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REVIEW

Superoxide Anion Radical Generation in Photosynthetic Electron Transport Chain

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Abstract—This review analyzes data available in the literature on the rates, characteristics, and mechanisms of oxygen reduction to a superoxide anion radical at the sites of photosynthetic electron transport chain where this reduction has been established. The existing assumptions about the role of the components of these sites in this process are critically examined using thermodynamic approaches and results of the recent studies. The process of O_2 reduction at the acceptor side of PSI, which is considered the main site of this process taking place in the photosynthetic chain, is described in detail. Evolution of photosynthetic apparatus in the context of controlling the leakage of electrons to O_2 is explored. The reasons limiting application of the results obtained with the isolated segments of the photosynthetic chain to estimate the rates of O_2 reduction at the corresponding sites in the intact thylakoid membrane are discussed.

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INTRODUCTION

Molecular O_2 is a byproduct of water oxidation in photosynthesizing organisms, which use it as a source of electrons for generating a reductant required in carbon metabolism reactions. At the same time, components of the photosynthetic apparatus in aerobic organisms can react with O_2 molecules. In 1951, Alan Mehler discovered that illumination of thylakoids leads to hydrogen peroxide (H_2O_2) formation, and concluded that molecular O_2 could serve as a direct electron acceptor from the reduced components of the photosynthetic electron transport chain (PETC) [1]. The process of electron transfer from PETC components to O_2 molecules, accompanied by their reduction, is known as the Mehler reaction. The main function of PETC is to reduce $NADP^+$, and vast majority of the research has been conducted to estimate the share of "non-productive" Mehler reaction in the overall electron flow in PETC under various operational conditions [2].

However, understanding oxidation processes of the PETC components by O_2 molecules is crucial, not only for assessing the impact of Mehler reaction on effectiveness of CO_2 fixation, but also for recognizing its role in facilitating this fixation. Synthesis of ATP, used in CO_2 fixation reactions, is driven by the build-up of proton gradient across the thylakoid membrane. This gradient arises not only during linear electron transport to the oxidized pyridine nucleotide, but also during electron transport to oxygen as an acceptor (known as pseudocyclic electron transport), and during cyclic electron transport. The latter requires a certain necessary level of

Abbreviations: Fd, ferredoxin; FNR, ferredoxin:$NADP^+$ oxidoreductase; DCPIP, 2,6-dichlorophenolindophenol; DNP-INT, dinitrophenyl ether 2-iodine-4-nitrotimol; E_m, midpoint redox potential, PETC, photosynthetic electron transport chain; PhQ, phylloquinone; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; Q_A and Q_B, primary and secondary quinone acceptors of photosystem II, respectively; Q_O and Q_R, quinol-oxidizing (Q_O site) and quinol-reducing (Q_R site) sites of the b_6f complex, respectively; ROS, reactive oxygen species.

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oxidation of the plastoquinone (PQ) pool (redox poising), which can be provided both by electron transfer to the acceptors of Photosystem 1 (PSI), particularly the Mehler reaction, or, possibly, by the direct oxidation of the PQ pool by oxygen (see below). The process of O_2 reduction in PETC plays a critical role in maintaining homeostasis of the photosynthesizing cell and in adapting the entire photosynthetic organism to environmental conditions. This role is realized via formation of Reactive Oxygen Species (ROS), such as the superoxide anion radical ($O^{\bullet-}_2$), and H_2O_2. These ROS serve as primary signaling molecules that trigger adaptive metabolic readjustments. It is exactly the generation of ROS that allows PETC to function as a sensitive sensor of environmental changes, such as light intensity, temperature, water availability, soil salinity, and so on.

Unsurprisingly, many studies have been devoted to determining from which PETC components the transfer of electrons to O_2 molecules is possible, and from which it predominantly occurs [3-7]. To date, new data have been accumulated about the mechanisms of O_2 reduction in PETC, and new ideas have emerged regarding the conditions under which this process occurs and how the components of PETC that can be oxidized by oxygen have evolved. This review focuses on these new findings, analyzing earlier results in each case. Primary attention is given to the O_2 reduction in PSI, which is generally considered as the main site in PETC where this process takes place.

CONDITIONS AND PATHWAYS OF O_2 REDUCTION IN PETC

Twenty years after discovery of the Mehler reaction, it was demonstrated that it begins as a one-electron oxidation of PETC components by O_2 molecules under illumination, resulting in formation of $O^{\bullet-}_2$ [8, 9]. From this point forward, the term "O_2 photoreduction" is used synonymously with the term "formation of $O^{\bullet-}_2$ during the electron transfer from the PETC components to the O_2 molecule". When assessing thermodynamic feasibility of O_2 reduction, it should be noted that PETC contains components dissolved in aqueous phase, membrane-associated components in contact with the aqueous phase, as well as components embedded in the hydrophobic zones of proteins and membrane. The midpoint redox potential, E_m, for the $O_2/O^{\bullet-}_2$ pair varies in different environments: −160 mV (measured against the normal hydrogen electrode, NHE) in water and approximately −550 to −600 mV in dimethylformamide, a model solvent for the membrane with dielectric constant of 36.7 [10]. The possibility of $O^{\bullet-}_2$ formation within the thylakoid membrane under the light was suggested in the works by K. Asada et al. [11], and experimentally confirmed in our studies with the EPR method, using the lipophilic cyclic hydroxylamine TMT-H when O_2 was the only final acceptor [12, 13]. Subsequent research showed that light can induce formation of $O^{\bullet-}_2$ within the thylakoid membrane even when ferredoxin (Fd) and $NADP^+$ were present. This suggests that the O_2 reduction can occur simultaneously with the $NADP^+$ photoreduction [14].

Under illumination, there may be more than one pathway for O_2 reduction active in the PETC. The table presents the rates of $O^{\bullet-}_2$ formation reported in the literature at the main PETC sites: Photosystem 2 (PSII), PSI, cytochrome b_6f complex, stromal pool of Fd, and membrane PQ pool. Properties and characteristics of each of the known O_2 reduction pathways in the chloroplast PETC are discussed below.

O_2 reduction in Photosystem II. Many studies have been devoted to investigation of oxygen photoreduction in PSII; analysis of these studies is presented in the reviews [2, 4]. Most of these works have been carried out

Rates of $O^{\bullet-}_2$ generation in the various segments of photosynthetic electron transport chain in higher plants

Structure	$O^{\bullet-}_2$ generation rate	$O^{\bullet-}_2$ generation rate normalized to PSII content[g]
Photosystem II	0.1[a]-0.75[b] e^- (PSII × s)$^{-1}$	0.1-0.75 e^- s^{-1}
Plastoquinone pool	2.3 e^- (PSII × s)$^{-1}$[c]	2.3 e^- s^{-1}
Cyt. b_6f complex	4.5 e^- (b_6f × s)$^{-1}$[d]	1.6 e^- s^{-1}
Photosystem I	3-10 e^- (PSI × s)$^{-1}$[e]	1.75-5.8 e^- s^{-1}
Ferredoxin	0.4-1.4 e^- (PSI × s)$^{-1}$[f]	0.2-0.8 e^- s^{-1}

[a] Calculated from the cytochrome *c* reduction rate in [15].

[b] Calculated from the O_2 uptake rate in BBY particles in [16], considering the Chlorophyll : P_{680} ratio = 350 [17].

[c] Calculated from the diuron-independent O_2 uptake rate in thylakoids in [16], considering the Chlorophyll : P_{680} ratio = 370 [18].

[d] From [19].

[e] From [20]; range of rates for different light intensities (from lowest to highest intensity).

[f] Calculated, using the values of oxidation rate constant of Fd by oxygen (0.08-0.28 s^{-1}) [21, 22] and the ratio of Fd:PSI in chloroplasts = 5 [23].

[g] Calculated using the stoichiometry b_6f : PSII = 0.35 and PSI : PSII = 0.58 [24].

with PSII preparations of varying integrity: fragments of thylakoid membranes enriched with PSII (BBY-particles), and PSII complexes in which PQ is absent. To date, the studies appeared, which have attributed $O^{\bullet}_2^-$ generating activity to PSII components not only in the isolated thylakoids but also in leaves. This has primarily been achieved by visualizing oxidized amino acid residues near the PSII cofactors [25-27] – an experimental approach based on the assumption that ROS produced by the electron transfer cofactors can first modify proximal residues in close proximity to the site of ROS generation [28]. However, this approach does not allow any quantitative evaluation of the rate of $O^{\bullet}_2^-$ formation, whereas working with the PSII preparations isolated from thylakoid membranes makes it possible to measure the rate of light-induced $O^{\bullet}_2^-$ generation. The rates for this process in the BBY-particles are given in the table.

Various components of PSII are considered as oxygen reductants. Formation of $O^{\bullet}_2^-$ was registered in the D1/D2/cytochrome *b559* complex, where quinones were absent at the Q_A and Q_B sites, suggesting that pheophytin, the primary electron acceptor in PSII, could reduce O_2 [29]. Pheophytin, with the lowest E_m value among the PSII cofactors (–610 mV), is capable of reducing O_2 in the hydrophobic part of the protein, where it is located. In this region, potential of the $O_2/O^{\bullet}_2^-$ pair is close to this value, or even slightly more positive (see above). Moreover, it was recently shown that in the *Arabidopsis vte1* mutant deficient in tocopherol biosynthesis, in which PSII lacks two tocopherol molecules characteristic of the wild type located near pheophytin and non-heme iron, generation of $O^{\bullet}_2^-$ is increased and oxidized amino acid residues were detected near pheophytin [27]. The authors suggested that at high illumination, pheophytin produces $O^{\bullet}_2^-$, which in the wild type PSII oxidizes the adjacent tocopherol molecule rather than the surrounding amino acid residues. It is usually assumed that under moderate illumination, reduction of O_2 by pheophytin is unlikely due to the short lifetime of its reduced form, 200-500 ps, during oxidation by the next electron carrier in PSII, PQ molecule at the Q_A site [2].

Reduction of O_2 by the tightly bound $PQ^{\bullet-}$ at the Q_A site has been suggested, in particular, based on the fact that low concentrations of the herbicide diuron, an inhibitor of electron transfer from Q_A to the next PETC component, the PQ molecule at the Q_B site, stimulated formation of $O^{\bullet}_2^-$ in the pea thylakoids [30]. Generation of $O^{\bullet}_2^-$ was recorded in the BBY complexes additionally treated to remove PQ molecules, thereby leaving the Q_B site vacant, using specific spin traps and reduction of exogenous cytochrome *c* [15]. Modified amino acid residues located closer to the Q_A site were also found in the spinach leaves and in the *Arabidopsis vte1* mutant [25, 27].

The question of thermodynamics of the reaction between O_2 and $PQ^{\bullet-}$ at the Q_A site remains, however, debatable. It was shown that E_m (Q_A/Q_A^-) depends on the presence of a bicarbonate ion near the non-heme iron: –70 mV and –145 mV in the absence and presence of HCO_3^-, respectively [31]. The E_m values of the pair ($Q_A/Q_A^{\bullet-}$) in the presence of HCO_3^- (–145 mV) and the pair $O_2/O^{\bullet}_2^-$ (–160 mV in water) are close, suggesting that from the thermodynamic point of view oxidation of $PQ^{\bullet-}$ at the Q_A site by oxygen, although not beneficial, is plausible. The question is whether we can consider E_m ($O_2/O^{\bullet}_2^-$) in water, since Q_A is located in a rather hydrophobic part of PSII? This assumption is plausible, as Q_A interacts with water channels through which HCO_3^-, in particular, arrives at the non-heme iron [15, 32], and there are many polar and ionogenic amino acid residues in this region. Therefore, reduction of O_2 by the tightly bound $PQ^{\bullet-}$ at the Q_A site appears more favorable, when HCO_3^- is present on the acceptor side of PSII. However, under conditions where the stable $PQ^{\bullet-}$ at the Q_A site induces release of HCO_3^-, the rate of $O^{\bullet}_2^-$ production increases [15]. Based on this, it was concluded that the presence of HCO_3^- limits access of O_2 to the Q_A site. The authors of the study [15] suggest that the molecule of O_2 that is reduced to $O^{\bullet}_2^-$ is bound to the non-heme iron, which increases the E_m value of the pair $O_2/O^{\bullet}_2^-$.

Reduction of O_2 at the Q_B site is considered thermodynamically improbable due to the E_m ($PQ/PQ^{\bullet-}$) at the Q_B site being +90 mV [33]. Indeed, even exposure of BBY particles to high light intensity causing photoinhibition did not result in appearance of the oxidized amino acid residues at the Q_B site [26]. It is likely that there is no generation of $O^{\bullet}_2^-$ at this site.

Based on the coupling of O_2 reduction with oxidation of cytochrome *b559*, which is part of the PSII complex and participates in the cyclic transport of electrons around PSII, it has been assumed in the literature that this cytochrome is responsible for O_2 reduction [34, 35], when it is in a low potential form (E_m = –40 – +80 mV [36]) or in a very low potential form (E_m = –150 – –200 mV [37]). However, O_2 reduction even by the low-potential forms of cytochrome *b559* is thermodynamically unfavorable due to the cytochrome location in the hydrophobic zone, where E_m value of the $O_2/O^{\bullet}_2^-$ pair is significantly lower than these values. It was suggested that either $PQ^{\bullet-}$, which is formed during the oxidation of PQH_2 by cytochrome *b559* at the PSII plastohydroquinone-binding Q_C site [38], or free $PQ^{\bullet-}$, which is produced in the comproportionation reaction (see below) of PQ with PQH_2 formed at the Q_C site during oxidation of cytochrome *b559* by the bound plastosemiquinone [2], act as the O_2 reductant.

Thus, $O^{\bullet}_2^-$ generation in PSII is possible via oxidation of pheophytin and $PQ^{\bullet-}$ at the Q_A site (and possibly at the Q_C site). However, quantitative estimates of this process available in the literature, obtained for the BBY particles (0.1 e^- (PSII × s)$^{-1}$ [15], 0.25 e^- (PSII × s)$^{-1}$ [39], 0.75 e^- (PSII × s)$^{-1}$ [16]), indicate its low efficiency.

Some estimates may even be exaggerated, as they were obtained for the BBY complexes, which retain 2 to 3 free PQ molecules per PSII reaction center, and these molecules, when reduced, can reduce $O^{\bullet}_2^-$ (see below). Additionally, in some works [15, 39], experimental conditions prevented reliable quantitative estimation of $O^{\bullet}_2^-$ generation due to the use of media with low pH values, at which the rate of spontaneous dismutation of $O^{\bullet}_2^-$ is high, and it is almost impossible to achieve saturating concentrations of the trap for $O^{\bullet}_2^-$, that would ensure registration of all $O^{\bullet}_2^-$ generated in the system [40]. In most experiments with PSII particles, O_2 was the only electron acceptor from PSII cofactors, but even in this case, the rate of $O^{\bullet}_2^-$ production was very low. Given the aforementioned evidence, it is unlikely that actual contribution of PSII to $O^{\bullet}_2^-$ production in chloroplasts is significant.

O_2 reduction in the plastoquinone pool of thylakoid membrane. The O_2-dependent oxidation of the PQ pool, observed in darkness after illumination of thylakoids [41], suggested the possibility of electron transfer from the components of this pool to O_2 molecules. Light-induced generation of $O^{\bullet}_2^-$ was demonstrated in the isolated pea thylakoids in the presence of dinitrophenyl-2-iodo-4-nitrothymol (DNP-INT), a highly effective competitive inhibitor of PQH_2 oxidation in the quinol-oxidizing site, Q_o-site, of the b_6f-complex [16, 30]. These conditions suggest that only PSII and components of the PQ pool can reduce O_2. It was shown in [16] that the BBY-particles generated $O^{\bullet}_2^-$ at a much slower rate than the thylakoids in the presence of DNP-INT (see table). This indicated that the light-induced generators of $O^{\bullet}_2^-$ in thylakoids were molecules of the PQ pool. Based on the similarity of dependencies between the increased generation of $O^{\bullet}_2^-$ in the PQ pool with increasing pH from 5.0 to 6.5 [16] and decrease in the redox potential differences between $PQ/PQ^{\bullet-}$ pair and the $O_2/O^{\bullet}_2^-$ pair (in water) to negative values in this pH range, it was suggested [3, 16] that $O^{\bullet}_2^-$ is formed in the reaction of O_2 with the molecules of free $PQ^{\bullet-}$ at the interface between the membrane and aqueous phase.

The source of free $PQ^{\bullet-}$ in thylakoids under light could be, firstly, the comproportionation reaction $PQH_2 + PQ \rightarrow 2PQ^{\bullet-} + 2H^+$. The steady-state concentration of $PQ^{\bullet-}$, produced in this reaction in the PQ pool, was calculated in [3]. It was in good agreement with the calculated concentration of $PQ^{\bullet-}$ required to ensure the rates of $O^{\bullet}_2^-$ production observed in [16]. It should be noted that in the case of $PQ^{\bullet-}$ generation in the comproportionation reaction, the maximum rates of $O^{\bullet}_2^-$ production in the PQ pool should be observed under conditions when the pool is half-reduced, while at high light intensities, when the PQ pool is almost fully reduced, the $PQ^{\bullet-}$ content significantly decreases as a result of this reaction. Secondly, free $PQ^{\bullet-}$ can also be formed as a result of PQH_2 oxidation by hydrogen peroxide and superoxide radical, which are formed both in the PQ pool [16] and at other sites of the PETC, primarily in PSI at high light intensity [42]. Thirdly, another potential source of free $PQ^{\bullet-}$ in the pool could be incomplete oxidation of PQH_2 at the quinol-oxidizing site (Q_o) of the b_6f complex, followed by the release of $PQ^{\bullet-}$ from this complex. The possibility of semiquinone leaving the quinol oxidation site in the bc_1 complex was suggested in [43].

Considering the above, it can be assumed that quantitative estimates of the PQ pool contribution obtained using inhibitors of enzymatic oxidation of PQH_2 do not completely reflect O_2 reduction in the PQ pool in chloroplasts. Firstly, nearly complete reduction of the PQ pool in the presence of DNP-INT is observed at significantly lower light intensities than in the case with the operation of the full PETC [44]. Secondly, the use of DNP-INT or another inhibitor of PQH_2 oxidation in the b_6f complex blocks the electron flow in the chain and minimizes production of $O^{\bullet}_2^-$ in the PSI, as well as in the b_6f complex (see below). Thirdly, inhibitors at saturating concentrations prevent PQH_2 oxidation at the Q_o-site and exclude the third described above possible source of free $PQ^{\bullet-}$. Therefore, it seems likely that the maximum rates of $O^{\bullet}_2^-$ production observed in the thylakoids treated with DNP-INT (see table) may be underestimated due to the use of inhibitors of enzymatic oxidation of PQH_2. It is possible that the rates of $O^{\bullet}_2^-$ production in the PQ pool of intact chloroplasts are higher than those reported in [16].

O_2 reduction in the cytochrome b_6f complex. Production of $O^{\bullet}_2^-$ was demonstrated in the isolated b_6f complexes (PQH_2-plastocyanin oxidoreductase) to which reduced decylplastoquinone and plastocyanin were added as an electron donor and acceptor, respectively [19]. Simultaneously, reduction of plastocyanin was recorded. The authors hypothesized that the most probable source of $O^{\bullet}_2^-$ in this system might be $PQ^{\bullet-}$, which forms at the Q_o site after one-electron oxidation of PQH_2 [19]. In this study, it was found that the rate of superoxide anion-radical production in the isolated b_6f complex as a percentage of the electron transport rate was nearly an order of magnitude higher than in the isolated mitochondrial bc_1 complex. It was estimated in another study [45] that at the Q_o site of the b_6f complex, E_m ($PQ/PQ^{\bullet-}$) was quite low, −280 mV, and its reaction with O_2 was thermodynamically possible.

First cofactor of the high-potential branch of the b_6f complex cofactors, Fe_2-S_2 Rieske cluster, which receives the first electron during the incipient oxidation of PQH_2 at the Q_o site, has high E_m (+330 mV) making its oxidation by O_2 thermodynamically unfavorable. Participation of the low-potential heme of cytochrome b_6 (b_6^L), the first cofactor of the low-potential branch of b_6f complex cofactors, which receives the second electron during oxidation of PQH_2 at the Q_o site and has a rather negative Em, −150 mV [46] in the $O^{\bullet}_2^-$ production

was suggested [19]. Oxidized amino acid residues were found at the Q_o site [28], indicating the possibility of $O^{\bullet}_2^-$ production there. However, precise interpretation of the reactions leading to oxidative modifications is complicated, since the b_6f complex contains a chlorophyll *a* molecule capable of producing $^1O_2$ [47], as well as Fe_2-S_2 cluster of the Rieske protein, which, like other Fe-S clusters [48], may potentially catalyze production of $HO^{\bullet}$ from H_2O_2 molecules. $^1O_2$ and $HO^{\bullet}$ have greater reactivity than $O_2^{\bullet -}$ and can modify amino acid residues more effectively than $O^{\bullet}_2^-$.

Role of ferredoxin in O_2 reduction. The stromal protein Fd contains a single Fe_2-S_2 cluster and has low E_m equal to −420 mV. In its reduced form it can effectively reduce O_2 to $O^{\bullet}_2^-$ in the aqueous phase. However, production of $O^{\bullet}_2^-$ involving Fd occurs at low rates: the first-order rate constant for oxidation of the reduced Fd by molecular O_2 is from 0.08 to 0.28 s^{-1} [21, 22, 49]. This is presumably due to the structure of its iron-sulfur active center; quinones with similar E_m values of the $Q/Q^{\bullet -}$ pair have O_2 reduction rate constants about 6 orders of magnitude higher [10]. Considering the values of the rate constants of the reaction and the ratio of Fd : PSI in the chloroplasts of higher plants [23], the rate of Fd-dependent photoreduction of O_2 in the chloroplast does not exceed 10% of the maximum rate of O_2 photoreduction in PSI (table).

Nevertheless, Fd was long considered as a primary participant in O_2 photoreduction in chloroplasts [50] based on the frequently observed significant stimulation of O_2 uptake and $O^{\bullet}_2^-$ production when Fd was added to the isolated thylakoids of spinach/pea/*Arabidopsis* deprived of stromal components during extraction [14, 51, 52]. In such experiments, the ratio of Fd to PSI was three orders of magnitude higher than *in vivo*. Due to the slow oxidation of reduced Fd, it led to significant accumulation of the reduced Fd ensuring the observed high rate of O_2 reduction. Addition of $NADP^+$, main electron acceptor from the reduced Fd, significantly decreased contribution of Fd to $O^{\bullet}_2^-$ production by the thylakoids [14, 53]. Apparently, efficiency of $NADP^+$ regeneration in the Calvin–Benson–Bassham cycle determines contribution of Fd to $O^{\bullet}_2^-$ production *in vivo*, due to the change in the number of reduced Fd molecules accessible to oxidation by oxygen.

In the literature, attempts have been made to assess involvement of Fd in the reduction of O_2 in chloroplasts (i.e., at the native Fd : PSI ratio). It was done by comparing Michaelis constants, $K_m(O_2)$, measured for the Mehler reaction in the isolated thylakoids and in the intact chloroplasts/cells/leaves. This approach has been thoroughly described in [54], and is based on the assumption that the higher $K_m(O_2)$ in more complex structures reflects involvement of multiple sites in O_2 photoreduction. The $K_m(O_2)$ values for chloroplasts and whole cells (50-95 μM) were one order of magnitude higher than the $K_m(O_2)$ for thylakoids (3-10 μM). An obvious conclusion drawn from such comparison was that Fd serves as an additional site in more complex structures. However, the situation is more complex than it seems at first glance.

Firstly, there are questions about the $K_m(O_2)$ values used when comparing different structures. The assumption that PSI is the only site of O_2 photoreduction in the conducted experiments with isolated thylakoids is likely not entirely accurate. The $K_m(O_2)$ value for O_2 photoreduction in PSI, 3 μM, obtained in one of the studies [55] was evidently underestimated due to the use of 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron donor to PSI [56] (see more details below). On the other hand, reliability of distinguishing the Mehler reaction and other O_2-consuming reactions in more complex structures including mitochondrial respiration, Rubisco oxygenase reaction (photorespiration), oxidation of the PQ-pool by plastid terminal oxidase (chlororespiration), O_2 uptake due to the production of $^1O_2$ and peroxidation of lipids, reduction of O_2 to water involving proteins containing two iron atoms in contact with the flavin group (flavodiiron proteins, Flvs or FDPs), which are absent in angiosperms, but present in cyanobacteria, green algae and other higher plants is also questionable. Thus, depending on the organism studied and conditions in which the measurements were carried out, the obtained $K_m(O_2)$ value may be associated not only with the Mehler reaction.

Secondly, in addition to the chloroplast stromal Fd, other stromal components may be involved in the direct photoreduction of O_2 *in vivo*. In the literature, there have been suggestions about the roles of nitrite reductase, Fd-reducible glutamate synthase [57], and monodehydroascorbate reductase [58] in this process. Contribution of these proteins to the light-induced O_2 reduction may affect the measured value of $K_m(O_2)$ for the intact systems, but their contribution to this process has not yet been determined due to the fact that the mentioned studies were not continued. Most likely, these enzymes could participate in O_2 reduction only under conditions of deficiency of their specific substrates [57].

Therefore, comparing $K_m(O_2)$ values for different structures with the aim of determining contribution of Fd to the reduction of O_2 in chloroplasts represents an approach that makes it difficult to draw reliable conclusions. Moreover, quantitative assessment of O_2 photoreduction pathways in the suspension of pea thylakoids in the presence of Fd showed that the increase in Fd concentration stimulated not only reduction of O_2 involving the reduced Fd, but also O_2 reduction by the membrane components [49, 53]. To explain the latter effect, it was hypothesized that the increase in the electron flow from PSI to Fd with the increase in its concentration might alter the ratio of direct electron transport pathways and charge recombination in PSI in such a way

that concentrations of the reduced forms of intermediate acceptors of this photosystem, phylloquinone in A_1 site and iron-sulfur center F_X, increases, and the electron flow from them to O_2 increases also [53]. An alternative assumption could be that Fd initiates or stimulates a certain pathway of O_2 photoreduction in thylakoids, which is inactive or minimally active in the absence of Fd. For example, such pathway could be O_2 reduction involving the membrane-bound FNR, which receives electrons from PSI only in the presence of Fd. It is known that the exogenous addition of FNR to thylakoids highly stimulates O_2 reduction [14, 58]. However, recent experimental results argue against significant participation of FNR in O_2 reduction in thylakoids [14]. In this study, production of the membrane-bound $O^{\bullet-}_2$ was measured in thylakoids from *Arabidopsis*, isolated from both wild-type plants and a mutant deficient in the FNR1 isoform [59]. This mutant is characterized by the absence of FNR in isolated thylakoids [60]. It turned out that the rates of $O^{\bullet-}_2$ production in the membrane, both in the presence and absence of Fd, were the same in both genotypes, ruling out direct involvement of FNR in the production of $O^{\bullet-}_2$ in the thylakoids from the wild-type plant.

Another site of O_2 reduction, which receives additional electrons in the presence of Fd, could be the cytochrome b_6f complex. Some authors consider this complex to be a Fd-PQ oxidoreductase that participates in the cyclic electron transport around PSI [61, 62]. Based on this model, Fd donates one electron to reduce PQ at the quinone-reducing site (Q_R) of the complex, while the second electron comes from the Q_o site. If this pathway is operating, it is possible that the presence of an electron flow from Fd to the b_6f complex could influence lifetime of $PQ^{\bullet-}$ at the Q_o site and probability of its reaction with O_2 (see above).

O_2 reduction in Photosystem 1. This photosystem has long been recognized as the principal site of $O^{\bullet-}_2$ generation during the Mehler reaction (see references in [63]), and indeed, among all PETC components, it is characterized by the highest rates of O_2 photoreduction (table). However, contradictory assessments of its activity are reported in the literature. This is most pronounced when the rate constants of the O_2 reduction reaction in PSI (k_2), published in various studies are compared. The range of k_2 values available in the literature is anomalously broad: from 7×10^2 M^{-1} s^{-1} to 10^7 M^{-1} s^{-1}.

The chronological first estimation of k_2 (10^7 M^{-1} s^{-1}) was obtained for the spinach thylakoids, in which PSI functioned in isolation (i.e., diuron was added to inhibit PSII activity, and artificial electron donors were added to reduce P^+_{700}) [55]. However, this estimation is close to the rate constant for the reduction of methyl viologen by the terminal cofactors of PSI (1.5×10^7 M^{-1} s^{-1}; [64]), which suggests similar efficiency of O_2 and methyl viologen as immediate electron acceptors from PSI. This is unlikely, since methyl viologen significantly enhances the electron flow "through" PSI [42, 56]. The value of 10^7 M^{-1} s^{-1} is likely an overestimation, probably due to the use of reduced DCPIP as an electron donor for P^+_{700} in the cited study [55]. It has been demonstrated with the isolated PSI complexes from Synechocystis and pea thylakoids that DCPIP acts as a redox mediator between PSI and O_2, similar to methyl viologen. That is, the singly reduced form of DCPIP on the acceptor side of PSI is effectively oxidized by oxygen [56, 65]. Therefore, estimations of k_2 and other characteristics of the PSI reaction with O_2 (for example, $Km(O_2)$) using DCPIP are erroneous, as they reflect the sum of reactions of O_2 photoreduction by PSI cofactors and reduced DCPIP.

The lowest values for k_2, 7.2×10^2 and 6.1×10^3 M^{-1} s^{-1}, presented in the study [66], were calculated from the experimental data with pea thylakoids [42]. However, the O_2 reduction rates in [42] were measured at atmospheric O_2 content, which is a saturating concentration for the reaction of O_2 reduction in PSI, whereas in order to correctly estimate the rate constant of the reaction, the use of the substrate at rate-limiting concentrations is required, i.e., in this case, when the oxygen reduction rate is dependent on its concentration. This is exactly how the measurements were conducted in the studies [20, 55, 67].

The most recently reported k_2 values, ranging from 0.6×10^5 to 3.7×10^5 M^{-1} s^{-1} (depending on light intensity; see below), were obtained using the natural electron donor for PSI, plastocyanin [20]. Moreover, these values were also obtained in the presence of Fd, FNR, and $NADP^+$, when the terminal acceptors of PSI reduced O_2 concurrently with photoreduction of Fd with subsequent electron flow to $NADP^+$, i.e., under conditions close to physiological. In the absence of Fd, the k_2 values did not change significantly. Thus, the k_2 values as well as the rate of O_2 reduction measured at atmospheric O_2 content presented in the study [20] are the closest characteristics of O_2 reduction in PSI *in vivo*.

Which PSI cofactors can reduce O_2? For a long time, it has been believed that electrons are transferred to O_2 from the terminal cofactors of PSI, Fe_4-S_4 clusters F_A/F_B, located at the PsaC subunit on the stromal side of the PSI complex [9, 64]. Later, it became clear that the intermediate cofactors of PSI electron flow also contribute to $O^{\bullet-}_2$ production. It was, in particular, demonstrated in the study of light-induced H_2O_2-dependent iodination of the thylakoid membrane proteins that during the first seconds of illumination, O_2 reduction is carried out by the cofactors situated at the PsaA and PsaB proteins, while longer illumination leads to the appearance of H_2O_2 in other parts of the thylakoids, including the protein area near F_A/F_B [11]. The authors suggested that O_2 is reduced by the cluster preceding the F_A/F_B clusters in the electron flow chain, the F_X cluster, located between the PsaA and PsaB subunits.

A hypothesis has been proposed regarding participation of the phylloquinone molecules, PhQ [68], a secondary cofactor of electron transfer, in O_2 reduction. These are located in the A_1 sites of the two pseudo-symmetrical branches of cofactors in PSI, A and B, and precede the F_X cluster. In that study, thylakoid membranes were treated with hexane, which led to extraction of all PQ pool molecules and one PhQ molecule from the PSI located in the A branch, PhQ_A. Such membranes did not demonstrate O_2 uptake in response to light flashes. Addition of PhQ in the form of vitamin K led to reappearance of O_2 uptake, but only in response to the first light flash. The authors suggested that the hexane treatment modified the A_1 site in such a way that its affinity for PhQ decreased [68].

Reduction of O_2 with participation of PhQ in the native PSI complexes under steady-state illumination was first studied using complexes isolated from the cyanobacterium *Synechocystis* sp. PCC 6803 [69], assuming that both the composition of PSI electron transfer cofactors and amino acid environment in the A_1 site are relatively conserved among cyanobacteria, algae, and higher plants. The study used a wild type strain and a strain with blocked PhQ biosynthesis (*menB* mutation). It was previously shown that PQ molecules were incorporated into the A_1 sites of the mutant causing increase in E_m ($Q/Q^{\bullet-}$) by ~100 mV relative to E_m in the wild type strain. It also led to a 1000-fold increase in the lifetime of semiquinone in both branches [70]. The PSI complexes from the mutant showed significantly lower rates of O_2 photoreduction compared to the complexes from the wild type strain [69], which was explained by the greater ability of $PhQ^{\bullet-}$ in the A_1 sites of the wild type to reduce O_2 compared to $PQ^{\bullet-}$ in the mutant sites.

Contribution of the individual PSI electron transport cofactors was revealed through investigation of the influence of light intensity on the k_2 value for the PSI complex, isolated from the unicellular alga *Chlamydomonas reinhardtii* [20]. Increase in the apparent value of k_2 with the increased light intensity was observed, and this was interpreted as evidence of several O_2 photoreduction sites operating in PSI, each characterized by its own rate constant for this process and achieving maximum efficiency at the specific for this site light intensity. Experimental analysis using methyl viologen, a highly effective acceptor of electrons from the terminal cofactors of PSI, demonstrated that involvement of the terminal cofactors F_A/F_B in O_2 reduction reaches its maximum at low light intensity, at which the F_A/F_B clusters are saturated with electrons. The apparent k_2 increase with the increased light intensity is likely associated with the increase in contribution to O_2 reduction from the preceding electron transport cofactors in PSI, when they are saturated with electrons. The roles of F_X and PhQ were elucidated using sequential removal of Fe_4-S_4 clusters through specific treatments: the removal of F_A/F_B led to a slight decrease in the rate of O_2 reduction across a wide range of light intensities, while additional removal of F_X, resulting in PhQ in the A_1 sites becoming the terminal cofactor, led to significant stimulation of O_2 reduction. The latter is in agreement with the hypothesis of PhQ playing a key role in O_2 reduction. PSI complexes, isolated from the mutant PsaA-F689N *C. reinhardtii*, in which Phe at position 689 of the PsaA protein was replaced by Asn, thereby increasing lifetime of $PhQ^{\bullet-}_A$ from 0.25 μs to 17 μs [71], exhibited much higher rates of O_2 photoreduction across a wide range of light intensities [20]. These data also indicate increase in the contribution of $PhQ^{\bullet-}$ to the generation of $O^{\bullet-}_2$ in PSI with increasing light intensity.

In the isolated PSI complexes, two O_2 reduction sites are active: the terminal F_A/F_B clusters and PhQ in the form of semiquinones at A_1 sites. Contribution of each site depends on the conditions. At low light intensity, the rate of O_2 photoreduction decreases in the presence of Fd, FNR, and $NADP^+$, as the electron flow from PSI diminishes accumulation of electrons on the F_A/F_B clusters [20]. At the same time, presence of Fd, FNR, and $NADP^+$ did not suppress O_2 photoreduction observed at high light intensity indicating that PhQ is responsible for O_2 reduction under conditions of parallel electron transport to $NADP^+$ in this circumstance.

Surprisingly, the approach based on detection of the oxidized amino acid residues, which was successfully applied to determine the $O^{\bullet-}_2$-generating activity of PSII and b_6f complex cofactors (see above), proved to be unsuitable for visualizing $O^{\bullet-}_2$ production in PSI. The oxidized residues were not found in close proximity of the F_A/F_B clusters in spinach PSI complexes grown under field conditions [72]. Also, no modified residues were found in the immediate vicinity of the F_X cluster and PhQ_A [72]. Conversely, two modified residues were detected near the PhQ in the B-branch (PhQ_B). However, interpretation of these results is complicated due to location of the chlorin ring in the chlorophyll *a* molecule between PhQ_B and these residues [72]. It could be hypothesized that the $O^{\bullet-}_2$ produced by the F_A/F_B clusters easily diffuses from the PsaC protein into the stroma (the F_B cluster is located 3-4 Å from the surface of PsaC) and does not modify amino acid residues. Possibly, $O^{\bullet-}_2$ from the A_1 sites also efficiently diffuses to the stromal side of the membrane, not reacting with the adjacent amino acid residues, since existence of the water-filled cavities leading from the A_1 sites was demonstrated for PSI from the cyanobacterium *Synechocystis* sp. PCC 6803 [73].

It should be noted that the rates of $O^{\bullet-}_2$ generation in the isolated PSI complexes (table) may not fully reflect actual $O^{\bullet-}_2$-generating activity of PSI in thylakoids and chloroplasts. O_2 reduction in the isolated PSI complexes from *Synechocystis* [69] and *C. reinhardtii* [20] did not reach saturation with increasing light intensity across

a wide range of light intensities (up to 2000 μmol photons m^{-2} s^{-1}), while O_2 reduction by the isolated, functionally active PSI in higher plant thylakoids (in the presence of diuron and artificial electron donors) tended to saturate at 500-600 μmol photons m^{-2} s^{-1} [42, 65]. On the one hand, this discrepancy might be due to introduction of the redox mediators such as *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) and sodium ascorbate to maintain PSI activity in the thylakoids. The oxidized forms of these compounds could accept electrons from the terminal cofactors of PSI [74-76], which should reduce accumulation of electrons on the PSI cofactors. On the other hand, the isolated PSI complexes might lack certain regulatory components that determine $O^{\bullet}_2^-$ generating activity of PSI in the thylakoids. In particular, existence of a protein that regulates O_2 reduction in PSI was postulated based on the comparison of the effects of short-day and long-day conditions on the Mehler reaction in tobacco plants [77]. It turned out that the short-day conditions favor higher rate of the PSI-dependent photoreduction of O_2 in thylakoids and leaves. The authors suggested that a certain protein binds to PSI under the short-day conditions and facilitates diffusion of O_2 to the site of its photoreduction, stimulating generation of $O^{\bullet}_2^-$. It has also been shown that the PsaE subunit, which together with the PsaC and PsaD subunits forms a docking site for Fd, could determine the degree of electron leakage to O_2 [78].

The study [77] suggested that FNR might be the hypothetical protein regulating photoreduction of O_2 in PSI. It has been shown that FNR and PSI, isolated from *C. reinhardtii*, interact with each other in a 1 : 1 stoichiometry, particularly involving the PsaE subunit [79], and the authors suggested that FNR might function as a subunit of PSI. FNR binding to PSI via the PsaE subunit was also demonstrated for barley [80]. In higher plants, the main proteins binding FNR are TROL and Tic62 [81, 82]. However, if the interaction with Tic62 and TROL is disrupted, FNR can interact with alternative, weaker binding sites on the thylakoid membrane, including PSI [60]. It is possible that the attachment of FNR to PSI could affect diffusion of O_2 to the cofactors of PSI and/or initiate redistribution of the O_2 reduction pathways within PSI.

EVOLUTIONARY ASPECTS

Effective electron transfer from PETC components to O_2 leads to excessive production of such reactive oxygen species as $O^{\bullet}_2^-$, H_2O_2, $HO^{\bullet}$, and also reduces quantum yield of the light reactions of photosynthesis. The PETC of oxygenic phototrophs evolved into its current form under conditions of ongoing O_2 production in light. It is logical to assume that minimizing the reactions of PETC components with O_2 was one of the directions in the evolution of photosynthetic apparatus. Evolution of various photosynthetic complexes as they adapted to an emergence of oxygen-rich atmosphere has been addressed in several recent reviews [6, 83-86]. These studies particularly emphasize evolutionary strategies that the photosynthetic apparatus might have developed to minimize formation of singlet oxygen ($^1O_2$), which definitely was among the evolutionary trajectories of these systems. In this review, we considered changes that could have occurred in the photosynthetic apparatus to minimize non-productive electron leakage to O_2 and excessive $O^{\bullet}_2^-$ production.

In our opinion, evolutionary changes should first affect the pools of mobile carriers, as their reduced state is necessary for productive electron transport. The E_m values of plastocyanin and cytochrome c_6, electron donors to PSI, are high enough to exclude the possibility of their reaction with O_2. However, Fd and the components of the PQ-pool, even in the modern PETCs possess low enough potentials for $O^{\bullet}_2^-$ generation, which has been experimentally observed (see above).

Final product of the light stages of photosynthesis, reduced Fd, serves as an electron donor not only for $NADP^+$ reduction but also for other metabolic pathways in the chloroplast [87]. To implement these pathways, the reduced Fd must diffuse in the stroma of the chloroplast. Obviously, this requires low efficiency of the Fd reaction with O_2. In modern phototrophs with oxygenic photosynthesis, prosthetic group of Fd is the Fe_2-S_2 cluster, which is sufficiently deeply embedded in the protein. In modern phototrophs with anoxygenic photosynthesis, which have type I reaction centers, the role of electron acceptor is performed by Fd with two Fe_4-S_4 clusters, one of which is located almost on the surface of the protein [83], making the cluster accessible to O_2 molecules. It has been suggested [83, 84] that embedding the cluster deeply within the protein restricts access of O_2 molecules to it and reduces the likelihood of electron transfer to O_2. Thus, replacement of the dicluster Fe_4-S_4·Fd with a monocluster Fe_2-S_2·Fd, with a relatively deeply embedded cluster in the protein, during the evolution of phototrophs could have been driven by adaptation to functioning in the presence of O_2.

In the modern PETC, dicluster Fd has been preserved in the form of the PsaC subunit carrying two Fe_4-S_4 clusters in the form of cofactors of PSI, intermediate F_A and terminal F_B, which reduce the mobile monocluster Fd. Rapid efflux of electrons from F_B to O_2 is undesirable, as it could reduce efficiency of Fd reduction, which depends on the diffusion exchange of the reduced Fd for the oxidized Fd on the acceptor side of PSI. A higher E_m (F_A/F_A^-) compared to E_m (F_B/F_B^-) ensures longer residence time of the electron on F_A than on F_B [88, 89]. Thus, inversion of the redox potentials of F_A and F_B could be an evolutionary adaptation to minimize reduction of O_2 by the F_B cofactor in the

absence of Fd [83, 84]. The F_A cofactor is embedded in the protein and located in an area with sufficiently low dielectric permeability [90]. Raising E_m (F_A/F_A^-) also reduces thermodynamic probability of its reaction with O_2.

In the energy-converting membranes of the very first organisms, menaquinone likely functioned as a liposoluble mobile carrier of protons and electrons, which to this day is present in a number of anaerobic phototrophs [91]. Redox potentials of the pairs ($Q/Q^{\cdot-}$) and (Q/QH_2) of menaquinone are approximately 100 and 180 mV lower, respectively, than the corresponding pairs of PQ, i.e., the reduced forms of menaquinone are significantly easier can be oxidized by O_2 than the forms of PQ. Therefore, in the presence of O_2, menaquinones are less efficient as electron carriers from the type II reaction centers to the *bc*-type cytochrome complexes than such high-potential quinones as PQ or ubiquinone. Thus, the evolutionary replacement of menaquinone with PQ, a quinone with a more positive E_m value, in the membranes of organisms with an oxygenic type of photosynthesis appears to be an important step in optimizing photosynthetic apparatus to the conditions of an oxygen atmosphere.

However, $PQ^{\cdot-}$ has an E_m allowing it to reduce O_2 molecules to form $O_2^{\cdot-}$ in aqueous environment (see above), albeit at low rates. Semiquinones generally react with O_2 molecules in a kinetically efficient manner [10]. In the PETC, there are several sites where PQ is consecutively reduced to PQH_2, and where PQH_2 is oxidized to PQ, forming in both cases an intermediate semiquinone form. Apart from $PQ^{\cdot-}$, the semiquinone form of the isoalloxazine portion of FAD is also transiently formed in FNR during sequential oxidation of two molecules of Fd and during reduction of $NADP^+$. An effective oxidation of these cofactor semiquinone forms by oxygen at the moment when they should receive or give the second electron could disrupt normal electron transfer. Therefore, in our view, the next global trend in adaptation of photosynthetic apparatus to operate in the presence of O_2 was to prevent O_2 reactions with the intermediate semiquinone forms of PETC components during their two-step reduction or oxidation.

The semiquinone form of FAD reacts with O_2 with high efficiency [92]. Possible mechanism for preventing oxidation of the semiquinone form of FAD by oxygen is discussed in the review [85]. It has been noted that the FNR of the phototrophs with oxygenic type of photosynthesis has two orders of magnitude greater catalytic activity than the FNR of anaerobic organisms [93], even though affinity of FNR for Fd may be similar [94]. High catalytic activity is achieved due to conformational changes induced by $NADP^+$ binding, which significantly enhance both oxidation of Fd [95] and dissociation of the oxidized Fd molecules from the complex with FNR [96]. Such increase in the FNR catalytic activity likely reduces the probability of both oxidation of the semiquinone form of FAD by oxygen and formation of the Fd/FNR^- complex in the absence of $NADP^+$.

$PQ^{\cdot-}$ is formed during the single reduction of PQ at the Q_B site of PSII and the Q_R site of the b_6f complex. Apparently, at the Q_B site of PSII, the issue of decreasing electron leakage to O_2 molecules is addressed on a thermodynamic level due to the high E_m ($Q_B/Q_B^{\cdot-}$), +90 mV [33], as a result of which the $Q_B^{\cdot-}$ reaction with O_2 is thermodynamically unfavorable even in aqueous phase. No $PQ^{\cdot-}$ generation has been demonstrated at the Q_R site of the b_6f complex, although semiquinone appearance was recorded at the similar site of the bc_1 complexes of purple bacteria [97] and mitochondria [98]. One of the key differences between the bc_1 and b_6f complexes is the presence of an additional heme in the cytochrome b_6 – covalently linked heme c_n. It has been suggested that, as part of the Q-cycle operation, the first of two electrons needed to reduce PQ at the Q_R site of the b_6f complex is transferred from the high-potential heme of the cytochrome b_6 (b_6^H) to the heme c_n [99], and from the heme c_n, an electron is transferred to the PQ molecule only simultaneously with the transfer of the second electron from the heme b_6^H [7]. Such mechanism minimizes lifetime of $PQ^{\cdot-}$ at the Q_R site and, accordingly, reduces the probability of its reaction with O_2.

At the Q_o site of the b_6f complex, it is believed that PQH_2 is sequentially oxidized to PQ through a concerted mechanism, i.e., successive acts of electron transfer to the Fe_2-S_2 Rieske center and to the low-potential heme b_6^L. Efficient operation of the Q-cycle ensures efficient outflow of electrons from $PQ^{\cdot-}$ at the Q_o site along the low-potential branch of b_6f complex cofactors. However, if there are few oxidized PQ molecules in the PQ pool, or if the heme b_6^L in the b_6f complex is already reduced, a reverse electron transfer could occur from the heme b_6^L (E_m (b_6^L/b_6^{L-}) = –150 mV) to $PQ^{\cdot-}$ (E_m ($PQ^{\cdot-}/PQH_2$) = +480 mV) [7]. This reduces the probability of reaction between O_2 and either heme b_6^L, or $PQ^{\cdot-}$ at the Q_o site. Disruption of the concerted oxidation could also occur under conditions of photosynthetic control, when the proton release from the Q_o site to the lumen slows down, and $PQH^{\cdot}$ cannot be deprotonated by the amino acid residue Glu78 of the subunit IV, which remains in a protonated state, as was recently hypothesized [100]. Since E_m ($PQ/PQH^{\cdot}$) is higher than E_m ($PQ/PQ^{\cdot-}$), electron leakage from the protonated plastosemiquinone to O_2 is less probable. We suggest that the retardation of proton removal from the Q_o site and increase in the lifetime of $PQH^{\cdot}$ by maintaining Glu78 in a protonated state comprise an important mechanism for preventing the $PQ^{\cdot-}$ reaction with O_2 at the Q_o site.

Replacing of the free menaquinone in the membrane pool with the higher potential quinone resulted in the increase of E_m (by about 110-150 mV) of all tightly bound cofactors in the partner proteins of the mobile membrane carrier pool, i.e., in the type II reaction

center and cytochrome bc_1 complex [101, 102]. This, in turn, led to the decrease in the probability of these cofactors being oxidized by O_2 molecules. Apparently, increase in E_m was enough to solve the problem of electron leakage to O_2 in the cytochrome b_6f complex. Among all its tightly bound cofactors, heme b_6^L has the lowest E_m value, −150 mV [103], which is insufficient to reduce O_2 in the hydrophobic regions of the membrane. Presence of an electron on the heme c_n should support efficient outflow of electrons from the heme b_6^L (see above) minimizing its reactions with O_2.

However, for PSII, increasing E_m may not be enough to minimize oxidation of its cofactors by oxygen. The tightly bound PQ at the Q_A site under normal operating conditions is singly reduced. Efficiency of the productive electron transfer from $PQ^{\bullet-}$ at the Q_A site to PQ at the Q_B site is determined by the presence of HCO_3^- near the non-heme iron, which reduces E_m (Q_A/Q_A^-) from −70 mV to −145 mV as shown in the study [31]. However, it has been shown in another work that HCO_3^- on the acceptor side of PSII blocks the potential channel through which O_2 molecules could diffuse to the Q_A site, thereby limiting access of O_2 molecules to the site [15], which reduces formation of $O^{\bullet}_2^-$.

Pheophytin has a sufficiently low E_m (−600 mV) to reduce O_2 even in hydrophobic parts of the protein. However, suppression of the non-productive electron leakage to O_2 is achieved at the kinetic level: short lifetime of the reduced pheophytin due to electron transfer to Q_A (200-500 ps) and recombination of the reduced pheophytin with P_{680}^+ (4-30 ns), apparently, significantly reduces the possibility of this cofactor reacting with O_2.

In the type I reaction centers of modern anaerobic organisms, there are no tightly bound quinones [104-106] and, in heliobacteria in particular, menaquinones function as a mobile, lipid-soluble electron acceptors, an alternative to ferredoxin [107]. Presence of two acceptor pools could be advantageous in terms of the efficiency of photosynthetic reactions and protection of the photosynthetic apparatus from excessive illumination. In PSI (a type I reaction center, inherent exclusively to phototrophs with oxygenic photosynthesis) menaquinone (or more accurately, in most organisms, its derivative – PhQ) remains a tightly bound cofactor serving as a single-electron carrier, i.e., it does not get reduced to hydroquinone [108]. Unlike electron transfer between Q_A and Q_B in PSII, stoichiometry of which is 1 : 1, in PSI, the quinones of the two A_1 sites transfer an electron to one Fe_4-S_4 cluster F_X. Under conditions of increased illumination and limited electron flow from the stromal acceptors of PSI, a situation may occur when the iron-sulfur clusters of PSI will be predominantly reduced, and the two A_1 sites will compete for one F_X cluster. In this case, there may be a risk of charge recombination of quinones with P_{700}^+, including by a mechanism leading to formation of $^3P_{700}$, as a result of which the risk of generating $^1O_2$ [84] would increase. Oxidation of $PhQ^{\bullet-}$ by O_2 in this case would be a potentially less dangerous process, decreasing over-reduction of the ETC and lowering the risks of generating $^1O_2$. Similar processes could occur in the Q_A site in PSII. However, given that E_m ($PhQ/PhQ^{\bullet-}$) is much more negative than E_m ($PQ/PQ^{\bullet-}$), it is significantly more challenging to minimize electron leakage from $PhQ^{\bullet-}$ to O_2 compared to $PQ^{\bullet-}$. At the same time, maintaining low E_m values for PSI cofactors is necessary to reduce such a low-potential electron carrier as ferredoxin.

Kinetic control (i.e., rapid electron transfer to the next cofactor in the chain) likely also cannot be fully implemented in PSI, where two PhQ are present, differing in E_m by 170 mV [90] and in the lifetime of the semiquinone form by one order of magnitude [109]. Two PhQ under steady-state illumination conditions could compete for one F_X cluster, which increases probability of the electron leakage to O_2 from the longer-lived $PhQ^{\bullet-}$ in the A-branch. Limiting of the O_2 accessibility to PhQ in the A_1 sites seems to be only marginally feasible evolutionary strategy. Unlike PSII, PSI does not have channels for either influx/efflux of the reaction substrates/products, or for bicarbonate ions. In the study modeling the structure of cyanobacteria PSI, presence of aqueous cavities connecting the A_1 sites to the acceptor side has been suggested [73] through which not only O_2 molecules are assumed to diffuse, but also much larger molecules such as methyl viologen.

Thus, we suggest that the reaction of O_2 with PhQ in the A_1 sites could not have been minimized sufficiently, and to this day, among all components of the PETC, the highest rates of $O^{\bullet}_2^-$ production are characteristic for PSI due to the presence of PhQ in it. Under conditions of over-reduction of the chain, when the intensity of light exceeds the capabilities of metabolic utilization of light energy, O_2 becomes an available additional electron acceptor, capable of sustaining electron transport and minimizing over-reduction of the PETC components, thereby mitigating photoinhibition. Therefore, it is likely that evolution of the photosynthetic apparatus proceeded not merely towards minimizing electron transfer to O_2, but towards regulating this process.

CONCLUSION

The above analysis considered potential mechanisms for the reduction of O_2 to $O^{\bullet}_2^-$ by various components of the PETC, as well as evolutionary transformations that the PETC might have undergone to reduce non-productive electron leakage to O_2. The table summarizes estimates of $O^{\bullet}_2^-$ generation rates at different segments of the PETC available in the literature. The table also includes rates normalized to the content of PSII, allowing comparison of the potential contributions

of different components to the production of $O^{\bullet}_{2}^{-}$ in chloroplasts.

However, such comparison requires consideration of the conditions under which these rates were measured. Firstly, the rates presented in the table were obtained using various experimental approaches under different conditions and for different organisms. Secondly, many of these rates were obtained for the isolated structures (for PSII, PSI, b_6f-complex) or in the presence of inhibitors (for the PQ-pool). Consequently, interrelations between the PETC components could have been altered and some components that are present in whole membranes might have been absent. Thirdly, in some cases (PSII, PQ-pool), O_2 served as the only available electron acceptor. It is difficult to definitively assess how this could affect the probability of $O^{\bullet}_{2}^{-}$ generation in these cases. For example, presence of $NADP^+$ reduces $O^{\bullet}_{2}^{-}$ production by the reduced Fd [53], whereas presence of Fd and $NADP^+$ does not significantly affect $O^{\bullet}_{2}^{-}$ production in PSI [20]. It should also be noted that normalization presented in the table is based on the relative content of the individual pigment–protein complexes in a specific organism (*Arabidopsis thaliana*) under specific conditions [24]. The content of PSII, b_6f-complex, PSI, and relative size of the photoactive PQ-pool, and their ratio, vary in plants depending on environmental conditions [110, 111], and relative contribution of these components to $O^{\bullet}_{2}^{-}$ production, evidently, could also vary.

Despite these limitations, comparison of the rates presented in the table provides a useful model and approximation for the comprehensive description of O_2 reduction in PETC. It is clear that contribution of PSII is the smallest, but even Fd, previously considered as the primary reducer of O_2 (see above), generates $O^{\bullet}_{2}^{-}$ at approximately the same rates as PSII. It should be noted that calculation of rates for Fd is based on the assumption that the reduced Fd is accumulated in the chloroplasts, and therefore the rates are close to maximum. The PQ-pool and the cytochrome b_6f-complex contribute nearly equally to the total rate of O_2 photoreduction in chloroplasts. Contribution of PSI depends on light intensity, and under high light intensity, PSI produces approximately half of all $O^{\bullet}_{2}^{-}$ generated in this PETC model.

PSI is commonly regarded as the main site of O_2 reduction in the PETC. This notion is supported by several indirect pieces of evidence (though these could also be interpreted in favor of other segments of PETC) and by a number of experimental results based on the use of mutants or inhibitors, which also allows for ambiguous interpretation. Our analysis shows that no single component of the PETC can generate $O^{\bullet}_{2}^{-}$ as effectively as PSI under high light intensity. But from these data, it is also clear that the components of the PETC without PSI can collectively generate $O^{\bullet}_{2}^{-}$ at the rate comparable to that in PSI. Therefore, while it is reasonable to consider PSI the place where $O^{\bullet}_{2}^{-}$ is produced at the highest rates under illumination, it may not be appropriate to consider PSI as the dominant site of its production under all circumstances.

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