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Oxygen production and consumption by chloroplasts in situ and in vitro as studied with microscopic spin label probes

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Abstract

A new spin-label oximetry approach able to measure the oxygen partial pressure in complex photosynthetic systems has been developed using bovine serum albumin (BSA)-coated light paraffin oil particles containing cholestane spin label (CSL). Paraffin oil particles protect the spin label against the action of chemically active metabolites. The amplitude of the electron paramagnetic resonance (EPR) signal from CSL measured at a saturating microwave power is sensitive to the concentration of oxygen. We demonstrate here the ability of this method to monitor the kinetics of light-induced oxygen production in situ, i.e., in the interior of a bean leaf. The oxygen release, observed during leaf illumination with continuous light, exhibits an overshoot that correlates with the well-known nonmonotonous behavior of the Photosystem I reaction center, P700. Short-term illumination of isolated bean chloroplasts, suspended in the presence of the electron mediator methylviologen, induces a reversible uptake of oxygen. However, after prolonged illumination, chloroplasts lose their ability to regenerate oxygen in the dark. The exhaustion of oxygen (and oxygen active forms) is accompanied by the loss of CSL paramagnetism and the capacity to photooxidize P700. Comparison of the kinetics of P700 redox transients with oximetric data demonstrates that oxygen concentration is the essential factor controlling electron transport in leaves and isolated chloroplasts.

Key words: Spin-label oximetry; Electron transport; Chloroplast

1. Introduction

Oxygen plays an essential role in the functioning of intact chloroplasts. Since the pioneering work by Mehler [1], who established that oxygen can act as an electron acceptor in chloroplasts, the interaction of oxygen with the photosynthetic electron transport chain has been investigated by many authors (see, for example, [2–11]). In class B chloroplasts oxygen is the terminal electron acceptor which is reduced by Photosystem I (PS I) ($O_2 + e^- \rightarrow O_2^-$), directly or via artificial low-potential electron mediators. In class A chloroplasts, in the course of photosynthetic electron transport leading to CO_2 fixation, the chloroplasts also can reduce oxygen [2–5]. It has been demonstrated with the electron paramagnetic resonance (EPR) technique [10] that the noncyclic electron transport in a leaf does not operate normally in an atmosphere containing the physiological concentration of CO_2, but lacking oxygen. In particular, electron flow from PS I to oxygen might supply extra ATP molecules needed for CO_2 fixation in Calvin cycle reactions. According to the data obtained in [7–9], cyclic electron flow around PS I via ferredoxin, which catalyzes so-called cyclic photophosphorylation, operates only under deoxygenated conditions. Thus, oxygen could control the distribution of electrons between the pathways of cyclic and noncyclic electron transport.

For many years, the conventional gas-exchange technique was the main tool for the investigation of the role of oxygen in photosynthesis of higher plants and algae, but this technique cannot measure photosynthetic oxygen production in the interior of the leaf. However, this measurement can now be made using an

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Abbreviations: PS I and PS II, Photosystems I and II, respectively; BSA, bovine serum albumin CSL, cholestane spin label; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; T_1, spin-lattice relaxation time.

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EPR spin-label oximetry technique. Additionally, a comparative study of the oxygen production or consumption and electron transport kinetics under the same experimental conditions can be done using an EPR method. Spin-label oximetry is based on the magnetic interaction between dissolved molecular oxygen and spin label molecules [12–15]. Heisenberg spin-exchange leads to the broadening of the EPR signal and shortening of the spin-lattice relaxation time (T_1) of the spin label nitroxide radical [16, 17]. Both effects depend on the concentration of oxygen in the surroundings of spin labels.

At present, there are three major methodological approaches used for spin-label oximetry: (i) molecular, (ii) macroscopic, and (iii) microscopic. Also a rapidly developing new approach, (iv) microscopic solid state in which crystals of paramagnetic materials are used, can be added to this list. The first approach is based on the use of spin labels directly dissolved in the system to be examined [18], while the second one is associated with the use of spin labels confined to an oxygen permeable plastic capillary, which prevents their mixing with biological material [19]. The 'molecular-probe' method provides sampling over the entire sample, however, the spin labels are not distributed selectively. In general, there is a fast response of appropriate molecular spin probes to changes in oxygen concentration. A disadvantage of the molecular-probe method results from Heisenberg spin exchange with other paramagnetic species such as metal ions. Also, nitroxides can undergo redox reactions with certain metabolites [20–23]. Furthermore, the rate of the chemical reduction of spin labels depends on oxygen concentration in the system [24]. The 'macroscopic' method provides a good isolation of spin labels from chemically active metabolites and paramagnetic ions. However, the sizes of macroscopic capsules containing spin probes are usually rather large. Their implantation into biological tissues might cause damages that would distort physiological processes. Macroscopic sensors respond relatively slowly to changes in oxygen concentration. All these difficulties might seriously complicate the use of spin labels in the investigation of photosynthetic processes in isolated chloroplasts [11, 22], as well as in chloroplasts in situ (i.e., in green leaves [23]).

Because of these problems, we have introduced another approach to spin-label oximetry, which falls into the third group of 'microscopic' approaches. This method is based on the use of microscopic oxygen probes, light paraffin oil particles containing cholestane spin label (CSL) coated with bovine serum albumin (BSA). Similar paraffin oil particles were previously used by Ito and collaborators in their investigation of the phagocytic process associated with the functioning of mouse peritoneal macrophages [25]. In this way, it is possible to isolate nitroxide radicals from water soluble reductants and paramagnetic ions that might interfere with spin-label oximetric measurements. In the present method, CSL is surrounded by a light paraffin oil environment, which dissolves oxygen very well. Therefore, the change in the oxygen partial pressure around the paraffin oil particles is the only factor that can influence the EPR spectrum from CSL molecules. Such microscopic probes are readily and uniformly distributed within the sample, thus giving fast response to changes in the partial pressure of oxygen in the system.

A similar 'microscopic' approach using liposomes loaded with the molecules of water soluble spin label has been suggested earlier [26]. As an oxygen sensitive parameter the authors used the superhyperfine structure of the EPR signal. However, the sensitivity of this method is limited by relatively low resolution of the superhyperfine structure parameter and the necessity of using a small modulation amplitude for the registration of the EPR spectra. Perdeuteration of the spin label increases the sensitivity of the method and a nitroxide-liposome system has been used for in vivo oximetric measurements [27]. Other microscopic solid-state probes, such as certain crystalline forms of lithium phtalocyanine [28] and a derivative of coal termed fusinite [29], also can be used for oximetric measurements. Swartz et al. [30] reported about the application of these probes for the measurements of oxygen concentrations in the tissue of intact animals.

The use of BSA-coated light paraffin oil particles loaded with CSL brings new advantages, facilitating their application to studying photochemically active photosynthetic systems. Being water insoluble, CSL molecules should have an extremely low rate of exchange (through a water bulk phase) between the paraffin oil particles and other hydrophobic regions of biological system as compared with the exchange rates for the other widely used amphiphylic spin labels (stearic acid and androstane spin labeled derivatives). The sensitivity of our method is enhanced for the following reasons: (i) a high T_1 value of CSL [17], (ii) a favorable partition coefficient of oxygen in paraffin oil as compared with that for aqueous media (by the factor of about 10 [31]), and (iii) the use of the hydrocarbon solvent of comparable (or larger) molecular weight than that of spin label. The latter results in favorable relationships between the rates of the translational and rotational diffusion of spin label and oxygen. Despite the relatively high viscosity of light paraffin oil (about 100-times higher than the viscosity of water [32]), oxygen diffuses in this medium as readily as in a water solution. On the other hand, the rate of CSL translational diffusion is expected to be significantly decreased, while rotational diffusion of CSL in the paraffin oil medium is relatively fast. With decreased spin label translational diffusion, high concentration of spin label (e.g., $2 \cdot 10^{-2}$ M of CSL in our experiments) can

be used without significant line broadening effects that could arise from Heisenberg exchange between spin labels.

Using the 'microscopic' method for measuring oxygen concentration, we were able to monitor the kinetics of the light-induced oxygen production in situ, i.e., directly in the interior of bean leaf. The comparison of the oximetric data with the light-induced kinetics of P700 redox transients has proved the important role of oxygen as the electron acceptor in chloroplasts.

2. Materials and methods

2.1. Reagents

Cholestane spin label (CSL) was purchased from Molecular Probes (Junction City, OR), paraffin oil (light) was obtained from MCB, Manufacturing Chemists (Darmstadt, Germany), and Hepes from Sigma (St. Louis, MO). Bovine serum albumin (BSA) (fraction V) and other reagents (analytical grade) came from P.O. Chem. (Gliwice, Poland).

2.2. Preparation of oxygen sensitive microscopic probes

An emulsion of light paraffin oil coated with BSA was prepared according to the method described earlier [25]. 0.3 mL of light paraffin oil containing 20 mM CSL was layered on 0.9 ml of 10 mM Hepes (pH 7.0) containing 2% (w/w) BSA. The tip (◇ 12 mm) of a sonicator (Type UD-11, Unitra, Poland) was placed above the oil/water interface, and the emulsion was collected after a 1 min treatment at 0°C with the sonicator operating at the minimal power level of about 25 W. The emulsion was uniform with the size of particles about 5 μm. The recovery of the EPR signal from CSL in the final suspension was about 80%. The emulsion could be stored at 4°C for a few weeks without loss of EPR signal, as well as without the traces of particles aggregation as tested with optical microscopy.

2.3. Preparation of chloroplasts

Class B chloroplasts were isolated from bean leaves (2–3 week) by the method described in [22], and were suspended in a medium containing 10 mM Hepes (pH 8.0), 2 mM $MgCl_2$, 5 mM inorganic phosphate, 200 mM sucrose. The concentration of PS I (P700) reaction centers in the chloroplast suspension was about 2 μM. In this suspension, 20 μM methylviologen was used as the mediator of electron transport from PS I to oxygen. Spin labeled emulsion (1% of total volume of the sample) was added to the chloroplast suspension immediately before the measurements. To monitor oxygen evolution inside the leaf, the emulsion was injected into the leaf through the midrib with the help of a microsyringe. Our control experiments have demonstrated that such a procedure, in itself, does not cause any damage in the leaf that might disturb the normal functioning of the electron transport chain of native chloroplasts located in bean leaves.

2.4. EPR measurements

EPR measurements were carried out at X-band. A Varian E-3 and Varian E-4, equipped with variable temperature controllers, were used. For calibrating the oxygen probes, a portion of the emulsion (about 10 μl was transferred to a capillary (0.7 mm i.d.) made of gas-permeable methylpentene polymer known as TPX [13]. This plastic is permeable to nitrogen, oxygen and carbon dioxide, but it is substantially impermeable to water. The concentration of oxygen in the sample was controlled by equilibrating the sample with the same gas that was used in the temperature controlling unit — a mixture of nitrogen and air adjusted with flowmeters. All measurements were performed at 25°C $\pm$1°C in darkness or dim light.

3. Results and discussion

3.1. Characterization of the spin-labeled system as an oximetric sensor

Fig. 1 demonstrates the EPR signal from CSL in the emulsion of light paraffin oil particles. Due to anisotropic rotational motion of the spin-label molecules in the viscous oil medium and the high CSL concentration inside the paraffin particles, all components of the spectrum are rather broad. Therefore, the

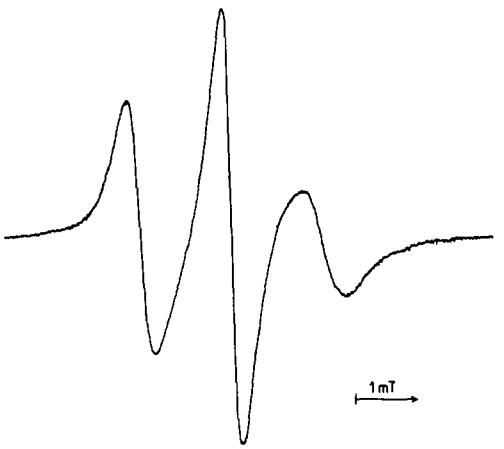

Fig. 1. The EPR spectrum of light paraffin oil particles emulsion injected into bean leaf. Local concentration of CSL in light paraffin oil particles was $2 \cdot 10^{-2}$ M.

shape of the EPR spectrum is relatively insensitive to variations in the concentration of oxygen. Therefore, the linewidths of the components of the spectra cannot be used as oxygen-sensitive parameters. To monitor the changes in oxygen concentration, we have used the so-called continuous wave saturation method [33]. In this method, the fixed high-incident microwave power (200 mW in our experiments) is applied, and the amplitude of the signal is used as the observable parameter sensitive to oxygen concentration. The concentration of CSL in the light paraffin oil does not influence the saturation curves in the range of $1 \cdot 10^{-2}$ M to $2.5 \cdot 10^{-2}$ M. At lower CSL concentrations, deoxygenated samples show less saturation with increasing microwave power (data not shown).

Fig. 2 shows the height of central components of the EPR signal vs the square root of the microwave power for the samples equilibrated with nitrogen (curve 1) and air (curve 2). The EPR signal from a deoxygenated sample shows saturation with increasing levels of microwave power, while the plot for an aerated sample deviates only slightly from linearity even at the highest level of the microwave power available in our spectrometers (200 mW). For the signals recorded at 200 mW, a sample equilibrated with air demonstrates almost two times higher amplitude than for a deoxygenated sample.

Fig. 3 shows the calibration curve which represents the dependence of the relative increase in the EPR signal amplitude (parameter $\Delta A/A_0$) on the partial pressure of oxygen in air/nitrogen mixture at 1 atm pressure. For brevity we will often use the term 'oxygen concentration' instead of 'oxygen partial pressure.' Our

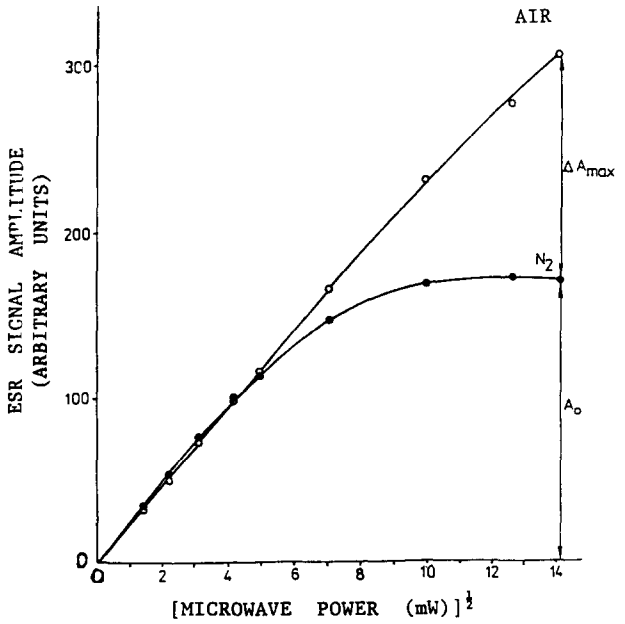

Fig. 2. The dependence of the amplitude of central component of the EPR spectrum in light paraffin oil particles on the microwave power.

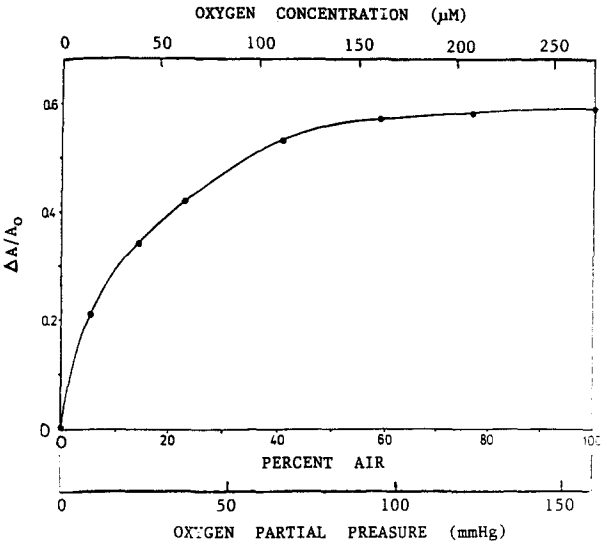

Fig. 3. The calibration curve for measuring the partial pressure of oxygen. The oxygen concentration in water saturated with oxygen at a given partial pressure at 25°C is also shown on the top of the figure.

method gives information only about the surrounding oxygen partial pressure, which could be transformed to the oxygen concentration if the solubility of oxygen concentration in the surrounding medium is known, as it is for water. Here, the values A_0 and $(A_0 + \Delta A)$ are the amplitudes of a central component recorded at 200 mW microwave power for deoxygenated and oxygenated samples, respectively. The sensitivity of the method appears to be higher at lower oxygen concentrations. The signal/noise ratio is sufficient to support a limiting sensitivity of 2–4% air, i.e., 5–10 μm of oxygen dissolved in water.

3.2. *Light-induced changes in oxygen concentration inside bean leaves*

In this section we consider an explicit example illustrating the applicability of paraffin oil particles for monitoring the processes of oxygen production in the interior of a bean leaf. The shape of the EPR signal from CSL in paraffin oil particles does not change with the injection of the emulsion into the leaf. Also, in contrast with the variety of other water soluble and amphiphylic spin labels injected into the leaves of higher plants without a protecting 'coat' [22], encapsulated CSL molecules have a very stable EPR signal whose amplitude does not change in the dark for a rather long period of time (tens of minutes or more). The stability of the EPR signal means that after the particles are injected into a leaf, the CSL molecules remain protected from reduction by the metabolites located inside the leaf.

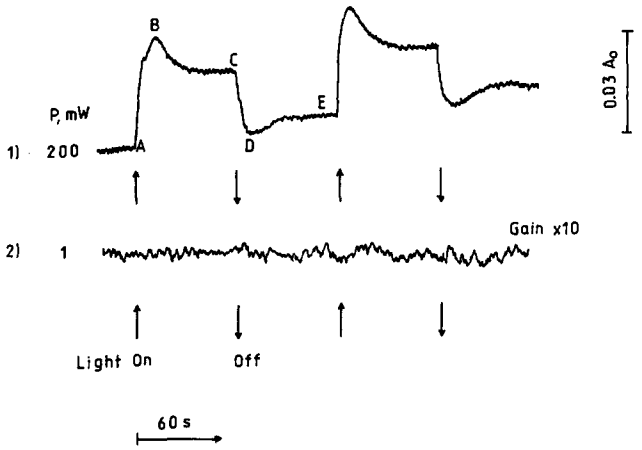

Fig. 4. The kinetics of the light-induced changes in the amplitude of the low-field peak of the EPR spectrum of CSL in light paraffin oil particles emulsion injected into a bean leaf. (1) microwave power 200 mW, (2) microwave power 1 mW, (↑) light on, (↓) light off. A_0, the amplitude of the low-field peak of the EPR spectrum in the dark for sample equilibrated with gas mixture of 30% air and 70% N_2.

The initial amplitude of the EPR signal recorded at high microwave power (200 mW) can be easily modulated by varying the gas mixture around the sample (a piece of bean leaf) placed in the cavity of the EPR spectrometer. With increasing N_2-flow, the height of the signal decreases indicating a drop in oxygen concentration in the leaf interior. After stopping the N_2–flow, oxygen diffusion from the atmosphere into the leaf leads to the concentration of oxygen in the surroundings of paraffin oil particles increases, which results in an enhancement of the EPR signal. The sensitivity of our method is limited at high concentrations of oxygen (Fig. 3). For this reason, to enhance the visualization of the light-induced evolution of oxygen, the initial level of oxygen in the leaf interior was usually reduced (by 50–70% of normal partial pressure of oxygen in the atmosphere) by a relatively weak flow of N_2 around the sample. Such a low reduction of oxygen partial pressure does not disturb the normal functioning of the electron transport chain in native chloroplasts.

Fig. 4 demonstrates the typical pattern of the light-induced response of the EPR signal from CSL in the bean leaf. In order to avoid interference between the EPR signals from CSL and the oxidized reaction centers $P700^+$ (so-called light-induced EPR signal 1 [34]), the magnetic field was centered on the low-field peak of the EPR signal from the spin label molecules. For this signal recorded at high level of microwave power (200 mW), switching on a white light induces the enhancement of the signal amplitude caused by the oxygen production in the leaf. The drop of the signal observed after ceasing the illumination (stage C-D) can be explained by the oxygen consumption due to certain metabolic processes, as well as by the diffusion of oxygen from the leaf tissue into the atmosphere. The following rise of the signal amplitude (stage D–E), may be accounted for by the oxygen production from the reaction of catalase with H_2O_2. Hydrogen peroxide, accumulated in the leaf as a result of light-induced oxygen reduction by chloroplasts, could partly compensate the oxygen consumption in the dark and its efflux from the leaf interior.

The light-induced increase in the amplitude of the EPR signal of CSL can be transformed into the change in the oxygen partial pressure with the use of a calibration curve (Fig. 3). It can be seen that in our experiment the light induces only a very small increase in the average oxygen partial pressure inside the leaf of about 7 mmHg, which corresponds to the increase in oxygen concentration in water of about 10 μM. This is a rather small change, which should not be damaging to the leaf tissue. This result indicates a good ventilation of the leaf (diffusion of O_2 from the leaf) and possible existence of other regulatory mechanisms, which keep the oxygen concentration low.

There is unambiguous evidence that it is changes in oxygen concentration and not the changes in nitroxide radical concentrations that cause the variations in the amplitude of the EPR signal from CSL. This follows from the fact that the fast light-induced response of the EPR signal was detected only at high microwave power ($P = 200$ mW, curve 1 on Fig. 4), while it was absent at rather low level of microwave power ($P = 1$ mW, curve 2 on Fig. 4). Additionally, we show in the control experiment that for deoxygenated samples, the signal amplitude of CSL in light paraffin oil recorded at 1 mW microwave power increases with an increase in CSL concentration up to $2.5 \cdot 10^{-2}$ M. Although at the highest CSL concentrations, deviation from linearity is observed.

Oxygen release is observed only during an illumination of the leaf by the light exiting both photosystems ($\lambda \leq 700$ nm or white light), while the far-red light ($\lambda \geq 700$ nm) exiting predominantly PS I is inefficient for oxygen production. As one can see from curve 1 on Fig. 4, the signal response to switching on continuous white light reveals characteristic 'overshoot' (A-B-C). This overshoot may be the result of some regulatory mechanisms. Indeed, such nonmonotonous changes in oxygen concentration inside the leaf also correlate with the well-known nonmonotonous behavior of the Photosystem I reaction center P700 observed earlier in the leaves of various species [34–38]. The typical pattern of the light-induced kinetics of P700 redox transients in dark-adapted bean leaf is shown on Fig. 5. Far-red illumination ($\lambda_{max} = 707$ nm) of the leaf causes the oxidation of P700 centers. After switching from far-red to white light and efficiently exciting both photosystems, the EPR signal from $P700^+$ nonmonotonously reaches a new steady state level (curve A-B-C). It has

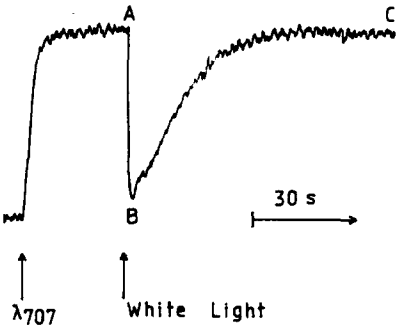

Fig. 5. The kinetics of the light-induced changes in the amplitude of the EPR signal from oxidized reaction centers $P700^+$ in bean leaf.

been demonstrated in [35–38] that in the leaves of higher plants such nonmonotonous response of the reaction centers P700 is determined mainly by the existence of regulatory mechanisms controlling the rate of electron transport at the acceptor side of Photosystem I (the activation of Calvin cycle enzymes in the course of the illumination of dark-adapted leaves) [39–46].

3.3. Light-induced uptake of oxygen by isolated chloroplasts

If the superoxide dismutase reaction ($2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$) and catalase reaction ($H_2O_2 \rightarrow 1/2O_2 + H_2O$) are not the rate-limiting factors in class B chloroplasts, the light-induced uptake of oxygen at the acceptor side of PS I ($2e^- + 2H^+ + 1/2O_2 \rightarrow H_2O$) should be stoichiometrically balanced by oxygen production in PS II ($H_2O \rightarrow 2e^- + 2H^+ + 1/2O_2$). It is a common knowledge that in the presence of sufficient amounts of catalase the illumination of chloroplasts does not lead to any visible changes in oxygen concentration. Our measurements, performed with the Clark electrode as well as the use of a spin labeling technique, are consistent with this fact (data not shown). On the other hand, if the catalase activity of chloroplasts limits the regeneration of oxygen (or its activity is artificially inhibited by KCN or NaN_3), in the course of functioning of the chain of the noncyclic electron transport (from H_2O to O_2) the net balance between oxygen consumption at the acceptor side of PS I and its production by PS II would correspond to the uptake of oxygen from the chloroplasts suspension.

Fig. 6 illustrates that the microscopic spin label probe is also an adequate sensor for monitoring oxygen in a suspension of isolated bean chloroplasts. In the presence of methylviologen (curve 1 on Fig. 6), switching on white light induces an oxygen consumption, which is indicated by the immediate decrease in the amplitude of the EPR signal from CSL (stage A-B). A few seconds after beginning the illumination, the system reaches a quasi-steady state corresponding to the rather low concentration of oxygen (stage B-C). The immediate light-induced uptake of oxygen is less evident when methylviologen is not present (curve 2), and can be totally suppressed in chloroplasts treated with noncyclic electron transport inhibitors (e.g., addition of $HgCl_2$, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)), or 10 min heating at 55°C) as shown with curve 3. Using a calibration curve (Fig. 3), we can calculate that in chloroplast suspensions, in the presence of methylviologen, the oxygen partial pressure drops during illumination from 157 mmHg (system saturated with air) to about 0 mmHg. The drop in the concentration of oxygen at the initial stage of chloroplasts illumination (A-B-C) is a reversible process: after stopping the illumination, the amplitude of the EPR signal increases (stage C-D), indicating a restoration of oxygen in the chloroplast suspension. The process of oxygen recovery in the dark may be accounted for by superoxide dismutase and catalase reactions. The rise of oxygen concentration in the dark indicates the presence of significant amounts of superoxide radi-

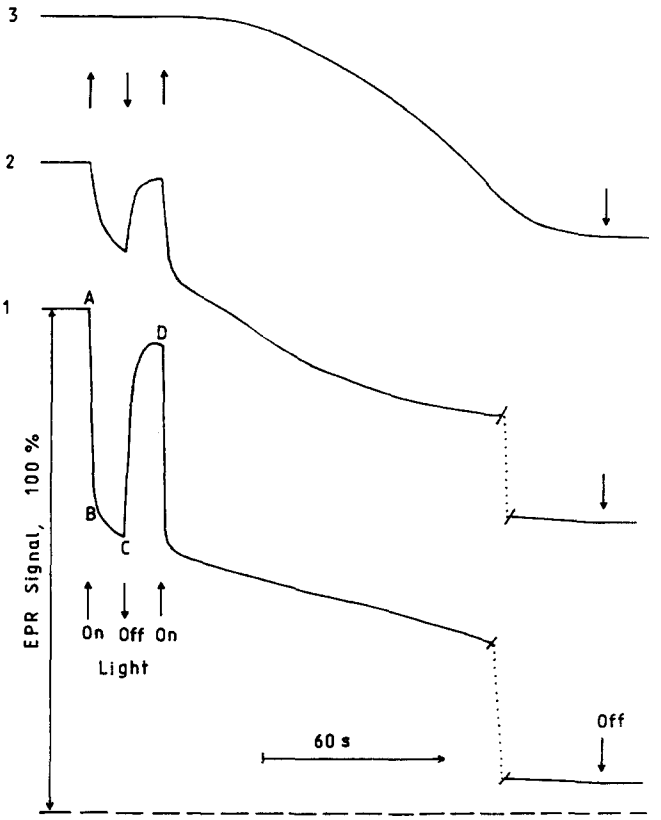

Fig. 6. The kinetics of the light-induced changes in the amplitude of the low-field peak of the EPR spectrum of CSL in light paraffin oil particles in the suspension of bean chloroplasts recorded at 200 mW microwave power. (1) in the presence of 20 μM methylviologen, (2) without added electron acceptors, (3) in the presence of 10 mM $HgCl_2$ and 20 μM DCMU. 100% correspond to the signal amplitude for sample saturated with air. The discontinuity on the time axis (dotted line) indicates a 10 min period of continuous illumination of the sample without changes in the signal amplitude being recorded.

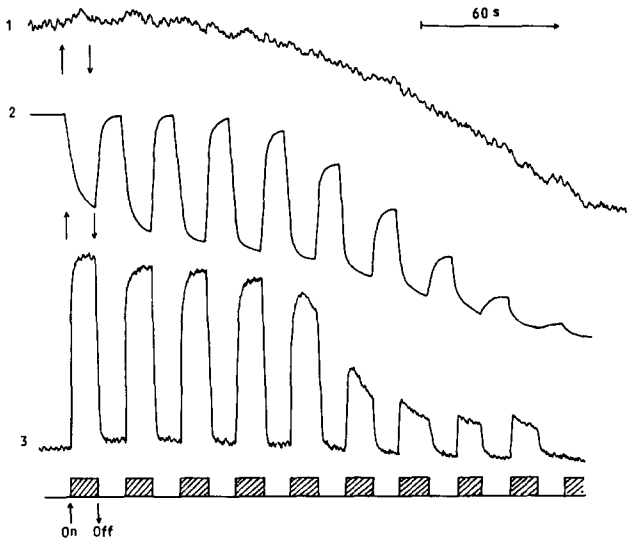

Fig. 7. The kinetics of the light-induced changes in the amplitude of the low-field peak of the EPR spectrum of CSL in light paraffin oil particles in the suspension of bean chloroplasts, (1) microwave power 1 mW, (2) microwave power 200 mW, and the kinetics of the light-induced changes in the amplitude the EPR signal from oxidized reaction centers $P700^+$ (3).

cals, $O_2^{\cdot-}$, and hydrogen peroxide, H_2O_2, that can be a source for oxygen production as a result of superoxide dismutase and catalase reactions.

The lack of corresponding light-induced responses of the signal from CSL recorded at low level of microwave power (curve 1 on Fig. 7) demonstrates that there is no concomitant redox transformations of nitroxide radicals. Thus, for a relatively long period of time (about one min in our experimental conditions) spin label molecules do not react chemically with electron intermediates produced in the course of chloroplast illumination. Reversible changes in the amplitude of the EPR signal, corresponding to fast light-induced oxygen consumption (A-B) and its production in the dark (C-D), can be repeated several times (curve 2 on Fig. 7). However, after a sufficiently long time of illumination the ability of chloroplasts to regenerate oxygen in the dark disappears. At this stage, essential exhaustion of oxygen is accompanied with the loss of CSL paramagnetism (caused, probably, by the reduction of nitroxide radicals). This is indicated by the decline in the amplitude of the EPR signal recorded at both levels of microwave power, 1 and 200 mW (compare curves 1 and 2 on Fig. 7). Redox transformations of CSL molecules suggests that nitroxide radicals could accept electrons from certain intermediates produced in the course of chloroplasts illumination. However, under normal conditions this process proceeds too slowly, being visualized only after practically full exhausting of oxygen in chloroplasts suspension. It is also likely that during the first few cycles of the illumination, effective reoxidation of CSL will take place because of the interaction with oxygen derived active forms, especially with $O_2^{\cdot-}$ [47,48]. As we discussed above, during chloroplasts illumination oxygen is transformed to $O_2^{\cdot-}$, and in the dark it is covered back to molecular oxygen. It can be concluded that the effective reduction of CSL occurs only after exhaustion of oxygen and $O_2^{\cdot-}$. Thus, we can conclude that oxygen is much more efficient electron acceptor than CSL molecules confined to BSA-coated light paraffin oil particles. This circumstance clearly demonstrates the advantage of our microscopic probe technique for oximetry as compared with the other conventional spin probe methods.

Earlier, using the traditional approach for spin-label oximetry based on the dependence of superhyperfine structure parameter of the EPR spectra of a spin probe 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl (CTPO) on oxygen concentration [11], we demonstrated that electron flow from PS I to oxygen (via methylviologen) does not limit the overall rate of electron transport over a wide range of oxygen concentrations (20–200 μM). However, with the significant exhaustion of oxygen in the sample, the outflow of electrons from PS I becomes the factor limiting the overall rate of the noncyclic electron transport. It follows from Fig. 7 that the kinetic behavior of the light-induced EPR signal from P700 correlates with the behavior of the EPR signal from CSL. Concomitant with the essential disappearance of oxygen (curve 2 on Fig. 7) is the decrease in the concentration of oxidized centers P700 (curve 3 on Fig. 7). Obviously, the lack of oxygen hinders the electron outflow from PS I, retarding the oxidation of P700 centers. This effect is reversible: after re-equilibrating the sample with the air, the light-induced response of the EPR signal from P700 increases, indicating the activation of electron flow from PS I to oxygen (data not shown).

Summing up the results of our study, we conclude that this new approach in spin-label oximetry appears to be fruitful for studying oxidative processes in photosynthetic systems. In concentrated suspensions of chloroplasts an application of the earlier method, based on using the superhyperfine structure parameter of the EPR spectra of a spin probe CTPO [11], is limited due to the fast reduction of the nitroxide radicals. The main advantage of our method is determined by the relatively high protection of spin probe molecules from the action of chemically active metabolites. Using this method, we were able to monitor photosynthetic oxygen production and its consumption in the interior of the leaf. The comparison of the kinetics of P700 redox transients with the oximetric data have demonstrated that oxygen serves the role of the essential factor in controlling electron transport in leaves and isolated chloroplasts.

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