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# Effects of Oxygen on the Dark Recombination between Photoreduced Secondary Quinone and Oxidized Bacteriochlorophyll in *Rhodobacter sphaeroides* Reaction Centers

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**Abstract**—The influence of duration of exposure to actinic light (from 1 sec to 10 min) and temperature (from 3 to 35°C) on the temporary stabilization of the photomobilized electron in the secondary quinone acceptor ($Q_B$) locus of *Rhodobacter sphaeroides* reaction centers (RC) was studied under aerobic or anaerobic conditions. Optical spectrophotometry and ESR methods were used. The stabilization time increased significantly upon increasing the exposure duration under aerobic conditions. The stabilization time decreased under anaerobic conditions, its dependence on light exposure duration being significantly less pronounced. Generation of superoxide radical in photoactivated aerobic samples was revealed by the ESR method. Possible interpretation of the effects is suggested in terms of interaction between the semiquinone $Q_B$ with oxygen, the interaction efficiency being determined by the conformational transitions in the structure of RC triggered by actinic light on and off.

*Key words*: purple bacteria, photosynthetic reaction center, ubiquinones, electron transport, oxygen

It was shown in the pioneering study of oxygen interaction with photosynthetic membranes (chromatophores) and isolated reaction centers (RC) of purple bacteria that exposure of chromatophores or isolated RC preparations from *Rhodospirillum rubrum* to actinic light was accompanied by oxygen uptake [1]. The process of light-induced oxygen uptake was found to be mediated by oxidation of photoreduced secondary quinone acceptor (ubiquinone-10, $Q_B$) of reaction center [1]. Preparations of RC were tested in the presence of exogenous electron donors for the photoactive RC bacteriochlorophyll (P), which mediated the light-induced reduction of $Q_B$. None of the redox components preceding $Q_B$ in the RC electron transport chain interact with oxygen. However, such interaction is observed in RC preparations isolated from photosynthetic membranes in the presence of detergent (lauryl dimethylamine oxide or Triton X-100), agents causing solubilization of membrane proteins, if the detergent concentration is increased to 0.3%. According to [1], in RC preparations with detergent-induced structural modification, oxygen is capable of accepting electron from both the primary quinone ($Q_A$, ubiquinone-10) and bacteriopheophytin.

It is well known that photoactivation of RC in purple bacteria is accompanied by fast (<1 msec) transfer of a photomobilized electron to $Q_B$. In the absence of external donors for photooxidized P, the electron from $Q_B$ during further dark period returns to $P^+$ due to the reversion of the direct transfer reaction. The characteristic time of the recombination process may exceed 1 sec. The electrostatic stabilization of the photomobilized electron in quinone acceptors of RC is associated with displacement of protons and surrounding groups in RC interior. Resulting modification of the charge state of quinone induces changes in the values of p*K* of protonated amino acid residues of RC protein at distances of up to 15-17 Å [2-4]. Reduction of the secondary quinone is accompanied by a 5 Å shift in its position in the RC interior toward the protein surface (this distance in the dark is about 10 Å), whereas the quinone ring plane is rotated through 180° relative to the isoprenoid chain. The fact of the quinone shift and its ring plane rotation is seen by the results of the X-ray diffraction analysis of the *Rhodobacter sphaeroides* RC crystals frozen to cryogenic temperature under conditions of exposure to actinic light [5]. The acceptors $Q_A$ and $Q_B$ in these RC preparations are also represented by ubiquinone-10 molecules. It was shown that the lifetime of stabilization of the photomobilized electron in $Q_B$

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depends on the intensity of actinic light and exposure time: an increase in the intensity of actinic light or exposure time was accompanied by a decrease in the rate of dark recombination between $P^+$ and $Q_B^-$ [6, 7]. This phenomenon was interpreted in [6, 7] as evidence of possible conformational changes of RC induced by $Q_B$ reduction and providing more effective prevention of electron recombination. Under conditions of long-term photoactivation, polarization of molecular groups surrounding the secondary quinone in the RC interior causes an effective decrease in the level of free energy of electron in $Q_B$, thereby decreasing the probability of back electron tunneling to $P^+$. However, there are reports in the literature that molecular oxygen can also contribute to stabilization of electron in quinone acceptors. However, according to [8], the mechanisms of the interaction (reoxidation of semiquinone) remain obscure.

The goal of this work was to elucidate the effects of molecular oxygen on the one-electron interaction between P and $Q_B$ in bacterial RC preparations isolated from *Rb. sphaeroides*. These preparations contained no exogenous electron donors. This study was of particular interest in the context of refinement of the role of conformational dynamics of RC in the functional activity of the quinone acceptor segment of the photosynthetic electron transport chain, which fulfils an important physiological function of coupling of extremely fast processes of light-induced intraprotein charge separation with further diffusion-controlled processes of transport of reducing equivalents in the photosynthetic membrane.

## MATERIALS AND METHODS

To obtain cell biomass, the non-sulfur purple bacteria *Rhodobacter sphaeroides* were grown in Ormerud liquid culture medium [9] under anaerobic conditions in a luminostat at a temperature of 30°C for 3-5 days. Cells were disrupted by sonication at 4°C using an UZDN-1 ultrasonic disintegrator. Chromatophores were isolated by centrifugation and incubated for 30 min at 4°C in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.5% zwitterionic detergent lauryl dimethylamine oxide (LDAO). After the incubation, chromatophores were centrifuged at 144,000*g* for 90 min at 4°C. The supernatant fraction containing RC was separated chromatographically on a column with hydroxyapatite as described in more detail in [10]. Concentration of RC preparations suspended in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.05% LDAO was about 10 μM. Light-induced reactions were studied either spectrophotometrically (by measuring absorption changes in the $Q_y$ absorption band of bacteriochlorophyll P at 870 nm) using a locally-made single-beam differential spectrophotometer or by monitoring the ESR signal of cation-radical $P^+$ with *g*-factor 2.0026 using a commercially available RE-1307 ESR spectrometer (microwave range, 3 cm; frequency of magnetic field modulation, 100 kHz). Kinetics of ESR signals of $P^+$ was recorded at the high-frequency modulation amplitude of 4 Gs. During spectrophotometric measurements, a 1- or 2-mm-thick cuvette with a sample was arranged at angles of 45° to beams of monitoring and actinic light, which were separated from one another with a rotary phosphoroscope. Intensity of continuous actinic light emitted by an incandescent lamp (spectral range of >600 nm was limited with a glass filter) was about 100 $J \cdot m^{-2} \cdot sec^{-1}$. A flat quartz cuvette (inner thickness, about 0.25 mm) with a sample was used in ESR measurements. Intensity of continuous actinic light (400-800 nm) was about 1 $kJ \cdot m^{-2} \cdot sec^{-1}$. The methods of optical and ESR measurements on these devices were described in more detail in [11]. To attain anaerobic conditions during optical measurements, a hermetically sealed cuvette with RC was flushed with argon for 5 min. Similar anaerobic conditions during ESR measurements were attained by addition of 6 mM glucose, 4 IU/ml glucose oxidase, and 400 IU/ml catalase to RC sample and further flushing with argon. Enzymatic oxidation of glucose provided more complete removal of oxygen from the RC sample.

## RESULTS AND DISCUSSION

Kinetic curves of light-induced absorption changes of bacteriochlorophyll P were measured in RC preparations of *Rb. sphaeroides* under aerobic or anaerobic conditions at different duration of photoactivation (Fig. 1). Kinetic curves of formation and decay of the light-induced ESR signal of the cation-radical P, as measured under similar conditions, are shown in Fig. 2. Kinetic parameters of dark reduction of photooxidized bacteriochlorophyll by photoreduced secondary quinone in RC *Rb. sphaeroides* at different duration of photoactivation are given in the table. Kinetic curves were represented as a sum of two exponential components. Values of characteristic time ($t_{1/2}$) of the two exponential components and relative amplitude of the first component (%) are given in the table. Preparations of reaction centers used in these experiments (both optical and ESR measurements) were isolated from different batches of bacterial culture. As noted above, actinic light intensity in ESR measurements was higher than in spectrophotometric measurements.

It follows from these data that under aerobic conditions there was a significant increase in the characteristic time of $P^+$ reduction upon increasing the photoactivation duration. This effect was particularly pronounced at short duration of photoactivation (less than 1 min). Oxygen removal from experimental samples caused a significant decrease in the characteristic time of $P^+$ reduction (table). This effect was particularly pronounced in ESR measurements. Perhaps this was due to more effective

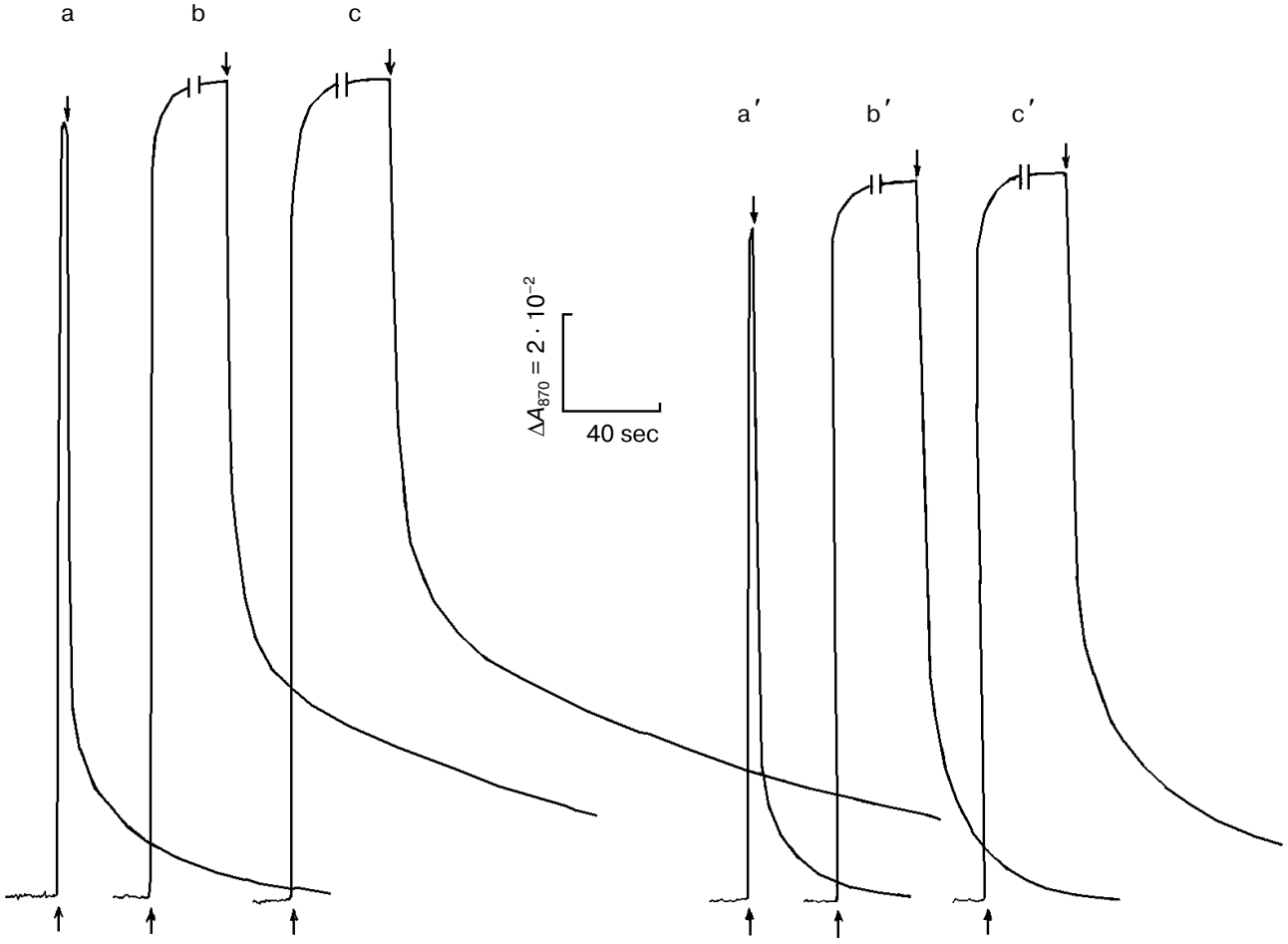


**Fig. 1.** Kinetic curves of light-induced absorption changes (at 870 nm) of bacteriochlorophyll P as measured using optical spectrophotometry in RC preparations of *Rb. sphaeroides* under (a, b, c) aerobic and (a′, b′, c′) anaerobic conditions at different duration of photoactivation: a, a′) 1 sec; b, b′) 1 min; c, c′) 5 min. Temperature, 22°C. Upward and downward arrows show moments of actinic light (100 $J \cdot m^{-2} \cdot sec^{-1}$) on and off, respectively.

removal of oxygen from the ESR cuvette (see "Materials and Methods"). The influence of the photoactivation duration on kinetic parameters of dark reduction of photooxidized bacteriochlorophyll $P^+$ under anaerobic conditions was significantly less pronounced than under aerobic conditions.

It follows from the results described above that the presence of oxygen in RC preparations exerts a significant effect both on the characteristic time of stabilization of photomobilized electron in $Q_B$ and on the dependence of the characteristic time of electron stabilization on the duration of photoactivation. To analyze hypothetical mechanisms of molecular oxygen involvement in prolongation of electron trapping in the acceptor site of RC we used ESR spectrometry for testing possible generation of superoxide radical under conditions of long-term exposure of experimental samples to light. It is well known from the literature that ubisemiquinone radicals can interact with molecular oxygen giving rise to generation of superoxide anion-radical [12]. Indeed, illumination of RC suspension containing $10^{-3}$ M Tiron (sodium 1,2-dihydroxybenzene-3,5-disulfonate), an indicator of the presence of superoxide anion-radical in the system tested [13], was accompanied by the appearance of signals typical of generation of these radicals. The light-induced signal of Tiron was characterized by longer rise time and shorter decay time (actinic light on and off, respectively) as compared to corresponding kinetic characteristics of the cation-radical P (corresponding signals are shown in Fig. 3). For example, the rise time of the light-induced bacteriochlorophyll cation-radical signal was less than 1 sec (resolution time of detection system). Light-induced generation of the Tiron signal had two-component kinetics: 1) component with rise time of less than 1 sec (amplitude contribution, 60%), and 2) component with rise time of 18 sec (40%). The processes of dark decay of both Tiron and bacteriochlorophyll signals, as measured after 3.5-min photoactivation, were character-

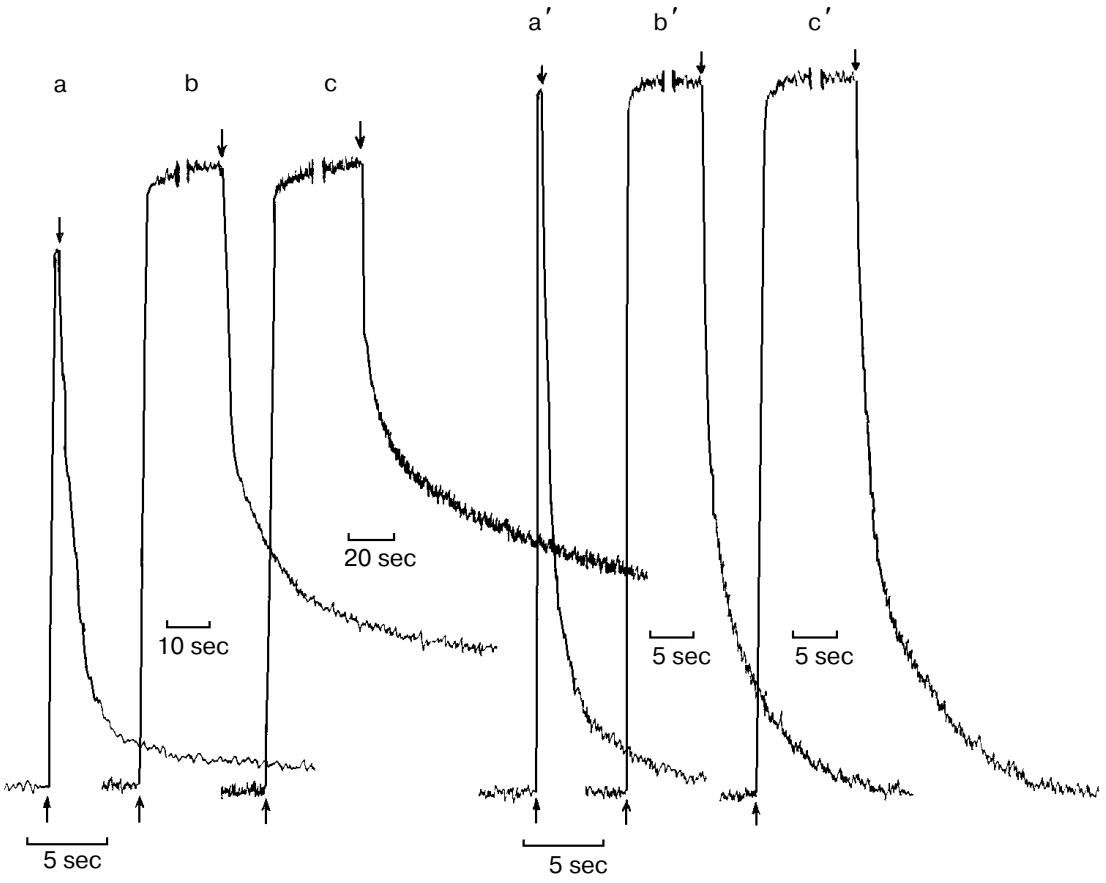


**Fig. 2.** Kinetic curves of formation and decay of the light-induced ESR signal of the cation-radical P, as measured using ESR spectrometry in RC preparations of *Rb. sphaeroides* under (a, b, c) aerobic and (a′, b′, c′) anaerobic conditions at different duration of photoactivation: a, a′) 1 sec; b, b′) 1 min; c, c′) 5 min. Temperature, 22°C. Upward and downward arrows show moments of actinic light (1 kJ·m$^{-2}$·sec$^{-1}$) on and off, respectively. Signal amplitude is given in arbitrary units.

Dependence of kinetic parameters of dark reduction of photooxidized bacteriochlorophyll in RC *Rb. sphaeroides* on the duration of photoactivation under aerobic and anaerobic conditions. Values of characteristic time ($t_{1/2}$) of two exponential components and relative amplitude (%) of faster component are given

| Duration of photoactivation | Optical spectrophotometry | | ESR spectrometry | |
|---|---|---|---|---|
| | Aerobic conditions | Anaerobic conditions | Aerobic conditions | Anaerobic conditions |
| 1 sec | ≅1 sec (80%) 28 sec | ≅1 sec (60%) 8 sec | ≅1 sec (95%) 40 sec | ≅1 sec (80%) 4.5 sec |
| 10 sec | | | 1.5 sec (90%) 75 sec | ≅1 sec (52%) 4.5 sec |
| 30 sec | ≅1 sec (77%) 70 sec | ≅1 sec (55%) 11 sec | 2.5 sec (80%) 140 sec | ≅1 sec (52%) 5 sec |
| 1 min | ≅1 sec (70%) 120 sec | ≅1 sec (40%) 11 sec | 3 sec (70%) 160 sec | ≅1 sec (50%) 5 sec |
| 2 min | | | 4 sec (60%) 175 sec | ≅1 sec (50%) 5 sec |
| 3 min | ≅1 sec (50%) 155 sec | ≅1 sec (68%) 20 sec | | |
| 5 min | ≅1 sec (60%) 140 sec | ≅1 sec (75%) 60 sec | 4 sec (55%) 200 sec | ≅1 sec (50%) 6 sec |
| 10 min | | | 4 sec (60%) 220 sec | ≅1 sec (50%) 9 sec |

ized by two-component kinetics. In case of Tiron signal, the first component of signal decay had characteristic time of 1.5 sec and amplitude contribution of 40%. The second component of Tiron signal decay had characteristic time of 40 sec. In case of bacteriochlorophyll, the signal characteristic times of two components of dark decay were 3.5 and 180 sec (amplitude contribution, 50% each).

It is well known from the literature that superoxide anion is capable of inducing deprotonation of certain organic compounds [14]. In addition to some other processes, this capacity underlies the mechanism of superoxide-induced generation of the ESR signal of Tiron. It may also be suggested that long-term photoactivation of macromolecular RC complexes gives rise to development of specific conformational changes. Redox characteristics of quinones in RC interior and position of their energy levels are very sensitive to the nature of closest protein surrounding and orientation of substituting side groups relative to the benzoquinone ring plane. These parameters can be substantially modulated by even weak internal structural perturbations of RC [15, 16]. Perhaps, the light-induced conformational changes and $Q_B^-$ displacement in RC structure toward the site of the channel, through which hydroquinone is exchanged by membrane pool quinone in functional membrane and molecular oxygen migrates to semiquinone, cause displacement of photomobilized electron toward oxygen. The electron displacement to oxygen modifies the state of mobile lateral proton in the Tiron molecule, which is indicated by the appearance of its light-induced signal. The channel connecting the $Q_B$ binding site with bulk water phase is thought to be easily permeable for Tiron molecule, because even larger molecules (e.g., *o*-phenanthroline) readily migrate to the $Q_B$ locus and displace the secondary quinone from its binding site in RC structure [17]. After actinic light had been off, reaction centers relaxed to the initial dark state. This relaxation was accompanied by structural changes in the microenvironment of $Q_B$, which modulate its redox properties and facilitate the reaction of the photomobilized electron transfer back to $Q_B$ and further tunneling to oxidized P. The pathway of these electron transport reactions and related events is schematically shown in Fig. 4. It seems that the process of the photomobilized electron transfer back to $Q_B$ does take place. This suggestion is confirmed by model experiments, in which benzoquinone ring reacted as an effective quencher of superoxide radical [18]. Experimental findings that freezing of RC to cryogenic temperature in the light with further switching actinic light off results in stabilization of a significant

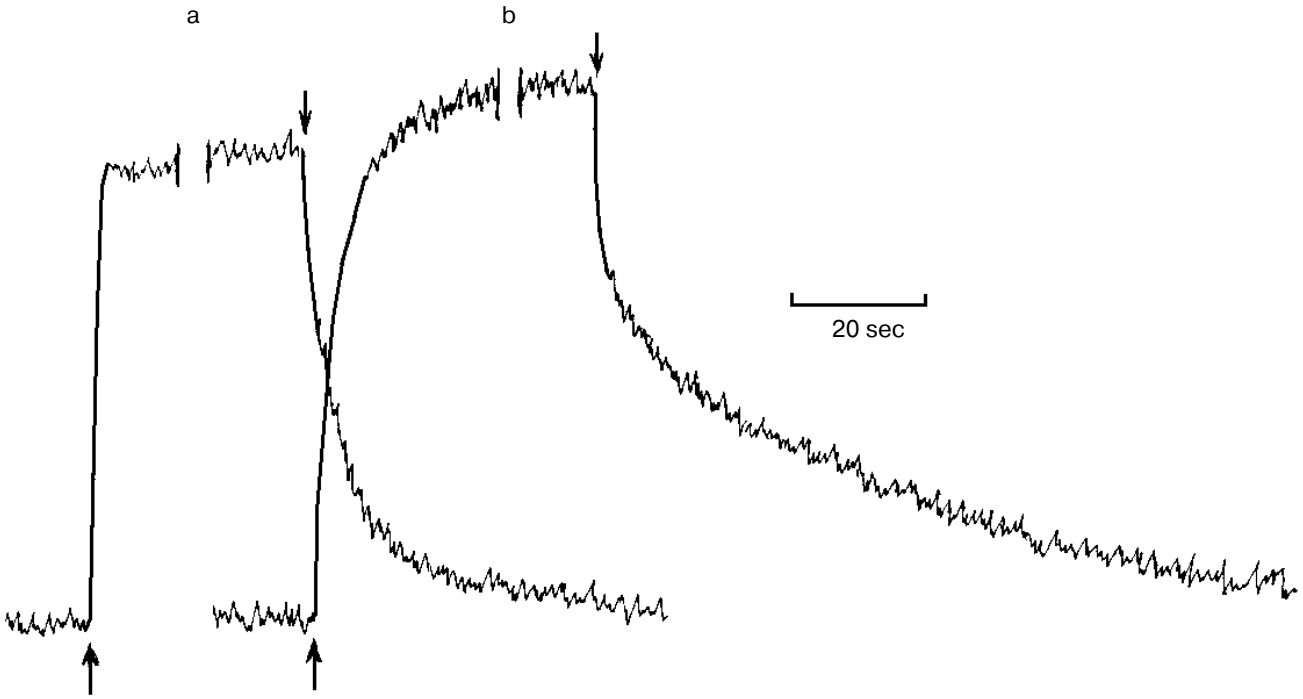


**Fig. 3.** Kinetic curves of formation and decay of the light-induced ESR signal of (a) bacteriochlorophyll cation-radical and (b) Tiron as measured using ESR spectrometry in RC preparations of *Rb. sphaeroides* under aerobic conditions at the duration of photoactivation of 3.5 min. Because signals of bacteriochlorophyll cation-radical and Tiron overlap with one another, the kinetic curve the light-induced ESR signal of Tiron was recorded at the magnetic field value corresponding to the first component of the signal at the high-frequency modulation amplitude of 0.2 Gs. Under these settings, the contribution of $P^+$ to recorded signal was minimal. Temperature, 22°C. Upward and downward arrows show moments of actinic light on and off, respectively. Signal amplitude is given in arbitrary units. Time scale: a) 180 sec; b) 20 sec.

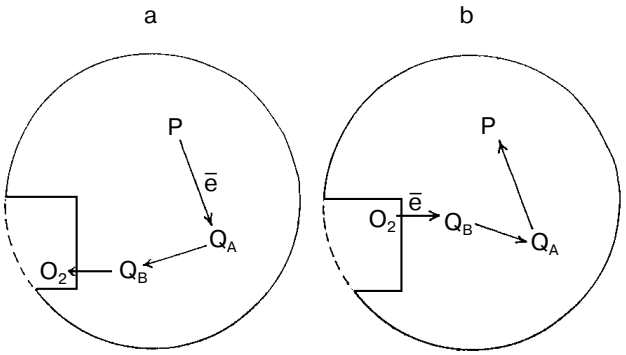


**Fig. 4.** Scheme of structural and functional states of pigment–protein complexes of RC under aerobic conditions: a) during exposure to actinic light; b) after switching actinic light off.

fraction (up to 70-80%) of reaction center for an indefinitely long period of time in the state with oxidized P can be regarded as evidence of the requirement of structural changes of RC for effective electron transfer back to $Q_B$ in the dark (after switching actinic light off). If this sample is then heated in the dark above −60°C (temperature threshold of activation of conformational mobility of RC [19]), the electrons "frozen" at the acceptor side of RC return to oxidized P.

In accordance with the scheme of electron transport reactions and related processes induced by long-term exposure to actinic light (Fig. 4), the ESR signal of the semiquinone $Q_B$ should disappear during photoactivation. This effect was indeed observed in our experiments. As suggested in [20], the processes of generation and decay of semiquinone anion were monitored by absorption changes at 335 nm. It should be noted that direct detection of semiquinone anion-radical in RC is substantially hampered by significant broadening of its ESR signal caused by dipole–dipole interaction with non-heme iron located in RC complex between primary and secondary quinone acceptors. The kinetic curve of light-induced absorption changes at 335 nm is shown in Fig. 5. Kinetic curves were measured in RC preparations without addition and in the presence of $10^{-2}$ M *o*-phenanthroline. As noted above, *o*-phenanthroline is an inhibitor of electron transfer from $Q_A$ to $Q_B$, which displaces the secondary quinone from its binding site in RC structure. It follows from Fig. 5 that in RC preparations with uninhibited electron transfer from $Q_A$ to $Q_B$ an increase in the light activation duration is accompanied by a gradual decrease in the amplitude of the light-induced signal of semiquinone anion-radical. On the other hand, virtually rectangular signals were observed in RC preparations tested in the presence of *o*-phenanthroline: the light-induced concentration of the semiquinone form of $Q_A$ was maintained at a virtually constant level, whereas the photomobilized electron rapidly ($t_{1/2}$, about 100 msec) returned back to oxidized P after the actinic light had been switched off.

In our opinion, the temperature dependence of the temporary stabilization of photomobilized electron in $Q_B$ can be regarded as indirect evidence in favor of the interpretation of experimental results suggested above. Similar

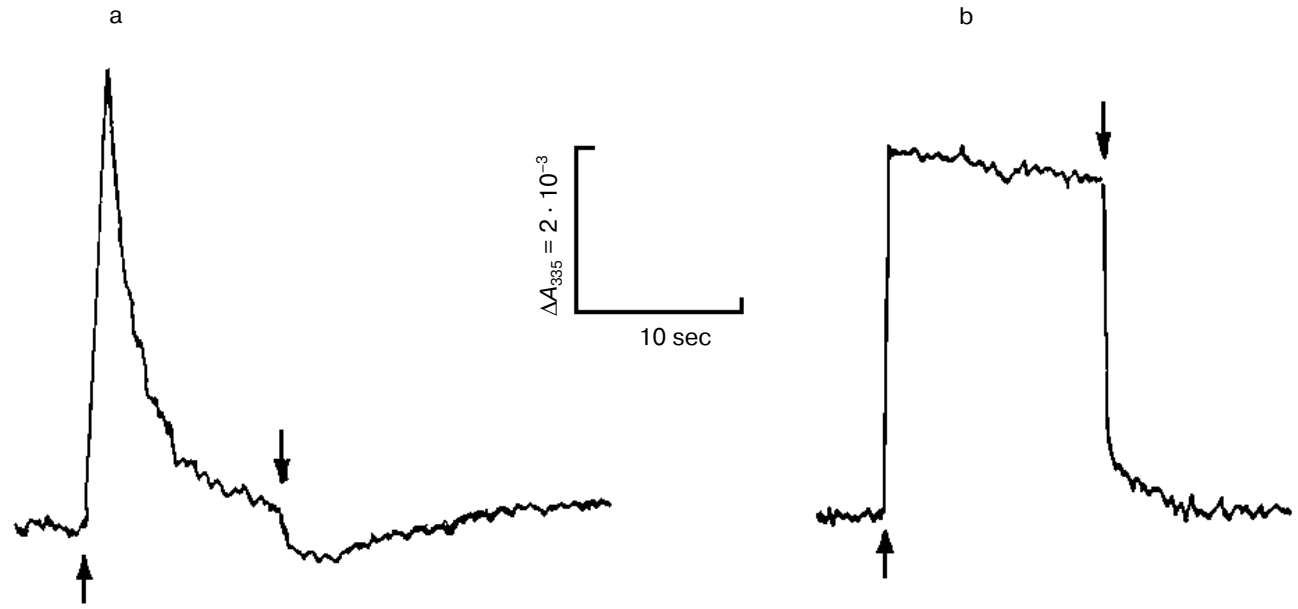


**Fig. 5.** Kinetic curve of light-induced changes in the absorption band of semiquinone anion-radical at 335 nm in RC *Rb. sphaeroides* under aerobic conditions. Temperature, 22°C. a) RC without addition; b) RC in the presence of $10^{-2}$ M *o*-phenanthroline. Upward and downward arrows show moments of actinic light on and off, respectively.

results were obtained at temperatures of 3 and 35°C. It was found that at short duration of photoactivation (<10 sec) under aerobic conditions kinetics of dark reduction of $P^+$ at 22°C was slower or faster than at 3 or 35°C, respectively. However, at long duration of photoactivation (>2 min) kinetic curves of dark reduction of $P^+$ from $Q_B^-$ at the three temperatures were similar to each other. Obviously, temperature decrease below or increase above the room level causes a decrease or an increase in the rate of conformational dynamics and diffusion of molecular oxygen to $Q_B$, respectively. At short duration of photoactivation, the photomobilized electron is stabilized at the acceptor side of RC at 3 and 35°C for a short and a long interval of time, respectively. However, this difference disappears upon increasing the photoactivation duration. An increase in the temperature from 3 to 35°C was also accompanied by a gradual decrease in the range of difference between the times of electron stabilization in $Q_B$ at different photoactivation duration.

As a result of addition of glycerol (up to 70 vol. %) to RC samples, the range of difference between the times of electron stabilization in $Q_B$ at short duration of photoactivation (<10 sec) and temperatures 22 and 35°C became larger than in samples without glycerol. Perhaps, this was due to the fact that upon increasing the temperature from 22 to 35°C, the viscosity of water–glycerol solution of RC decreases more steeply than viscosity of similar glycerol-free sample under otherwise identical conditions. According to the literature, the ratio of viscosity of 70% glycerol solution in water at temperatures 20 and 30°C is 1.6, whereas in pure water this ratio is 1.25 [21].

Thus, the results of this study confirm the role of molecular oxygen in the processes of long-term stabilization of photomobilized electron in the RC $Q_B$ locus of purple bacteria under conditions of one-electron photoreduction of the secondary quinone. Under physiological conditions in a functionally active cell, the semiquinone $Q_B$ can receive the second electron within hundreds of microseconds from the photoactive bacteriochlorophyll P prereduced by cytochromes. After that fully reduced quinone $Q_B$ takes up two protons from cytoplasm medium and migrates to the membrane, being substituted by a neutral quinone molecule from the membrane pool. Because the rate of electron transfer from photoactive pigment to photosynthetic membrane under normal conditions is maintained at a high level, the physiological role of molecular oxygen in this particular stage of the photosynthetic electron transport chain of purple bacteria seems to be negligible. However, it follows from the results considered above that the effects induced by molecular oxygen under specific experimental conditions (e.g., in isolated RC preparations capable of mediating cyclic reversible intraprotein one-electron transfer) can give valuable information for further elucidation of the role of structural dynamic organization and functional activity of bacterial RC.

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