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Oxygenic photosynthesis: EPR study of photosynthetic electron transport and oxygen-exchange, an overview

Alexander N. Tikhonov^{1,*} and Witold K. Subczynski²

¹Department of Biophysics, Faculty of Physics, M. V. Lomonosov Moscow State University, Moscow, Russia

²Department of Biophysics, Medical College of Wisconsin, Milwaukee, USA

Abstract

In this review, we consider the applications of electron paramagnetic resonance (EPR) methods to the study of the relationships between the electron transport and oxygen-exchange processes in photosynthetic systems of oxygenic type. One of the purposes of this article is to encourage scientists to use the advantageous EPR oximetry approaches to study oxygen-related electron transport processes in photosynthetic systems. The structural organization of the photosynthetic electron transfer chain and the EPR approaches to the measurements of molecular oxygen (O₂) with O₂-sensitive species (nitroxide spin labels and solid paramagnetic particles) are briefly reviewed. In solution, the collision of O₂ with spin probes causes the broadening of their EPR spectra and the reduction of their spin-lattice relaxation times. Based on these effects, tools for measuring O₂ concentration and O₂ diffusion in biological systems have been developed. These methods, named “spin-label oximetry,” include not only nitroxide spin labels, but also other stable free radicals with narrow EPR lines, as well as particulate probes with EPR spectra sensitive to molecular oxygen (lithium phthalocyanine, coals, and India ink). Applications of EPR approaches for measuring O₂ evolution and consumption are illustrated using examples of photosynthetic systems of oxygenic type, chloroplasts *in situ* (green leaves), and cyanobacteria.

Keywords

Photosynthesis; Chloroplasts; Cyanobacteria; Electron transport; EPR; Spin-label oximetry; Oxygen exchange

1. Introduction: oxygenic photosynthesis

Oxygenic photosynthesis is one of the most important biological processes. Thanks to oxygenic photosynthetic organisms (cyanobacteria, green algae, and higher plants), the earth's atmosphere is filled with molecular oxygen (O₂). The molecular structure of the photosynthetic apparatus (PSA) is well known (for review, see [1–12]). In plant cells, the PSA is localized in the energy transducing organelles, termed chloroplasts, in which numerous photosynthesis reactions occur. Two pigment-protein complexes, Photosystem I

*Corresponding Author: Alexander N. Tikhonov, Department of Biophysics, Faculty of Physics, M. V. Lomonosov Moscow State University, Moscow, 119899, Russia, Tel: +7(495) 939-2973; an_tikhonov@physics.msu.ru; an_tikhonov@mail.ru.

(PSI) and Photosystem II (PSII), are embedded into the membranes, which form closed flattened vesicles termed *thylakoids* (see sketch of chloroplasts in Fig. 1a). In oxygenic photosynthetic systems, the energy of light quanta absorbed by the light-harvesting antenna is used to assimilate the atmospheric carbon dioxide (CO₂), providing synthesis of organic products and evolution of O₂ from water molecules split by the water-oxidizing complex of PSII. Operating in tandem, PSII and PSI complexes drive electron transfer from the H₂O molecule, oxidized by PSII, to NADP⁺, the terminal electron acceptor of PSI. The light-induced electron transfer between spatially separated PSII and PSI complexes occurs with the help of the mobile electron carriers, plastoquinone (PQ) and plastocyanin (Pc), through the membrane-bound cytochrome (Cyt) *b₆f* complex: H₂O → PSII → PQ → *b₆f* → Pc → PSI → NADPH⁺. In cyanobacteria, electron transfer between the Cyt *b₆f* complex and PSI is mediated by Cyt *c₆*. Photosynthetic electron transport provides the generation of the *trans*-membrane difference in electrochemical potentials of protons (P₇₀₀), which serves as the driving force for operation of the membrane-bound ATP synthase (ADP + P_i → ATP + H₂O) [13, 14]. Reduced NADP molecules (NADPH) and ATP are used mainly to provide the reduction of CO₂ and formation of organic compounds in the Calvin-Benson cycle (CBC) reactions of photosynthesis. The arrangement of electron transport and ATP synthase (CF₀–CF₁) complexes in the thylakoid membrane is depicted schematically in Fig. 1b.

The oxidized NADP molecule (NADP⁺) serves as a physiological acceptor of electrons donated by PSI. Along with the mainstream electron flux to NADP⁺, termed *non-cyclic* electron flow (H₂O → PSII → PSI → NADP⁺), there are alternative pathways: *cyclic* electron transport around PSI [15, 16] and *pseudo-cyclic* electron transport (also named the “water-water” cycle, H₂O → PSII → PSI → O₂ → H₂O [17]). Numerous works in the field of photosynthesis have revealed different routes of electron transfer associated with the light-induced reduction of molecular oxygen (for review, see [17–23]). In addition to the “water-water” cycle, O₂ is involved in other metabolic processes in photosynthetic organisms (for instance, respiration of plant cell mitochondria and cyanobacteria) [18–21]. In oxygenic photosynthetic systems, molecular oxygen evolves due to the splitting of H₂O molecules in PSII. On the other hand, O₂ molecules are consumed due to their reduction by the electron transport chain (ETC). In chloroplasts, O₂ can accept an electron: (i) at the acceptor side of PSI [17, 18] (shown as pathway 1 in Fig. 1b), (ii) due to the oxygenase reaction catalyzed by ribulose-diphosphate-carboxylase (Rubisco, [19]) (pathway 2), and (iii) due to the O₂ reduction by the plastoquinol terminal oxidase (PTOX [20–22]) (pathway 3). Alternative routes of electron transport, associated with the O₂ reduction by the electron transport chain, allow photosynthetic organisms to show the flexibility to cope with variable environmental conditions and survive under solar stress. Variations of O₂ concentration in the atmosphere can significantly influence the functioning of photosynthetic organisms [24, 25].

The main purpose of this article is to summarize information about use of the electron paramagnetic resonance (EPR) approaches to study the interplay between electron transport and oxygen-exchange processes in oxygenic photosynthetic systems. The EPR technique allows an experimenter to monitor the electron transport and oxygen-exchange processes under the same experimental conditions. One of the advantages of using this technique is that it allows the noninvasive monitoring of the oxygen-exchange processes in native

photosynthetic systems (*e.g.*, in cuttings of green leaves [24–27]). Our paper is structured as follows: We start by considering of some pictorial examples of EPR applications to register electron transport in chloroplasts *in situ* (green leaves) and in cyanobacterial cells that demonstrate the important role of O₂ in regulation of oxygenic photosynthesis. Then, we briefly outline a variety of the EPR approaches to measure the content of O₂ with different kinds of O₂ sensors (nitroxide spin labels, microscopic probes, and solid particles). After summarizing the results of these studies, we conclude that under the normal physiological conditions oxygen-exchange reactions in photosynthetic systems of oxygenic type are well-balanced, providing the electron transport chain an efficient and safe mode functioning.

2. Photosynthetic electron transport in chloroplasts

2.1. EPR measurements of P₇₀₀ redox transients *in situ*

The EPR method provides convenient means for monitoring the redox state of P₇₀₀ (the primary electron donor in PSI) in plant leaves, because oxidized centers P₇₀₀⁺ give a characteristic light-induced EPR signal (Fig. 2a) [28]. Figure 2b shows the time-course of P₇₀₀ photooxidation in dark-adapted (5 min) *Hibiscus rosa-sinensis* leaves. In response to the far-red light (FRL, $\lambda_{\text{max}} = 707$ nm) preferentially exciting PSI, reaction centers P₇₀₀ become oxidized (the O-A rise of the EPR signal given by P₇₀₀⁺). After turning on the white light, which excites both PSI and PSII, a concentration of P₇₀₀⁺ changes nonmonotonously: after the initial drop (phase A-B), the level of P₇₀₀⁺ gradually grows toward a steady-state level (the B-C-D-E phase). Such a multiphase behavior of P₇₀₀⁺ is explained by the light-induced regulatory processes that take place during the illumination of dark-adapted leaves (for review, see [29–31]). Some other examples, illustrating the influence of pre-illumination history on the induction of P₇₀₀ in higher plants, can be found in [32–34]. The basic feedback that determines the nonmonotonous kinetics of P₇₀₀ oxidation follows: (i) the light-induced activation of the CBC, which accelerates the outflow of electrons from PSI [35–38], (ii) pH-dependent attenuation of PSII activity caused by non-photochemical quenching of Chl *a* excitation in the light-harvesting antenna of PSII [39, 40], (iii) deceleration of PQH₂ oxidation within the Cyt *b₆f* complex [41–43], (iv) the light-induced redistribution of light quanta between PSI and PSII [41], and (v) redistribution of electron fluxes between the alternative pathways of electron transport [32–34, 44–47].

The light-induced modulation of the CBC activity is one of the crucial factors of electron transport control in chloroplasts. In dark-adapted chloroplasts, the key enzymes of the CBC are inactive; therefore, the outflow of electrons from PSI to the CBC should be limited. Illumination of chloroplasts activates the CBC reactions. At high rates of CO₂ assimilation in the CBC, the consumption of NADPH accelerates. This facilitates the outflow of electrons from PSI, which is one of the reasons for a gradual increase in P₇₀₀ during illumination (the B-C-D-E phase, Fig. 2, curve 1). Molecular oxygen serves as an alternative electron acceptor (the Mehler reaction; for review, see [17]), which releases the electron outflow limitations from PSI. This can be illustrated by experiments with the injection into the leaf of the artificial redox-mediator methylviologen (MV), which efficiently accepts an electron from PSI and then donate it to O₂ [23, 26, 48]. As one can see from Fig. 2b (curve 2),

administration of MV into the leaf accelerates the re-oxidation of P_{700} during illumination. Acting as an efficient mediator of electron transfer from PSI to O_2 ($PSI \rightarrow MV \rightarrow O_2$), MV promotes rapid outflow of electrons from PSI, accelerating photooxidation of P_{700} . We did not observe EPR signals from MV radicals under aerobic conditions [48]; therefore, it is highly likely that the re-oxidation of MV by O_2 occurs more rapidly than the reduction of MV by PSI.

The reduction of O_2 can lead to generation of superoxide radicals ($O_2^{\bullet -}$), which then convert to H_2O_2 and O_2 by superoxide dismutase (SOD) [17, 18]. In plants, the transformation $H_2O_2 \rightarrow H_2O$ can occur due to H_2O_2 reduction by ascorbate and/or glutathione [49]. The participation of ascorbate in “detoxication” of ROS in plants finds visual evidence in experiments with MV-treated leaves. Figure 3 shows the diagrams of participating MV and O_2 in electron transport beyond PSI. As one can see from Fig. 3b, the MV-mediated stimulation of electron flow from PSI to O_2 promotes the formation of ascorbate free radicals ($Asc^{\bullet -}$). These radicals revealed themselves as the light-induced doublet EPR signal observed in the presence of MV injected into *Hibiscus rosa-sinensis* leaves (see right panel in Fig. 3b), thereby demonstrating that ascorbate participates in detoxication of $O_2^{\bullet -}$ radicals produced due to MV-mediated electron flow from PSI to O_2 [48].

2.2. Oxygen-exchange processes in photosynthetic systems

In order to illustrate the importance of the O_2 interaction with photosynthetic ETC, we consider below the kinetics of P_{700} photooxidation in the cyanobacteria *Synechocystis* sp. PCC 6803, as studied earlier by Trubitsin et al. [50, 51]. Figure 4 shows the typical patterns of the time courses of P_{700}^+ induction in dark-adapted cells *Synechocystis* sp. PCC 6803. The kinetics of P_{700} photooxidation strongly depend on the illumination pre-history. A cell suspension was either placed into a gas-permeable plastic tube (curve 1, aerated cells) or confined inside a gas-impermeable cuvette (curve 2). Under aerobic conditions, illumination of dark-adapted cells induced nonmonotonous redox transitions of P_{700} . After a relatively fast rise of the P_{700}^+ signal (the O-A phase), there was a small dip (phase A-B) followed by a signal rise to a steady-state level. For cells incubated inside a gas-impermeable cuvette, we observed another pattern of the multiphase induction of P_{700} . In response to switching the light on, a fast jump of the P_{700} signal (the O-A rise) occurred. This was followed by insignificant decay (phase A-B) and a subsequent, relatively slow rise of P_{700} up to a steady-state level (phases B-C and C-D). The retardation of P_{700} oxidation correlates with deprivation of O_2 inside a quartz cuvette due to cell respiration [52–54]. Actually, the rate of P_{700} photooxidation in aerated suspension of cells (curve 1) was much faster than that for cells placed into a gas-impermeable quartz cuvette (curve 2). A substantial difference between kinetic curves 1 and 2 is caused by O_2 deprivation inside a quartz cuvette due to the operation of terminal oxidases localized in the thylakoid and cytoplasm membranes of cyanobacterial cells (for review, see [52–54]). According to [48, 50, 51], under the O_2 deficit, the efflux of electrons from PSI decelerates whereas electron flow to P_{700} from the reduced PQH₂ pool (via the Cyt *b₆f* complex and Cyt *c₆*) is maintained. A high level of PQH₂ under anaerobic conditions can be explained by the inefficient operation of PTOX

(Fig. 1b, electron transfer pathway 3). In the meantime, over the course of illumination, the O_2 concentration in the cell suspension confined to the quartz cuvette begins to increase due to the functioning of PSII. The light-induced aeration of the cell suspension promotes a slow rise of P_{700}^+ . Experiments with the O_2 -sensitive spin label CTPO confirmed a correlation between the rates of P_{700} oxidation and O_2 evolution. Below, these results are described in more detail, with consideration given to the spin-label oximetry approaches to investigate the oxygen-exchange processes in photosynthetic systems (Sect. 4.1).

Taken together, the above-considered examples of the light-induced induction of P_{700} in green leaves (Fig. 2) and in cyanobacteria (Fig. 4) clearly demonstrate that the presence of O_2 in the external media markedly influences the operation of photosynthetic ETC. We would like to stress that the notion of a photoprotective role for O_2 as an alternative electron sink is widespread in the literature (for review, see [55]). Molecular oxygen plays the role of a sink for electrons donated by ETC, which can adjust an optimal redox status of electron transport chain. In the meantime, the reduction of O_2 by photosynthetic ETC may lead to generation of reactive oxygen species (ROS), which can be harmful for PSA and other components of the plant cell [56–58]. Under normal physiological conditions, the formation of ROS in green leaves is suppressed due to the protective action of ascorbate and glutathione, which operate as efficient scavengers of ROS [49, 58]. Below, we outline the EPR-based methods for measuring the O_2 concentration in biological systems and illustrate some of them in the context of O_2 interaction with PSA in plants and cyanobacteria.

3. Nitroxide radicals as molecular sensors for O_2

3.1. Nitroxide radicals as the probes for O_2

The earlier finding [59] that the resolved proton superhyperfine structure of the EPR spectrum of a small water soluble nitroxide radical 2,2,5,5-tetramethyl-3-imidazoline-3-oxide-1-oxyl-4-carbonic acid (spin label I shown in Fig. 5) depends on its collisions with O_2 formed a basis of the spin-label oximetry, developed later into the “EPR oximetry.” The spin-label oximetry was finally established when the spin label 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy (spin label II in Fig. 5, termed later as CTPO [60]) was introduced. The EPR spectra of CTPO, with a well-resolved superhyperfine structure, were calibrated, allowing measurements of O_2 uptake in cellular suspensions [61, 62]. In these approaches (called the T_2 -sensitive oximetry methods), the resolved superhyperfine structure disappears in the presence of O_2 due to the line broadening induced by collisions with paramagnetic O_2 molecules. The spin label CTPO was widely used to measure O_2 uptake in cellular suspensions, by cell organelles, as a function of the cellular cycle, and during biochemical processes [62–69]. An example of the use of this approach to determine O_2 uptake in the dark- and the light-induced evolution of O_2 in the suspension of cyanobacterial cells is illustrated in Fig. 7.

The alternative T_1 -sensitive oximetry methods are based on the fact that a Heisenberg spin-exchange between a slow-relaxing spin label and a fast-relaxing O_2 leads to enhanced spin-lattice relaxation of the nitroxide radical. These methods have advantages over T_2 -sensitive methods, because T_1 (1–10 μ s) is markedly longer than T_2 (from one to three orders of

magnitude). Additionally, these methods can be applied to any spin-probed (or spin-labeled) investigated system, without the need for a resolved superhyperfine structure in the EPR spectra. Saturation recovery [70–72] and continuous wave (CW) saturation [73–75] are most widely used.

An experimenter can use the spin-lattice relaxation time of spin labels (T_1 -sensitive methods) or the linewidth of the EPR spectrum of spin labels (T_2 -sensitive methods) as a parameter sensitive to O_2 concentration. The changes in T_1 and T_2 values are proportional to the collision rate between the nitroxide spin label and the O_2 . It is significant that both the T_1 - and T_2 -sensitive approaches can be applied as quantitative oximetry methods, because every collision of an oxygen molecule with a spin-label nitroxide moiety affects the EPR spectrum [73, 76–78]. Thus, the spin-label oximetry approach provides the local values of the product of the oxygen diffusion coefficient and oxygen concentration ($D_O C$) around the nitroxide moiety.

Bimolecular collisions between O_2 and the nitroxide spin label can be described by the simplified Smoluchowski equation,

$$\omega_{\text{exp}} = 4\pi p r_o D_O \cdot C. \quad (1)$$

Here, r_o is an interaction distance between oxygen and nitroxide radical spin labels; C is the local O_2 concentration, D_O is the diffusion coefficients of O_2 , and p is the probability that an experimentally observable event (change in the linewidth or T_1) occurs when the collision occurs. It was shown for spin labels that $pr_o = 4.5 \text{ \AA}$ [70, 77, 78]. It is also assumed that the diffusion coefficient of O_2 is much greater than the diffusion coefficient of spin label. With all these assumptions and simplifications, we can state that the EPR spin-label oximetry method is quantitative, yielding the true oxygen diffusion-concentration product, $D_O \cdot C$.

3.2. “Local” and “averaged” oximetry: O_2 concentration and partial pressure of O_2

If spin labels are distributed uniformly across the sample, the experimentally measured parameter of the EPR signal is proportional to the integrated O_2 concentration over the total volume of the sample. For instance, for homogenous fluids, the average concentration is easily measured [62]. Otherwise, an unambiguous interpretation of the EPR measurements may be complicated in the case of the nonuniform systems such as cells. In the latter case, using the molecular probes, one will get only qualitative results. Consider, for instance, molecular probes dissolved in the aqueous solution spread over the whole cell. In this case, the O_2 -probe molecules will serve as the reporters of the local $D_O \cdot C$, providing averaged values throughout all the sample of C (and/or O_2 partial pressure) (see schematic diagram in Fig. 6).

If a spin label is attached to the certain site of the protein (site-directed spin labeling) or introduced at a specific depth in the lipid bilayer, the local oximetry measurement can be performed. In the case of spin-labeled proteins, the spin label molecule will report the accessibility of O_2 molecules to a certain amino acid [79]. Local oximetry measurements for spin labels located at a certain depth in the lipid bilayer allowed the profiles of the $D_O C$ to

be obtained across the membrane. Having measured the transmembrane profile of the D_{O_2} , one can evaluate the membrane permeability coefficient for oxygen [72], including the thylakoid membranes [75, 80].

O₂ concentration and oxygen partial pressure are related values. In equilibrium, the O₂ concentration in solution is determined by the partial pressure of oxygen with which the sample is equilibrated and the solubility coefficient. In all EPR oximetry approaches (the use of molecular spin labels, microscopic spin label probes, or particulate materials sensitive to O₂), the measured value is the O₂ partial pressure. This, for the system in equilibrium, is schematically illustrated in Fig. 6.

4. Experimental approaches to EPR oximetry

In this section, we consider some methodological aspects of EPR-based oximetry, as illustrated by two examples of studying oxygen exchange processes in photosynthetic systems. In spin-label oximetry (EPR oximetry), three major approaches are used, namely the molecular, microscopic, and solid-state particulate approaches. We start our consideration with brief review of molecular and microscopic probes. Then we consider the oximetry methods based on the use of O₂-sensitive particulates.

4.1. Molecular and microscopic probes

The *molecular probe method* uses water-soluble small spin labels such as CTPO or TEMPONE (see their structures in Fig. 5), which rapidly tumble in a water solution. TEMPONE (structure IV in Fig. 5), when dissolved in a deoxygenated water, shows a very narrow EPR line ($H_{pp} \approx 0.25$ G) without a resolved proton superhyperfine structure in the EPR spectrum. The deuteration of TEMPONE decreases the linewidth to 0.15 G. Specific deuteration can also change the pattern of the superhyperfine structure of the EPR spectrum. This was the case with CTPO. Two resolved superhyperfine lines were observed in the EPR spectrum of a CTPO analog in which four methyl groups were deuterated (see structure III in Fig. 5). The interaction of an unpaired electron with the remaining ring proton is responsible for the observed splitting. This new feature gives a 20-fold increase in the sensitivity of the EPR spectrum to O₂-induced line broadening [81].

It should be noted that a stable free radical, other than nitroxide free radicals, namely triaryl methyl radical (TRITYL) (see structure V in Fig. 5), was developed [82] and was used as a new oxygen-sensitive EPR molecular probe. Because of its extremely narrow linewidth (~25 mG in deoxygenated solution), TRITYL offers a great improvement in the signal-to-noise ratio. TRITYL's improved signal-to-noise ratio improved resolution and sensitivity for *in vivo* EPR imaging [83].

In the molecular probe approach, it is assumed that the spin labels are located mainly in the water phase and the "contamination" of spectral parameters coming from spin labels located in another environment, with different oxygen solubility and diffusion coefficient, is negligible. An additional side effect that could influence the EPR spectra of spin labels is the interaction with other water soluble paramagnetic molecules (like paramagnetic metal ions). Another complication can arise from their interaction with chemically active reducing

agents: accepting electrons from water soluble or other reductants inside the cell will cause a spin label to lose its paramagnetism, thereby converting to EPR silent hydroxylamines. The chemical reactivity of a spin label may restrict its application as an O₂-sensor. On the other hand, this property of nitroxide radicals can be used to test the reducing potential of the media, for instance, in suspensions of mitochondria and chloroplasts where the spin labels are dissolved (for references, see [84]).

Experiments using the spin label CTPO as an oxygen sensor confirmed that O₂ is necessary for cyanobacteria to function normally. Figure 7 illustrates how CTPO can be used to monitor oxygen-exchange processes in a suspension of cyanobacterial cells. Figure 7a demonstrates the EPR signals (their low-field parts) coming from CTPO dissolved in water equilibrated with air or argon. In a deoxygenated sample (produced by bubbling with Ar gas for 20 min), the EPR CTPO signal showed a well-resolved proton superhyperfine structure. The equilibration with air causes a broadening of the EPR lines and disappearance of the superhyperfine structure. All of this can be explained by line broadening due to collisions with O₂ molecules. As indicated in previous works (for review, see [27, 78]), we can take the ratio $a = h_1/h_2$ as the spectral parameter sensitive to O₂ concentration in the cell solution (for definition, see Fig. 7a).

Figure 7b depicts the changes of the O₂-sensitive parameter a of CTPO measured in the suspension of cyanobacterial cells placed in either an O₂-impermeable quartz cuvette or an O₂-permeable plastic tube. For cells incubated inside the plastic tube in the dark and during illumination, parameter a did not change. This demonstrates that the cell suspension is well aerated through the oxygen-permeable wall of the plastic container. Upon cyanobacteria incubation in an O₂-impermeable quartz cuvette, we can observe the uptake of O₂ in the dark and O₂ transient evolution during illumination. During incubation of cells in the dark, parameter a gradually increased, indicating a consumption of O₂ as the result of respiration of the cyanobacterial cell. The illumination of the cell suspension caused the superhyperfine lines of the EPR spectrum to disappear due to the light-induced restoration of the O₂ level (a decrease in a).

The insert box in Fig. 7b shows a correlation between the kinetics of P₇₀₀ photooxidation (the time-course of the EPR signal from P₇₀₀⁺) and O₂ evolution (the light-induced changes in parameter a) for cell suspensions incubated inside the quartz cuvette. This demonstrates a close relationship between the O₂ production and kinetics of P₇₀₀ redox transients, which can be seen by the response of P₇₀₀ oxidation to the light switching on after the dark adaptation. The explicit delay in response correlates with the lag-phase observed for O₂ evolution. This demonstrates that electron transport is controlled by O₂ concentration in the investigated cell suspension, which first increased during illumination and then decreased. According to [50, 51], this might be caused by the photoinhibition of PSII due to the prolonged exposure of cells to white light.

In the *microscopic probe method*, an experimenter uses the hydrophobic nitroxide radicals surrounded by the hydrophobic solvent (*e.g.*, spin probes encapsulated within paraffin particles [85, 86], sonochemically synthesized microspheres [87], or liposomes [88]). In this case, the above-mentioned problems are solved. Additionally, as follows from equation (1),

hydrophobic (hydrocarbon) solvents with a high solubility for molecular oxygen and a high D_O will enhance the sensitivity of spin labels to changes in O_2 concentration. Hydrocarbons solvents dissolve O_2 4–10 times greater than water [89]. The diffusion of oxygen in these solvents is similar to that in water [77]. Helpful examples of microscopic probes include paraffin oil particles that are a few micrometers in diameter and are coated with BSA containing cholestane spin labels [85, 86], or hexane particles coated with BSA containing stearic acid spin labels [87]. In these methods, which belong to the T_2 -sensitive approaches, the O_2 sensitive parameters were the EPR linewidth or the EPR line height. The authors claim that the increase in sensitivity to oxygen is as much as 40-fold. A major concern is the possibility of leaking nitroxides as well as the leaking of solvents from the microspheres.

A recently designed microscopic probe made of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) small unilamellar vesicles containing 1% of 1-palmitoyl-2-(16-doxylstearoyl)phosphatidylcholine spin label (16-PC) [88] has all the advantages of microscopic probes, including the major advantage that the partial pressure of O_2 is the only factor that can influence the EPR spectrum parameters. Additionally, all of the above-indicated problems encountered for previously prepared microscopic probes are absent here. This microscopic probe (~30 nm in diameter) is stable in cell suspensions. It is also distributed uniformly through the sample and reacts rapidly to the changes in O_2 partial pressure. The saturation-recovery EPR approach was used. It is notable that, in this molecular probe, the measured spin-lattice relaxation rate is a linear function of O_2 partial pressure up to 159 mmHg (100% air).

4.2. Solid-state O_2 -probes based on insoluble particulate materials

Paramagnetic particulates provide “localized” data about the partial pressure of O_2 at the places where they are located. The similarities between microscopic spin-label oximetry and particulates may be considerable, especially when measurements are performed with a suspension of small particles. For *in vivo* measurements, macroscopic particles are placed at few sites of interest and repeated data are obtained from the same sites. Coals, charcoals, or India ink are particulate paramagnetic probes based on carbonaceous material [90, 91]. Another paramagnetic particulate material is based on certain forms of lithium phthalocyanine [92]. EPR lines of these insoluble particulate materials are very narrow, which significantly increases sensitivity to collisions with O_2 as compared with the sensitivity of the soluble materials used for oximetry measurements. Particulate materials are principally used for *in vivo* measurements [93]. They also were used to measure O_2 partial pressure in cellular systems [92, 94]. Below, we consider in more details the use of fusinite particles to monitor the O_2 production and consumption in the interior of intact leaves [95].

The functioning of the photosynthetic water-splitting complex can enhance the local concentrations of O_2 inside the thylakoids, thereby stimulating the production of toxic ROS (superoxide radicals, hydrogen peroxide) caused by O_2 reduction by electron carriers of PSI. Using O_2 -sensitive particles injected into the leaf tissue, we demonstrated that under the normal physiological conditions the leaf interior is subjected to good ventilation that precludes an increase in O_2 concentration, thereby diminishing the production of dangerous ROS. Below, we illustrate this point using fusinite particles as sensors for O_2 . EPR spectra

of fusinite particles are very sensitive to O₂ partial pressure in their surroundings. Figure 8a shows the EPR spectrum of dry fusinite particles equilibrated either with pure argon (curve 1) or pure oxygen (curve 2). Interaction of paramagnetic O₂ molecules with fusinites causes a broadening of the fusinate signal. Having a calibration curve (amplitude of the EPR signal *versus* the percentage of O₂ in the atmosphere, Fig. 8b), an experimenter can determine a concentration of O₂. Figure 8c shows the time courses of the amplitude of the EPR signal from fusinite particles located inside a bean leaf [95]. Curves 1 and 2 demonstrate the kinetic curves recorded during the first and the second cycles of illumination, respectively. Samples were placed into the EPR cavity using a closed chamber (curves 1 and 2) or were kept in the open holder and were exposed to the surrounding atmosphere (curve 3). In the first case, O₂ molecules accumulated within the leaf and in the water-filled closed cell. We observed two processes: (i) O₂ *consumption* in the dark, which led to the increase in the magnitude of the EPR signal, and (ii) O₂ *production* during illumination, which caused a decrease in the magnitude of EPR signal. In response to the first turn of illumination, the light-induced production of O₂ in dark-adapted leaves was visible only after the lag phase (≈ 2 min), while the second exposure to light resulted in the production of O₂ without the lag phase. This difference reflects the regulatory events in intact photosynthetic systems (the so-called state 1 $\rightarrow$ state 2 transition) particular to intact chloroplasts (for review, see [29]). When the leaf specimen was exposed to air (curve 3), we did not observe any marked changes in the amplitude of the EPR signal from the particles located inside the leaf, neither in the dark nor during illumination. This is because the light-induced production of O₂ in chloroplasts was compensated by the rapid efflux of O₂ from the leaf to the atmosphere. The good ventilation of the leaf interior can be explained by rapid, long-distance diffusion of O₂ from chloroplasts to leaf stomata. Thus, the efficient ventilation of a leaf interior prevents its overoxygenation, thus protecting against injuries in the leaf photosynthetic apparatus.

One of the factors that facilitate a rapid outflow of O₂ molecules from the thylakoids is a relatively high coefficient of diffusion through the thylakoid membrane. We reached this conclusion in our earlier work [80], using lipid-soluble spin labels to probe the local oxygen diffusion concentration product across the thylakoid membranes of spinach chloroplasts (*Spinacia oleracea*). From the membrane profile of this product, using the procedure developed in [72], we estimated the coefficient for O₂ permeability through the lipid domains as 39.5 cm s⁻¹ (at 20°C), the value of which was 20% higher than the oxygen permeability coefficient of a water layer of the same thickness as the thylakoid membrane. Such a high permeability of a thylakoid membrane for O₂ should preclude any significant increase in concentration in local chloroplast compartments (c_{O_2} μ M). Thus, taking together the results of O₂ measurements in the leaf interior and a high permeability of thylakoid membranes for O₂, we can conclude that under normal physiological conditions the light-induced production of O₂ itself should not be damaging to thylakoids.

5. Concluding remarks and future trends

Research during the past three decades has revealed the importance of O₂ interaction with the photosynthetic electron transport chains of chloroplasts and cyanobacteria (for review, see [17–23, 96–100]). The main purpose of this article is to review the advantages of using the EPR oximetry approaches to study oxygen-related processes in photosynthetic systems.

One of the basic advantages of this approach is that an experimenter has the opportunity to measure the processes of photosynthetic electron transport and O₂ production/consumption under the same experimental conditions, using the same EPR spectrometer (see, for instance, Fig. 7). Another advantage is that it allows us to perform a noninvasive study of photosynthesis processes in native systems (*e.g.*, in cuttings of green leaves; see Fig. 8). In addition, spin-labeling techniques provide the means to investigate structure–function relationships in photosynthetic membranes and to measure the *trans*-thylakoid pH difference, which is the driving force for ATP synthesis (for references, see [23, 101–103]). Thus, the multiple EPR spectroscopy approaches, which are based on monitoring the redox transients of electron carriers and the use appropriate paramagnetic O₂-sensors and pH-probes, provide efficient tools for scrutinizing different aspects of energy transduction processes in photosynthetic systems. In particular, after analyzing the results of our previous research, we conclude that under the normal physiological conditions the light-induced production of O₂ itself should not be damaging to thylakoids. This conclusion is based on the following observations obtained by the EPR method (for illustration, see Figs. 2–4, 7, and 8):

- i. Molecular oxygen plays the role of an electron sink and can adjust the optimal redox status of an electron transport chain. Alternate pathways of O₂-mediated electron transport help support a high rate of electron flow through PSI, thereby precluding the over-reduction of the acceptor side of PSI and the PQ pool between PSII and PSI.
- ii. An efficient ventilation of the plant cell interior prevents a significant rise of the internal concentration of O₂ that might provoke strong generation of ROS that is harmful to the PSA and other components of the plant cell.
- iii. The light-induced generation of the ascorbate free-radical signal observed in green leaves under the oxidative stress conditions suggests that ascorbate, operating as an efficient scavenger of ROS, is involved in protecting PSA from solar stress.

We have to note, however, that the use of spin labels is not always straightforward. Along with the above-mentioned advantages of nitroxide spin labels as probes for O₂ in biological systems, there may be unexpected problems. One of the major problems is their chemical reactivity, *i.e.*, the reduction of radicals to the EPR-silent hydroxylamines. These reduction processes are intensive when nitroxide spin labels contact with functioning ETCs of chloroplasts or mitochondria [84, 104]. The loss of paramagnetism can mask changes in EPR spectra induced by changes in O₂ concentration or the local surrounding of radicals. Strzalka et al. [67] suggested discriminating between the changes in the signal intensity (amplitude) caused by the reduction of nitroxides or their interaction with O₂. This approach, based on recording the spectral changes at low and saturating microwave powers, was successfully used to study interactions of pH-sensitive nitroxide radicals with chloroplasts [101–103]. In particular, it was possible to reliably distinguish the light-induced changes in the EPR spectra caused by generation of the *trans*-membrane pH difference and the reduction of nitroxides due to their interaction with the chloroplast ETC. Although the microscopic probe oximetry method practically cancelled the reduction of nitroxide radicals

hidden in a hydrophobic local environment, it can be only applied to obtain total (average) information about oxygen consumption or evolution. Microscopic probes, even as small as 30 nm [88], cannot replace local molecular measurements. For local measurements of O₂, a promising approach is based on the use of lipid-soluble spin labels, which can locate nitroxide moiety at a special location in the membrane [75, 80]. If such a location is possible in close proximity to the oxygen evolution center, it could give information about O₂ accumulation in that local environment and the rate of its diffusion out.

Spin-label oximetry also allows determinations of the O₂ concentration when the medium (water) is oversaturated with oxygen (up to 2–3 mM of O₂). At normal pressure (760 mmHg), water saturated with pure oxygen contains ~1 mM oxygen. Spin-label oximetry allows measurement of such oversaturated states before oxygen bubbles are formed. Thus, spin-label oximetry allows very low and very high (for biological systems) oxygen concentrations to be measured.

The application of spin-label oximetry to study biological systems began in 1977 with the paper of Backer et al. [59], which showed that the O₂-induced broadening of the EPR spectrum and the disappearance of the superhyperfine structure can be used to monitor mitochondrial respiration. During the subsequent four decades, the spin-label oximetry (extended to the EPR oximetry) developed tremendously and was intensively applied in biological and biomedical studies [102], including studies related to photosynthesis [26]. In the last decade, new approaches in noninvasive oximetry were developed that are strongly linked to trityl radicals and lithium phthalocyanine crystals, both of which possess an extremely narrow single EPR line. A new type of oxygen sensitive macroscopic probe (oxygen-sensing paramagnetic lithium crystals encapsulated in a biocompatible polymer, called OxyChips) was developed [106] and is already at the stage of preclinical evaluation for long-term EPR oximetry [107, 108]. All these approaches were successfully used for *in vivo* oximetry [109, 110] and *in vivo* imaging [109, 111]. They also present new opportunities for studies of photosynthesis but were not yet applied for these studies. However, the last decade added only few papers in which EPR oximetry was applied to photosynthetic research. In this review, we discuss the advantages of the EPR oximetry approaches and hope to encourage scientists to use these methods to study oxygen-related processes in oxygenic-type photosynthetic systems.

Additionally, new advances in the EPR technique and designed loop-gap resonators with sample volumes as small as 30 nL open the possibilities of making oximetric measurements within extremely small-volume samples (for illustration of this approach, see [90]).

Otherwise, we have to note that advanced EPR spectroscopy techniques, based on the use of L-band EPR spectrometers, allow an experimenter to examine O₂ distribution in whole organisms [112].

In summation, we hope that the methodological and scientific aspects of the EPR method applications considered above will encourage scientists to use EPR oximetry approaches to study oxygen-related electron transport processes in photosynthetic systems of the oxygenic type.

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Fig. 1.

A sketch of a chloroplast (a) and photosynthetic noncyclic electron transport (b).

Abbreviations: CBC, Calvin-Benson cycle; Fd, ferredoxin; FeS, iron-sulfur acceptors of PSI; FNR, ferredoxin-NADP-oxidoreductase; PTOX, plastoquinone terminal oxidase. “Up” and “down” arrows indicate evolution and consumption of O_2 , respectively.

Fig. 2. EPR spectra of isolated bean chloroplasts (a). Panel (b) depicts the kinetics of the light induced redox transients of P_{700} in *Hibiscus rosa-sinensis* leaves adapted to the dark for 5 min: curve 1 – control (untreated) sample, curve 2 – methylviologen (100 mM)-treated sample. Modified figure 1 from [25].

Fig. 3.

Alternative O_2 -dependent routes of electron transport in chloroplasts (a) and the influence of methylviologen (a redox-mediator of electron transfer from PSI to O_2) on the light-induced generation of ascorbate anion-radical in *Hibiscus rosa-sinensis* leaves (b). The right bottom panel demonstrates the light-induced EPR signals in *Hibiscus rosa-sinensis* leaves: 1 – control; 2 – MV (500 μ M)-treated sample; 3 – MV (500 μ M) + monensin (100 μ M)-treated sample; 4 – DCMU (100 μ M) + MV (500 μ M) + monensin (100 μ M)-treated sample. EPR spectra were recorded at the microwave power 10 mW and modulation amplitude 0.4 G. Modified Fig. 7 from [48]. Abbreviations: APX, ascorbate-peroxidase; SOD, superoxide dismutase.

Fig. 4. Kinetics of light-induced changes in the amplitude of the EPR signal from P_{700} in the cyanobacterium *Synechocystis* sp. PCC 6803 cells incubated in an O_2 -permeable plastic tube (curve 1) or placed into an O_2 -impermeable quartz cuvette (curve 2). Before switching the light on, cells were adapted to the dark for 10 min. Modified Fig. 2 from [51].

Fig. 5.

Chemical structures of spin-label molecular probes. I – (2,2,5,5-tetramethyl-3-imidazoline-3-oxide-1-oxyl-4-carboxylic acid); II – (3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy, named CTPO); III – deuterated CTPO; IV – (2,2,6,6-tetramethylpiperidine-N-oxyl, named TEMPONE); V – (triaryl methyl radical, named TRITYL).

Fig. 6.

Schematic illustration of the purported local oxygen concentration (---) across the sample (aqueous environment, cell membrane, cell cytoplasm). The sample is in equilibrium with the set oxygen partial pressure (the partial pressure of oxygen (.....) across the sample is the same). Molecular probes measure the local oxygen concentration which can significantly vary across the sample. Concentration of oxygen inside microscopic probes is the same, independently of their locations (red broken lines inside green rings). The molecular probe approach measure oxygen concentration (correctly, oxygen diffusion-concentration product as indicated in eq. (3)) averaged throughout the sample. If the spin-label molecule can be attached to a specific site of a protein or placed at a certain depth in the membrane, local oximetry measurement is possible. The microscopic probe approach measure always local oxygen partial pressure which, for samples in equilibrium, is the same in all points of the sample (blue dotted lines inside green rings).

Fig. 7.

Panel **a** illustrates the influence of O_2 on the low-field component of the EPR signal from CTPO in water solution. The low-field component was used because this component does not overlap with the EPR signal of P_{700}^+ . Panel **b** demonstrates the time courses of the spectral parameter α for the low-field component of the EPR signals from CTPO in glycerol-treated cyanobacterial cells placed either in a quartz cuvette or an O_2 -permeable plastic tube, as indicated. The insert box shows a correlation between the kinetics of P_{700}^+ photooxidation (parameter P, as measured from the EPR signal of P_{700}^+) and O_2 evolution (parameter α). Modified Figures 5A and 9 from [51].

Fig. 8. *In situ* measurements of oxygen production and consumption using paramagnetic fusinite particles injected into a bean leaf (modified after [99]).

Panel (a) shows EPR spectra of dry fusinite particles: 1 – in the atmosphere of pure argon (Ar); 2 – in the atmosphere of pure oxygen (O₂). EPR spectra were recorded at the microwave power 10 mW and modulation amplitude 0.5 G. The receiver gain for spectrum 2 was 12.5 times higher than for spectrum 1. A , the measured amplitude of the EPR spectrum, as indicated.

Panel (b) demonstrates the relative amplitude of the EPR spectra of fusinite particles versus concentration of O₂, parameter $A(\text{O}_2)/A(\text{Ar})$. Fusinite particles were placed in water equilibrated with different mixtures of pure argon and oxygen adjusted with flow meters. Panel (c) shows the typical patterns of the light-induced changes in the amplitude of the EPR signal from fusinite particles injected into a leaf as described in [96]. Curves 1 and 2 correspond to the first and the second cycles of illumination, respectively. Before the first illumination, a sample was adapted to the dark for about 30 min. A_0 is the amplitude of the EPR spectrum of fusinite injected into the leaf and equilibrated with air. Samples were placed either in a closed (curves 1 and 2) or open chamber (curve 3).