text{C} - \text{X} \quad \text{(47)} \\
\text{O}_2^- + \text{R} &\rightarrow \text{ROO}^- + \text{C} - \text{OO}^- \quad \text{(48)}
\end{align*}
\]

In addition to nucleophilic reactions with organic molecules, \( \text{O}_2^{-}\) can bind to both transition metals and to metal complexes. For example, in PSII, the interaction of \( \text{O}_2^{-}\) with a ferrous heme iron leads to the formation of a ferric–peroxo ((Fe3+)–OOH) complex which can be protonated to a ferric–hydroperoxo ((Fe3+)–OOH) complex, Reaction (49) and (50) [72].

\[
\begin{align*}
\text{O}_2^- + \text{L-(Fe}^{2+}) &\rightarrow \text{L-(Fe}^{3+})\text{-OO}^- + \text{H}^+  \\
\text{L-(Fe}^{3+})\text{-OO}^- + \text{H}^+ &\rightarrow \text{L-(Fe}^{3+})\text{-OOH}  \\
\end{align*}
\]

where L is a ligand.

2.2.3. Lifetime and Diffusion Distance of \( \text{O}_2^{-}\)

The lifetime of \( \text{O}_2^{-}\) is controlled by dismutation (Reaction (5)). Thus, in the absence of SOD, the lifetime of \( \text{O}_2^{-}\) depends on pH in aqueous solutions and on the presence of a proton donor in aprotic media. \( \text{O}_2^{-}\) is more stable in alkaline aqueous solutions \((t_{1/2} = 50 \text{ s} \text{ at pH 14})\), and the lifetime of \( \text{O}_2^{-}\) decreases with decreasing pH \((t_{1/2} = 0.2 \text{ s} \text{ at pH 10})\). The lifetime of \( \text{O}_2^{-}\) prepared in a two-electrode cell in DMF was found to be 76 min at 0 °C for 0.1 M \( \text{O}_2^{-}\), and around 35 h for the \( \text{O}_2^{-}\) concentrations from 0.001 to 0.01 M [54]. In cells, the lifetime of \( \text{O}_2^{-}\) is efficiently controlled by SOD, and the lifetime will depend on SOD activity. In the periplasm of \textit{Escherichia coli}, the lifetime of \( \text{O}_2^{-}\) was estimated to be longer than 0.6 s using the rate of \( \text{O}_2^{-}\) formation and the rate constant of its dismutation. The diffusion distance was calculated as 35 μm, assuming a general diffusion coefficient of small molecules of about \(10^5 \text{ cm}^2 \text{ s}^{-1}\) [73].
2.3. Hydrogen Peroxide, H\textsubscript{2}O\textsubscript{2}

H\textsubscript{2}O\textsubscript{2} is the result of two-electron reduction of O\textsubscript{2} and considered a major biological ROS. In cells, H\textsubscript{2}O\textsubscript{2} is mostly present in the neutral form because its pKa is 11.8. H\textsubscript{2}O\textsubscript{2} is a strong, two-electron oxidant with a standard redox potential ($E_0$) of 1.32 V at pH 7.0. However, H\textsubscript{2}O\textsubscript{2} reacts slowly or does not react with most biological molecules, including low-molecular-weight antioxidants, due to a high activation energy barrier [74]. Even if a reaction with H\textsubscript{2}O\textsubscript{2} is thermodynamically favorable, it may be very slow.

Low-potential compounds reduce H\textsubscript{2}O\textsubscript{2} with one electron, as the redox potential of H\textsubscript{2}O\textsubscript{2}/HO\textsuperscript{*} is 0.3 V [12]. H\textsubscript{2}O\textsubscript{2} can also act as an electrophile due to the polarizability of the O–O bond. H\textsubscript{2}O\textsubscript{2} has a permanent dipole moment of 2.26 Debye. The O–O bond is relatively weak and susceptible to homolysis. H\textsubscript{2}O\textsubscript{2} is decomposed by heating, radiolysis, photolysis, or by reaction with redox active transition metals [74].

2.3.1. Formation of H\textsubscript{2}O\textsubscript{2}.

Reduction of O\textsubscript{2} to O\textsubscript{2}•\textsuperscript{-} followed by its dismutation (Reaction (5)) is the main pathway for the formation of H\textsubscript{2}O\textsubscript{2} in cells.

H\textsubscript{2}O\textsubscript{2} can be formed via oxidation of a quinol by O\textsubscript{2}. For example, hydroanthraquinone is widely used for the commercial synthesis of H\textsubscript{2}O\textsubscript{2}, Reaction (51) [75].

\[
\begin{align*}
\text{OH} & \quad \text{R} \quad \text{OH} \\
\text{OH} & \quad \text{R} \quad + \text{O}_2 \rightarrow \\
\text{OH} & \quad \text{R} \quad + \text{H}_2\text{O}_2
\end{align*}
\]

H\textsubscript{2}O\textsubscript{2} can be produced by a reaction of 1\textsubscript{O}\textsubscript{2} or O\textsubscript{2}•\textsuperscript{-} with an electron donor, like AscH\textsubscript{2}, Reactions (24) and (34), respectively [47,64].

The main reaction of 1\textsubscript{O}\textsubscript{2} with PQH\textsubscript{2} in methanol was found to result in the formation of PQ and H\textsubscript{2}O\textsubscript{2}. Reaction (25) and the amount of H\textsubscript{2}O\textsubscript{2} produced was essentially the same as the amount of oxidized PQH\textsubscript{2} [48].

The direct formation of H\textsubscript{2}O\textsubscript{2} in a reaction of O\textsubscript{2} and H\textsubscript{2} can occur over catalysts containing palladium (PdAu, Pd-SiO\textsubscript{2}, PdZn and others), Reaction (52) [76].

\[
\text{O}_2 + \text{H}_2 \xrightarrow{\text{Pd-catalyst}} \text{H}_2\text{O}_2
\]

No direct formation of H\textsubscript{2}O\textsubscript{2} from H\textsubscript{2} and O\textsubscript{2} is expected in aerobic cells because the production of hydrogen requires anaerobic conditions [77]. H\textsubscript{2} is consumed by the bidirectional hydrogenase in green algae [78], but an enzyme-catalyzing Reaction (52) has not been found.

2.3.2. Reactions of H\textsubscript{2}O\textsubscript{2}

Most biological molecules that do not bind transition metal ions do not react directly with H\textsubscript{2}O\textsubscript{2}. However, thiol and cysteine residues of proteins, as well as low-molecular-weight thiols, can directly react with H\textsubscript{2}O\textsubscript{2} [67]. The reaction of H\textsubscript{2}O\textsubscript{2} with thiols (RS) depends strongly on the pK\textsubscript{a} value of the thiol, because the reaction exclusively proceeds via the thiolate anion to form sulfenic acid (RSOH), Reaction (53).

\[
\text{RS}^- + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{RSOH} + \text{H}_2\text{O}
\]
The rate constants of Reaction (53) range from 0.16 to 10^7 M^{-1} s^{-1}. Sulfenic acids have a lower pKa than the corresponding thiols [79]. Sulfenic acid can react with another thiol or H_2O_2 to give the corresponding disulfide (RSSR) or sulfenic acid (RSO_2^-), Reactions (54) and (55), respectively. However, the second-order rate constant of Reaction (55) is about 10^3 times lower than that of Reaction (54) [74,80].

\[
\text{RSO}^- + \text{H}^+ + \text{H}_2\text{O}_2 \rightarrow \text{RSO}_2^- + \text{H}_2\text{O} \quad (55)
\]

The second-order rate constants of Reaction (53) for free GSH, cysteine and thioredoxin (TRX) are 0.89 M^{-1} s^{-1}, 2.9 M^{-1} s^{-1} and 1.05 M^{-1} s^{-1}, respectively [74,81]. However, H_2O_2 can react efficiently with peroxiredoxins (PRX); the second order rate constant is 10^7–10^8 M^{-1} s^{-1} [74]. H_2O_2 reacts with pyruvate to form acetate and CO_2 with a rate constant of 2.2 M^{-1} s^{-1}. The reaction of H_2O_2 proceeds via a two-electron pathway: (i) reversible formation of a tetrahedral intermediate; (ii) irreversible decomposition of the intermediate to CO_2, acetate, and water, Reaction (56) [82].

\[
\text{H}_2\text{O}_2 + \text{CO}_2 \rightarrow \text{HCO}_3^- + \text{H}^+ 
\]

H_2O_2 reacts with carbon dioxide to form peroxymonocarbonate (HCO_3^-) with a second-order rate constant for the forward reaction of 2 \times 10^{-3} M^{-1} s^{-1}, Reaction (57) [74].

\[
\text{H}_2\text{O}_2 + \text{CO}_2 \rightarrow \text{HCO}_3^- + \text{H}^+ 
\]

Carbonic anhydrase significantly accelerates Reaction (57) [83]. Transition metals (M) like iron and copper react with H_2O_2 via the Fenton mechanism, in which the transition metal cleaves the O=O bond to form HO^* and HO^-, Reaction (58).

\[
\text{M}^{(n-1)+} + \text{H}_2\text{O}_2 \rightarrow \text{M}^{+n} + \text{OH}^- + \text{HO}^* 
\]

Rate constants of the Fenton reaction depend on the metal or metal complex and are in the 5–20 \times 10^3 M^{-1} s^{-1} range [84–87]. In addition to Reaction (58), the interaction of H_2O_2 with transition metals or metal complexes leads to the formation of a higher oxidation state of the metal as L-M(H_2O_2)^{(n+)}^+, L-M^{(n+2)+} or L-MO^{(n+2)+} Reactions (59)–(61) respectively, where L is a ligand of the metal [74,88,89].

\[
\begin{align*}
\text{L-M}^{(n)+} + \text{H}_2\text{O}_2 & \rightarrow \text{L-M}-(\text{H}_2\text{O}_2)^{(n)+} \\
\text{L-M}-(\text{H}_2\text{O}_2)^{(n)+} & \rightarrow \text{L-M}^{(n+2)^+} + 2\text{HO}^- \\
\text{L-M}-(\text{H}_2\text{O}_2)^{(n)+} & \rightarrow \text{L-MO}^{(n+2)^+} + \text{H}_2\text{O}
\end{align*}
\]

H_2O_2 reacts rapidly with heme peroxidases, for example, myeloperoxidase and lactoperoxidase, with a second order rate constant in the range of 10^9–10^{10} M^{-1} s^{-1} [90]. The rate constant of the reaction of H_2O_2 with ascorbate peroxidase (APX) was estimated to be 10^7 M^{-1} s^{-1} with K_m for H_2O_2 of 80 \mu M [91,92].

The scavenging of H_2O_2 by peroxidases (PX) proceeds via the peroxidase mechanism (Reactions (62)–(64)) [93].

\[
\begin{align*}
\text{PX-Fe(III)-porphyrin} + \text{H}_2\text{O}_2 & \rightarrow \text{PX-Fe(IV)=O-porphyrin}^+ + \text{H}_2\text{O} \\
\text{PX-Fe(IV)=O-porphyrin}^+ + \text{AH} & \rightarrow \text{PX-Fe(IV)=O-porphyrin} + \text{A}^* + \text{H}^+ 
\end{align*}
\]
PX-Fe(IV)=O- porphyrin + AH → PX-Fe(III)- porphyrin + A• + OH⁻,  (64)

where A is a reductant, e.g., AscH₂.

Catalase (CAT)-dependent scavenging of H₂O₂ occurs via a ping-pong mechanism, Reaction (65) and (66), where one H₂O₂ molecule is used as an electron donor.

\[ H₂O₂ + Fe(III)-CAT → H₂O + Fe(IV)=O-CAT⁺ \]  (65)

\[ H₂O₂ + Fe(IV)=O-CAT⁺ → H₂O + Fe(III)-CAT + O₂ \]  (66)

2.3.3. Lifetime and Diffusion Distance of H₂O₂

H₂O₂ is a small and neutral molecule that can readily diffuse from the site of its production. However, the diffusion of H₂O₂ through membranes is hindered [94–96] and aquaporins may regulate the diffusion of H₂O₂ across membranes. H₂O₂ is rather stable and its lifetime in cells is limited by scavenging enzymes and other substances reacting with H₂O₂. The lifetime of H₂O₂ is 1–3 min in mammalian cells [97] and around 10 s in Arabidopsis guard cells [98].

2.4. Hydroxyl Radical, HO•

HO• has one unpaired electron and is one of the most powerful oxidizing agents. HO• is able to react unselectively with surrounding organic molecules due to the very high positive redox potential, of the pair HO•/H₂O, \( E₀′ \) of 2.3 V [99]. The rate constants of reactions of HO• with many molecules are estimated to be larger than 10⁹ M⁻¹ s⁻¹ [100–102].

2.4.1. Formation of HO•

The best-known reaction producing HO• is the Fenton reaction, in which the O–O bond of H₂O₂ is cleaved by reaction with a transition metal ion (Reaction (58)). HO• is produced via the same mechanism in the reaction of H₂O₂ with O₂•− (metal-catalyzed Haber–Weiss reaction), Reaction (3).

Another well-known means of HO• generation is through the photolysis of oxygen-containing species. In aqueous solution, the nitrate anion (NO₃⁻) can absorb UV radiation and produce HO•, Reactions ((67) and (69). The formation of HO• is also observed upon photolysis of the nitrite ion (NO₂⁻), Reactions (68) and (69) [102,103]. Due to the requirement of short-wavelength UV radiation, this process does not occur in biological systems.

\[ NO₃⁻ + hν ⇌ [O•−•NO₂] → O•− + •NO₂ \]  (67)

\[ NO₂⁻ + hν → O•− + •NO₂ \]  (68)

\[ O•− + H⁺ ⇌ HO• \]  (69)

The photolysis of a H₂O₂ molecule gives two HO• with a quantum yield of approximately 0.5 in aqueous solutions, Reaction (70). H₂O₂ photolysis requires UV-C radiation because the molar absorption coefficient of H₂O₂ is very low above 300 nm. H₂O₂ photolysis is an effective way of generating HO• in aqueous solutions [102,104].

\[ H₂O₂ \overset{Δ}{→} [HO••HO•] → 2HO• \]  (70)

Another potential source of HO• is O₃. The addition of an electron to an O₃ molecule leads to the decomposition of O₃ to HO• and O₂ via the formation of an ozonide anion radical [105]. O₃ can also be decomposed to O₂ and HO• via reduction by exited chlorophyll (Chl*), Reaction (71) [102].

\[ O₃ + Chl* + H⁺ → O₂ + HO• + Chl⁺ \]  (71)

However, O₃ has not been found inside plant cells.
O₃ reacts with O₂⁻⁻ to form HO• and O₂ as final products, Reaction (72)–(74)

\[
\begin{align*}
O_3 + O_2^{-} & \rightarrow O_3^{-} + O_2 \quad (72) \\
O_3^{-} + H^+ & \rightarrow HO_3^{+} \quad (73) \\
HO_3^{+} & \rightarrow HO^{+} + O_2 \quad (74)
\end{align*}
\]

HO• can be also produced in a radical–radical reaction of HO₂• with RO₂•, Reaction (75) [102].

\[
RO_2^{+} + HO_2^{+} \rightarrow RO^{+} + HO^{+} + O_2 \quad (75)
\]

2.4.2. Reactions of HO•

HO• participates in several typical reactions:

- abstraction of hydrogen atom from an organic molecule (RH) with the formation of H₂O and radical (R*) of substrate (76);

\[
HO^{+} + RH \rightarrow H_2O + R^{+} \quad (76)
\]

- addition to double bonds with the formation of a hydroxylated radical (77);

\[
\text{HO}^{+} + \text{substrate} \rightarrow \text{hydroxylated radical} \quad (77)
\]

- electron transfer reactions leading to the formation of a neutral radical (78) or a cation radical (79) [106]; SCN⁻ is the thiocyanate ion.

\[
HO^{+} + SCN^{-} \rightarrow OH^{-} + SCN \quad (78)
\]

Formation of an aromatic-OH adduct due to a reaction of an aromatic compound with HO• is one of the methods for HO• detection with high-performance liquid chromatography–mass spectrometry. For example, HO• can react with phenylalanine to form isomers of tyrosine, Reaction (80) [107]. Isomers of tyrosine are rather stable and not normally present in proteins, and can serve as HO• traps in biological samples [108].
HO• interacts with many metal (M) cations via an electron transfer Reaction (81), with a rate constant of ~$10^8$ M$^{-1}$ s$^{-1}$ [100].

$$\text{HO•} + \text{M}^{n+} \rightarrow \text{M}^{(n+1)+} + \text{OH}^-$$

HO• initiates lipid peroxidation, resulting in hydrogen abstraction from a pentyl group of an unsaturated fatty acid, and the formation of a radical that interacts with O$_2$ to form an ROO• with a rate constant of ~$10^8$ M$^{-1}$ s$^{-1}$ [109], Reaction (82).

2.4.3. Lifetime and Diffusion Distance of HO•

The lifetime of HO• in aqueous solution has been estimated to range from picoseconds to nanoseconds. The self-diffusion coefficient of HO• in water has been estimated to be $2.8 \times 10^{-5}$ cm$^2$ s$^{-1}$, and consequently the diffusion distance of HO• is a few molecular diameters from the site of origin [110,111].
2.4.3. Lifetime and Diffusion Distance of HO•

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3. Production of ROS in Chloroplasts

Chloroplasts have a high metabolic activity accompanied with intensive formation of redox active compounds, which are able to react with oxygen to produce ROS. Most ROS production in the chloroplast occurs by the components of the light reactions. Photorespiration is responsible for 70% of total H$_2$O$_2$ production in the leaves of C3 plants [112,113], but this reaction runs in peroxisomes outside of the chloroplast.

3.1. ROS Production in Chloroplast Stroma

3.1.1. Formation of $\text{O}_2^\bullet$ in the Stroma

The chloroplast stroma is not considered as a significant source of $\text{O}_2^\bullet$, although disintegration of the antenna complexes under stress conditions and disturbances in Chl synthesis and the accumulation of its precursors may lead to $\text{O}_2^\bullet$ production in the stroma [114]. The lack of FLU, a nuclear-encoded chloroplast protein that plays a key role during the negative feedback control of Chl biosynthesis, leads to the accumulation of protochlorophyllide in plastids and, consequently, to photosensitized generation of $\text{O}_2^\bullet$ [115]. It has been recently shown that lipoxygenase localized in the chloroplast is responsible for $\text{O}_2^\bullet$ formation [116]. Lipoxygenase initiates lipid oxidation to corresponding lipid peroxides, which decompose to lipid peroxyl radicals (LOO•). LOO• can react with each other, forming a cyclic endoperoxide (dioxetane) intermediate. Dioxetane, in turn, can decompose via the Russel mechanism to form $\text{O}_2^\bullet$, Reaction (9).

Theoretically, the Haber-Weiss mechanism (Reactions (3) and (4)) can cause the formation of $\text{O}_2^\bullet$ in the stroma, but the rate of this reaction is expected to be low due to very efficient scavenging of O$_2$•− by chloroplasts [12] and the low rate constant of Reactions (3) and (4) [21].

3.1.2. Formation of Reduced Forms of Oxygen, O$_2$•−, H$_2$O$_2$, HO•, by Fd in the Stroma

Ferredoxin (Fd) and free flavins (FL) and flavoenzymes are considered as the main stromal components involved in O$_2$ reduction and ROS formation. Fd is involved in electron transfer from the acceptor side of PSI to NADP$^+$ in a reaction catalyzed by Fd-NADP$^+$ reductase (FNR) (EC 1.18.1.2) [117]. Fd is a soluble 10 kDa protein [118] containing a 2Fe-2S center [119]. The leaf-type Fd from higher plants has an $E_m$ vs. NHE (at pH 8.0) from −390 to −425 mV [120]. The redox potential of Fd$_{ox}$/Fd$_{red}$ is much more negative than the redox potential of O$_2$/O$_2$•− (−160−−180 mV) in aqueous solutions, and the reduction in O$_2$ by Fd is thermodynamically very favorable. It was suggested that the oxidation of Fd can occur via 2-step reaction with the formation of the Fd-O$_2$•− complex [121], Reactions (83) and (84).

\[
\text{Fd(II)} + \text{O}_2 \Delta \text{Fd(II)-O}_2 \quad \text{(83)}
\]

\[
\text{Fd(II)-O}_2 \Delta \text{Fd(III)-O}_2^\bullet \quad \text{(84)}
\]

The subsequent dissociation of the Fd(III)-O$_2$•− yields O$_2$•−. The involvement of Fd in O$_2$ reduction has been studied by the addition of Fd to a suspension of thylakoid membranes in the light. In this case, Fd is reduced by PSI. Fd increased the rate of O$_2$ consumption in response to an increase in Fd concentration. The rate of O$_2$ consumption was saturated at 60 µM Fd and became sevenfold higher than in the absence of Fd [122,123]. On the other hand, Fd did not stimulate the formation of O$_2$•− measured using O$_2$•− traps such as epinephrine or cytochrome c [124]. This suggests that the formation
of $O_2^{•−}$ by exogenous Fd is very slow in comparison to the formation of $O_2^{•−}$ by thylakoid-bound photoreductants. This correlates with the finding that the rate of direct oxidation of Fd by $O_2$ was rather low, with a second-order rate constant of $10^3 \text{ M}^{-1} \text{s}^{-1}$ for chemically (dithionite as reductant) reduced Fd [125]. Recent studies show that $O_2$ reduction in a thylakoid suspension in the presence of Fd is a result of $O_2$ reduction by both a membrane-bound reductant and Fd. The distribution of electron flow from Fd and membrane-bound reductant to $O_2$ is sensitive to light intensity and NADP+ but not to Fd concentration. Furthermore, Fd stimulates the reduction in $O_2$ by membrane-bound reductants [126]. Interestingly, NADP+ very strongly inhibits $O_2$ reduction by Fd but stimulates $O_2$ reduction by a thylakoid-membrane-bound reductant [126]. These results suggest that Fd has a minor role in the direct reduction of $O_2$ in vivo.

Catalase, added to a suspension of illuminated thylakoid membranes, almost completely suppressed Fd-dependent $O_2$ consumption, suggesting that $H_2O_2$ is the final product of $O_2$ reduction by Fd. This is clear from the well-known stoichiometry between $O_2$ consumption and $O_2$ evolution in isolated thylakoids when a reduction in $O_2$ occurs by electrons originally arising from water-splitting in PSII without any electron acceptors or ROS traps, Reactions (85)–(89). In this case, $H_2O_2$ is produced via dismutation of $O_2^{•−}$ [127,128].

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^- \quad \text{water splitting in PSII} \quad (85)$$

$$4Fd(III) + 4e^- \rightarrow 4Fd(II) \quad \text{Fd reduction in PSI,} \quad (86)$$

$$4Fd(II) + O_2 \rightarrow 4Fd(III) + 4O_2^{•−} \quad \text{$O_2^{•−}$ formation,} \quad (87)$$

$$4O_2^{•−} + 4H^+ \rightarrow 2H_2O_2 + 2O_2 \quad \text{$O_2^{•−}$ dismutation,} \quad (88)$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad \text{$H_2O_2$ decomposition,} \quad (89)$$

Reaction (84) is included only to describe an experiment with isolated thylakoids. Chloroplasts do not contain CAT and, in chloroplasts, $H_2O_2$ is scavenged by APX (Reactions (62)–(64)) [12]. Fd-mediated photosynthetic $O_2$ consumption is inhibited by SOD, suggesting that autoxidation of Fd is involved in both the reduction of $O_2$ to $O_2^{•−}$ and of $O_2^{•−}$ to $H_2O_2$. Reactions (90) and (91) [129].

$$Fd(II) + O_2 \rightarrow Fd(III) + O_2^{•−} \quad (90)$$

$$Fd(II) + O_2^{•−} + 2H^+ \rightarrow Fd(III) + H_2O_2 \quad (91)$$

The reduction in $O_2^{•−}$ by Fd can produce $H_2O_2$ in the chloroplast stroma. The very low stimulation of $O_2^{•−}$ production by Fd in comparison to $O_2$ consumption [124,127] could result from Reaction (91). It is also possible that Fd stimulates $O_2$ reduction in chloroplasts through a more complicated process than the direct reduction in $O_2$.

### 3.1.3. Formation of Reduced Forms of Oxygen, $O_2^{•−}$, $H_2O_2$, $HO^•$, by Flavins in the Stroma

The reduction in $O_2$ by FLs can also produce ROS in the stroma, as FLs react with $O_2$ to form $O_2^{•−}$ and $H_2O_2$. Free FLs are oxidized by $O_2$ via the formation of an intermediate complex containing a radical pair that can decompose with the formation of a flavin semiquinone radical ($FLH^•$) and $O_2^{•−}$, Reaction (92). The radical pair may also be transformed to a flavin hydroperoxide ($FLHOOH$) that can decompose to a FL and $H_2O_2$, Reaction (93) [130–134].

$$FLH^- + O_2 \rightarrow [FLH^•-OO^•−] \rightarrow FLH^• + O_2^{•−} \quad (92)$$

$$[FLH^•-OO^•−] + H^+ \rightarrow FLHOOH \rightarrow FL + H_2O_2 \quad (93)$$

The second-order rate constant of Reaction (92) is estimated to be only $2.5 \times 10^2 \text{ M}^{-1} \text{s}^{-1}$ [133,134]. The formation of $FLH^•$ (Reaction (92)) can also initiate complex autocatalytic FL oxidation. In solution,
some amount of the FLH• is formed in a mixture of oxidized and reduced FL (FLH2) via an equilibrium reaction, Reaction (94).

\[
\text{FLH}_2 + \text{FL} \rightleftharpoons 2\text{FLH}^• \tag{94}
\]

With free FLs, the second-order rate constants for forward and backward Reaction (94) are \(10^6 \text{ M}^{-1} \text{s}^{-1}\) and \(5 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\), respectively [133]. Thus, the equilibrium constant of Reaction (94) is \(2 \times 10^{-3}\). Only 1–5% of the radical is stabilized in an equimolar mixture of oxidized and reduced FL [134,135]. The flavin semiquinone radical can exist in neutral (FLH•) or anionic form (FL•−), with a pKa of \(\approx 8.5\) (Reaction (95)).

\[
\text{FLH}^• \rightleftharpoons \text{FL}^•− + \text{H}^+ \tag{95}
\]

The next steps of the autocatalytic process are described by Reactions (96)–(101), in which the reaction of O2 with FLH• or FL•− by O2 produces O2•−, Reaction (96) and (97), and then O2•− or HO2• can react with FLH2 to form FLH• or FL•− and H2O2, Reaction (98) and (99). O2•− and HO2• can also react with FLH• to form FL and HO2− or H2O2, Reaction (100) and (101).

\[
\text{FLH}^• + \text{O}_2 \rightarrow \text{FL} + \text{O}_2•− + \text{H}^+ \rightarrow \text{FL} + \text{HO}_2 \tag{96}
\]

\[
\text{FL}^•− + \text{O}_2 \rightarrow \text{FL} + \text{O}_2•− \tag{97}
\]

\[
\text{FLH}_2 + \text{O}_2•− \rightarrow \text{FL}^•− + \text{H}_2\text{O}_2 \tag{98}
\]

\[
\text{FLH}_2 + \text{HO}_2• \rightarrow \text{FLH}^• + \text{H}_2\text{O}_2 \tag{99}
\]

\[
\text{FLH}^• + \text{O}_2•− \rightarrow \text{FL} + \text{HO}_2− \tag{100}
\]

\[
\text{FLH}^• + \text{HO}_2• \rightarrow \text{FL} + \text{H}_2\text{O}_2 \tag{101}
\]

The second-order rate constant for the reaction of O2 with FLH• (Reaction (96)) is around \(10^4 \text{ M}^{-1} \text{s}^{-1}\) and that of the reaction of O2 with FL•− (Reaction (97)) is much larger, around \(10^8 \text{ M}^{-1} \text{s}^{-1}\) [133]. The \(E_m\) at pH 7 for FL/FLH• of FL mononucleotide is estimated to be \(-313 \text{ mV}\) vs. NHE, which is more negative than the redox potential of O2/O2•− (\(-160 \text{ mV}\)) in aqueous solution [134,136]. As predicted from the redox potentials, the reaction of FL•− with O2 is thermodynamically favorable and the rate of oxidation of FLs by O2 via autocatalytic mechanisms can strongly depend on both the stability and pKa value of FLH•. Free flavins can therefore be involved in the formation of ROS in the chloroplast stroma.

Flavoenzymes can also be involved in the production of O2•− and H2O2 in chloroplasts. The reactivity of FLs in flavoenzymes is modulated by the protein environment of reduced FLs and the second-order rate constant of O2 reduction by flavoenzymes varies from 2 M\(^{-1}\) s\(^{-1}\) to \(2 \times 10^6\) M\(^{-1}\) s\(^{-1}\) [134]. The redox potential of flavoenzymes vs. NHE varies from \(-16\) to \(-263\) mV and \(-60\) to \(-231\) mV for FL/FLH• and for FLH•/FLH2, respectively [134]. The reactivity of flavoenzymes towards O2 may differ by several orders of magnitude between flavoenzymes having similar redox potentials [134]. Such very high differences are due to the protein environment, which affects both O2 movement and the binding of O2 to the active site. In addition, the polarity of the protein environment in the active site can change the redox potential of O2/O2•− because the redox potential of O2/O2•− becomes very negative (\(\approx -600\) mV vs. NHE) in a non-polar solvent [55]. The reduction in O2 by a FL in a non-polar active site is thus unlikely [134]. The reactivity of flavoenzymes towards O2 depends on the stabilization of the semiquinone in the active site because semiquinones show higher reactivity with O2 than fully reduced FLs [133]. The reactivities of flavoenzymes with O2 can be limited by a substrate that acts as a specific electron acceptor of the flavoenzyme. Some flavoenzymes, like glucose oxidase and xanthine oxidase, employ O2 as a natural acceptor, forming both H2O2 and O2•− with high efficiency [137,138]. The mechanism of O2 reduction by flavoenzymes has recently been reviewed in detail [139].
Some stromal flavoenzymes, such as FNR, monodehydroascorbate reductase (MDAR), glutathione reductase (GR) and glycolate oxidase, can efficiency reduce O$_2$ to O$_2^{•−}$ in the absence of the specific substrate [12,140]. The flavoenzymes are reduced and oxidized by their specific electron donors and acceptors with high rates. For example, MDAR is reduced by NAD(P)H with a second-order rate constant of $1.8 \times 10^8$ M$^{-1}$ s$^{-1}$ [141], and the reduced MDAR can be oxidized by monodehydroascorbate radical (AscH$^+$ and its anionic form (Asc$^{•−}$), abbreviated as MDA) with a second-order rate constant of $2.6 \times 10^8$ M$^{-1}$ s$^{-1}$ [142]. The flavoenzymes MDAR, GR, FNR and glycolate oxidase can, after reduction by Fd or NAD(P)H, efficiently reduce O$_2$ to form O$_2^{•−}$ as a primary product with a maximum rate of 300 µmol of O$_2^{•−}$ (mg Chl)$^{-1}$ h$^{-1}$. The K$_m$ value for O$_2$ in the reaction with MDAR was 100 µM, while the K$_m$ for O$_2$ reduction by thylakoid membranes was 10 µM [140]. In the work of Goetze and Carpenter [143] the addition of FNR to a thylakoid suspension increased H$_2$O$_2$ formation by 33%. The rates of O$_2$ consumption in the absence and presence of FNR were 28 and 37 µmol (mg Chl)$^{-1}$ h$^{-1}$, respectively, which imply (in a system that produces O$_2$ at PSI) O$_2^{•−}$ formation rates of 112 µmol (mg Chl)$^{-1}$ h$^{-1}$ and 148 µmol (mg Chl)$^{-1}$ h$^{-1}$, respectively [143]. However, the autoxidation rates of the MDAR, glutathione reductase and FNR that can be reduced by NAD(P)H are extremely slow [141,144], suggesting that the high rates of O$_2$ reduction by photooxidized flavoenzymes result from the formation of a stable semiquinone. Photoreduction of the flavoenzymes, added to thylakoid membranes, can occur at the F$_{A}/$F$_{B}$ (4Fe-4S clusters of PSI) [140]. The prosthetic group of MDAR can be reduced by F$_{A}/$F$_{B}$ to the semiquinone form, as the $E_m$ of F$_{A}/$F$_{A}^−$ and F$_{B}/$F$_{B}^−$ pairs are ~479 and ~539 mV, respectively [145]. The stable semiquinones of flavoenzymes can be oxidized by O$_2$ with a high rate [133]. In leaves, the photoreduction rate of O$_2$ was estimated to be 18–26 µmol O$_2$ (m$^{-2}$ of leaf area) s$^{-1}$ [146] which gives a rate of O$_2^{•−}$ production of 240–350 µmol (mg Chl)$^{-1}$ h$^{-1}$ assuming 0.6 mmol Chl (m leaf area)$^{-2}$ [147]. Thus, flavoenzymes may contribute to the high rates of O$_2$ photoreduction in chloroplasts.

In cyanobacteria [148,149], flavodiiron proteins reduce oxygen to water without ROS production. A substantial fraction of the total photosynthetic electron flow may be directed to this route [150]. Flavodiiron proteins have later been found from all oxygenic phototrophs except for angiosperms and some non-green algae (for review, see [151]).

3.1.4. Formation of H$_2$O$_2$ in the Stroma

In most cases, the reduction in O$_2$ by Fd or by flavoenzymes yields O$_2^{•−}$ as the primary product [127,140] and H$_2$O$_2$ is usually formed via the dismutation of O$_2^{•−}$ [152]. The dismutation of O$_2^{•−}$ in Reaction (5) is a major mechanism of H$_2$O$_2$ formation in the stroma. At physiological pH, the rate constant for O$_2^{•−}$ dismutation is about $10^5$ M$^{-1}$ s$^{-1}$ [68]. SOD catalyzes the disproportionation of O$_2^{•−}$ at a diffusion-controlled rate, as the second-order rate constant was estimated to be $2.2 \times 10^8$ M$^{-1}$ s$^{-1}$ for stromal conditions [152]. H$_2$O$_2$ can also be formed via a reaction of O$_2^{•−}$ with a reduced stromal compound like AscH$_2$, GSH or Fd [127,152]. The yields of these reactions are very small in the presence of SOD. The second-order rate constants of reduction of O$_2^{•−}$ with AscH$_2$ and GSH were estimated to be $3.3 \times 10^8$ M$^{-1}$ s$^{-1}$ and $10^5$ M$^{-1}$ s$^{-1}$, respectively [62,66].

In chloroplasts, H$_2$O$_2$ is efficiently scavenged by an enzyme-catalyzed reduction in H$_2$O$_2$ by AscH$_2$ to form H$_2$O and DHA (see Section 5.1). The reaction is catalyzed by both stromal and thylakoid-bound APXs [5,152]. Accumulation of H$_2$O$_2$ can lead to the generation of HO$^•$ via the Fenton reaction (Reaction (58)) if the scavenging of H$_2$O$_2$ by the antioxidant enzymes is not fast enough for the efficient removal of H$_2$O$_2$. The Fenton reaction is possible in the chloroplast because up to 80% of cellular Fe in leaf cells is found in chloroplasts [153]. The involvement of free Fe in Fenton reaction is limited, since the Fe is stored in a redox inactive form as ferritin [154,155]. However, Fe can be activated and released from ferritin via interaction of ferritin with O$_2^{•−}$ [156]. In addition to free transition metals, Fd can be involved in the production of HO$^•$ [157,158]. The second-order rate constant for the reaction of reduced Fd with H$_2$O$_2$ was found to be $5 \times 10^7$ M$^{-1}$ s$^{-1}$ [121], which is two orders of magnitude higher than the second-order rate constant of HO$^•$ production in the Fenton reaction, 84 M$^{-1}$ s$^{-1}$ [84].
3.2. Formation of ROS in Thylakoid Membranes

The PETC employs three membrane protein complexes: PSI, PSII, their respective light-harvesting complexes (LHCI and LHCl), and the cytochrome b6/f complex (Cyt b6f, a plastoquinol-plastocyanin-oxidoreductase). Electron transfer between the complexes involves two mobile electron carriers, PQ and plastocyanin (PC). The liposoluble PQ mediates electron flow from PSII to Cyt b6f complex, and the water-soluble lumenal protein PC mediates electron flow from Cyt b6f to PSI. ROS are formed in several sites of the PETC, including PSII, PSI, the PQ pool and the light-harvesting complexes (LHC).

3.2.1. Formation of \( O_2 \) in Thylakoids

The production of \( O_2 \) in plants occurs mainly by the interaction of \( O_2 \) with excited states of Chls (where \( ^1\text{Chl}^* \) and \( ^3\text{Chl} \) are the excited singlet and triplet states of Chl, respectively) via spin-conserved reactions, (Reactions (102) and (103)).

\[
\begin{align*}
^1\text{Chl}^* + O_2 & \rightarrow ^3\text{Chl} + ^1O_2 \\
^3\text{Chl} + O_2 & \rightarrow ^1\text{Chl} + ^1O_2
\end{align*}
\]

(102) \hspace{2cm} (103)

Reaction (102) is negligible because the lifetime of \( ^1\text{Chl}^* \) is very short (~10\(^{-8}\) s) [137]. The lifetime of \( ^3\text{Chl} \) is around 10\(^{-3}\) s under anaerobic conditions [16,45]. In solution, the quenching of \( ^3\text{Chl} \) by \( O_2 \) mostly occurs via \( O_2 \) generation with a second-order rate constant of 2 \( \times 10^9 \) M\(^{-1}\) s\(^{-1}\) [16]. \( ^3\text{Chl} \) is formed both in the LHCs and in the reaction centers (RC) of PSII and PSI. In the LHCs, \( ^3\text{Chl} \) is formed by intersystem crossing (ISC) from \( ^1\text{Chl}^* \) [114,159] and in the RCs by charge recombination. In PSII, the charge recombination between \( P680^+ \) (the primary donor) and \( Q_A^- \) (bound quinone) produces \( ^3P680 \) [114,160]. \( ^3P680 \) is formed through a time-dependent “virtual triplet state” of the primary radical pair \( P680^+ \) Pheophytin (Pheo)\(^- \) [161]. A triplet state of P700, the primary donor of PSI, can also be formed via charge recombination [162].

Chls are mostly bound to LHClII and CP47 and CP43 proteins of PSII and the PSA A/B proteins of PSI. According to the high concentration of Chl in chloroplasts, around 60 mM [163], a significant formation of \( ^3\text{Chl} \) via ISC in LHCs should be observed. However, there is no experimental evidence for the production of \( O_2 \) by the formation of \( ^3\text{Chl} \) in LHCs in vivo [114]. The formation of both \( ^3\text{Chl} \) and \( ^1O_2 \) has been observed in isolated LHCs. The formation of \( ^1O_2 \) was found in isolated LHClII with an electron paramagnetic resonance (EPR) measurement of 2,2,6,6-tetramethylpiperidine as a spin trap of \( ^1O_2 \) [164,165]. The appearance of long-lived \( ^3\text{Chl} \) in LHCs has been suggested to result from a small population of Chls that are substantially uncoupled from the matrix of LHC [166,167]. In a reconstructed Chl-protein complex, light-dependent \( ^1\text{O}_2 \) formation is lower by a factor of four compared to free Chl [168]. As the isolated protein does not contain pigments that would effectively quench \( ^3\text{Chl} \) or \( ^1O_2 \), it was suggested that the low \( ^1O_2 \) formation is caused by the tight packing of Chl molecules inside the hydrophobic zone of the pigment–protein complex where the interaction of \( ^3\text{Chl} \) with \( O_2 \) is limited [168]. The same situation can probably be realized in LHCs where Chls are tightly packed. Furthermore, LHCs contain carotenoids that efficiently quench \( ^3\text{Chl} \). In addition, the highly efficient transfer of excitation energy to the RC lowers the probability of ISC. Thus, \( ^1O_2 \) can only be formed in LHCs in sites where Chl is weakly bound to the protein matrix and \( ^3\text{Chl} \) cannot be efficiently quenched by carotenoids.

The main source of \( O_2 \) appears to be \( O_2 \) reacting with \( ^3P680 \). As in the case of antenna complexes, the formation of \( ^1O_2 \) in the RC also depends on two factors: the lifetime of \( ^3P680 \) and the probability that \( O_2 \) reacts with \( ^3P680 \). Assuming that the accessibility of \( O_2 \) to \( ^3P680 \) is not changed significantly in different conditions; the yield of \( ^1O_2 \) generation in the RC of PSII is mainly limited by the rate of \( ^3P680 \) formation. The formation of \( ^3P680 \) proceeds via charge separation and charge recombination. The formation of the excited singlet state \( ^1P680^* \) is followed by the formation of the radical pair
The methods of

In thylakoid membranes where both the donor and acceptor side are functional, electron transfer to QA and then to Q is prevented from the formation of a long-lived primary radical pair [172]. However, pairs [P680+Pheo−] and [P680+PheoQ−] recombine when forward electron transfer is impossible. A fraction of the recombination of the pair [P680+PheoQ−] produces [P680+Pheo−QA] [173]. Originally, [P680+Pheo−] is formed from [1P680+Pheo] in a virtual singlet state [2P680+Pheo−] that recombines to 1P680. However, the long lifetime of the state [P680+PheoQ−] destroys spin correlation, and the recombination [P680+PheoQ−] to [P680+Pheo−QA] often produces a virtual triplet state of the primary radical pair [3P680+Pheo−QA] that has such a spin configuration that its recombination to an excited state of the primary donor produces a triplet, 3P680 [174]. At 40 K, the triplet state is mainly localized on the monomeric chlorophyll ChlD1 [175], while, at 25 °C, about 30% of this state is associated with the chlorophyll P1D1 [176]. Thus, the site of O2 production is mainly localized in the D1 protein of PSII. The formation of 3P680 has a high probability because the two β-carotene molecules of the PSII RC are located far from ChlD1 and P1D1, at 19.9 Å and around 30 Å, respectively [174,177]. Such a long distance does not allow for the direct quenching of 3P680 [178].

1O2 generation by photosynthetic samples has been measured using several methods, including the spin traps 2,2,6,6-tetramethylpiperidine [179–185], 2,2,6,6-tetramethyl-4-piperidone [183,184,186], 3-[N-(β-diethylaminoethyl)-N-dansylaminomethyl]-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole [187–189], trans-1-(2′-methoxyvinyl)pyrene [190], histidine [191–193] and Singlet Oxygen Sensor Green [190,194,195]. The 1270 nm luminescence has been used to measure 1O2 generation by isolated RC complexes [171,196]. The methods of 1O2 measurement were recently reviewed [23].

The absolute rate of O2 production can be estimated by comparing the signal strength (e.g., 1270 nm luminescence intensity, fluorescence yield, the yield of 2,2,6,6-tetramethylpiperidine-1-yl) oxyl or EPR signal amplitude) with the signal obtained from a sensitizer chemical with a known 1O2 yield. The main limitation of such an estimation is that, in photosynthetic material, 1O2 may be effectively quenched before reacting with the sensor, and therefore all estimates of 1O2 yield of photosynthetic material represent a lower limit. At the PPFD (photosynthetic photon flux density) of 2000 μmol m−2s−1, histidine-dependent O2 uptake measurements showed that isolated PSII RCs (6 Chl/RC, [197]) produce 1O2 at a rate of 4000 μmol 1O2 (mg Chl)−1 h−1, with a quantum yield 0.16 [191]. The yield of 1O2 per 3P680 is very high, as the quantum yield of 3P680 formation in the same preparations was 0.3 [191,198]. 2,2,6,6-tetramethylpiperidine measurements at the same PPFD showed that isolated thylakoid membranes produced 3.73 × 10−7 1O2 molecules per Chl molecule s−1, and the quantum yield of 1O2 formation was 2.59 × 10−4 [185]. Assuming 490 and 173 Chls per PSII and PSI, respectively [197], and a PSI:PSII ratio of 1 [199], the ratio of Chl to RC of PSII is 663 for a plant thylakoid membrane. Thus, isolated RCs and thylakoids produce, at PPFD 2000 μmol m−2s−1, 21,600 and 0.89 1O2 per RC per h, respectively. The large difference probably reflects differences in both the actual 1O2 production rate and in the experimental method. In cyanobacteria illuminated at PPFD 2300 μmol m−2 s−1 in deuterium oxide, a decrease in O2 concentration in the presence of histidine showed 1O2 production of approximately 27 μmol (mg Chl)−1 h−1 [192], suggesting that 1O2 production in vivo may actually be of the same order of magnitude as the maximum rate of O2 evolution. A similar conclusion was drawn from measurements with isolated RCs [191].

Inactivation of the oxygen-evolving complex (OEC) of PSII leads to an increase in the redox potential of the QA/Q− pair, so that 3P680 is no longer formed, and therefore virtually no 1O2 can be produced through recombination reactions. However, even in this situation, some 1O2 production can be expected because inactivation of the Mn-cluster leads to the oxidation of organic molecules, presumably by P680+ or TyrZ+ (the redox active tyrosine residue in the D1 protein), and the formation of organic hydroperoxides [200]. Recently, 1O2 formation has been detected in Mn-depleted PSII.
membranes and correlated with R* formation on the donor side of PSII. It was proposed that O2 is formed via the Russell mechanism, Reactions (9), (104) and (105) [201,202].

\[
P680^{**} \text{(or TyrZ*)} + RH \rightarrow P680 \text{(TyrZ)} + R^* \quad (104)
\]

\[
R^* + O_2 \rightarrow \text{ROO*} \quad (105)
\]

Thus, the formation of O2 associated with PETC can proceed both via the interaction of O2 with Chl and decomposition of ROOOOR (Reaction (9)). The formation of O2 can occur in at least three sites of PETC: (1) LHCs; (2) RC of PSII; and (3) the donor side of the PSII (Figure 1).

**Figure 1.** Singlet oxygen (O2) generation in the antenna complex (A) and in the reaction center of PSII via charge recombination reactions (B). Proposed mechanism for the formation of O2 on the donor side of PSII via formation of organic peroxyl radicals (C). (A) In the antenna complex, the absorption of a photon by a molecule of Chl leads to the formation of a singlet excited state, Chl*, that can transform to the triplet state Chl via intersystem crossing. The formation of O2 via intersystem crossing has been demonstrated to occur in isolated LHCl [164,165]. (B) In the reaction center of PSII, [P680*Pheo0] is originally formed via electron transfer from P680* to Pheo in a virtual singlet state [P680] that combines to P680*. Charge recombination of P680*PheoQA− causes the formation of [P680*Pheo0]. The long lifetime of the state [P680*PheoQA−] destroys spin correlation, and therefore the recombination [P680*PheoQA−] to [P680] often produces a "virtual triplet state" of the primary radical pair, i.e., a radical pair with such a spin configuration that, in its recombination to an excited state of the primary donor, produces a triplet, [P680] via the Russell mechanism [172–174]. (C) On the donor side of PSII, carbon centered radicals (R*) can be formed via the oxidation of lipids and proteins by P680 if electron donation from Mn-cluster to P680 does not function. R* can react with O2 to form peroxy radical (ROO*). Two peroxy radicals react with each other to form linear tetraoxide (ROOOOR) that decomposes to O2, carbonyl (R=O) and alcohols (R–OH) via the Russell mechanism [200–202].
PSI is not considered as a site of $^1$O$_2$ production, although theoretically the formation of $^3$Chl can occur through charge recombination between P700$^+$ and its electron acceptors. In isolated PSI membrane fragments, the recombination of [P700$^+$A$_5^-$] in the presence of dithionite was found to lead to the formation of the triplet state in PSI RCs with a quantum yield of approximately 30% [203]. In PSI particles, the flash-induced absorption changes at 820 nm are attributed to the formation of $^3$P700 via conversion of the cation–anion biradical pair [P700$^+$A$_5^-$], with a yield approaching approximately 50% for 10 ns [204]. It was found that an increase in absorption at 820 nm is immediately followed by a multiphasic decay, including a major fast phase within 5–10 µs and an intermediate phase (about 10–15% of the signal) within 2 ms [204]. Interestingly, O$_2$ does not affect the decay. It can be speculated that this indicates that O$_2$ is unable to efficiently quench $^3$P700 in isolated PSI complexes and thereby produce $^1$O$_2$. It seems that, even if $^3$P700 is formed in PSI, its quenching by O$_2$ is minimized. It has also been suggested that charge recombination mainly occurs between P700$^+$ and phyloquinone A$_1^-$, which minimizes the formation of $^3$P700 triplet [205]. $^1$O$_2$ could be detected in PSI membrane fragments and PSI core complexes but not in PSI particles under the same conditions [181]. However, isolated PSI-LHCI supercomplexes of Arabidopsis produced $^1$O$_2$ at a rate of approximately one tenth of that measured in PSII-LHCI supercomplexes, and the rate of $^1$O$_2$ production by PSI-LHCI supercomplexes of the low-carotene szl1 mutant was approximately one fourth of that measured in PSII-LHCI supercomplexes [206]. There is also some evidence that the Fe-S centers of PSI produce $^1$O$_2$ [207].

3.2.2. Oxygen Reduction in PETC

The first evidence that O$_2$ can accept electrons from PETC was observed by Mehler who found that O$_2$ was consumed and H$_2$O$_2$ evolved under the illumination of broken chloroplasts [208]. Light-dependent O$_2$ consumption as an indicator of Mehler’s reaction has been reported also in vivo in algae and cyanobacteria [209,210], and in isolated intact chloroplasts with the capacity for CO$_2$ fixation [211,212]. Later studies have shown that O$_2$ reduction occurs in different sites of the PETC (including both PSII and PSI), and illumination of thylakoids triggers the appearance of several forms of reduced O$_2$, including O$_2$$^•$-, HO$_2$$^•$, H$_2$O$_2$ and HO$^*$ [213]. Because the acceptor side of PSI is the major site of O$_2$ reduction in thylakoid membranes (see reviews [213–215]), the term “Mehler’s reaction” has become synonymous with O$_2$ reduction at the acceptor side of PSI, with H$_2$O$_2$ as the final product.

3.2.3. Formation of Reduced Forms of Oxygen, O$_2$$^•$-, H$_2$O$_2$, HO$^*$, in PSII

Although $^1$O$_2$ is the main ROS produced in PSII, O$_2$$^•$-, H$_2$O$_2$ and HO$^*$ have also been found to be formed [160,174]. The contribution of PSII to the generation of O$_2$$^•$- in the intact chloroplast is generally small [216]. The O$_2$ consumption associated with O$_2$ reduction by PSII membranes capable of water-splitting is about 1 µmol O$_2$ (mg Chl)$^{-1}$ h$^{-1}$ or 4 µmol O$_2$$^•$- (mg Chl)$^{-1}$ h$^{-1}$ when O$_2$ is the only electron acceptor [128]. The rate of O$_2$ reduction is higher in disintegrated PSII complexes, which might suggest that, during stress conditions in vivo, when the structure and functional activity of PSII are disturbed, more O$_2$$^•$-, H$_2$O$_2$, or HO$^*$ is produced in PSII [217,218].

Of the redox active components of PSII, the Pheos and QA and QB may be able to reduce O$_2$. Formation of 21 µmol O$_2$$^•$- (mg Chl)$^{-1}$ h$^{-1}$, measured as an SOD-dependent cytochrome c reduction, was observed in D1/D2/cytochrome b559 (cyt b559) complexes illuminated at 200 W m$^{-2}$ in the presence of an artificial electron donor [217]. D1/D2/cyt b559 complexes lack QA and the Mn-cluster, and therefore the result suggests that Pheo$_{D1}$$^•$- (Pheo bound to the D1 protein) can be involved in the generation of O$_2$$^•$- . However, the reaction of O$_2$ with Pheo$_{D1}$$^•$- is expected to be negligible in native RCs because of the very short lifetime of Pheo$_{D1}$$^•$- (Table 2). The redox potential of Pheo$_{D2}$/Pheo$_{D2}$$^•$- (Pheo bound to the D2 protein) is 80–210 mV more negative than that of Pheo$_{D1}$/Pheo$_{D1}$$^•$- [219] and therefore, if Pheo$_{D2}$$^•$- is formed, it would have a low enough redox potential to reduce O$_2$. 

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It is difficult to tell whether QA^- can reduce O_2, as the E_m of the pair O_2/O_2**^- depends on the hydrophobicity of the environment [12,54,58–60]. If the environment of O_2 is equivalent to an aqueous solution, QA^- would have a low enough potential to reduce O_2, whereas the redox potential of O_2/O_2**^- in a hydrophobic environment is so low that QA^- would be a poor reductant, although its lifetime is long enough for chemical reactions (Table 2). However, the participation of QA^- in O_2 reduction has been suggested [160,234–236]. O_2**^- production was found to increase in the presence of an inhibitor of electron transfer at the QB site of PSII, DCMU (3-(3,4-di-chlorophenyl)-1,1-dimethyl urea), which was explained by the fact that DCMU increases the lifetime of QA^- [236].

The production of O_2**^- by both PSII with a functional Mn-cluster and by Ca^{2+} and Cl^- depletred PSII was detected using 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide as a spin trap [235]. The generation of O_2**^- at QA may occur due to the flexibility of the redox potential of QA/QA^- via forward electron transfer. Reduced QA^- via electron transfer from QA**^- to Q_B and removal of HCO_3^- by PQ that binds to an empty QA site. Removal of QA^- by PQ that binds to an empty QA site. Reduction of QA^- via charge recombination with oxidized TyrZ. Reduction of QA^- via charge recombination with S2, removing HCO_3^-.

Double-reduced QA has a very negative redox potential (Table 2) and can reduce O_2. However, QA^-2 can only be formed by chemical treatment or by strong illumination in the absence of O_2 [238]. Thus, the involvement of QA^-2 in O_2 reduction seems unlikely.

Reduced QB is less likely to reduce O_2 than QA^- because the redox potential of QB/QB^- is around -45 mV (Table 2). Although QB^- has a much longer lifetime than QA^- when electron donation from QA^- does not occur (Table 2), the quinone in the QB site is involved in proton-coupled electron transfer, and the redox potential of QB^- becomes positive when the quinone is protonated (Table 2). The possible generation of O_2**^- by quinones at QA and QB pockets is illustrated in Figure 2A.

| Redox Active Cofactors | Midpoint Redox Potential vs. Normal Hydrogen Electrode (NHE), mV | Lifetime, s | Remarks |
|------------------------|---------------------------------------------------------------|-------------|---------|
| QA^-/QA**^-            | ~-610 [220,221]                                               | (2-5) × 10^{-6} [222] | Reoxidation of QA^- via forward electron transfer to QA**^- |
|                        | -588 [219]                                                    | (4-30) × 10^{-4} [222] | Reoxidation of QA^- via recombination of [P680^’/Pheo^-] |
| Q_B/Q_B^-              | ~-80—-200 [224,225]                                           | (0.1-0.2) × 10^{-4} [224,226] | Reoxidation of QA^- via forward electron transfer to QA**^- |
|                        | (0.3-0.5) × 10^{-3} [224,226,228]                             | (2-4.6) × 10^{-3} [226,228,229] | Removal of HCO_3^- bound to acceptor side of PSI. |
|                        | (0.2-2) × 10^{-4} [222]                                      | 1-2 [226] | Redoxiation of QA^- via forward electron transfer to QA^- and protonation. |

| QA^-/Q_A^-            | ~-500 [230]                                                   | Double reduction achieved either by chemical treatment or by strong illumination in anaerobic conditions. No doubly reduced QA accumulates during aerobic light treatment. |

| Q_B/Q_B^-              | ~-45—-60 [220,231,232]                                    | (0.3-0.5) × 10^{-3} [224,226,228] | Reduction of QA^- via electron transfer from QA**^- |
|                        | >0.4 [222]                                                    | 0.4 [222] | Reoxidation of QA^- via charge recombination with oxidized TyrZ. |
|                        | 30 [222]                                                     | 30 [222] | Removal of QA^- by PQ that binds to an empty QA site. |

| Q_B/Q_B H              | 100 [220]                                                     | (0.3-0.5) × 10^{-3} [224,226,228] | Assuming the same time as for QA^- |
|                        | >0.4 [222]                                                    | 0.4 [222] | Removal of QA^- by PQ that binds to an empty QA site. |
|                        | 30 [222]                                                     | 30 [222] | Removal of QA^- by PQ that binds to an empty QA site. |

| Q_B/Q_B H              | ~-200—-464 [233]                                              | 290-373 [233] | Removal of QA^- by PQ that binds to an empty QA site. |
Plants from intact chloroplasts may best reflect the situation in vivo. HP:IP:LP was estimated as 44:31:25, with redox potentials of 375, 228 and 57 mV, respectively [243,244]. In untreated PSII membranes, the ratio modification of the donor side of PSII by removal of the Mn-cluster leads to the conversion of the HP • form: 150–260 mV; • LP form: 350–450 mV; • IP form: 200–300 mV [241]. In intact chloroplasts, the ratio of HP to LP forms was found to be 58 to 31, with respective redox potentials of 383 and 77 mV [242].

Cyt b559 has been suggested to be involved in cyclic electron transfer around PSII, where PQ Cyt b559 acts as both an electron donor and an electron acceptor [239]. This idea is supported by the finding that PQH₂ can reduce cyt b559 in both intact chloroplasts and isolated thylakoids and was inhibited by DCMU [248]. In Triton X-100-solubilized PSII particles, the ratio between the forms is variable and depends on isolation procedure. For example, modification of the donor side of PSII by removal of the Mn-cluster leads to the conversion of the HP form to the LP form [241]. In intact chloroplasts, the ratio of HP to LP forms was found to be 58 to 31, with respective redox potentials of 383 and 77 mV [242]. In untreated PSII membranes, the ratio HP:IP:LP was estimated as 44:31:25, with redox potentials of 375, 228 and 57 mV, respectively [243,244]. In isolated thylakoid membranes, 85% of cyt b559 was in the HP form [245]. The values measured from intact chloroplasts may best reflect the situation in vivo.

In untreated chloroplasts, cyt b559 is found in high potential (HP), intermediate potential (IP) and low potential (LP) forms [239]. A very low potential (VLP) form was observed in isolated RCs of PSII [240] and seems to be an isolation artefact. The ratio of HP to LP forms is variable and depends on isolation procedure. For example, modification of the donor side of PSII by removal of the Mn-cluster leads to the conversion of the HP form to the LP form [241]. In intact chloroplasts, the ratio of HP to LP forms was found to be 58 to 31, with respective redox potentials of 383 and 77 mV [242]. In untreated PSII membranes, the ratio HP:IP:LP was estimated as 44:31:25, with redox potentials of 375, 228 and 57 mV, respectively [243,244]. In isolated thylakoid membranes, 85% of cyt b559 was in the HP form [245]. The values measured from intact chloroplasts may best reflect the situation in vivo.

Cyt b559 has been suggested to be involved in cyclic electron transfer around PSII, where PQ bound in the specific binding pockets Qb and QC acts as both an electron donor and an electron acceptor [239]. This idea is supported by the finding that PQH₂ can reduce cyt b559 in both intact chloroplasts and isolated thylakoids and was inhibited by DCMU [248]. In Triton X-100-solubilized PSII particles, the ratio between the forms is variable and depends on isolation procedure. For example, modification of the donor side of PSII by removal of the Mn-cluster leads to the conversion of the HP form to the LP form [241]. In intact chloroplasts, the ratio of HP to LP forms was found to be 58 to 31, with respective redox potentials of 383 and 77 mV [242]. In untreated PSII membranes, the ratio HP:IP:LP was estimated as 44:31:25, with redox potentials of 375, 228 and 57 mV, respectively [243,244]. In isolated thylakoid membranes, 85% of cyt b559 was in the HP form [245]. The values measured from intact chloroplasts may best reflect the situation in vivo.

Figure 2. Formation of reactive oxygen species (ROS) in PSII. (A) Formation of superoxide (O₂•−) can occur with the interaction of O₂ with a semiquinone anion radicals at the QA and QB sites, when the electron flow from Qₐ to the PQ pool is limited. The low potential form of cyt b559 can reduce O₂ to O₂•− inside the thylakoid membrane [234–236]. (B) Formation of H₂O₂ and HO•. Cyt b559 can catalyze the formation of the H₂O₂ inside the thylakoid membrane by a O₂•− dismutation mechanism [72,160]. O₂•− can reduce cyt b559 (Fe³⁺) to cyt b559 (Fe²⁺). O₂•− + cyt b559 (Fe³⁺) ΔO₂ + cyt b559 (Fe²⁺). The following interaction of HO₂• with Cyt b559 (Fe²⁺) leads to the formation of a ferric–hydroperoxo intermediate of cyt b559 (Fe³⁺–OOH) which can spontaneously decompose to cyt b559 (Fe³⁺) and H₂O₂. HO• + cyt b559 (Fe²⁺) → cyt b559 (Fe³⁺–OOH) + H⁺ → cyt b559 (Fe³⁺) + H₂O₂. The formation of H₂O₂ in a cyt b559-dependent way requires the protonation of O₂•− to form HO₂•. The interaction of O₂•− with Fe²⁺ on the acceptor side of PSII can result in the formation of a ferric–peroxo intermediate [Fe³⁺–OO⁻] that can be protonated to a ferric–hydroperoxo intermediate [Fe³⁺–OOH]. O₂•− + [Fe²⁺] → [Fe³⁺–OO⁻] + H⁺ → [Fe³⁺–OOH]. [Fe³⁺–OOH] can be reduced by an electron from QA−, which causes its decomposition to ferric–oxy intermediate [Fe³⁺–O⁻] and HO•. QA− + [Fe³⁺–OOH] → QA + [Fe³⁺–O⁻] + HO•. (C) Formation of organic hydroperoxides on the donor side of PSII. Charge separation when the OEC is inactive leads to the formation of P680•+ and TyrZ• which have a long lifetime and are therefore able to interact with surrounding molecules such as Chls, carotenoids and amino acids. The interaction of P680•+ or TyrZ• with an organic molecule (RH) can proceed via a radical chain mechanism [200–202].
chloroplasts [246] and in PSII RC preparations [247]. The photoreduction of cyt b559 was found in isolated thylakoids and was inhibited by DCMU [248]. In Triton X-100-solubilized PSII particles, which mostly have the LP form of cyt b559, short-chain PQs stimulated both photoreduction and dark oxidation of cyt b559 [248].

The involvement of cyt b559 in electron transfer reactions of PSII indicates that cyt b559 is a redox active component that can potentially reduce O₂. It has been shown that fast, dark reoxidation of the PQ pool in thylakoid membranes is not caused by direct oxidation of PQH₂ by O₂, and it was suggested that the LP form of cyt b559 can transfer an electron to O₂ and thereby act as a PQH₂–O₂ oxidoreductase [248]. In isolated thylakoid membranes, O₂ has been shown to compete with prenylquinones for oxidation of the LP form of cyt b559, suggesting that LP cyt b559 can form O₂•− [249]. Exogenously added short-chain quinones significantly enhance O₂•− production by PSII [245]. This finding was interpreted to indicate that these quinones reduce LP cyt b559, which then undergoes spontaneous autoxidation, resulting in O₂•− formation.

However, the reduction in O₂ by LP cyt b559 is thermodynamically unfavorable, taking into account that the redox potential of the LP form is usually within 20–110 mV in untreated membranes, although sometimes an LP form with a negative potential is observed [239]. To explain O₂ reduction via an apparently thermodynamically unlikely reaction, it has been suggested that the Eₐ’ of O₂/O₂•− should be calculated by the Nernst equation, due to differences in concentrations of O₂ and O₂•− [160]. According to the Nernst equation, and assuming concentrations of 250 μM and 500 nM for O₂ and O₂•−, respectively, the redox potential becomes close to 0 mV. Thus, the electron transfer from LP cyt b559 to O₂ becomes feasible. However, it seems that the comparison of standard middle point potentials is more correct, as cyt b559 is bound within a protein matrix, and a considerable difference in the local concentrations of O₂ and O₂•− is questionable.

A possible alternative solution is that cyt b559 mediates the formation of semiquinones at Qᵥ sites. Experimental evidence of the reduction in O₂ by a loosely bound plastosemiquinone anion radical (PQ•−) at the Qᵥ site was provided by Yadav et al. [250]. The authors showed that PQ•− can be formed by a one electron reduction in PQ at the Qₛ site and one electron oxidation of PQH₂ by cyt b559 at the Qᵥ site. Because a PQ molecule has been crystallographically detected in the Qᵥ site [251], PQ might be tightly bound within the Qᵥ pocket and act as an electron carrier from cyt b559 to P680. The environment of the Qᵥ pocket is probably flexible and lipophilic and can facilitate a PQ/PQH₂ exchange. In this case, the PQ pool can serve as an electron donor for cyt b559. It might be proposed that the formation of O₂•− in a cyt b559-dependent pathway couples cyt b559 and quinones and depends on the redox state of the PQ pool (Figure 2A). The rate constant of cyt b559-mediated reduction of O₂ was estimated to be about 10⁻⁶ M⁻¹ s⁻¹ inside the thylakoid membrane, assuming that the reaction proceeds as a second-order chemical reaction [252].

The formation of O₂•− in PSII causes the formation of H₂O₂ via spontaneous dismutation (Reaction (5)) [234] or via a cyt b559-dependent catalytic reaction [253]. In isolated RCs of PSII, cyt b559 was found to exhibit SOD activity [217]. As proposed by Pospišil [160], the catalytic formation of H₂O₂ by cyt b559 proceeds as a two-step reduction–oxidation reaction involving two molecules of O₂•−. The first step is reduction of cyt b559 (Fe³⁺) to cyt b559 (Fe²⁺), Reaction (106). The second step is the oxidation of cyt b559 (Fe³⁺) by HO₂•, the protonated form of O₂•−, with formation of cyt b559 (Fe³⁺) and H₂O₂, Reaction (107).

\[
\begin{align*}
O₂•− + \text{cyt b559 (Fe³⁺)} & \rightarrow O₂ + \text{cyt b559 (Fe²⁺)} \\
\text{HO₂•} + \text{cyt b559 (Fe²⁺)} & \rightarrow H₂O₂ + \text{cyt b559 (Fe³⁺)}
\end{align*}
\]

(106) (107)

According to this mechanism, the catalytic disproportionation of O₂•− should be pH-dependent because the protonated form of O₂•− is needed.

In addition to its formation by the dismutation of O₂•−, H₂O₂ might also appear during incomplete oxidation of H₂O by the Mn-cluster. It has been suggested that incomplete oxidation of H₂O can occur
during the two-electron oxidation of water by the Mn-cluster at the transition from the S2 state to the S0 state [234, 254]. However, the two-electron oxidation of water during the transition of the Mn-cluster from S3 to S1 does not result in the formation of H2O2 [255].

In PSII, the Fenton mechanism involving a metal (M) cation, Reaction (58), can also function, leading to the formation of HO•.

In PSII, HO• can be formed both in the dark and in the light. HO• formation was shown when PSII membrane particles were heated in the dark [256]. The authors suggested that this process is associated with heat-induced changes of the PSII donor side and proceeds via the Fenton mechanism. The formation of HO• was suppressed by CAT and metal chelators, indicating that the appearance of HO• is related to the decomposition of H2O2. However, a high concentration of CAT, around 5000 U/mL, was required to suppress the appearance of HO•. Exogenous calcium and chloride prevented the appearance of HO•. Furthermore, no HO•-related EPR signal was observed after removal of the Mn-cluster by Tris-treatment of PSII membranes [257]. These data confirm that the Mn-cluster is likely involved in HO• formation in PSII under heat stress in the dark.

The light-dependent formation of HO• occurs in untreated PSII membranes [235, 258–260]. Experimental results suggest that HO• can be produced in the light by two pathways: firstly, by the well-known Fenton mechanism and secondly, by the reduction of a peroxide bound to the non-heme iron on the acceptor side of PSII [72]. The formation of HO• at the non-heme iron is initiated by the binding of O2•− and formation of an O2•−-iron complex that can be protonated to a ferric-hydroperoxo complex, Reactions (49) and (50). The ferric-hydroperoxo complex can be decomposed via reaction by QA− with the formation of HO• and a ferric-oxo ((Fe3+-O•−) complex, Reaction (108).

\[
L-(Fe^{3+})-OOH + QA^- \rightarrow L-(Fe^{3+})-O^- + HO^+ + QA^- \quad (108)
\]

where L is a ligand. Reaction (108) can be considered as a Fenton reaction proceeding with a bound hydroperoxide. The possible sites of formation in PSII are shown in Figure 2B.

The formation of bound hydroperoxides has been found to occur on the donor side of the PSII (Figure 2C). The mechanism is associated with the formation of a long-lived species, having a high positive redox potential in PSII. In PSII membranes holding an intact Mn-cluster, the O2 consumption rate is very low, around 1 µmol O2 (mg of Chl)−1 h−1 [128], but the rate becomes 6-fold higher in alkaline-treated and Mn-depleted PSII membranes [261]. O2 consumption was found to be associated, at least partially, with the generation of a component with positive charge(s) on the donor side of PSII, as the electron donors diphenylcarbazide and ferrocyanide suppressed the rate of O2 consumption caused by disruption of the donor side of PSII. A further study revealed that the removal of Mn from the OEC of PSII leads to O2 photoconsumption with a maximum at the first flash, with a yield comparable to the yield of O2 evolution on the third flash measured in the PSII samples before Mn removal [262]. Inactivation of the OEC can lead to the formation of both P680•+ and TyrZ•. In the absence of electron donation from the OEC, both will have a long lifetime, and will therefore be able to interact with surrounding molecules such as Chls, carotenoids and amino acids. Based on these results, it has been proposed that the formation of peroxides on the donor side of PSII proceeds via a radical chain mechanism, starting with P680•+ (or TyrZ•), Reactions (104), (105) and (109).

\[
\text{ROO}^* + \text{RH} \rightarrow \text{ROOH} + \text{R}^* \quad (109)
\]

The evidence of ROOH production on the donor side of PSII was obtained using the specific fluorescence probe SPY-HP [200]. In this work, highly lipophilic peroxides (LOOH) and relatively hydrophilic ones (ROOH), were distinguished by the rate of reaction with Spy-HP. The formation rates of both LOOH and ROOH were estimated to be 0.022 µmol LOOH (µmol RC)−1 s−1 and 1.11 µmol ROOH (µmol RC)−1 s−1, respectively [200]. The formation of carbon centred radicals, in turn, was found in PSII membranes with EPR spin-trapping technique when PSII membranes were treated by high light and heating. It has recently been found that exposure of Mn-depleted PSII membranes to
high light results in the formation of protein radicals located mainly in the D1, D2, CP43 and CP47 proteins [202]. The formation of protein radicals is suppressed by diphenylcarbazide, indicating that protein radicals were formed by the oxidation of proteins by P680⁺ or TyrZ⁺. The formation of protein radicals was correlated with the formation of hydroperoxides measured with the SPY-HP probe. The formation of R⁺ can initiate chain propagation reactions, and thereby lead to accumulation of ROOH (Reactions (105) and (109)).

3.2.4. Formation of Reduced Forms of Oxygen, O₂⁺⁻, H₂O₂, HO•, in PSI

The acceptor side of PSI is believed to be the predominant site of O₂ reduction in thylakoid membranes, as O₂ reduction depends on the PSI activity (see reviews [12,213–215]). It has been shown that both the photoreduction of cytochrome c and photooxidation of epinephrine, which have been used as traps for O₂⁺⁻, were inhibited by SOD. This indicates that the reduction in O₂ proceeds via univalent reduction, and O₂⁺⁻ was identified as the primary product in illuminated thylakoids [124,263]. The predominant role of PSI in O₂ reduction was shown in experiments with specific inhibitors that block PETC at different sites, and using a PSI-deficient mutant. The photoproduction of O₂⁺⁻ in thylakoids is inhibited by DCMU and can be restored by the addition of Asch₂ and N₅N₆N₇,N₈-tetramethyl-p-phenylenediamine, to provide electron donation to PC and P700, respectively [124,263,264]. This indicates that the contribution of PSII to the photoproduction of O₂ in thylakoids is small. A slight influence of O₂ on the steady-state level of Chl fluorescence in a PSI-deficient mutant of Oenothera sp. was attributed to insignificant leakage of electrons from PETC to O₂, due to the suppression of Mehler’s reaction [265]. On the other hand, a significant rate of O₂ reduction by thylakoids was observed in the presence of dibromothymoquinone (DBMIB) and dinitrophenylether of 2-iodo-4-nitrothymol (DNP-INT), inhibitors of PQH₂ oxidation by Cyt b₆f, [128,266]. It was found that the contribution of other sites of PETC, besides PSI, to O₂ reduction increased with an increase in light intensity, and at high intensities achieved 60% of total O₂ reduction in PETC. These data suggest that PSI is not the only site of O₂ reduction in thylakoid membranes, but other sites of PETC can contribute to O₂ reduction [128]. Thus, experiments with isolated PSI membranes can provide more correct measurements of activity of PSI in the photoproduction of O₂⁺⁻.

The electron transport chain within PSI (Figure 3) consists of two quasissymmetrical branches (A and B) containing six Chl, two phylloquinones (A₁), and three 4Fe-4S clusters (Fₓ, Fₐ, and Fₐ). Two Chl a molecules have been assigned to the spectroscopically characterized primary acceptor A₀. Another pair of Chl a molecules is located between P700 and A₀ and assigned as accessory Chls that may participate in excitation and/or electron transfer (for more details, see review [267]).

![Figure 3](https://example.com/fig3.png)

**Figure 3.** (A) Forward electron transfer chain, lifetimes and midpoint redox potentials of the cofactors of PSI; (B) charge recombination reactions and recombination lifetimes of the cofactors of PSI; the values were taken from [145,268–271]; (C) possible means of ROS formation in PSI [152,266,272–275]. PC is plastocyanin; P700 is a dimer of Chl a molecules, the primary electron donor; A₀A and A₀B are Chl a molecules located in branches A and B, respectively, both act as primary electron acceptors; A₁A and A₁B are phylloquinone molecules located in branch A and B, respectively, both acting as electron acceptors; Fₓ, a 4Fe-4S cluster, a secondary electron acceptor; Fₐ and Fₐ, 4Fe-4S clusters, terminal electron acceptors.
The mechanism of O$_2$ reduction in PSI is still under debate. It was suggested that O$_2^{•−}$ production within the thylakoid membranes most likely occurs via autoxidation of the membrane-bound primary electron acceptors in PSI, possibly 4Fe-4S clusters (F$_X$, F$_{A'}$, and F$_B$) [152]. The $E_m$ of F$_X$/F$_{A'}^−$ and F$_B$/F$_B^−$ and F$_X$/F$_X^−$ vs. NHE were estimated to be −479, −539, and −650 mV, respectively (Figure 3) [145]. The reduction in O$_2$ by F$_X$ is thermodynamically favorable but kinetically less likely than a reduction in O$_2$ by F$_{A'}^−$ or F$_B^−$, as the lifetime of F$_X^−$ is less than 50 ns (Figure 3). When F$_{A'}$ and F$_B$ clusters are reduced, the lifetime of F$_X$ is limited by charge recombination [P700$^+$F$_X^−$] and estimated to be ~250 µs [276]. Electron transfer from F$_B^−$ to Fd occurs within 1 µs, and therefore the oxidation of F$_A^−$ and F$_B^−$ by O$_2$ in an aqueous region is not kinetically favorable in the presence of oxidized Fd (Figure 3). The lifetimes of F$_A^−$ and F$_B^−$ become much longer if Fd is mostly reduced or its diffusion to the F$_A$ and F$_B$ sites is limited. The charge recombination of [P700$^+$F$_{A'}^−$] and [P700$^+$F$_B^−$] has a lifetime of about 50 ms when no extrinsic electron acceptors and donors are present [277]. The rate of O$_2^{•−}$ production by PSI in both isolated thylakoids and isolated PSI complexes ranges from 15 to 30 µmol O$_2^{•−}$ (mg Chl)$^{−1}$ h$^{−1}$, corresponding to 2.5–4.5 O$_2^{•−}$ per P700 s$^{−1}$ if the ratio of P700 to total amount of Chl is 1 to 600 for isolated thylakoid membranes [124,264]. The rate of O$_2^{•−}$ production is at least one order of magnitude higher in PSI subchloroplast fragments in the presence of the surfactant Triton X-100 than in its absence [278]. The $K_m$ value for O$_2$ in photoreduction by PSI was estimated to be 2–3 µM in both thylakoid membranes and PSI subchloroplast fragments and the second order rate constant for O$_2$ reduction by the electron acceptors of PSI was calculated to be 1.5 × 10$^{7}$ M$^{−1}$ s$^{−1}$ [278]. In another work, the $K_m$ value for O$_2$ was estimated to equal to ~8 and ~3 µM for thylakoids, in the absence and in the presence of Triton X-100, respectively [279].

Experiments with O$_2^{•−}$-dependent protein iodination showed that O$_2^{•−}$ can also be produced in the aprotic interior of the thylakoid membrane close to the RC of PSI [272]. Thus, not only F$_A$ and F$_B$, but also F$_X$ and A$_J$, might be involved in O$_2^{•−}$ production within the thylakoid membrane. It has been suggested that O$_2^{•−}$ mediates cyclic electron transfer by donating electrons to Cyt b6f or to P700$,^+$, and this cycle would explain why the observed rate of O$_2^{•−}$ production is low in intact PSI [12]. The increase in O$_2^{•−}$ production in PSI subchloroplast fragments in the presence of Triton X-100 could result from the prevention of the putative O$_2^{•−}$ mediated cyclic electron flow around PSI, due to the disintegration of the supermolecular structure of PSI by Triton X-100 [12]. The increase in O$_2^{•−}$ production in the presence of ammonium ions, and amines is considered as evidence of an O$_2^{•−}$-mediated cyclic electron flow in PSI [272], as these substances supply protons to the membranes and accelerate the dismutation of O$_2^{•−}$. The dismutation of O$_2^{•−}$ prevents the O$_2^{•−}$-mediated cyclic electron flow, thereby increasing the detectable production of O$_2^{•−}$. Thus, dismutation of O$_2^{•−}$ in thylakoid membranes and cyclic electron flow in isolated PSI complexes would explain why the rate of O$_2^{•−}$ production is similar in these two preparations.

It has recently been suggested that the reduction in O$_2^{•−}$ by F$_A$, F$_B$ and F$_X$ occurs in a lipophilic region [273]. As the dielectric constant in the immediate environment of the F$_A$ and F$_B$ centers is 5.4 [280], the redox potential of O$_2$/O$_2^{•−}$ in aprotic medium (−550 and −600 mV vs. NHE [54] in DMF) should be used in comparisons of the redox potentials of PSI cofactors and O$_2$/O$_2^{•−}$. Thus, the difference in the redox potentials of O$_2$/O$_2^{•−}$ and PSI redox cofactors F$_A$/F$_{A'}^−$ and F$_B$/F$_B^−$ would make the reduction of O$_2$ by F$_A$ and F$_B$ thermodynamically less favorable. If we assume that O$_2^{•−}$ is produced via O$_2$ reduction by F$_A$ and F$_B$ in a lipophilic environment, then the differences in redox potentials would easily explain why the rate of O$_2^{•−}$ production is low in PSI subchloroplast fragments. In this case, the effect of Triton X-100 would be to make the immediate area of F$_A$ and F$_B$ less lipophilic, which would shift the redox potential of O$_2$/O$_2^{•−}$ toward positive values.

According to the $E_m$, F$_X$ and A$_J$ would be favorable reductants of O$_2$, even in an aprotic environment, as the $E_m$ values of the pairs A$_J$/$A_1^+$ located on the A- and B-branches of PSI electron transfer chain are −0.7 and −0.81 mV, respectively (Figure 3). Indeed, phylloquinone A$_1$ stimulated the flash-induced photoconsumption of O$_2$ when added to thylakoid membranes from which A$_1$ had been partially removed [266]. It was suggested that A$_1$ could be the main reductant in O$_2^{•−}$ production in
PSI. However, results regarding the importance of the phylloquinone in O2•− production vary. Firstly, the stimulation of O2 photoconsumption by addition of A1 was observed only on the first flash [266]. The appearance of O2•− on the outside and inside of thylakoid membranes was tested with hydrophilic and lipophilic cyclic hydroxylamines that react with O2•−, forming nitroxide radicals with a specific EPR spectrum [274]. In this work, a significant effect of SOD on the formation of both hydrophilic and lipophilic nitroxide radicals suggested that 90% of O2•− is formed at the membrane surface or outside of the membrane. On the other hand, evidence of the participation of A1 in O2•− formation was obtained with PSI complexes isolated from menB mutant, a phylloquinone-less knockout strain of the gene encoding 1,4-hydroxynaphthoyl-CoA-synthase of the cyanobacterium Synechocystis sp. PCC 6803. The mutant contains PQ at the phylloquinone-binding site A1 [275]. In the mutant, the redox potential of PQ bound to the A1 site was −553−693 mV, close to the redox potential of FX/FX− and about 100 mV more positive than that of A1/A1− [281]. O2 photoconsumption in isolated PSI complexes of the mutant was found to be slower than in the wild type [275]. The low rate of O2 photoconsumption in the mutant was explained by the difference in the redox potentials of PQ and A1, and the results suggest that A1 is the main site of O2 reduction in PSI. N,N,N′,N′-Tetramethyl-p-phenylenediamine and AscH2 were used as electron donors.

A1−, located in the B-branch of PSI, decays within 20 ns by electron transfer to FX, whereas electron transfer from the A-branch A1− takes 170 ns (Figure 3). A1− can accumulate in high light when electron transfer from A1− to FX is limited. In this case, the electron flow from FA and F2 to the MDA radical can prevent the accumulation of A1−, which minimizes the interaction of O2 with A1−. MDA is formed mainly by a reaction between APX and AscH2 (Reactions (62)–(64)). MDA can also be formed by a reaction between AscH2 and O2•−, and MDA is an effective electron acceptor of PSI, effectively competing with methyl viologen [282]. The reduction in MDA by PSI occurs via reduced Fd [283]. In summary, a number of AscH2-related reactions can influence the photoconsumption of O2 by PSI (Reactions (34), (62)–(64) and (110)–(113)), and the large number of reactions and reactants makes it difficult to estimate the importance of AscH2/MDA/DHA in O2 reduction in PSI.

\[
\text{PSI}^{\text{red}} + O_2 \rightarrow \text{PSI}^{\text{ox}} + O_2^{•−} \quad (110)
\]

\[
O_2^{•−} + \text{AscH}_2 \rightarrow H_2O_2 + \text{MDA} \quad (111)
\]

\[
\text{PSI}^{\text{red}} + \text{MDA} + H^+ \rightarrow \text{PSI}^{\text{ox}} + \text{AscH}^− \quad (112)
\]

\[
\text{MDA} + \text{MDA} + 2H^+ \rightarrow \text{AscH}_2 + \text{DHA} \quad (113)
\]

From data presented by Kozuleva et al. [275], the rate of O2•− production by PSI can be estimated to be 2.5 O2•− per P700 s−1 according to the O2 consumption rate (250 μmol O2 (mg Chl)−1 h−1) assuming 40 molecules of Chl per P700 in isolated PSI complexes [278]. If the production of O2•− by PSI proceeds as an elementary second-order reaction, then the second-order rate constant is about 10^4 M−1 s−1 for a saturated concentration of O2 in aqueous solution. 4Fe-4S clusters of PSI can have a low efficiency toward O2 reduction, and the second-order rate constant of the reaction of O2 with Fe-S proteins like Fd is about 10^3 M−1 s−1 [121]. However, the rate of O2 reduction by PSI was saturated to above 20 μM of O2, with the second-order rate constant 1.5 × 10^7 M−1 s−1 at a high light intensity [278]. The reaction of O2 with semiquinones having low redox potential proceeds with rate constants in the range of 10^8–10^9 M−1 s−1 [61]. These data may suggest that cooperation between 4Fe-4S clusters and phylloquinones A1 can provide flexibility for the O2•− formation inside and outside of the thylakoid membrane. In high light, O2•− formation by A1 becomes more important, which leads to the accumulation of O2•− within the membrane.

In the aqueous phase, the dismutation of O2•− is catalyzed by SOD (Reaction (5)). Intramembranous formation of O2•−, in turn, can lead to the formation of H2O2 within the thylakoid membrane due to the reaction of O2•− with PQH2, Reaction (114) [128,264,266].
whereas the formation of H\textsubscript{2}O\textsubscript{2} was suggested that HO\textsuperscript{+} is predominantly produced in PSI via the reduction in H\textsubscript{2}O\textsubscript{2} by protein-bound iron in PSI, as the metal chelator Desferal did not suppress HO\textsuperscript{+} production [157]. The formation of HO\textsuperscript{+} via the reaction of O\textsubscript{2}•− with the terminal acceptors F\textsubscript{X}, F\textsubscript{A}, and F\textsubscript{B} of PSI was recently suggested [285]. However, this route of HO\textsuperscript{+} formation requires the dismutation of O\textsubscript{2}•− to form H\textsubscript{2}O\textsubscript{2} as an intermediate. In the presence of PQH\textsubscript{2}, the production of O\textsubscript{2}•− would lead to the accumulation of H\textsubscript{2}O\textsubscript{2} within the thylakoid membrane. The redox potential of H\textsubscript{2}O\textsubscript{2}/(HO\textsuperscript{+}, −OH) is 400–600 mV in organic solvents [286], and therefore the presence of H\textsubscript{2}O\textsubscript{2} in the vicinity of the A\textsubscript{1} site, with a much more negative redox potential (Figure 3), would lead to the formation of HO\textsuperscript{+} in a Fenton-type reaction of H\textsubscript{2}O\textsubscript{2} with phyloquinone A\textsubscript{1}•− (Reaction (115)) [252].

A\textsubscript{1}•− + H\textsubscript{2}O\textsubscript{2} → A\textsubscript{1} + HO\textsuperscript{+} + −OH

(115)

3.2.5. Formation of Reduced Forms of Oxygen, O\textsubscript{2}•−, H\textsubscript{2}O\textsubscript{2}, HO\textsuperscript{+}, in the PQ Pool and by Cyt b6f

PQ is a prenyl lipid consisting of 2,3-dimethyl-1,4-benzoquinone and a side chain of nine isoprenyl units attached to Position 5. The total amount of PQ in leaves is in the range 25–40 molecules per P700 [248,287–289]. PQ has been found in thylakoid membranes, the envelope of the chloroplast and plastoglobules. [290–294]. The ratio of PQ in the envelope and PQ in the thylakoid membrane was found to be 2:5 [293]. The PQ involved in electron transfer in the thylakoid membrane is called the photoactive PQ and its amount is in the range 6–15 PQ per P700, assuming that the ratio of P700 and Chl is 1/600 [248,288,295–298]. PQ can be present in three forms: PQ, PQ•−, and PQH\textsubscript{2}. Both reduced forms can exist in protonated and deprotonated forms: PQH\textsuperscript{+} or PQ\textsuperscript{•−}, and PQH\textsubscript{2}, PQH\textsuperscript{−} or PQ\textsuperscript{2−}. The pK\textsubscript{1} and pK\textsubscript{2} values of PQH\textsubscript{2} in aqueous solutions are 10.8 and 12.9; the pKa value of PQH\textsuperscript{+} is 5.9 [299]. The above data were measured for plastocyanin-1, which has only one prenyl group attached to Position 5 of 2,3-dimethyl-1,4-benzoquinone.

Significant PSI-independent O\textsubscript{2} reduction was observed in a thylakoid suspension in the presence of the DNP-INT, that prevents the oxidation of PQH\textsubscript{2} by Cyt b6f [128,236,266]. In the work of Kruk et al. [266], significant O\textsubscript{2} reduction was also demonstrated in the presence of DBMIB, another inhibitor of oxidation of PQH\textsubscript{2} by the Cyt b6f. DBMIB was found to strongly inhibit O\textsubscript{2}•− production, whereas the formation of H\textsubscript{2}O\textsubscript{2} was only partially inhibited. Furthermore, the rate of H\textsubscript{2}O\textsubscript{2} production increased with the concentration of DBMIB [300]. On other hand, the removal, by a repeated freeze-thaw procedure, of PC, suppressed O\textsubscript{2} reduction by thylakoid membranes. In addition, the PC-inhibitor HgCl\textsubscript{2} significantly suppressed O\textsubscript{2} reduction [266]. These data may suggest that the suppression of PSI-independent O\textsubscript{2} reduction requires a strong inhibition procedure that may cause unspecific damage to the photosynthetic apparatus. In the work of Cleland and Grace [236], the production of O\textsubscript{2}•− in the presence of DNP-INT was attributed to O\textsubscript{2} reduction by QA−. However, Khorobrykh and Ivanov [128] showed that PSI-independent O\textsubscript{2} consumption in thylakoids was suppressed by DCMU, and O\textsubscript{2} consumption by isolated PSII membranes was low. Thus, PSI-independent O\textsubscript{2} consumption in thylakoid membranes in the presence of DNP-INT was interpreted as O\textsubscript{2} reduction occurring in the PQ pool. The amount of detectable O\textsubscript{2}•−, measured using cytochrome c as a trap of O\textsubscript{2}•−, was found to be about 25% of the amount of O\textsubscript{2}•− estimated from the O\textsubscript{2} consumption rate [128]. This indicates that O\textsubscript{2}
reduction occurs mainly inside the thylakoid membrane, where O$_2$•− can be consumed in concomitant reactions. It has been proposed that O$_2$ reduction in the PQ pool develops as a two-stage autocatalytic process that starts by the production of PQH$^+$ via dismutation (Reaction (116)) and is followed by the deprotonation of PQH$^+$ and subsequent oxidation of PQ$^{•−}$ by O$_2$ with the formation of O$_2$•− and PQ. Furthermore, PQH$_2$ can be oxidized by O$_2$•− with the formation of PQ•− that would again react with O$_2$ to produce O$_2$•− and PQ (Figure 4) [128].

Thylakoid membranes have also been shown to accumulate H$_2$O$_2$ in the presence of cytochrome c that reacts with O$_2$•− and prevents the formation of H$_2$O$_2$ via superoxide dismutation (Reaction (31)) [284]. These data suggest that a considerable amount of H$_2$O$_2$ is generated inside the thylakoid membrane in the reaction of O$_2$•− with PQH$_2$ ([284], Figure 4), as earlier suggested by Khorobrykh and Ivanov [128]. These results contradict with the results of Asada et al. [124], where cytochrome c completely inhibited H$_2$O$_2$ formation by thylakoids. However, in a later work of Takahashi and Asada [272], the formation of H$_2$O$_2$ in the presence of cytochrome c was shown. It is possible that different light intensities caused the contradiction, as H$_2$O$_2$ formation appears to increase with light intensity [284].

The mechanism and efficiency of O$_2$ reduction in the PQ pool are under debate. Autoxidation of PQH$_2$ is one possible mechanism (Figure 4) but is it biologically significant? According to the redox potential, the reduction in O$_2$ by both PQ$^{•−}$ and PQ$^{2−}$ is thermodynamically favorable in aqueous solution since the redox potentials of PQ/PQ$^{•−}$ and PQ$^{•−}$/PQ$^{2−}$ are −165 and −274 mV, respectively [299]. The deprotonation of PQH$_2$ or PQH$^+$ is essential for O$_2$ reduction. Since PQ$^{2−}$ is mostly protonated under physiological pH, PQ$^{•−}$ was considered the main form of reduced PQ that could be involved in O$_2$ reduction in thylakoids. The reactions of semiquinones with O$_2$ with formation of O$_2$•− are equilibrium reactions where the quinone can be reduced by O$_2$•− (Reaction (31)).

The equilibrium constant for the reaction of O$_2$ with PQ$^{•−}$, as determined by the equation (RT/F)lnK = E(O$_2$/O$_2$•−) − E(Q/Q$^{•−}$), where R is the gas constant, T is temperature and K is the equilibrium constant, and F is the Faraday constant, is 1.56 if the redox potentials of PQ/PQ$^{•−}$

![Figure 4. Autocatalytic oxidation of reduced plastoquinone (PQH$_2$) by O$_2$ in the thylakoid membrane.](image-url)
and $O_2/O_2^{**}$ are $-165$ and $-160$ mV, respectively [61]. The forward and reverse second-order rate constants for the formation of $O_2^{**}$ by PQ$^{**}$ (Reaction (31)) are $k_{forward}$ ~ $10^8$ M$^{-1}$ s$^{-1}$ and $k_{reverse}$ ~ $7 \times 10^7$ M$^{-1}$ s$^{-1}$ [61]. The equilibrium constant for Reaction (116) was estimated to be $10^{-9.2}$ [301], which shows that the formation of PQH$^*$ via Reaction (116) is negligible. Thus, the apparent rate of $O_2^{**}$ production in the PQ pool is determined by the rate of PQ$^{**}$ appearance, rate of $O_2^{**}$ production via reaction of $O_2$ with PQ$^{**}$ (Reaction (31)) and the rate of $O_2^{**}$ removal from the equilibrium Reaction (31). In solvents with pure of PQH$_2$ and PQ, the apparent rate of $O_2^{**}$ production obviously results from the following reactions:

\[
PQH_2 + PQ \Delta 2PQH^* \tag{116}
\]

\[
PQ^{**} + H^+ \rightarrow PQH^* \tag{117}
\]

\[
Q^{**} + O_2 \Delta Q + O_2^{**} \quad \text{Reaction (31)}
\]

\[
PQH_2 + O_2^{**} \rightarrow PQ^{**} + H_2O_2 \quad \text{Reaction (114)}
\]

\[
O_2^{**} + O_2^{**} + 2H^+ \rightarrow H_2O_2 + (O_2 \text{ or } O_1O_2) \quad \text{Reaction (5)}
\]

In thylakoid membranes, PQ is reduced by PSI in PQH$_2$ in the light. This lowers the concentration of PQ, thereby preventing reaction (31) and leading to an increase in PQH$_2$ oxidation by $O_2^{**}$.

In organic solvents, the redox potentials of both PQ/PQ$^{**}$ and $O_2/O_2^{**}$ become more negative. The redox potentials of PQ/PQ$^{**}$ and $O_2/O_2^{**}$ were estimated to be $-400$ [302,303] and $-600$ mV vs. NHE in DMF [54] and around $-640$ mV in acetonitrile [59,60], respectively. According to the redox potentials, the equilibrium constant is $10^{-7.8}$, and therefore a reaction of PQ$^{**}$ with $O_2$ is thermodynamically unfavorable in an aprotic medium. Thus, efficient $O_2^{**}$ production via Reaction (31) can be observed only in an aqueous solution or at the membrane–water interface, or in a protein pocket where the redox potentials of $O_2/O_2^{**}$ and PQ/PQ$^{**}$ can be equal. The second-order rate constants for the autoxidation of PQH$_2$ in different solvents, estimated from the initial rates, were found to range from $10^{-2}$ to $10^{-3}$ M$^{-1}$ s$^{-1}$ for both aqueous and aprotic solvents [248,252]. However, fast PQH$_2$ oxidation in organic solvent was observed after the addition of KOH [252]. This reaction likely results from the formation of PQ$^{**}$ with a very negative redox potential, $-1.1$ V for PQ/PQ$^{**}$ [301]. The rate constant of PQH$_2$ oxidation associated with $O_2$ reduction by the PQ pool in thylakoids was estimated to be $10^3$ M$^{-1}$ s$^{-1}$ if the reaction occurs inside the thylakoid membrane [128], and a later work calculated this rate constant to be $1.21 \times 10^{-3}$ M s$^{-1}$ while the rate of PQH$_2$ autoxidation was $10^{-8}$ M s$^{-1}$ [252]. These rates were calculated assuming that PQH$_2$ oxidation by $O_2$ is a second-order chemical reaction and the oxidation of PQH$_2$ occurs in the volume of thylakoid membrane. The steady-state concentration of PQ$^{**}$ inside the thylakoid membrane produced via reaction (116) can be estimated to be about $10^{-8}$ M. The following values were used in the calculations: amount of photoactive PQ, $14 \times 10^{-3}$ mol PQ (mol Chl)$^{-1}$ [248]; volume of thylakoid membrane, $4.6 \times 10^{-6}$ L (mg Chl)$^{-1}$ [304]; molar mass of Chl, 894 g mol$^{-1}$ [304]; the equilibrium constant for reaction (116) was taken as $10^{-10}$; and the ratio of PQ and PQH$_2$ was taken as 1/9. If $O_2^{**}$ is very rapidly removed and therefore Reaction (31) can be considered an irreversible reaction with a second order rate constant of about $10^8$ M$^{-1}$ s$^{-1}$, then the rate of PQH$_2$ oxidation by $O_2$ inside the thylakoid membrane can be estimated to be $2.4 \times 10^{-3}$ M s$^{-1}$. This estimated rate is close to the rate of PQH$_2$ oxidation by $O_2$ inside the thylakoid membrane, calculated from the $O_2$ consumption rate [252]. Thus, $O_2$ reduction by the PQ pool via an autoxidation mechanism (Figure 4) occurs when $O_2^{**}$ is efficiently consumed and the second-order rate constant is about $10^8$ M$^{-1}$ s$^{-1}$. The consumption of $O_2^{**}$ can occur via the reaction of $O_2^{**}$ with PQH$_2$, Reaction (114). The second-order rate constant for the reaction of $O_2^{**}$ with PQH$_2$ has recently been estimated to be $4 \times 10^6$ M$^{-1}$ s$^{-1}$ [252]. The second-order rate constant for the oxidation of PQH$_2$ by $O_2$ in illuminated thylakoids is within $10^2$–$10^3$ M$^{-1}$ s$^{-1}$. Thus, the autoxidation of PQH$_2$ by $O_2$ can explain $O_2$ consumption in the light in the presence of an inhibitor of Cyt b6f only with some assumptions. In addition, the rate of PQH$_2$ oxidation in thylakoids in the light is over 20 times as fast as the rate of oxidation of PQH$_2$ in the dark.
after photoreduction [248], suggesting that light-dependent reaction(s) dominate in the O₂-dependent oxidation of the PQ pool. The PQ pool has also been found to scavenge \(^1\)O\(_2\) in thylakoid membranes [305–307], with the second-order rate constant of the reaction of \(^1\)O\(_2\) with PQH\(_2\), 0.97 × 10\(^8\) M\(^{-1}\) s\(^{-1}\) in acetonitrile [50]. The reaction of \(^1\)O\(_2\) with PQH\(_2\) in methanol was found to lead to the formation of \(H_2O_2\) (Reaction (119)) [48]. It was suggested that the reaction of \(^1\)O\(_2\) with PQH\(_2\) is initiated by the formation of \(^1\)O\(_2\) in PSII and can also proceed inside the thylakoid membrane [48]. The formation of \(H_2O_2\) via oxidation of PQH\(_2\) by \(^1\)O\(_2\) may occur in two ways. In the first one, \(^1\)O\(_2\) reacts with PQH\(_2\) to form an unstable hydroperoxide adduct of the quinone ring (PQH\(_2\)-OO), which directly decomposes to form \(H_2O_2\) and PQ (Reaction (119)). In the second way, the hydroperoxide adduct decomposes to form \(HO_2^-\) and PQH\(_2\) (Reaction (120)). This indirect mechanism would be similar to that proposed for the oxidation of Asch\(_2\) by \(^1\)O\(_2\) [47]. In the indirect mechanism, \(H_2O_2\) is produced by the oxidation of PQH\(_2\) to PQH\(^*\) by \(HO_2^-\) (Reaction (121)).

\[
\begin{align*}
3\text{P680} + O_2 & \rightarrow \text{P680} + ^1\text{O}_2 \\
^1\text{O}_2 + \text{PQH}_2 & \rightarrow [\text{PQH}_2\text{-OO}] \rightarrow \text{PQ} + \text{H}_2\text{O}_2 \\
^1\text{O}_2 + \text{PQH}_2 & \rightarrow [\text{PQH}_2\text{-OO}] \rightarrow \text{PQ}^* + \text{HO}_2^- \\
\text{PQH}_2 + \text{HO}_2^- & \rightarrow \text{PQH}_2^* + \text{H}_2\text{O}_2 \\
\end{align*}
\]

Thus, the PSI-independent O\(_2\) reduction in the PQ pool may depend on \(^1\)O\(_2\) production in PSII, and this reaction can cause the formation of \(H_2O_2\) inside the thylakoid membrane.

O\(_2\) reduction, associated with the PQ pool, also occurs without any inhibitors [264]. The ratio of the rate of O\(_2\) reduction in PSI and the rate of O\(_2\) reduction in the PQ pool, in the absence of any inhibitors, reaches 1:1 at a high light intensity [264]. However, the rate of O\(_2\) reduction by the PQ pool in the presence of DNP-INT is saturated at a low light intensity [128,264,308]. These data confirm that O\(_2\) reduction in a PQ pool in thylakoids without any inhibitors can occur parallel to O\(_2\) reduction in PSI. Interestingly, efficient O\(_2\) reduction by the PQ pool is observed at pH 5.0 in the absence of inhibitors, but not in the presence of DNP-INT [128,264]. The simplest explanation for differences in O\(_2\) reduction by the PQ pool in the absence and presence of DNP-INT, is to assume that the formation of O\(_2^*\) occurs in PSI. O\(_2^*\) can react with PQH\(_2\) to form \(H_2O_2\) via Reaction (114) [264].

The autoxidation of PQH\(_2\) does not imply the participation of any enzymes. However, the oxidation of the PQ pool with PTOX is widely discussed [309]. PTOX is a non-heme diiron quinol oxidase that oxidizes PQH\(_2\) and reduces O\(_2\) to H\(_2\)O. PTOX is localized in the non-appressed regions of the thylakoid membrane [310]. It has been suggested that PTOX provides an alternative electron flow from the PQ pool to O\(_2\) to prevent photo-inhibition of PSI [311]. However, in higher plants, PTOX-mediated electron flow to O\(_2\) is negligible [312] or its contribution is less than one percent of the total electron flow through PETC [313,314]. However, PTOX may depend on conditions, as high PTOX content and high PTOX activity were induced in the alpine species Ranunculus glacialis L. during growth in strong light [315]. The rate of PTOX-mediated electron flow is approximately 0.3 e\(^-\) s\(^{-1}\) (P680\(^-\))\(^{-1}\) [313]. This makes the rate of PQH\(_2\) oxidation equal to 0.15 PQH\(_2\) (P680\(^-\))\(^{-1}\) s\(^{-1}\), or 0.35 \(\mu\)mol PQH\(_2\) (mg Chl\(^-\))\(^{-1}\) h\(^{-1}\), assuming that the ratio of PSI to Chl is 1:420. Thus, the rate of PQH\(_2\) oxidation can be estimated to be 8.68 × 10\(^{-5}\) M s\(^{-1}\) inside the thylakoid membrane [252]. The second-order rate constant of PTOX-mediated oxidation of PQH\(_2\) inside the thylakoid membrane is 10.6 M\(^{-1}\) s\(^{-1}\). In the light, the oxidation of PQH\(_2\) by PTOX, associated with the reduction of O\(_2\) to \(H_2O_2\), would not lead to the consumption of O\(_2\) because of its matching stoichiometry with O\(_2\) production by PSII, Reactions (122) and (123).

\[
\begin{align*}
2\text{PQH}_2 + O_2 & \rightarrow 2\text{PQ} + 2H_2O \\
\text{PSII} + 2H_2O + 2\text{PQ} & \rightarrow 2\text{PQH}_2 + O_2
\end{align*}
\]
PTOX-mediated electron flow to O\(_2\) is assumed to produce no ROS. However, isolated PTOX can oxidize decylPQH\(_2\) with the formation of O\(_2^{•−}\) or H\(_2\)O\(_2\) at pH 8.0 or in substrate-limiting concentrations [316]. The efficiency of ROS production by PTOX was estimated to be around 17% of the total O\(_2\)-reduction activity of PTOX [316]. The rate of PTOX-mediated PQH\(_2\) oxidation associated with the formation of H\(_2\)O\(_2\) was estimated to be \(1.47 \times 10^{-5}\) M s\(^{-1}\), with the second-order rate constant of 1.8 M\(^{-1}\) s\(^{-1}\) inside the thylakoid membrane [252]. Thus, the estimated rate of PTOX-mediated O\(_2\) reduction is 100 times less than the rate of O\(_2\) reduction by the PQ pool in illuminated thylakoids. Furthermore, if O\(_2^{•−}\) is formed by PTOX, PQ\(^{•−}\) might also be formed.

PQ\(^{•−}\) is also considered a source of O\(_2^{•−}\) production by Cyt b6f via the reaction of O\(_2\) with PQ\(^{•−}\). It has been suggested that PQ\(^{•−}\), generated via one-electron oxidation of PQH\(_2\) at the Q\(_O\) site by the 2Fe-2S cluster of the high-potential, Rieske iron–sulfur protein of the Cyt b6f (Reaction (124)), can be oxidized by the conversion of O\(_2\) to O\(_2^{•−}\) [317].

\[
PQH_2 + 2\text{Fe-2S}_{\text{ox}} \rightarrow PQ^{•−} + 2\text{Fe-2S}_{\text{red}} + 2\text{H}^+ \quad (124)
\]

Isolated Cyt b6f complexes have been shown to produce H\(_2\)O\(_2\) when decylPQH\(_2\) and PC were used as an electron donor and electron acceptor, respectively [317]. It was suggested that H\(_2\)O\(_2\) appeared via O\(_2^{•−}\) dismutation. No detectable O\(_2^{•−}\) formation is observed in the presence of DBMIB, which has been shown to bind to an iron-sulfur binding site and at a position distal to the iron–sulfur binding site in Cyt b6f. This indicates that the mechanism of H\(_2\)O\(_2\) production is related to the oxidation of PQH\(_2\) at the Q\(_O\) site of Cyt b6f. The production of O\(_2^{•−}\) in Cyt b6f was also shown with EPR spectroscopy [317]. O\(_2^{•−}\) can be formed via the interaction of O\(_2\) with PQ\(^{•−}\) in the Q\(_O\) pocket or/and with the interaction of O\(_2\) with the reduced form of p-side heme b\(_p\) [317]. The rate of O\(_2^{•−}\) production by the isolated Cyt b6f is 4.5 (Cyt b6f)\(^{-1}\) s\(^{-1}\). This gives a rate of O\(_2^{•−}\) production inside thylakoid membrane of about 2.6 \(\times\) \(10^{-3}\) M s\(^{-1}\), assuming that the ratio of Cyt b6f and Chl is 1:420 and the volume of the thylakoid membrane is 4.6 \(\times\) \(10^{-6}\) L (mg Chl)\(^{-1}\) [304]. This rate is close to the rate of O\(_2^{•−}\) production by the PQ pool in the presence of DNP-INT. As DNP-INT blocks the oxidation of PQH\(_2\) at the Q\(_O\) site, the oxidation of PQH\(_2\) in the Q\(_O\) site cannot be responsible for O\(_2\) reduction in the PQ pool in the presence of DNP-INT. The formation of PQ\(^{•−}\) at the Qi site of Cyt b6f appears to cause O\(_2\) reduction in the PQ pool in the presence of DNP-INT (S. Khorobrykh and E. Tyystjärvi, unpublished data). The possible means of the reduction in O\(_2\) in Cyt b6f are shown in Figure 5.

The formation of HO\(^{•}\) has never been detected in the PQ pool, although it is supposed to happen. In the chloroplast stroma, H\(_2\)O\(_2\) is efficiently scavenged, which would limit HO\(^{•}\) formation. However, H\(_2\)O\(_2\) formed inside membranes by the PQ pool is not efficiently scavenged, and may therefore react with PQ\(^{•−}\) to form HO\(^{•}\) via the Fenton mechanism (Reaction (125)).

\[
PQ^{•−} + \text{H}_2\text{O}_2 \rightarrow \text{PQ} + \text{HO}^{•} + \text{OH}^{−} \quad (125)
\]
Photoinhibition in vivo in the green alga *Plants* 2020

Arabidopsis mutants of reaction of photoinhibition [193], whereas the same reaction is not more rapid in slower in CO anaerobic conditions [321], and photoinhibition in lincomycin-treated spinach leaf disks is only slightly of thylakoid membranes does not depend on O$_2$ PSII is the agent of damage in the photoinhibition of PSII (for reviews, see [319,320]). The photoinhibition of illuminated with the O$_2$ core complexes, electron transfer activity is lost and pigments are bleached only in the presence of DNP-INT. The formation of PQ•− in the presence of DNP-INT (S. Khorobrykh and E. Tyystjärvi, unpublished data). The possible means of the reduction in O$_2$ in Cyt b6f are shown in Figure 5. The possible means of the reduction in O$_2$ in Cyt b6f are shown in Figure 5. 

**Figure 5.** The proposed generation of O$_2$•− in Cyt b6f. (A) In the Q$_O$ site of Cyt b6f, PQH$^+$ is generated (Table 2) by the 2Fe-2S cluster of the high-potential Rieske iron--sulfur protein. PQ$^{•−}$ that has a long residence time within the Q$_O$ pocket, and cytochrome $b_L$ can also serve as a reductant for the generation of O$_2$•− in Cyt b6f. In addition, HO$_2$•, can be reduced by cytochrome $b_L$ to form H$_2$O$_2$ [317]. (B) The proposed generation of O$_2$•− in Cyt b6f in the presence of DNP-INT, an inhibitor of PQH$_2$ oxidation by Cyt b6f. The oxidation of PQH$_2$ does not occur in Q$_O$ site, formation of PQH$^{•−}$ and its deprotonation can occur in the Qi site. PQH$^+$ can be oxidized in subsequent reactions with O$_2$ or with hemes $b_H$ or $b_L$. H$_2$O$_2$ can be formed via the reaction of O$_2$•− with PQH$_2$ or via the reaction of HO$_2$• with cytochrome $b_H$ or cytochrome $b_L$. 

### 4. Damage Caused by ROS in the Chloroplast

#### 4.1. Damage to PSII

PSII is the main producer of $^1$O$_2$ in the chloroplast and a minor producer of other ROS (see Section 3.2), and therefore it is of great interest whether PSII is damaged by $^1$O$_2$. In isolated PSII core complexes, electron transfer activity is lost and pigments are bleached only in the presence of O$_2$, suggesting an effect of ROS [318]. Furthermore, PSII is sensitive to damage caused by externally applied $^1$O$_2$, as shown by a decrease in the quantum yield of PSII in lincomycin-treated tobacco leaves illuminated with the $^1$O$_2$ sensitizer Rose Bengal [189].

The above results indicate that PSII can be damaged by $^1$O$_2$ but do not prove that $^1$O$_2$ produced by PSII is the agent of damage in the photooinhibition of PSII (for reviews, see [319,320]). The photooinhibition of thylakoid membranes does not depend on O$_2$ and has a similar action spectrum under aerobic and anaerobic conditions [321], and photooinhibition in lincomycin-treated spinach leaf disks is only slightly slower in CO$_2$ doped N$_2$ than in air [322]. The effects of both deuterium oxide and ROS scavengers (reviewed by [319]) are variable and may depend on the type of complex. Similarly, effects of intrinsic $^1$O$_2$ quenchers and scavengers vary, as overproduction of the xanthophyll zeaxanthin protects against photooinhibition in vivo in the green alga *Chlamydomonas reinhardtii* [323] and the carotenoid-rich mutant ΔSigCDE of the cyanobacterium *Synechocystis* sp. PCC 6803 show protection against the damaging reaction of photooinhibition [193], whereas the same reaction is not more rapid in α-tocopherol-deficient mutants of *Arabidopsis* [324] and *Synechocystis* [325]. Further indirect evidence on the participation of $^1$O$_2$ in photooinhibition of PSII also varies, as the modification of the recombination reactions of PSII...
toward non-\( \text{O}_2 \)-producing direction provides protection against photoinhibition [326], whereas the protection offered by NPQ is very limited [327], suggesting that the photoinhibition of PSII may not depend only on the excitation of Chl [320,328]. The photoinhibition-tolerant green alga \textit{Chlorella ohadii} exhibits a recombination reaction model that is expected to lead to low \( \text{O}_2 \) production [329].

Apart from their direct effect on PSII electron transfer activity, ROS have been shown to cause loss [318] and fragmentation [330–333] of the D1 protein in isolated PSII core complexes [330] and PSII membranes [333]. Both \( \text{O}_2 \) [330] and \( \text{H}_2\text{O}_2 \) [332] cause fragmentation of the D1 protein. Miyao [333] concluded, on the basis of the protective effects of ROS scavengers, that several ROS, including \( \text{O}_2^{•−} \), \( \text{H}_2\text{O}_2 \), \( \text{H}_2\text{O}_2 \) and \( \text{HO}^{•} \), participate in protein damage in PSII. The connection of these results to what happens in vivo is unclear, as the proteases responsible for the degradation of the D1 protein in vivo (for review, see [334]) have not been shown to be present in isolated PSII preparations. Kale et al. [335] detected the formation of \( \text{O}_2^{•−} \) and \( \text{HO}^{•} \) in illuminated PSII membranes. Furthermore, the oxidative modifications of several amino acid residues of the D1 and D2 proteins were found to be associated with the formation of the radicals. Interestingly, both radicals were formed by PSII membranes throughout the illumination period, suggesting that they could contribute to the \( \text{O}_2 \)-dependent part of photoinhibition of PSII.

4.2. Damage to PSI

PSI has long been known to become inhibited, at least in certain plants, at chilling temperatures [336], in a reaction that depends on electron transfer from PSII to PSI [337]. The damage targets the iron–sulfur centers of PSI, and the remaining inactive PSI still functions as an excitation energy quencher [338]. The dependence of the photoinhibition of PSI on electron transfer, and the ability of PSI to reduce \( \text{O}_2 \) to \( \text{O}_2^{•−} \), strongly suggest that \( \text{O}_2^{•−} \), \( \text{H}_2\text{O}_2 \) or \( \text{HO}^{•} \) participate in the damage [339]. However, neither the identity of the inhibitory ROS nor the exact site and the mechanism of production are known. Damage to PSI can be specifically induced by the application of fluctuating light, either in the form of short (10–300 ms) strong flashes [340], or in the form of few-seconds-long, saturating but not very strong flashes fired on top of short-term exposure of the plant to weak, PSII-specific light [341].

4.3. Oxidation of Membrane Lipids by ROS

Unsaturated fatty acids of membrane lipids can become peroxidated in a reaction with \( \text{O}_2 \) (Reaction (20)) or \( \text{HO}^{•} \) (reaction (77)). Peroxidation by \( \text{O}_2 \) dominates the non-enzymatic formation of lipid peroxides in leaves, whereas radical-induced peroxidation is more common in non-photosynthetic tissues [4].

Fatty acid peroxides, in turn, decompose either spontaneously or enzymatically to oxylipin carbonyls [342]. Tri-unsaturated fatty acids especially fragment to malondialdehyde that is highly reactive in its protonated dialdehyde form (O=CH-CH2-CH=O) [343]. Both malondialdehyde and acrolein, another highly reactive fragmentation product, are produced under non-stressed conditions but their concentrations increase during stress [344,345]. Lipid–peroxide-derived aldehydes and ketones like malondialdehyde function both as agents of damage and signaling molecules in \textit{Arabidopsis} [344,346]. Due to their reactivity towards ROS, tri-unsaturated fatty acids may function as ROS sinks [347], and signaling by products of lipid oxidation may be essential for plant cells’ ability to survive oxidative stress [346].

4.4. Damage to Stromal Proteins

The production of ROS in the chloroplast is expected to damage proteins of the compartment of origin. In thylakoid membranes, light-induced damage primarily targets the photosystems. In the stroma, several proteins are known to be targets of ROS damage. The inhibitory effects are often ascribed to the oxidation of cysteine residues.
ROS have a strong inhibitory effect on translation in cyanobacteria [348]. The mechanism of the inhibition by H$_2$O$_2$ is the oxidation of cysteine residues and the subsequent formation of an intramolecular disulfide bond in translation elongation factor G [348], and the formation of a sulfenic acid and an intermolecular disulfide bond in elongation factor Tu [349,350]. The inhibition of translational elongation exerts its effect on the activity of PSII by inhibiting or slowing down the turnover of the D1 protein [351]. Similar ROS effects are expected in chloroplasts.

The Calvin–Benson cycle is inhibited by H$_2$O$_2$ [352] with the ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco) as the most important target of oxidation [353]. Analysis of the proteome of H$_2$O$_2$-treated chloroplasts revealed modified cysteine residues in both subunits of rubisco, Fd-dependent glutamate synthase, ferredoxin-NADP$^+$ oxidoreductase 1 (FNR1) and glyceraldehyde 3-phosphate dehydrogenase subunit B, and a similar analysis after methyl viologen treatment revealed oxidative changes in 24 chloroplast proteins and modified cysteines in rubisco large subunit, FNR1, myrosinase and NAD(P)-binding Rossman-fold-containing protein [353]. The authors suggested that, due to its large amount, rubisco functions as a redox buffer in the chloroplast.

### 4.5. Damage to Chloroplast DNA

ROS are known to react with DNA [2], and chloroplast DNA is not an exception. A comparison of the integrity of DNA of the chloroplasts of mesophyll and bundle sheath cells of maize, a C4 plant, offers insight into ROS damage within the chloroplasts [354]. In C4 plants, mesophyll cells carry out the photosynthetic electron transfer reactions that produce NADPH and ATP, but also ROS, whereas the bundle sheath chloroplasts are almost devoid of PSII that produces O$_2$. A drastically larger amount of DNA damage, analyzed with a long-sequence-specific variant of polymerase chain reaction, was found in the chloroplast DNA of light-grown maize plants in mesophyll cells than in bundle sheath cells [354]. Interestingly, mitochondrial DNA showed a similar difference between mesophyll and bundle sheath mitochondria. Doping soil with Cr(VI) that causes ROS production in leaves also caused damage, visualized by staining with 4′,6-diamidino-2-phenylindole, in the chloroplast DNA [355].

### 5. Detoxification of ROS in Plant Chloroplasts

#### 5.1. Detoxification of O$_2^{•−}$ and H$_2$O$_2$

Plants have evolved a multitude of enzymatic and non-enzymatic ROS-scavenging and quenching mechanisms. ROS-mediated signaling and ROS detoxification are coupled, as signaling is generally initiated by the oxidation of target molecules, that therefore also act as antioxidants (see reviews [10,356,357]). Here, we discuss the main scavenging mechanisms and antioxidant molecules controlling ROS in the chloroplasts, with emphasis on ROS detoxification in the thylakoid membrane, or stromal-scavenging mechanisms in its immediate vicinity.

O$_2^{•−}$, produced in chloroplasts, is scavenged efficiently by copper/zinc SODs residing on the stromal face of the thylakoid membrane [12,358]. The dismutation reaction, catalyzed by CuZnSODs, is described in Reactions (5), (44) and (45). While SOD is the main catalyst, the dismutation reaction can also be catalyzed by redox reactive metals such as manganese [359,360], or it can occur non-catalytically [68]. O$_2^{•−}$ can also oxidize two highly important chloroplast antioxidants, AscH$_2$ [62,64] (Reaction (34)) and GSH [66,67] (Reaction (37)).

H$_2$O$_2$ is reduced by AscH$_2$ in a reaction catalyzed by APXs (Reactions (62)–(64)) [93]. The net reaction of H$_2$O$_2$ scavenging by AscH$_2$ can be summarized as (Reaction (126)).

\[
    \text{H}_2\text{O}_2 + 2\text{AscH}_2 \rightarrow 2\text{H}_2\text{O} + 2\text{MDA} + 2\text{H}^+ \quad (126)
\]

The reaction produces water and MDA. Different APX isoenzymes are found in different chloroplast compartments. Stromal APXs and thylakoid APXs have specific roles in, e.g., plant development, but exhibit functional redundancy in ROS detoxification in mature leaves [361–363]. The rest of the...
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ascorbate–glutathione cycle regeneratesAscH$_2$ [12,364]. The first step is the reduction of MDA to
AscH$_2$ by Fd$_{red}$ (Reaction (127)),

\[
\text{MDA} + \text{Fd}_{\text{red}} + 2\text{H}^+ \rightarrow \text{AscH}_2 + \text{Fd} \quad (127)
\]
or by NADPH in a reaction catalyzed by MDA reductase (Reaction (128)).

\[
2\text{MDA} + \text{NADPH} + 3\text{H}^+ \rightarrow 2\text{AscH}_2 + \text{NADP}^+ \quad (128)
\]

The complete description of the catalytic cycle of reduction of MDA to AscH$_2$ is described in [12].
The MDA molecules that are not immediately reduced dismutate non-catalytically, forming
AscH$_2$ and DHA (Reaction (113)).

GSH donates electrons to DHA either non-catalytically or through catalytic oxidation mediated
by DHA reductase, forming AscH$_2$ and glutathione disulfide (GSSG) (Reaction (129)).

\[
\text{DHA} + 2\text{GSH} \rightarrow \text{AscH}_2 + \text{GSSG} \quad (129)
\]

NADPH, formed by the PETC, is then used by glutathione reductase to reduce GSSG back to GSH
(Reaction (130))

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + 2\text{GSH}, \quad (130)
\]

thereby completing the ascorbate–glutathione cycle. The functions of APXs in plants have been
reviewed by [365]. Because the electrons utilized in the reduction of O$_2$ to O$_2$•− by PSI originate from
water molecules broken down by PSII, and the end product of the production and scavenging of H$_2$O$_2$
is water, the whole scavenging system is often referred to as the water–water cycle [12].

PRXs, particularly two-cysteine peroxiredoxins (2-Cys PRX), have been shown to function in
conjunction with thylakoid APXs in downplaying H$_2$O$_2$ accumulation during conditions causing
oxidative stress in plants [366–368]. 2-Cys PRXs facilitate a peroxidative reduction of H$_2$O$_2$, utilizing
electrons from NADPH in a reaction catalyzed by thioredoxin reductase C or, less efficiently, from
reduced TRXs [366–368]. The catalytic cycle of peroxide detoxification by 2-Cys PRXs and their
subsequent regeneration is described in detail in [369]. NADPH is produced both in the light, by
PETC, and in the dark, by the oxidative pentose phosphate pathway [370], whereas TRXs are recycled
to their reduced form by Fd produced in the light by PSI; the reduction in TRXs is catalyzed by
thioredoxin reductases [371]. 2-Cys PRXs are not the only enzymes that can facilitate TRX-dependent
H$_2$O$_2$ detoxification, as glutathione peroxidases also utilize TRXs as substrates instead of reduced
glutathiones in plant chloroplasts, and can likely initiate a similar cycle to 2-Cys PRXs [372–375].
Many other components have been suggested to take part in the recycling of the PRX/glutathione
peroxidases-initiated H$_2$O$_2$ detoxification cycle, such as glutaredoxin, cyclophilins and AscH$_2$ [369].

5.2. Detoxification of $^1$O$_2$

Carotenoids and tocopherols are the main antioxidants against $^1$O$_2$ in chloroplasts [43].
Carotenoids function in the NPQ of singlet excited Chl (reviewed in [376,377]), quench $^3$Chl and quench scavenge $^1$O$_2$. Each LHClI subunit contains two luteins, a neoxanthin and a
violaxanthin/zeaxanthin [378]. All eight Chl $a$ molecules of an LHClI subunit are positioned within
close proximity to either of the two luteins or neoxanthin, which facilitates efficient $^3$Chl-quenching
especially by lutein, and lowers the probability of $^3$Chl interaction with O$_2$, quenching 95% of $^3$Chl in
LHClI [43,369–381]. Violaxanthin and zeaxanthin are not likely to be involved in $^3$Chl quenching
in LHClI, as they are bound far from the Chl molecules [382,383]. However, zeaxanthin can quench
$^3$Chls in the monomeric Lhcb antenna subunits of PSI (Lhcb4–6) and in the dimeric Lhca subunits of
PSI antennae [384]. In LHClII, zeaxanthin is specifically involved in NPQ. In high light, zeaxanthin is produced by violaxanthin de-epoxidase from violaxanthin through the intermediate antheraxanthin,
and the newly formed zeaxanthin replaces violaxanthin in LHCII. A switch back to moderate light or darkness induces the epoxidation of zeaxanthin back to violaxanthin and the subsequent replacement of zeaxanthin with violaxanthin in LHCII [385].

The PSII core, consisting of the proximal antennae CP43 and CP47, the Mn-cluster and the RC (D1/D2/Cyt b559) [386,387], binds 11 β-carotenes, two of which are located in the RC [386]. The distance between these two β-carotenes and the RC Chl P680 is too long to allow the participation of the β-carotenes in quenching of 3P680 [170,191,386,388]. However, there are indications that β-carotenes in other parts of the isolated PSII core are likely to quench 3Chls [386].

The detoxification of 1O₂ itself [192] by carotenoids occurs mainly through physical quenching via electronic energy transfer mechanism (Reaction (17), where A is a carotenoid). The resulting triplet state of the carotenoid (³Car) dissipates its excitation energy via a nonradiative transition to its ground state [23,41,43,389]. Carotenoids can also take part in the chemical scavenging of 1O₂ [43,390]. Oxidation products of β-carotene found in plants in high light suggest that 1O₂ can oxidize the β-carotenes of PSII reaction centre [301,390]. β-cyclocitril (β-CC), a volatile product of oxidation of β-carotene by 1O₂, has been shown to be involved in cell signaling [391] (see Section 6.1). In LHCII, 1O₂ produced by the interaction between O₂ and the residual ³Chl that is not quenched by carotenoids, is rapidly inactivated, due to the abundance of carotenoids in LHCII and free carotenoids such as zeaxanthin in the surrounding lipid matrix [388,392].

Other antioxidants in the thylakoid membrane are not bound to LHChs or, in stroma, offer an even greater capacity for the physical or chemical quenching of 1O₂. Tocopherols, or specifically α-tocopherol, are considered as important antioxidants against 1O₂ [393]. The rate constants of the physical quenching of 1O₂ by tocopherols in organic solvents are significantly higher than those of chemical scavenging [389], suggesting that, similarly to carotenoids, the main quenching mechanism by α-tocopherol is physical quenching (Reaction (17)) [43]. However, the oxidation of α-tocopherol by 1O₂ produces 8-hydroperoxy-tocopherone that can be re-reduced to α-tocopherol by AscH₂ [394,395], which lends the recyclability of the stromal ascorbate-glutathione cycle to 1O₂ detoxification of the lipid phase. AscH₂ also has the capacity to scavenge 1O₂ (reaction 24) that reaches the stroma [47]. Chloroplasts contain flavonoids in the envelope membrane, and they have the potential to quench 1O₂ both physically and chemically [396,397]. Even though the relatively remote location from the most prominent 1O₂ production sites does put their role as 1O₂ antioxidants in question, flavonoids have been shown to be involved in lowering the amount of 1O₂ in high light in vivo [397]. Other potential 1O₂ antioxidants include polyunsaturated fatty acids [4], PQH₂ [48,305–307,396] and isoprene [399,400].

6. ROS Produced by Plant Chloroplasts Function as Signaling Molecules

ROS are known to participate in retrograde signaling, acclimation to biotic or abiotic stresses, programmed cell death (PCD) and many other processes (for recent reviews, see [9–11,401–407]). Here, we aim to briefly summarize what is known (and what is not) about how chloroplast-derived ROS are sensed and how the signaling cascades are initiated. Signaling by ROS produced by enzymes like NADPH-oxidase (reviewed in [408]) will not be discussed here.

6.1. Signaling by 1O₂

The lifetime of 1O₂ in plant cells has not been measured, but is generally assumed to be too short (for review, see [23]; Section 2.1.4) to enable diffusion out of chloroplasts and, consequently, 1O₂ itself is unlikely to function as a messenger molecule. Instead, the accumulation of β-CC (a reaction product of β-carotene and 1O₂) has been shown to induce gene expression, leading to stress (e.g., high light) acclimation [391,409,410]. In theory, β-CC could directly travel to the nucleus and activate 1O₂ responsive genes (for discussion, see [411]), however, direct evidence is lacking. Methylene Blue Sensitivity 1 protein might participate in transferring the signal from cytosol to the nucleus [412,413]. In addition, β-CC can be converted to water-soluble β-cyclocitric acid, which also could function as a signaling molecule [410]. Other oxidation products of 1O₂ might have signaling functions, too [346,414].
Another $^1\text{O}_2$-induced pathway involves the Executer1 (EX1) (and possibly Executer2) proteins [415,416]. The oxidation of a tryptophan residue of EX1, presumably by $^1\text{O}_2$ [417], leads to the degradation of EX1 by FtsH, a protease that is also important to the repair cycle of PSII [418]. Afterwards, a signaling cascade leading to PCD is activated [419]. EX1 is not simply a repressor of the PCD pathway [407], however, it is not understood how the degradation of EX1 leads to the induction of PCD. In addition to cell death, EX1 is important in systemic acquired acclimation [420].

The $\beta$-CC and EX1 pathways are thought to operate independently [391]. A possible explanation of the need for two pathways is that small amounts of $^1\text{O}_2$ lead to acclimation responses while larger amounts initiate PCD (and still higher amounts cause damage and unregulated cell death [403]). Accordingly, under severe stress, the $\beta$-CC pathway, through Oxidative Signal-Inducible 1 kinase, may also lead to PCD, but even this route is EX1-independent [421,422]. Most $\beta$-CC is produced from the $\beta$-carotene located in the RC of PSII [390,423], and therefore in the grana core [424], whereas EX1 is located in grana margins [425]. As $^1\text{O}_2$ is not expected to diffuse far, the site of production rather than the amount may determine which signaling pathway is activated.

Why do plants need to react differently to $^1\text{O}_2$ produced in different sites? PSII repair occurs mainly in grana margins (for a review, see [426]), and it has been speculated that EX1 would activate PCD if PSII repair is impaired, possibly under adverse environmental conditions when loose Chls might produce $^1\text{O}_2$ [425]. Chl turnover was shown to associate with the repair of PSII [427], implying $^1\text{O}_2$ generation, even though Chl synthesis and degradation are tightly regulated and loose Chls are thought to be bound to specific proteins that prevent $^1\text{O}_2$ production [428]. During a low light to high light transition, the FtsH-protease may get transiently inactivated (possibly indirectly by $^2\text{H}_2\text{O}_2$; [429]), thus preventing activation of the EX1-induced PCD. This is in agreement with the view that the EX1 pathway does not respond to high light stress, but it is the $\beta$-CC pathway that initiates high light acclimation. Alternatively, the EX1 pathway might be important in plant defense against pathogens [402,430]. $^1\text{O}_2$ produced by Chl catabolites has been proposed to be involved in the hypersensitive response [431], and similarly to the flu-mutant [115], $^1\text{O}_2$ produced by Chl catabolites has been suggested to initiate the EX1 pathway also in the wild type [432]. Interestingly, NADPHprotochlorophyllide oxidoreductases were shown to associate with EX1 and FtsH [425], though the interactions may be weak or transient, as they are not always observed [433]. PSII is a target of many pathogens [434] and a non-functional PSII repair cycle might also be involved in plant immunity [435]. However, the physiological role of the EX1 pathway is still unclear.

### 6.2. Signaling by $\text{H}_2\text{O}_2$

In contrast to $^1\text{O}_2$, the long lifetime of $\text{H}_2\text{O}_2$ enables its function as a messenger molecule. Exposito-Rodriguez et al. [436] observed that photosynthesis-derived $\text{H}_2\text{O}_2$ rapidly accumulated in the nuclei, and the addition of cytosolic $\text{H}_2\text{O}_2$ scavengers did not prevent this. The authors proposed that $\text{H}_2\text{O}_2$ originated from chloroplasts closely associated with the nucleus. The diffusion of $\text{H}_2\text{O}_2$ through membranes is not extremely rapid [95], but the transport may be facilitated by (specialized?) aquaporins [95,437,438]. The formation of stromules has been observed under stress [439], and they have been suggested to allow for direct contact between chloroplasts and the nucleus [440]. Another hurdle that chloroplast-originated $\text{H}_2\text{O}_2$ needs to overcome is that the powerful antioxidant systems of stroma (see Section 5.1) are believed to efficiently scavenge $\text{H}_2\text{O}_2$. Accordingly, it has been proposed that $\text{H}_2\text{O}_2$ produced inside the thylakoid membranes (see Section 3.2) might have a great importance in signaling [441]. On the contrary, a meta-analysis of 79 transcriptomic studies concluded that ROS responses are determined by timing rather than the site of origin [442]. Therefore, $\text{H}_2\text{O}_2$ may participate in multiple pathways, some of which are sensitive to the site of $\text{H}_2\text{O}_2$ production [443]. $\text{H}_2\text{O}_2$ is involved in many signaling pathways. For example, photosynthesis-derived ROS, probably $\text{H}_2\text{O}_2$, may induce enzymatic $\text{O}_2^{**}$ production by cytosolic NADPH-oxidases 408. In addition, a reduced PQ pool was proposed to cause stomatal closure via $\text{H}_2\text{O}_2$ accumulation [444].
Borisova-Mubarakshina et al. [445] showed evidence that H$_2$O$_2$ regulates PSII antenna size in barley during long-term acclimation to high light.

It is not clear what senses H$_2$O$_2$ in plant cells. SAL1 (an inositol polyphosphate 1-phosphatase) degrades phosphoadenosine phosphate (PAP) in chloroplasts. The oxidation of cysteine residues of SAL1, e.g., under high light, probably by H$_2$O$_2$, leads to the inactivation of SAL1 and accumulation of PAP [446]. PAP can be transported into the nucleus and activate genes protecting plants from oxidative stress [447,448]. In addition, it has been proposed that a glutathione peroxidase [449], heat shock transcription factors [450], APX [362] and protein phosphatases (reviewed in [451]) might function as H$_2$O$_2$ sensors.

In general, genes responding to 1O$_2$ were found to differ from those known to be regulated by H$_2$O$_2$ [391]. The available data suggest that H$_2$O$_2$ actually antagonizes EX1-mediated 1O$_2$ signaling [452,453]. On the other hand, H$_2$O$_2$ and the β-CC-mediated 1O$_2$ signaling pathways may converge at Oxidative Signal-Inducible 1 kinase [407,454]. β-CC also down-regulates SAL1 and up-regulates genes generating PAP [391,423], supporting the view that both H$_2$O$_2$- and β-CC-signaling pathways induce stress acclimation.

6.3. Signaling by O$_2$•−

In plant cells, SOD rapidly converts O$_2$•− to H$_2$O$_2$. The reactivity of O$_2$•− may also limit its specificity in signaling. However, the literature suggests that a set of genes is specifically induced by O$_2$•− [455–457]. For example, Zinc-Finger Protein 12 was shown to be induced more strongly by O$_2$•− than by H$_2$O$_2$ [458].

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Abbreviations

β-CC β-cyclocitral
1Chl, 1Chl* and 3Chl respectively, singlet state, singlet excited state and triplet excited state of chlorophyll
1O$_2$ singlet oxygen (1AgO$_2$)
2-Cys PRX two-cysteine peroxiredoxin
A acceptor
A$_1$ phylloquinone of PSI
APX ascorbate peroxidase
AscH$_2$ ascorbate, ascorbic acid
Car and 3Car respectively, singlet and triplet state of carotenoid
CAT catalase
Chl and Chl* respectively, chlorophyll and excited chlorophyll
Chl a chlorophyll a
cyt cytochrome
cyt b559 cytochrome b559
Cyt b6f cytochrome b6/f complex
DBMIB 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone
DCMU 3-(3,4-di-chlorophenyl)-1,1-dimethyl urea
DHA dehydroascorbate; DMF, dimethylformamide
DNP-INT 2-(2,4-dinitrophenoxy)-3-iodo-4-methyl-1-(1-methylethyl)-5-nitro-benzene
Em midpoint redox potential
E$^0$′ standard redox potential
EPR electron paramagnetic resonance
EX1 Executor1
Fd, Fd$_{ox}$ and Fd$_{red}$ ferredoxin, and oxidized and reduced ferredoxin, respectively
FL flavin
FL* anion form of flavin semiquinone
FLH•
FLHOOH
FLU
FNR
GSH
GSSG
H₂O₂
HO•
HO₂•
HO₂−
HP, IP, LP and VLP
hv
ISC
k_{forward} and k_{reverse}•
LHC
LOO•
MDA
MDAR
MenB
NHE
NPQ
O₂•−
OEC
P680
P700
PAP
PC
PCD
PETC
Pheo
PheoD₁ and PheoD₂
PPFD
PQ
PQ•−
PQH₂
PRX
Pst80
Ptox
PX
Q
Q•−
R•
RC
ROO•
ROOH
ROOOOR
ROS
RS
rubisco
S
SAL1
SOD
TRX
TyrZ
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