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Effects of Oxygen, Heavy Water, and Glycerol on Electron Transfer in the Acceptor Part of *Rhodobacter sphaeroides* Reaction Centers

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Abstract—The kinetics of electron transfer between primary and secondary quinone acceptors of the photosynthetic reaction center (RC) of the purple bacterium *Rhodobacter sphaeroides* wild type was studied at the wavelengths 400 and 450 nm. It was shown that removing of molecular oxygen from RC preparations slowed down the fast phase of the process from 4-4.5 μsec to tens of microseconds. Similar effects were observed after the incubation of RC in heavy water for 72 h or glycerol addition (90% v/v) to RC preparations. The observed effects are interpreted in terms of the influence of these agents on the hydrogen bond system of the RC. The state of this system can determine the formation of different RC conformations that are characterized by different rates of electron transfer between quinone acceptors.

Key words: purple bacteria, photosynthetic reaction center, quinone acceptors, electron transport, oxygen, heavy water, glycerol

The effect of molecular oxygen on the temporary stabilization of an electron at the secondary quinone acceptor (Q_B) of photosynthetic reaction centers (RC) of the purple bacterium *Rhodobacter sphaeroides* was studied in the preceding work [1]. In the absence of exogenous donors of an electron, photoactivation of pigment–protein complexes of RC isolated from chromatophore membranes of *Rb. sphaeroides* induces fast (characteristic time, ~200 psec) transfer of an electron from the photoactive bacteriochlorophyll dimer (P) to the primary quinone acceptor (Q_A). Then, within about 200 μsec the electron is transferred to Q_B. In the RC of *Rb. sphaeroides*, the two quinone acceptors are molecules of ubiquinone-10. In further dark reactions the electron returns back to oxidized P. Electrostatic stabilization of the electron in quinone acceptors of RC is due to proton displacement in the RC microenvironment. Changes in the charge state of quinone modify the p*K* value of the protonated amino acid residues of the RC protein located within the vicinity of 15-17 Å [2-4].

The characteristic time of electron stabilization at Q_B is ~1 sec after activation with short light flashes. However, this time is substantially longer (tens of seconds) in the case of RC activation with continuous actinic light. It was shown in [5, 6] that such a decrease in the rate of dark reduction of P^+ after activation with continuous light was due to light-induced conformational changes in the RC. The magnitude of the conformational changes rises upon increasing the time of RC exposure to light. This process is accompanied by a corresponding decrease in the rate of dark reduction of P^+. It was shown in [1] that removal of molecular oxygen from RC preparations caused a significant decrease in the time of dark recombination between P^+ and Q_B^-. On the other hand, the rate of recombination between P^+ and Q_B^- in anaerobic samples still depends on the time of photoactivation. It was concluded that temporary stabilization of the electron in the locus Q_B of the *Rb. sphaeroides* RC exposed to long photoactivation was regulated by the RC conformation, molecular oxygen providing additional stabilization and further decrease in the rate of dark recombination between P^+ and Q_B^-.

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It was found that light-induced anion-semiquinone Q_B generated in aerobic RC samples decayed during illumination. This was accompanied by generation of superoxide anion, which disappeared during dark reduction of P^+ when the actinic light was off. A possible model of electron-transport events in RC under conditions of light-induced reversible transfer of one electron between the photoactive pigment and Q_B was suggested. Light-induced conformational changes in RC were suggested to control the reactivity of the secondary quinone acceptor. This mediates incorporation of electron from reduced Q_B into RC structure and reversible interaction with molecular oxygen. After cessation of actinic light the electron returns back to Q_B and then to P^+.

However, it is well known that direct photoinduced electron transfer from photoreduced Q_A to Q_B is also regulated by fast conformational changes in RC structure, the mechanism of the process being yet unidentified [7]. It was interesting to reveal whether or not the presence of oxygen had any effect on the rate of this reaction. The goal of this work was to study the effect of molecular oxygen on the rate of direct electron transfer from photoreduced Q_A to Q_B in *Rb. sphaeroides* RC.

MATERIALS AND METHODS

The cells of the wild-type nonsulfur purple bacterium *Rb. sphaeroides* were grown on liquid Ormerod culture medium [8] under anaerobic conditions in a luminostat at ~30°C for 4-6 days. Chromatophores (membranes containing photosynthetic apparatus) were isolated from sonicated cells by centrifugation and incubated in the dark for 30 min at 4°C in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.5% zwitterion detergent lauryl dimethylamine oxide (LDAO). After that, the chromatophore suspension was centrifuged (144,000*g*, 60 min, 4°C). The supernatant was brought to 22% saturation with solution of ammonium sulfate. The precipitate containing RC was dissolved in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.05% LDAO and subjected to chromatography on a column with hydroxyapatite. These procedures were described in more detail in [9]. The resulting RC preparation was suspended in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.05% LDAO and dialyzed against 0.01 M sodium phosphate buffer (pH 7.8) containing 0.1% anionic detergent sodium cholate. Following this procedure, RC preparations were more tolerant to long-term storage.

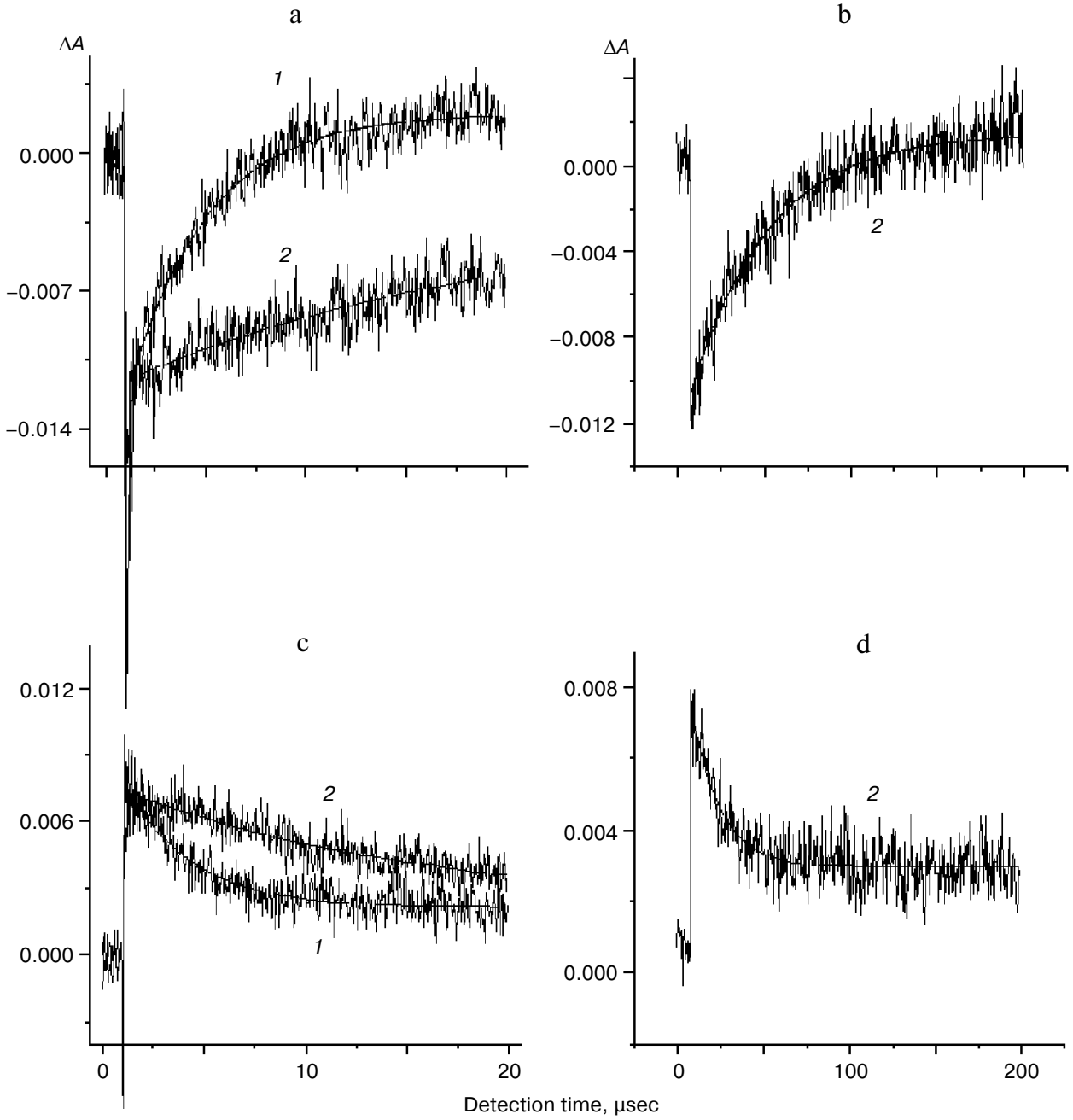
Absorption spectra of RC preparations were studied using a Shimadzu UV-VIS 2400-PC computer-assisted spectrophotometer (Japan). Photochemical activity of RC preparations within the microsecond time range was studied spectrophotometrically using an experimental set-up containing a Nd:YAG-laser (Spectrom Laser System; Warwickshire, England), λ = 532 nm (pulse energy, ~30 mJ; half-peak duration, ~10 nsec) and a xenon flash lamp (150 W). Pulse repetition frequency was 0.2 Hz. Kinetic curves of optical density changes were recorded using a Hewlett-Packard model 54510B digital oscilloscope connected to a personal computer. Five experimental kinetic curves were averaged. Optical cuvettes with 1 cm path length were used. The concentration of RC in samples was ~1 μM.

RESULTS AND DISCUSSION

In experimental research, the rate of electron transfer from Q_A to Q_B is mainly measured by two methods [10]. The first method is based on the use of two sequential light flashes inducing oxidation of hemes of cytochrome *c*, which is the secondary electron donor for photoactive RC bacteriochlorophyll dimer. This process is accompanied by light-induced reduction of RC acceptors. According to this method, the time interval between the two flashes should be long enough to allow the photomobilized electron to be transported from Q_A^- to Q_B. After that, the second heme of cytochrome can be oxidized [10, 11]. The second method is based on measurement of kinetics of electrochromic bandshift of RC bacteriopheophytin. This bandshift is due to electric environment changes in RC caused by electron transfer from Q_A^- to Q_B [10, 12, 13]. On the other hand, it is well known that intrinsic absorption bands of the semiquinone forms of RC ubiquinones are in the near UV and Soret spectral ranges [14]. However, overlap with spectral bands of other RC cofactors (primarily, photoactive bacteriochlorophyll) and electrochromic bandshifts of carotenoids makes it difficult to use absorption bands of these semiquinone forms for measuring the rate of electron transfer from Q_A^- to Q_B.

Spectral and amplitude similarity between optical changes associated with formation of semiquinones Q_A^- and Q_B^- presents additional difficulty for these measurements. Nevertheless, the characteristic absorption maximum of ubisemiquinone anion at 450 nm was used to study the processes of two-electron reduction of Q_B in the system containing exogenous electron donors for photooxidized P [15]. Substitution of the primary quinone (ubiquinone-10) by menaquinone was used in [16] to increase spectral difference between the semiquinone forms of Q_A and Q_B in the *Rb. sphaeroides* RC. The menaquinone primary acceptor Q_A has lower redox potential than the ubiquinone one. However, because the rate of electron transfer from Q_A^- to Q_B is controlled by the conformational "latch" [7] but does not depend on the "driving force" of the process, which is determined by the redox potential of Q_A, the method of quinone substitution proved to be a promising approach to direct studies of this reaction in the spectral bands of semiquinones.

The estimates of the rate constant of electron transfer from Q_A to Q_B obtained in isolated RC of purple bacteria using the method of double light flashes and method based on electrochromic shift of the absorption Q_Y-band of the RC bacteriopheophytin near 760 nm were found to be close to one another (characteristic time, ~200 μsec) [10, 12, 13]. On the other hand, the kinetics of the process in membrane preparations (chromatophores) contains faster components (characteristic time, tens of microseconds or <10 μsec) [17]. Detailed kinetic analysis of the process of the electron transfer from Q_A^- to Q_B in the spectral region of semiquinones of RC *Rb. sphaeroides* containing the menaquinone Q_A revealed that the isolated RC preparations were also characterized by three kinetic components of this reaction [16]. The characteristic times and amplitudes of these components are: $\tau_1 = 3.5 \pm 0.9$ μsec ($A_1 \sim 45\%$), $\tau_2 = 80 \pm 15$ μsec ($A_2 \sim 25\%$), $\tau_3 = 260 \pm 50$ μsec ($A_3 \sim 30\%$). In the authors opinion, the



Experimental curves of absorption changes at 400 (a, b) and 450 nm (c, d) induced by laser pulses in aerobic (*1*) and anaerobic (*2*) preparations of *Rb. sphaeroides* RC

Characteristic time (time constant τ_1) and amplitude (A_1) of fast kinetic component of electron transfer in the quinone acceptor site of aerobic and anaerobic RC preparations of *Rb. sphaeroides*, aerobic samples containing 90% (v/v) D_2O as measured immediately after addition and 72 h after addition, and in samples containing 90% glycerol. Photoinduced signals in samples were measured at wavelengths 400 and 450 nm; detection time intervals ranged from 20 to 200 μsec

Sample	400 nm		450 nm		Detection time range, μsec
	τ_1, μsec	A_1, %	τ_1, μsec	A_1, %	
Aerobic RC	4	100	3	71	20
	4	100	4.5	78	200
Anaerobic RC	20	100	10	70	20
	50	100	20	66	200
RC + ^2H_2O	3	100	3	47	20
	4.5	100	4	58	200
RC + ^2H_2O (after 72 h)	4	100	9.5	70	20
	10	100	9.5	70	50
RC + glycerol	7	100	10	50	50
	20	100	30	60	200

event of electron transfer from Q_A^- to Q_B itself is associated with the first component alone, whereas slower kinetic components are due to other concomitant processes in RC protein, e.g., displacement of protons and relaxation of protein structure.

In this work we studied the process of electron transfer in the quinone acceptor site of the *Rb. sphaeroides* RC preparations by analyzing the absorption changes at 400-450 nm induced by nanosecond light flashes. The wavelength 400 nm is close to the isobestic point of the absorption changes associated with photobleaching of bacteriochlorophyll P [10]. The contribution of these changes to the detected signal at this point is minimal. The spectral area near 450 nm is typical of the maximal absorption difference between the semiquinone forms of Q_A and Q_B [16].

The kinetics of the absorption changes in aerobic and anaerobic samples of *Rb. sphaeroides* RC are shown in the figure. The kinetics of dark relaxation of the photoinduced signal near 400 nm was approximated by a monoexponential curve. The characteristic time of the curve in aerobic samples was 4 μsec. More accurate kinetic analysis was impossible because of limited time range of detection (200 μsec) and low signal/noise ratio. Removal of oxygen from the sample caused a decrease in the rate of dark relaxation (figure). In this case, the characteristic time of the exponential signal decay was tens of microseconds (table).

The kinetics of dark relaxation of the photoinduced signal near 450 nm was approximated by the sum of a monoexponential component with characteristic time 3-4.5 μsec and a constant component with ~30% contribution to total signal amplitude. This constant component is perhaps due to absorption of the oxidized form of bacteriochlorophyll P. This form within the given spectral range is characterized by a broad unstructured differential absorption band [10].

It was suggested in [16] that experimentally detected kinetic components with τ_1 and τ_2 equal to several microseconds and tens of microseconds, respectively, could be attributed to two conformational states of RC, transitions between them being controlled by the protonation state of the amino acid Glu L-212 in the vicinity of Q_B. This amino acid is incorporated in a network of hydrogen bonds with other protonated amino acids and water molecules in RC structure with cell cytoplasm and plays an important role in the donation of the first proton to Q_B during formation of hydroquinone Q_BH_2 after two-electron reduction of this acceptor [7]. It is assumed that in the RC fraction with unprotonated Glu L-212 the rate of the electron transfer from Q_A to Q_B is higher than in the RC fraction with protonated Glu L-212 [16, 18]. Lower rate of electron transfer from Q_A to Q_B can be explained by the protonation of Glu L-212, which precedes the electron transfer event itself. It is conceivable that the transition between the RC states with different rates of electron transfer Q_A–Q_B is coupled with conformational changes affecting this process [18].

Small changes in orientation of molecular groups producing a chain of hydrogen bonds can modify the equilibrium position of the bonds [19]. Theoretical calculations showed that conformation-modulated dynamic changes in the geometry of amino acid residues producing hydrogen bonds could modify the p*K* value of inter-

acting groups, mediating thereby targeted proton transfer [19]. Perhaps the effects of oxygen on the electron transfer from Q_A to Q_B observed in our experiments in RC preparations were due to its influence on proton equilibrium in the system of hydrogen bonds near Q_B involved in electron stabilization on acceptor, e.g., its effect on the ratio of protonated and unprotonated forms of Glu L-212. Deoxygenation decreases the extent of protonation of the amino acid in the sample. This suggestion is consistent with results reported in [20]: deoxygenation of *Rb. sphaeroides* RC decreased the time of electron stabilization on Q_B after activation of RC with short light flashes. As noted above, this time (time of dark recombination between Q_B^- and P^+) is associated with electrostatic stabilization of an electron on Q_B caused by changes in the proton position in the vicinity of the acceptor [2-4].

Results of our studies of the effects of deuteration and glycerol on electron transport also confirmed an obvious role of the state of RC hydrogen bonds in modulation of electron transfer rate in the system of quinone acceptors. RC preparations were deuterated by adding heavy water (up to 90%) to the initial suspension in aqueous buffer. The final concentration of glycerol added to RC was 90%. Like in the case of deoxygenation, these factors also decreased the rate of electron transfer from Q_A to Q_B (table). It should be noted that although the effect of deuteration was virtually absent immediately after addition of D_2O, it developed during further prolonged (72 h) incubation of RC in D_2O. This fact is evidence that the effect of heavy water is not merely reduced to the solvent effect, because otherwise it should be manifested immediately after dissolving [21]. Obviously, it develops during incubation as a result of slow exchange of intraprotein protons by deuterons. This exchange apparently takes rather a long time interval (a few days) [21]. It was perhaps a secondary isotope effect [22], in which deuteration-induced modification of characteristics of proton bonds (e.g., decrease in the frequency and amplitude of vibrations of bound atoms [22], increase in the bond strength [23]) induces partial modification of the state of the system of RC hydrogen bonds or conformational state of the macromolecular complex.

The influence of substitution of H_2O by D_2O in RC preparations of purple bacteria on the rates of the intraprotein electron transport reactions was studied earlier. These studies revealed that isotope substitution caused a decrease in the rate of initial photoinduced charge separation in the porphyrin complexes of RC and further electron transfer to the primary quinone Q_A [24-26] and an increase in the rate of dark recombination between photooxidized bacteriochlorophyll P and Q_A^- under conditions of inhibited electron transfer from Q_A^- to Q_B [27]. We explained this effect in terms of deuteration-induced modification of the structural dynamic state of RC. Glycerol at high concentration exerted an effect similar to the deuteration-induced effect on charge separation and electron transfer to Q_A [24]. This agent is widely used as a cryoprotector and it also modifies the system of hydrogen bonds of protein molecules. It follows from the table that glycerol at high concentration causes a decrease in the rate of electron transfer at the quinone acceptor site of RC. Thus, the experimental data obtained in this work are in conformity with the conclusion that RC of purple bacteria is a macromolecular system with fine conformation regulation of specific high-efficiency electron-transport activity [1, 5, 6, 24-27].

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REFERENCES

1. Knox, P. P., Lukashev, E. P., Timofeev, K. N., and Seifullina, N. Kh. (2002) *Biochemistry (Moscow)*, **67**, 901-907.
2. Okamura, M. Y., and Feher, G. (1992) *Annu. Rev. Biochem.*, **61**, 861-896.
3. Lancaster, C. R. D., Michel, H., Honig, B., and Gunner, M. R. (1996) *Biophys. J.*, **70**, 2469-2492.
4. Miksovska, J., Maroti, P., Tandori, J., Schiffer, M., Hanson, D. K., and Sebban, P. (1996) *Biochemistry*, **35**, 15411-15417.
5. Abgaryan, G. A., Christophorov, L. N., Goushcha, A. O., Holzwarth, A. R., Kharkyanen, V. N., Knox, P. P., and Lukashev, E. P. (1998) *J. Biol. Phys.*, **24**, 1-17.
6. Goushcha, A. O., Manzo, A. J., Scott, G. W., Christophorov, L. N., Knox, P. P., Barabash, Yu. M., Kapustina, M. T., Berezetska, N. M., and Kharkyanen, V. N. (2003) *Biophys. J.*, **84**, 1146-1160.
7. Okamura, M. Y., Paddock, M. L., Graige, M. S., and Feher, G. (2000) *Biochim. Biophys. Acta*, **1458**, 148-163.
8. Pimenova, M. N., Grechushkina, N. N., Azova, L. G., Semenova, E. V., and Myl'nikova, S. I. (1983) *Practical Handbook of Microbiology* [in Russian], MGU Publishers, Moscow.
9. Zakharova, N. I., and Churbanova, I. Yu. (2000) *Biochemistry (Moscow)*, **65**, 149-159.
10. Clayton, R. K. (1981) *Photosynthesis: Physical Mechanisms and Chemical Patterns*, Cambridge University Press, London-New York.
11. Parson, W. W. (1969) *Biochim. Biophys. Acta*, **189**, 384-396.
12. Wraight, C. A. (1979) *Biochim. Biophys. Acta*, **548**, 309-327.
13. Tiede, D. M., Vazquez, J., Cordova, J., and Marone, P. A. (1996) *Biochemistry*, **35**, 10763-10775.
14. Bensasson, R., and Land, E. J. (1973) *Biochim. Biophys. Acta*, **325**, 175-181.
15. Shinkarev, V. P., and Wraight, C. A. (1993) in *The Photosynthetic Reaction Center*, Vol. 1 (Deisenhofer, J., and Norris, J., eds.) Academic Press, San Diego, pp. 193-255.
16. Li, J., Gilroy, D., Tiede, D. M., and Gunner, M. R. (1998) *Biochemistry*, **37**, 2818-2829.
17. Tiede, D. M., Gallo, D. M., and Hanson, D. K. (1997) *Biophys. J.*, **72**, A248.

18. Hienerwadel, R., Grzybek, S., Fogel, C., Kreutz, W., Okamura, M. Y., Paddock, M. L., Breton, J., Nabedryk, E., and Mantele, W. (1995) *Biochemistry*, **34**, 2832-2843.
19. Scheiner, S., and Hillenbrand, E. A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2741-2745.
20. Tandori, J., Hideg, E., Nagy, L., Maroti, P., and Vass, I. (2001) *Photosynth. Res.*, **70**, 175-184.
21. Cioni, P., and Strambini, B. (2002) *Biophys. J.*, **82**, 3246-3253.
22. Lobyshev, V. I., and Kalinichenko, L. P. (1978) *Isotope Effects of D_2O in Biological Systems* [in Russian], Nauka, Moscow.
23. Scheiner, S., and Cuma, M. (1996) *J. Am. Chem. Soc.*, **118**, 1511-1521.
24. Paschenko, V. Z., Korvatovskii, B. N., Logunov, S. L., Kononenko, A. A., Knox, P. P., Zakharova, N. I., Grishanova, N. P., and Rubin, A. B. (1987) *FEBS Lett.*, **214**, 28-34.
25. Paschenko, V. Z., Gorokhov, V. V., Grishanova, N. P., Goryacheva, E. A., Korvatovsky, B. N., Knox, P. P., Zakharova, N. I., and Rubin, A. B. (1998) *Biochim. Biophys. Acta*, **1364**, 361-372.
26. Paschenko, V. Z., Knox, P. P., Chamorovsky, S. K., Krasilnikov, P. M., Mamedov, M. D., Semenov, A. Yu., Zakharova, N. I., Renger, G., and Rubin, A. B. (2001) *Bioelectrochemistry*, **53/2**, 233-241.
27. Knox, P. P., Kononenko, A. A., and Rubin, A. B. (1980) *Biofizika*, **25**, 239-241.