ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 13, pp. 1465-1483. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © M. M. Leonova, T. Yu. Fufina, L. G. Vasilieva, V. A. Shuvalov, 2011, published in Uspekhi Biologicheskoi Khimii, 2011, Vol. 51, pp. 193-232.

REVIEW

Structure–Function Investigations of Bacterial Photosynthetic Reaction Centers

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Received March 18, 2011
Revision received May 16, 2011

Abstract—During photosynthesis light energy is converted into energy of chemical bonds through a series of electron and proton transfer reactions. Over the first ultrafast steps of photosynthesis that take place in the reaction center (RC) the quantum efficiency of the light energy transduction is nearly 100%. Compared to the plant and cyanobacterial photosystems, bacterial RCs are well studied and have relatively simple structure. Therefore they represent a useful model system both for manipulating of the electron transfer parameters to study detailed mechanisms of its separate steps as well as to investigate the common principles of the photosynthetic RC structure, function, and evolution. This review is focused on the research papers devoted to chemical and genetic modifications of the RCs of purple bacteria in order to study principles and mechanisms of their functioning. Investigations of the last two decades show that the maximal rates of the electron transfer reactions in the RC depend on a number of parameters. Chemical structure of the cofactors, distances between them, their relative orientation, and interactions to each other are of great importance for this process. By means of genetic and spectral methods, it was demonstrated that RC protein is also an essential factor affecting the efficiency of the photochemical charge separation. Finally, some of conservative water molecules found in RC not only contribute to stability of the protein structure, but are directly involved in the functioning of the complex.

DOI: 10.1134/S0006297911130074

Key words: bacterial photosynthesis, photosynthetic reaction center, site-directed mutagenesis, photosynthetic electron transfer, purple bacteria

The transmembrane electron transfer occurring in photosynthetic reaction centers (RCs) of bacteria and eukaryotes is one of the main stages of photosynthesis, which leads to generation of membrane potential. The quantum efficiency of light conversion during the initial stages of photosynthesis is close to 100%. Such high efficiency of light harvesting and light energy transformation without destruction of RC or any harmful side reactions is achieved by fine adjustment of the cofactor–protein interactions. Studies of the reaction centers from purple bacteria are important for understanding of principles and mechanisms of solar energy transformation during photosynthesis. These bacterial RCs are well characterized and have been used for many years as a structural and functional model for studying more complex photosystem 2 (PS2) of green plants and algae. During recent years significant progress has been made in crystallization and X-ray analysis of photosynthetic proteins. At present the influence of the RC protein and its conformational changes on the efficiency of the initial charge separation is a focal point of studies [1-3]. Our review is devoted to analysis of the studies of chemical and genetic modifications of purple bacteria RC structure, particularly those that used method of site-directed mutagenesis to study

Abbreviations: (B)Pheo, (bacterio)pheophytin; (B)Chl, (bacterio)chlorophyll; B_A and B_B, monomeric BChl of A or B branch of electron transfer cofactors; β, BChl molecule in BPheo binding site; cyt, cytochrome; D, BChl/BPheo heterodimer; E_m P/P^+, primary electron donor redox potential; EPR, electron paramagnetic resonance; Φ, BPheo molecule in monomeric BChl binding site; H_A and H_B, BPheo of A or B branch of electron transfer cofactors; LH-1 and LH-2, light-harvesting complexes of core and peripheral antenna; P, BChl homodimer, primary electron donor, special pair; P_A and P_B, BChl molecules forming primary electron donor; PGC, photosynthetic gene cluster; PS, photosystem; Q_A and Q_B, primary and secondary quinone electron acceptors; RC, reaction center; *Blc.*, *Blastochloris*; *Rba.*, *Rhodobacter*; *Rsb.*, *Roseobacter*; *Rsp.*, *Rhodospirillum*; *Rvi.*, *Rubrivivax*.

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the interactions between protein and RC cofactors. Among recent reviews on this topic one should note specialized chapters in the book series "Advances in Photosynthesis and Respiration", edited by Govindjee, released in 2006 and 2009, and also reviews [4-6].

COMPOSITION AND STRUCTURE OF REACTION CENTERS, SPECTRAL PROPERTIES OF THE COFACTORS

RC from the purple bacterium *Blastochloris* (*Bcl.*) *viridis* was the first membrane pigment–protein complex to be structurally characterized [7, 8]. The authors of this study received a Nobel Prize in 1988 (Fig. 1a). The structures of *Rhodobacter* (*Rba.*) *sphaeroides* [9, 10] and *Thermochromatium tepidum* [11] RC were also obtained later. The most studied RC from *Rba. sphaeroides* is formed by three polypeptides (L, M, and H) and 10 cofactors including four bacteriochlorophylls (BChl) *a*, two bacteriopheophytins (BPheo) *a*, two ubiquinone molecules, an ion of non-heme iron Fe^{2+}, and a carotenoid molecule (Fig. 1b) [12].

The L- and M-subunits of the complex each containing five transmembrane α-helices (*A*, *B*, *C*, *D*, *E*) [8, 13, 14] revealed pseudo-twofold rotational structural symmetry. They are noncovalently bound with the RC cofactors [15]. On the periplasmic side of the membrane two BChl molecules, P_A and P_B, form a special pair P, the chromophores of which overlap on the level of the first tetrapyrrole rings. The BChl macrocycles in the P dimer are nearly parallel to each other and perpendicular to the membrane surface. The distance between the P macrocycle planes is 3.4 Å. Due to such small distance the electron densities of the BChl molecules become shared [16-18], leading to a change in properties of tetrapyrrole (in particular, the properties of the P dimer are different from those of other RC bacteriochlorins). The remaining cofactors (monomeric BChl B_A and B_B, BPheo H_A and H_B, ubiquinones Q_A and Q_B) form two nearly symmetrical A and B branches, each starting from the BChl P dimer and continuing to the Fe^{2+} ion on the cytoplasmic side of the membrane. The only carotenoid (spheroidene) is bound to the M-subunit in close vicinity to BChl B_B. The RC symmetry axis is perpendicular to the membrane surface and passes through the center of the BChl P dimer and the iron ion. The H-subunit includes one transmembrane α-helix and a large cytoplasmic domain that separates the quinone-binding sites from the aqueous phase. About 400 water molecules are incorporated in the RC structure [12, 13, 19], and some of them play an important role in the functioning of the complex [20-22]. Additionally, many known structures of *Rba. sphaeroides* RC provide crystallographic data about one or more cardiolipin molecules, which apparently stabilize the complex [5].

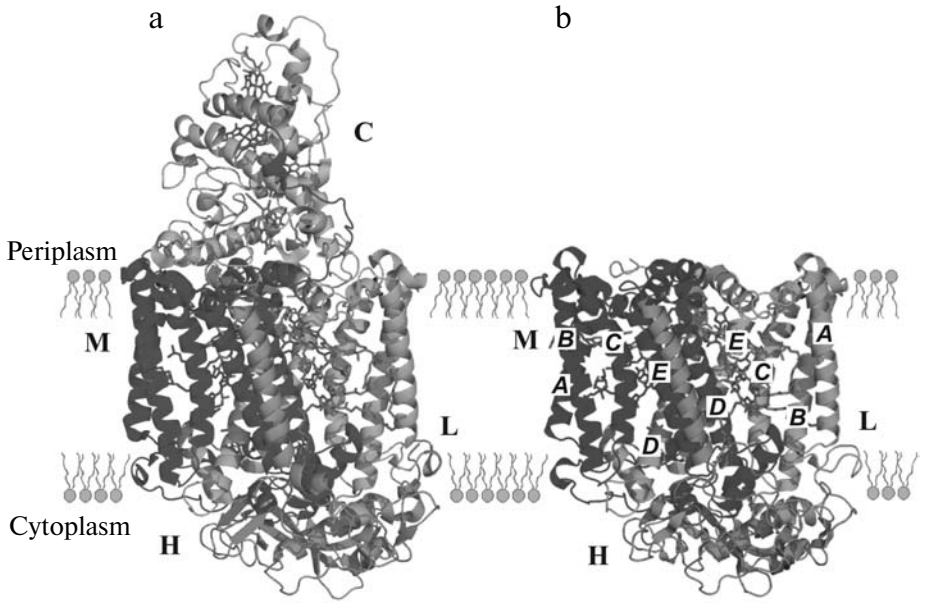

Fig. 1. Structure of photosynthetic reaction centers (RCs): a) *Blastochloris* (*Blc.*) *viridis* (Protein Data Bank code 1PRC [13]); b) *Rhodobacter* (*Rba.*) *sphaeroides* (1M3X [12]). The RC of *Blc. viridis* consists of four protein subunits (L, M, H, and C), and the RC of *Rba. sphaeroides* consists of three proteins (L, M, and H). The L- and M-subunits contain five transmembrane α-helices (labeled *A*, *B*, *C*, *D*, and *E*) that form the binding sites of electron transfer cofactors. The H-subunit consists of a single transmembrane α-helix (in the foreground of structure) and a cytoplasmic domain. The C-subunit is located on the periplasmic surface of the LMH complex of *Blc. viridis* RC and carries four cytochrome hemes.

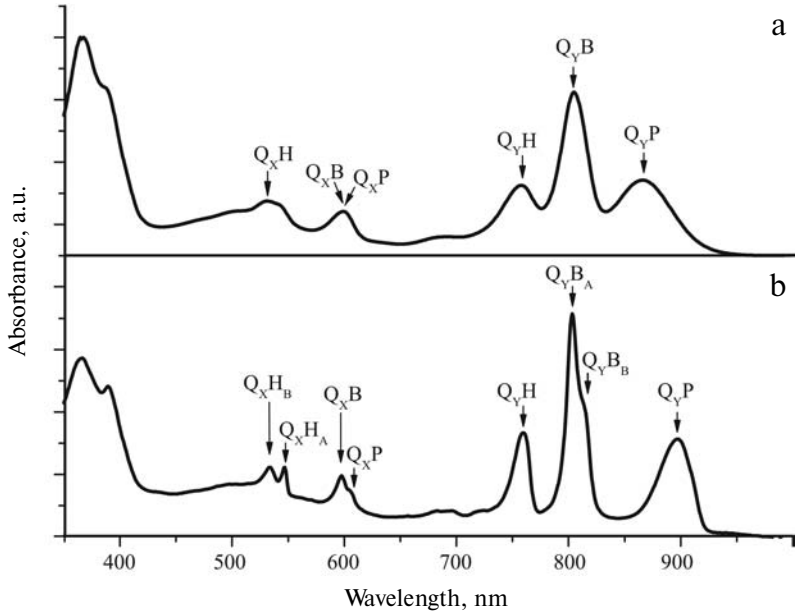

Fig. 2. Absorption spectra of RC from *Rhodobacter sphaeroides* measured at 293 K (a) and 10 K (b). The arrows indicate the absorption bands corresponding to Q_X- and Q_Y-transitions of electron transfer cofactor molecules: P – bacteriochlorophyll dimer (BChl); B_A and B_B – BChl monomers of active and inactive branches, respectively; H_A and H_B – bacteriopheophytins of active and inactive branches of electron transport, respectively.

The majority of the cofactors have distinctive absorption bands in the RC absorption spectrum. Bacteriochlorine cofactors (P, B_A, B_B, H_A, and H_B) absorb in the near infrared, visible, and near ultraviolet regions. In the optical absorption spectrum of RC from *Rba. sphaeroides* measured at room temperature three absorption bands are detected in the infrared region with absorption maximums at 865, 805, and 760 nm. They are assigned to Q_Y-transitions of the BChl dimer, monomeric BChls and BPheos, respectively (Fig. 2a). An absorption band in the visible region with maximum at 600 nm corresponds to the Q_X-transition of four BChl molecules, and a band at 540 nm corresponds to absorption of two BPheo molecules [15, 23-26]. At room temperature the absorption bands of cofactors of the same type but from the different branches (A and B) overlap. The absorption spectrum measured at 10 K displays narrowing of bands, which cause the absorption band of BPheo to split into two bands with maximums at 533 and 546 nm that are attributed to Q_X-transitions of BPheo H_B and H_A, respectively, while the 805-nm band gains a shoulder with maximum near 813 nm corresponding to the Q_Y-transition of BChl B_B and high-energy transition of the BChl dimer [25, 27, 28] (Fig. 2b). Tetrapyrrole pigments also absorb in the 370-390-nm region (Soret band). The short-wavelength part of the band corresponds to the BPheo absorption, and the BChl P dimer supposedly absorbs in the long-wavelength part [29]. The carotenoid absorption band is near 500 nm, where the other cofactors absorb weakly.

PATHWAY AND ELECTRON TRANSFER RATES

Only the A-branch is functionally active in bacterial RC (Fig. 3). In the RC of *Rba. sphaeroides* transmembrane electron transfer begins with the transition of the P dimer to the excited state P* and the electron transfer from P* to BChl B_A occurs in ~3 psec at 293 K. The recombination time of the $P^+B_A^-$ state is about 1 nsec, which is three-fold slower than the lifetime if P* (300 psec) [30]. An important step in the study of the primary charge separation between P* and B_A was the discovery of nuclear wave packet formation by measurement of stimulated emission [31] and oscillations in the state $P^+B_A^-$ [1, 22, 32, 33]. These facts were interpreted as indicating a transfer of wave packets from the P* state to the photoreaction product $P^+B_A^-$, and particular attention was devoted to oscillations at 32 cm^{-1} and its harmonics. In further studies it was suggested that this oscillation was attributed to a rotation of water molecule HOH55 located between P and B_A [22].

Then the electron is transferred from BChl B_A^- to BPheo H_A in ~0.9 psec, and the recombination time of the $P^+H_A^-$ state is 15 nsec at 293 K [34, 35]. All these processes are accelerated 2-3 times when the temperature drops below 77 K. Electron transfer from H_A^- to ubiquinone Q_A occurs in 200 psec at 293 K and in 100 psec at 5 K. The state with separated charges $P^+Q_A^-$ recombines to the ground state in 120 msec at 293 K and in 30 msec at a temperature below 77 K. Forward and

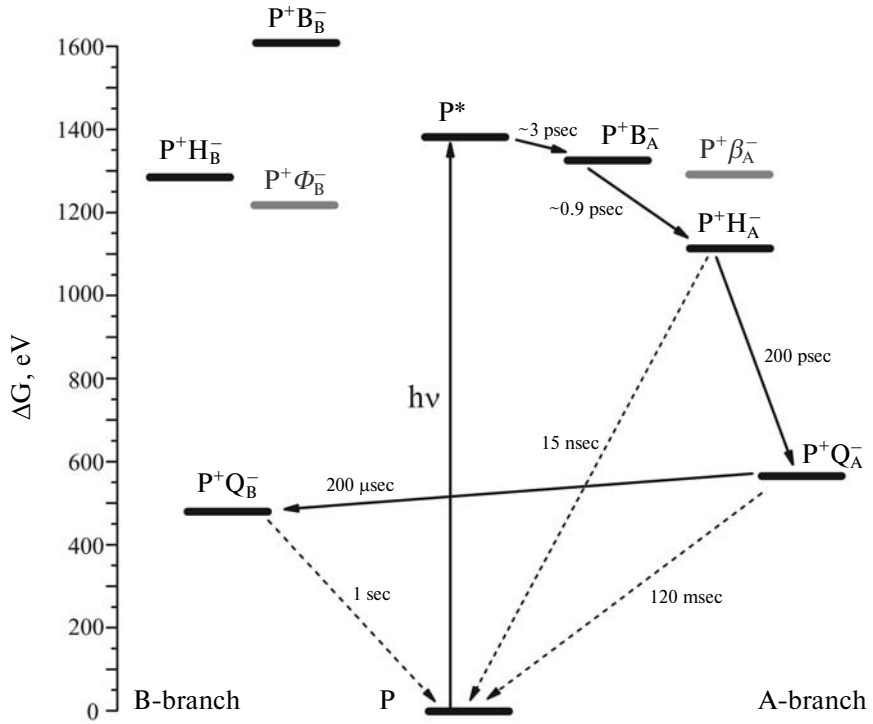

Fig. 3. Electron transfer scheme in reaction center (RC) of *Rhodobacter sphaeroides*. The diagram shows the calculated free energy levels of all ion-radical pairs that can theoretically be formed in wild type RC in black. Electron transfer goes through the A-branch from the primary electron donor, the excited bacteriochlorophyll dimer (BChl), and P* to the secondary quinone acceptor ubiquinone Q_B via the successive formation of several intermediate charge-separated states: $P^+B_A^-$, $P^+H_A^-$, and $P^+Q_A^-$, where B_A is the primary electron acceptor, BChl monomer; H_A – secondary electron acceptor, bacteriopheophytin (BPheo); Q_A – primary quinone electron acceptor, an ubiquinone molecule. The process is accompanied by a decrease in free energy levels of subsequent separated charge states [35, 38, 39, 43, 73]. The gray color indicates the calculated free energy levels of the $P^+\Phi_B^-$ and $P^+\beta_A^-$ states in mutant RC H(M182)L [144, 209] and L(M214)H [166, 211], where Φ_B is the BPheo molecule located at the binding site of monomeric BChl B_B of the inactive B-branch; β_A is the BChl molecule located at the binding site of BPheo H_A.

reverse electron transfer rates are determined by the free energy gap ΔG of charge-separated states [36, 37]. Energy gap during transition of dimer P from the ground to excited (P*) state is 1240 meV in RC of *Blc. viridis* and 1380 meV in RC of *Rba. sphaeroides* [38]. Thereafter the electron transfer proceeds with gradual decrease in energy levels of charge-separated states (Fig. 3) [39-43]. During the formation of P* state bleaching of P bands is observed at 870 and 600 nm, and there is stimulated fluorescence at 920 nm. As the electron is transferred from P* to B_A the stimulated emission is reduced, which is accompanied by bleaching of the B_A band at 800 nm and the formation of the B_A^- band at 1020 nm [1, 30, 44]. Further electron transfer from the primary quinone Q_A to the secondary quinone Q_B with the formation of ubisemiquinone occurs in the time scale of tens of microseconds and slows with decreasing temperature. The recombination time of this state is more than 1 sec [42, 44]. The subsequent photoinduced electron transfer from P* leads to a complete reduction and double protonation of ubisemiquinone Q_B, which causes formation of ubiquinol and removal of two protons from the cytoplasm. Reduced ubiquinol leaves the RC and is replaced by another molecule of oxidized ubiquinone from the intramembrane pool. The P^+ state is reduced with either exogenous cytochrome (cyt) of *c*-type (as in *Rba. sphaeroides*) [45] or with cytochrome of integrated C-subunit (as in *Blc. viridis*) [42], to which electrons are transferred from ubiquinol via cytochrome bc_1 complex. At the same time, protons, which have been taken from cytoplasm, are released on the periplasmic side of the membrane, and so proton gradient employed by the cell for synthesis of reducing equivalents is generated.

CHEMICAL MODIFICATIONS OF COFACTORS

There are different approaches to changing properties of cofactors for studying their role in RC functioning. Chemical substitution of quinone in the Q_A binding site was one of the first approaches to a serious modification of the RC structure in purple bacteria [46]. The modified RCs were used to study the processes of electron transfer to quinones Q_A and Q_B, the properties of radical pairs

$P^+Q_A^-$ and $P^+Q_B^-$ and their recombination, as well as many other properties of the RC and the parameters of photochemical reactions involving quinones [47-54].

Scheer et al. first proposed methods for biochemical treatment of RCs to replace natural BChl or BPheo with other bacteriochlorins [55, 56], which allowed substitution of monomeric BChl [57] and BPheo [58-61] in RCs from *Rba. sphaeroides*, *Rhodospirillum* (*Rsp.*) *rubrum*, and *Blc. viridis* [55]. Natural Mg-bacteriochlorins were replaced by Zn- and Ni-analogs of BChl *a* in order to change the redox potentials of the cofactors [55]. Only a few derivatives of BChl and BPheo can be integrated into the RC, indicating the selectivity of protein binding sites [62]. Despite the great similarity of the structures of these two molecules, so far no one has been able to replace BChl *a* by chlorophyll (Chl) *a* in bacterial RC, but at the same time natural BPheo *a* has been successfully replaced with pheophytin (Pheo) *a*, Pheo *b*, and a number of other Pheo and BPheo derivatives [55, 59, 63].

RCs with substituted monomeric BChl were used to study ultrafast electron transfer reactions from P* to monomeric BChl [64], to determine the absorption bands' assignment for different RC BChls [65], to confirm the role of B_A in the primary charge separation [66, 67], and participation of B_B in triplet state energy transfer to the carotenoid [68, 69]. Studies of RCs with BPheo replaced by plant Pheo allowed to prove that the charge separation between BChl dimer P and BPheo H_A is a two-step process involving the formation of the $P^+B_A^-$ state. Due to substitution of BPheo by Pheo the free energy of the $P^+H_A^-$ state has increased almost up to the energy level of the $P^+B_A^-$ state, which extended the lifetime of the latter and made it possible to study its properties [30, 36, 58, 67, 70, 71].

Energy gap between the P* and $P^+B_A^-$ states plays an important role in electron transfer. In the works of Shuvalov and Yakovlev [72] and Novak et al. [73] RCs with BPheo replaced by the plant Pheo were used, which greatly slowed the rate of electron transfer from B_A^- to Pheo. As a result, the temperature dependence of the stimulated P* emission revealed that the energy level of P* is higher than the energy of $P^+B_A^-$ by 300-550 cm^{-1}, i.e. the two levels are rather close and easily exchange energies especially at room temperature.

So far, attempts to substitute chemically the special pair of the BChls have been unsuccessful. Only the work of Kobayashi et al. described a partial (30%) substitution of the BChl dimer by Zn-BChl *a* in the RC from *Rba. sphaeroides* R-26 [74].

Experiments on reconstruction of non-natural carotenoids were carried out on RC from carotene-free strains of the purple bacteria *Rba. sphaeroides* R-26 and R-26.1, and *Rsp. rubrum* G9, in order to study the influence of side groups and the pigment electronic bond system on ability to perform its photoprotection function [75-77, 54]. The role of the monomer BChl B_B in the triplet transfer [68, 69], the effect of carotenoid on the protein electrostatic environment of the primary electron donor P [78], and the structural factors that cause unidirectional binding of the carotenoid with its protein site [79] were also investigated.

To study the structure and properties of the anion-radicals Q_A^- and Q_B^- by such spectroscopic methods as EPR, electron nuclear double resonance (ENDOR), and electron spin echo envelope modulation (ESEEM), the Fe^{2+} ion is often removed from the RC and replaced with another bivalent metal such as Zn^{2+} since the paramagnetic properties of iron strongly affect the natural reaction center EPR spectra [80]. Removal of Fe^{2+} from the RC leads to a 20-fold reduction in the rate of electron transfer from H_A to Q_A [81] and has only a minor effect on the rate of electron transfer from Q_A to Q_B [82]. In the Zn-substituted RCs the magnitudes of these rates are usually restored to their original values [83, 84]. The Zn-substituted RCs were used to study the quinone binding to the protein [85-87], the reorganization of the RC protein during formation of the $P^+Q_A^-$ state [88-91], to study the properties of the $P^+H_A^-$ state [92], photoinduced conformational changes near and within the Q_B binding site [93], spin–spin interactions of cofactors [94-96], and other characteristics of the photochemical reactions.

GENETIC MODIFICATIONS OF THE RC PROTEIN

Photosynthetic Gene Cluster, *puf*-Operon and Systems for Directed Mutagenesis

In purple bacteria the majority of the genetic information necessary for the construction of the photosynthetic apparatus is grouped in the so-called "photosynthetic gene cluster" (PGC) of about 40-50 thousand base pairs, which contains almost all the structural genes for biosynthesis of the photosystems, as well as genes coding the latter stages of carotenoid and BChl synthesis [97]. *Rsp. rubrum* and *Methylobacterium* are exception, as they have three PGCs located in different parts of the bacterial chromosome (Fig. 4) [98]. Figure 4 shows the layout of genes in the PGC of a number of purple bacteria. The organization of the genes within the cluster can vary considerably in representatives of different genera.

The photosynthetic gene clusters of *Rba. capsulatus* and *Rba. sphaeroides* are similar. They contain all the photosynthetic operons except for the *puc*-operon (Fig. 4) [99]. Operons with genes of bacteriochlorins and carotenoids biosynthesis (the *bch*- and *crt*-operons) are grouped in the center of the cluster, and the *puf*- and *puh*-operons encoding polypeptides of light-harvesting core complex (LH-1) and RC [100-103] are located at the edges. Genes of the *puc*-operon encode the α- and β-subunits of the peripheral antenna complex LH-2. The *puf*-operon of *Rba. capsulatus* and *Rba. sphaeroides* includes

the *puf*QBALMX genes [104]. Bacteria, whose RCs contain a fourth subunit, cytochrome *c* (e.g. *Rubrivivax* (*Rvi.*) *gelatinosus*, *Blc. viridis*), have an additional *puf*C gene (Fig. 4). The representatives of the *Rhodobacter* genus have *puf*Q (or *orf*Q) as the first gene of the *puf*-operon, which is required for BChl synthesis. The *orf*Q gene also contains *puf*-promoter [105, 106]. The *puf*B and *puf*A genes encode β- and α-subunits of LH-1, and the *puf*L and *puf*M genes encode L- and M-subunits of the RC [99]. The *puf*X gene encodes a polypeptide that is required for optimal phototrophic growth [99] and formation of RC–LH-1 dimers [107]. The role of PufX protein is to hold LH-1 in the open state, which is a prerequisite for rapid movement of quinone Q_B between the RC and cytochrome bc_1 complex [108]. So far the *puf*X gene was only found in the *Rhodobacter* genus [109], but W-polypeptide with similar function was found in the structure of the RC–LH-1 complex from *Rhodopseudomonas palustris* [110].

A number of genetic systems for mutagenesis were created to study the structure and function of photosynthetic genes and proteins encoded by them. Such a system includes the parent bacterial strain in which the gene group to be studied is replaced in the chromosome with a gene encoding resistance to a particular antibiotic. The second component of the system is a plasmid vector for complementation, which carriers the gene group for the study [111]. After introducing mutations the studied genes are transferred to the parent strain within the vector for complementation for expression *in vivo*. Recombinant strains with different phenotypes can be obtained by combining the different parental strains and plasmids.

The first works on mutagenesis of photosynthetic complexes were carried out by Douglas Youvan et al. on *Rba. capsulatus* [112-115]. Almost at the same time, such studies were begun on *Blc. viridis* [116] and *Rba. sphaeroides* [117, 118]. The design of *Rba. sphaeroides* and *Rba. capsulatus* genetic systems for mutagenesis that provided recombinant cells containing RC as the only BChl-binding complex played an important role in the study of mutant RCs. The most widely used strains of this type are the U43 strain of *Rba. capsulatus*, first obtained by Youvan et al. [113], as well as strains DD13 and DD13/G1 of *Rba. sphaeroides*, first described by Jones et al. [119, 120].

Change in Composition and Properties of Cofactors

Symmetry of the reaction center complex and mutations for its recovery. One important feature of the RC is a combination of relatively strict structural symmetry and functional asymmetry. These two properties are interrelated since the degree of amino acid sequence homology of L- and M-subunits that bind cofactors is only about 30% [121], and the differences in the protein environ-

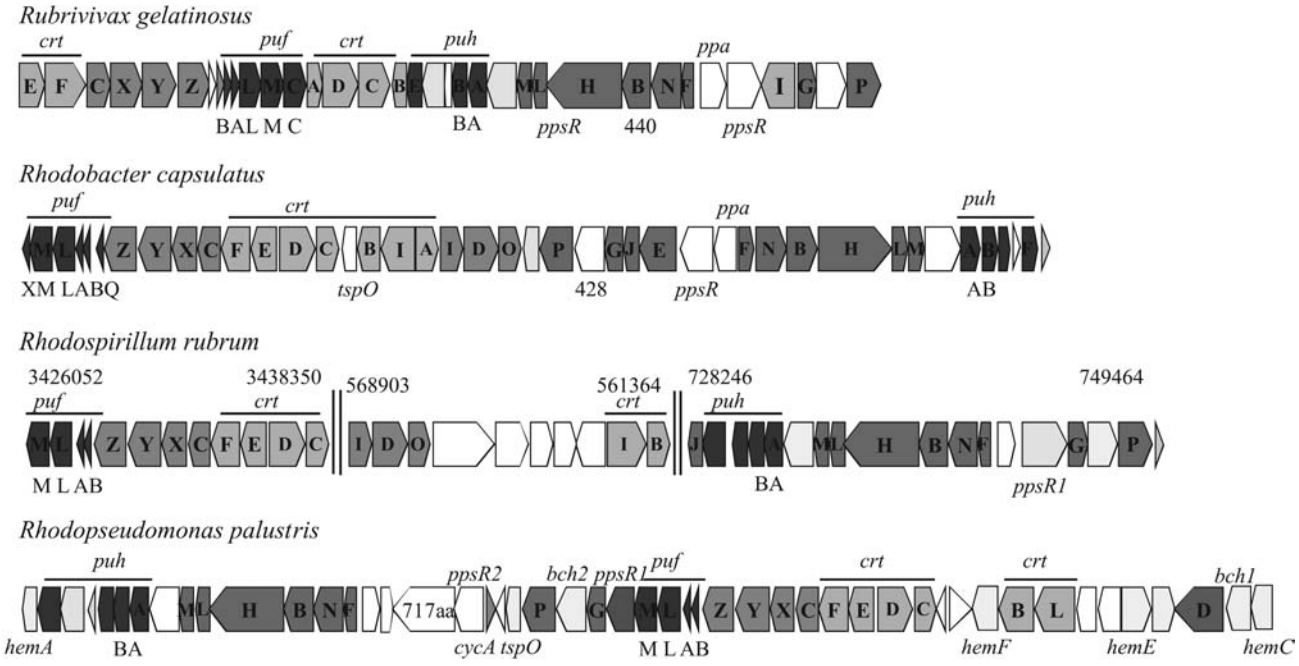

Fig. 4. Schematic comparison of photosynthetic gene clusters of different purple bacteria. Genes are colored as follows: the *puf*- and *puh*-operons containing the structural genes of the reaction center and light-harvesting complex LH-1 are shown in black, light gray color shows the genes of the biosynthesis of carotenoids, dark gray color shows genes for bacteriochlorophylls, and white indicates other structural genes of the photosynthetic apparatus as well as regulatory genes. Lines indicate the different positions of the same gene groups in adjacent clusters. Numbers above the gene groups of *Rhodospirillum rubrum* show the localization of the selected segment in the genome [98].

ment of cofactors in the two branches of electron transfer is the basis of the functional activity of the A-branch and inactivity of the B-branch. There are a number of works aimed at artificial RC "symmetrization", increasing the homology of particular parts of the L- and M-subunits. The most studied mutant RC in this area of research is the so-called D_{LL} mutant of *Rba. capsulatus*, in which 27 amino acid residues of the M-subunit (M192-217) are replaced by the symmetrical in RC structure L-subunit segment (L165-190) [122]. Each of these parallel sections of M- and L-polypeptides contains a part of α-helix D and loop that goes on the periplasmic surface of the complex. The resulting RCs are characterized by higher redox potential of P/P^+ and do not contain BPheo H_A. As there was a significant decrease in the RC stability, their properties were investigated in membranes of an antenna-less strain [123, 124]. As a result of H_A loss, electron transfer is blocked in the A-branch of D_{LL} RC and the lifetime of the primary electron donor excited state P* increases significantly, which allows to study the properties of this state. When incubated under light, the D_{LL} mutant regains its ability for phototrophic growth after the appearance of spontaneous mutations that lead to the return of BPheo H_A. Mutant RCs with genetic constructs D_{MM} and D_{LM} were unstable even in membranes [122]. A recent study by Carter et al. showed that tyrosine reduction at the position corresponding to M208 (as in the wild type RC) is accompanied by the formation of a short-lived (<1 psec) separated charge state $P^+B_A^-$ in the D_{LL} mutant of *Rba. capsulatus* RC [125]. These data confirm the important role of tyrosine residue M208 (M210 in *Rba. sphaeroides*) in the formation and stabilization of $P^+B_A^-$ [33]. The role of this tyrosine is discussed in more details in the section "Changing the Protein Environment of the Bacteriochlorophyll Dimer".

A similar mutant RC of *Rba. capsulatus* (so-called *sym-1* mutant) with replacements of a shorter polypeptide of 17 amino acids, M187-203, with L160-176 segment, were described in Taguchi et al. [126]. As in the D_{LL} mutant, increase in redox potential of the P/P^+ couple was observed in these RC, related, apparently, to the substitution of phenylalanine M195 with histidine and the formation of a new hydrogen bond between the latter and the acetyl group of P_B BChl [127-130]. Later, nine mutant RCs were created similar to *sym-1*, with replacements of the polypeptides in the M162-280 region by structurally equivalent parts of the L-polypeptide. Five of these mutants can grow phototrophically, and the RC was not detected in the membrane in the four other mutants. Mutations near the quinone were found to be especially critical for photosynthetic function [131].

As a result of the most large-scale segment replacement in order to "symmetrize" the RC of *Rba. capsulatus*, made in the region of weak L- and M-subunits sequence homology, so-called Q_AQ_A RC was created. A segment of the L-polypeptide of 35 amino acids (L193-227) was replaced by 42 amino acids of the M-polypeptide (M220-261) [132]. In each polypeptide, these sequences corresponded to two loops and a short α-helix connecting the cytoplasmic ends of transmembrane helices D and E and forming part of the contact surface between the LM- and H-polypeptides. Through this procedure, the authors replaced the polypeptide domain of the Q_B protein-binding pocket for the appropriate area of Q_A binding. Among the substituted amino acid residues were those that play a key role in Q_B functioning, in particular, Glu L212, Asp L213, and Ser L223. Because of this, the recombinant strain containing Q_AQ_A RC was incapable of phototrophic growth [132].

Removal of cofactors. Some of the RC cofactors can be removed without damaging the structure of the complex. For example, with a single substitution of glycine M71 for leucine, a carotenoid-less strain of *Rba. sphaeroides* can be obtained, similar to the above-mentioned strain R-26. This mutation blocks the access of carotenoid to its binding site on the RC protein surface [133].

While the occupancy of the Q_B quinone site in purified RCs usually varies due to the fragility of the cofactor binding with the protein, quinone Q_A is often lost after modifications of its protein environment. For example, using different spectroscopic methods [133-135], as well as X-ray analysis [136], it was shown that the RC A(M260)W mutation created assembly without quinine Q_A. In such RCs Q_B is the sole quinone, that activates electron transfer through the B-branch. As a result of a similar replacement of A(M248)W, RCs without Q_A were also obtained [133]. Introduction of the T(M250)V mutation in RCs from *Rba. capsulatus* [137] or substitution of tryptophan M252 with other residues in RC of *Rba. sphaeroides* [138] caused the Q_A protein binding site to be occupied in the membrane-bound reaction center. But RC loses the quinone during purification of the RC. The replacement of Trp M252 by Phe or Tyr leads to reversible loss of Q_A, but the occupancy of the site cannot be restored if Trp M252 is replaced with a non-aromatic amino acid residue.

Some bacteriochlorine cofactors can also be excluded from the RC as a result of a single amino acid substitution. For example, after replacement of Ala M149 with Trp a RC of *Rba. sphaeroides* was obtained having no BPheo in the B-branch [139]. The absence of BPheo in the mutant RC was confirmed by X-ray crystal diffraction data and the absorption spectrum of the RC in the membrane, but it did not affect the functioning of the complex. In the D_{LL} mutant of *Rba. capsulatus* mentioned above there is no BPheo in the active branch, which leads to the blocking of electron transfer in this branch [122].

Mutant RCs are known that have no BChl dimer. For example, the absorption spectrum of RC with L157 valine to arginine amino acid substitution lacks the Q_Y P band completely. The BChl/BPheo ratio in these RCs was 1.25,

indicating the loss of most of the BChl dimer [140]. In RC with a triple substitution of histidines by glycine, H(L168)G+H(L173)G+H(M202)G, the dimer is clearly lost as a result of missing ligands and hydrogen-bond donors in the surrounding protein [141]. The absorption spectrum of this RC also lacks the Q_Y P band, and the ratio of BChl/BPheo in isolated RC is 1 : 1.

The H(L153)Y mutant RC from *Rba. sphaeroides* was described in which the substitution of histidine, a ligand of the B_A BChl magnesium atom, to tyrosine leads to destabilization of the complex and the loss of monomeric BChl in the active branch. No B_A BChl absorption band is observed in the absorption spectrum of H(L153)Y RC, as well as in the absorption spectrum of RC with double mutation H(L153)Y+H(M182)L, where BPheo is contained instead of B_B BChl. Study of membrane-bound mutant RC H(L153)Y using ultrafast spectroscopy showed 14-fold reduction in the quantum yield of $P^+Q_A^-$ radical pair formation, and the lifetime of P* increased to 200 psec [142-144].

Substitution of cofactors. As mentioned in the section "Chemical Modifications of Cofactors", many cofactors of electron transfer in bacterial reaction center can be replaced to their derivatives or molecules of the same class chemically. Site-directed mutagenesis allows biological substitution, since the biosynthesis of a reaction center complex with altered pigment composition is achieved in bacterial cells. Replacement of the cofactors in this case is limited to BChl and BPheo molecules and is achieved by removal or introduction of amino acid residues forming the coordination bond with the Mg atoms.

Site-directed mutagenesis was used to replace each of the bacteriochlorophylls in the P dimer in RCs of *Rba. capsulatus* and *Rba. sphaeroides* with BPheo. The RCs were obtained with BPheo in P_B site as a result of the replacement of His M200 (M202 for *Rba. sphaeroides*) with Leu, Phe, or Glu [145-149], and in P_A site as a result of the replacement of His L173 with Leu [145, 147, 150]. Heterodimeric RCs are in use for studying the effect of electronic asymmetry of the primary donor on unidirectional electron transfer. Structural and functional asymmetry arises in heterodimers because of significant differences in the properties of BPheo and BChl, in particular because of their different redox potentials. It was shown that the BPheo molecule is 150-300 mV more difficult to oxidize *in vitro* than the BChl molecule, and it is also easier to reduce [151, 152]. Accordingly, after charge separation within the heterodimer (D), the $D^+_{BChl}D^-_{BPheo}$ state is more stable than the $D^-_{BChl}D^+_{BPheo}$ state [153]. In RC of *Rba. sphaeroides* the oxidation potential of D is 140-160 meV more positive than the potential of homodimer P [148, 154], and, at the same time, the D* state is slightly lower in energy than P*. These differences lead to the fact that the free energy of the charge separated states is increased while the free energy of D* is practically unchanged in heterodimer RC, so the formation of the $D^+B_A^-$ state is energetically unfavorable. Therefore, in both heterodimer RC electron transfer to H_A occurs in a one-step superexchange mechanism within 45 psec in H(M202)L and within 85 psec in H(L173)L [146]. The properties of heterodimeric mutants have been described in details in reviews [137, 155] and subsequent studies [149, 156-158]. Analysis of H(M202)L RC X-ray crystal diffraction data, obtained with a resolution of 3.0 and 2.55 Å [159, 160], suggests that the overall structure of the dimer is preserved, and changes in its protein environment are negligible, except for the loss of the conserved water molecule located in wild type RC near amino acid residue His M202 and the keto group of B_A [21], which plays an important role in primary charge separation [21, 22]. So, it was concluded that the effect of the H(M202)L mutation appears to be composed of two factors. One is a change in the properties of dimer P and the second is loss of the water molecule from the environment of the primary electron donor.

Monomeric bacteriochlorophylls in RC can also be substituted by site-directed mutagenesis. Changing His M182 to Leu in the B-branch of *Rba. sphaeroides* RC (M180 in RC of *Rba. capsulatus* and *Blc. viridis*) leads to the appearance of a BPheo molecule (Φ_B) in the B_B binding site (so-called φ mutant) [115, 161]. More details about the properties of this mutant are described in section "Electron Transfer through the B-Branch". The replacement of His L153, a ligand to the Mg atom of B_A BChl, by Leu caused the appearance of a BPheo molecule in the B_A binding site only in the RC of *Blc. viridis* [162]. The mutant RC was observed to have a long-lived $P^+\Phi_A^-$ state due to changes in the free energy level of the primary electron acceptor. In RCs of *Rba. sphaeroides* and *Rba. capsulatus* none of a large number of His L153 substitutions led to replacement of B_A BChl with BPheo [115, 144, 163]. Spectra of mutant RCs with replacements of His L153 by Thr [115], Gly and Val [163], Cys and Met [144] were observed to have significant changes in the region of bacteriochlorophyll absorption due to changes in the nature of the B_A BChl ligand [164]. It was shown that mutation at the L153 position affects not only the spectral properties of monomeric BChl, but P dimer also, as well as the stability of the RC as a whole. During the purification process of a number of mutant RCs, the appearance of a fraction without P dimer was observed, together with the fraction containing the complete set of pigments ([163], Leonova, unpublished data). The H(L153)L substitution was shown to lead to RC destabilization, which hampers study of the RC not only in the isolated state [163], but also in antenna-less chromatophores [144]. It has been suggested that the absence of significant structural damage in the H(L153)L RC of *Blc. viridis* can be explained by the presence of the cytochrome subunit on the periplasmic side of the RC, which contributes to the structural stabilization of the complex [54].

The reverse substitution of a BPheo molecule to BChl is also possible as a result of a number of mutations. The so-called β-mutant RC from *Rba. sphaeroides*, containing BChl β_A in place of BPheo H_A, was obtained by substitution of Leu M214 (Leu M212 in *Rba. capsulatus*) with His [165, 166] and as a result of a single mutation A(L124)H and double mutation F(L121)H+F(L97)V/C [167]. In the first mutant RC histidine M214 is situated at coordination bond distance to the Mg atom of the BChl β_A molecule, and no additional conformational changes in the protein environment are observed [168]. In the second case magnesium coordination apparently involves a water molecule (for details, see "Mutations Affecting Water Molecules") [167]. Much slowed rate of electron transfer and decreased quantum yield of the $P^+Q_A^-$ radical pair formation are observed in the β-mutant RC. Substitution of Leu L185 with His in the corresponding position of the B-branch also leads to the substitution of H_B BPheo with β_B BChl, but this mutation does not alter the kinetics of electron transfer through the A-branch [139].

The RCs from the *Rba. sphaeroides* strain with mutation of magnesium chelatase (bchD) were studied recently [169, 170]. It was shown that these RCs have six molecules of Zn-BChl in the binding sites of all bacteriochlorins. Four of them are located in place of BChls and demonstrate Q_X-transition at 600 nm, and the other two, substituting BPheo, absorb at 560 nm. The authors explain the difference in Q_X-bands positions of Zn-BChl by the presence or absence of the ligand to the zinc atom. The efficiency of electron transport in these RCs is more than 95% of that in the wild type RC [170].

Changing the protein environment of the bacteriochlorophyll dimer. Using spectral methods and genetic approaches it has been demonstrated repeatedly that the interaction of BChl P dimer with protein plays an important role in the functioning of the RC [54, 171, 172]. The heterodimeric mutants described above are remarkable confirmation of this fact.

The energy of the P^+ cation-radical obviously can be affected by electrostatic interactions with charged amino acid residues in the immediate protein environment of the dimer. A number of studies have been carried out in which ionizable amino acid residues in different positions at a distance of 10-15 Å from P were introduced or removed [173-175]. For some of these mutant RCs the presence of electrostatic interactions between the dimer P and the protein environment was confirmed by dependence of E_m P/P^+ on pH [173, 176]. The value of the midpoint potential of the P/P^+ pair was generally found to decrease by a maximum of 60 mV if the additional negative charge appeared at a distance of 10 Å from the BChl dimer molecule, and it increased by a maximum of 50 mV when a positive charge was introduced.

The bacteriochlorin molecule has two carbonyl groups – acetyl group of the ring I and a keto-group of the ring III. Both groups are included in the π-conjugated system of BChl and can serve as a hydrogen bond donor. Mutant RCs with altered amino acid residues close to these groups were designed to focus on the effect of hydrogen bonds on the BChl dimer structure. For example, the H(L168)F substitution removes the hydrogen bond formed by histidine with the acetyl group of P_A, and F(M197)H substitution in the symmetrical position M197 (Fig. 5) leads to the appearance of hydrogen bond with P_B [127, 162, 177, 178]. The introduction of His amino acid residues in places of Leu L131 and Leu M160 leads to formation of hydrogen bonds with the keto-groups of P_A and P_B, respectively (Fig. 5) [179]. Combining mutations can vary the number of hydrogen bonds between 0 and 4 [180], and the appearance or disappearance of such bonds at each position can be monitored by Fourier transform infrared and Raman spectroscopy [130, 181]. In a recent paper Deshmukh et al. have studied 11 mutants with different number and positions of hydrogen bonds formed by the BChl dimer P with its protein environment. Optical and Stark spectroscopy showed the correlation between the dielectric relaxation of the protein near P and photoinduced conformational changes [182].

Changing the number of hydrogen bonds of the BChl dimer with protein has a significant influence on the redox potential of P [54, 172]. The redox titration indicates that each new hydrogen bond increases the E_m P/P^+ value, which is about 500 mV in wild type RC. Removal

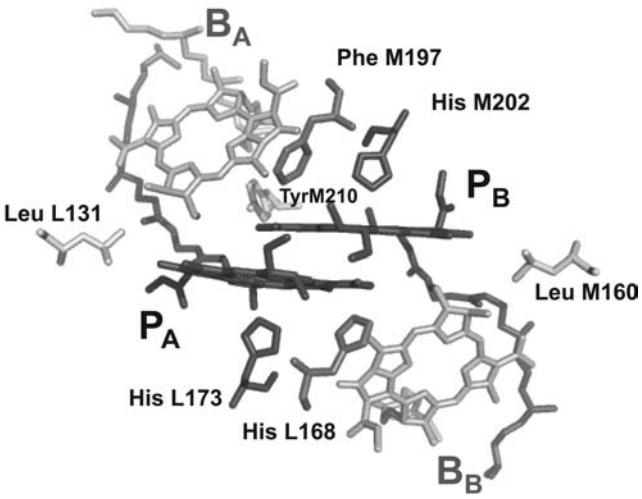

Fig. 5. Environment of bacteriochlorophyll (BChl) dimer P in the reaction center of *Rhodobacter sphaeroides*. Coordinates are taken from Protein Data Bank, access code 1M3X [12]. Electron transfer cofactors are marked on the figure: P_A and P_B – BChl molecules forming the dimer (the planes of macrocycles are perpendicular to the plane of the figure); B_A and B_B – monomeric BChl from active and inactive branches of electron transfer, respectively, and amino acid residues His M202, His L173, Leu L131, Leu M160, His L168, Phe M197, and Tyr M210.

of a hydrogen bond by the H(L168)F mutation, in contrast, lowers the value of E_m P/P^+ by 90 mV. Due to additive effect of several mutations, E_m P/P^+ can be altered from 410 mV (P without hydrogen bonds) to 765 mV (four hydrogen bonds of P with protein). According to X-ray structure analysis of the RCs with the mutations L(L131)H, L(M160)H, F(M197)H, and H(L168)F, no significant structural changes occurred except for 20-27° rotation of the acetyl group and a small shift of P_A in the H(L168)F RC [178, 183].

Close overlapping of the first rings of the special pair tetrapyrroles leads to the sharing of electronic orbitals and, consequently, to the change of the dimer P properties as compared to monomeric BChl. One of the changes is the red shift of the long-wavelength absorption band of the dimer relative to the absorption bands of monomeric BChl. In the wild type reaction center electron density measurements of the unpaired electron using ENDOR spectroscopy give 2 : 1 ratio on the L-side of the dimer P compared to the M-side. It was shown that by changing the number of hydrogen bonds between P and the protein the electron density distribution between the two molecules of the BChl dimer can be shifted. The percentage of electron density at P_A BChl can vary from 22 to 83% [184].

It is known that tyrosine occupies position M197 in RC of *Blc. viridis* and *Chromatium tepidum*, and it forms a hydrogen bond with the oxygen of the P_B BChl acetyl group [185, 186]. In *Rba. sphaeroides* RC the position M197 is occupied by phenylalanine, and this hydrogen bond is absent. The F(M197)H mutation leads to its appearance, and, consequently, to an increase of the redox potential of P [187, 188]. It is noteworthy that despite these facts, the values of E_m P/P^+ in the native RCs from *Blc. viridis* and *Rba. sphaeroides* are identical (about 500 mV) [180, 189]. The reason for this is apparently the presence of the C-subunit in the RC of *Blc. viridis*, which separates the special pair of BChl from the aqueous phase, leading to a redox potential shift. An additional hydrogen bond with one of the P BChls counterbalances this effect [190]. The simultaneous presence of tyrosine M197 and C-subunit has been proven for 16 species of purple bacteria [191].

In a study of Holden-Dye et al. mutant RCs with altered numbers of hydrogen bonds between the BChl dimer P and protein were used to study the relationship between the *Rba. sphaeroides* RC structure and its thermal stability [192]. The formation of the hydrogen bond between BChl P_B and His M197 was shown to increase the thermal stability of the protein, and the loss of the bond between BChl P_A and His L168 after substitution of this residue with Phe reduced the thermal stability of the reaction center complex. However, the stability was not increased if Tyr or Asp, capable of forming hydrogen bonds with acetyl groups of BChl P_B, were located in M197 position. Using X-ray spectral diffraction and additional mutations, the authors were able to determine the cause of this contradiction. It was found that His M197 and L168 residues form two additional hydrogen bonds each with Asn M195 and L166, respectively, which significantly increases the stability of the RC. Apparently, not every hydrogen bond between BChl and protein promotes stability of the complex as placing histidines in positions M160 and L131, which leads to the formation of such bonds with keto groups of BChl P_B and P_A, respectively, do not increase but in fact decreases the thermal stability of the RC [192].

Reaction centers from *Rba. sphaeroides* with substitutions of Tyr residue M210 are among the most thoroughly studied mutant RCs concerning electron transfer rates, properties of the primary donor, and other aspects of the photochemical charge separation. In addition, X-ray crystal diffraction data were obtained for the Y(M210)W RC [136, 193, 195]. Parson mathematically proved the assumption that the OH-group of tyrosine M210 (Fig. 5) in RC from *Rba. sphaeroides* (RC M208 in *Blc. viridis* and *Rba. capsulatus*) is one of the factors that create the difference in energy levels of the $P^+B_B^-$ and $P^+B_A^-$ states, and that the orientation of this group contributes to the stabilization of $P^+B_A^-$ [196, 197]. The Y(M210)W and Y(M210)L substitutions affect the primary donor redox potential, increasing it by 55 and 30 mV, respectively [198]. At the same time, free energies level difference of the P* and $P^+H_A^-$ states decreases by 60 mV [199]. The lifetime of P* increases from 3 psec in the wild type RC to several tens of picoseconds in Y(M210)W RC and 190 psec in Y(M210)L RC [194, 200]. In the works of Yakovlev et al. the role of the Tyr M210 residue in the separation and stabilization of charges between the primary electron donor P and the primary electron acceptor B_A has been studied by femtosecond spectroscopy in RC of *Rba. sphaeroides* [33]. It was shown both in the native and mutant RCs that reversible coherent femtosecond population of the $P^+B_A^-$ state occurs, but stabilization of $P^+B_A^-$ charge state in the picosecond time scale is almost completely absent in the RC Y(M210)L. According to the results of the study, the interaction of the polar OH-groups of Tyr M210 with charged BChl molecules P^+ and B_A^- accelerates the charge separation between them and stabilizes the charge-separated state $P^+B_A^-$. Using site-directed mutagenesis it has been demonstrated that the absence of tyrosine at the M210 position in the mutant RCs is not compensated by Tyr introduction at the M197 position or by significant increase in the free energy difference between the P* and $P^+B_A^-$ states in the mutant Y(M210)L+H(L168)L RC [201, 202].

Electron transfer through the B-branch. In RC from purple bacteria the $P^+B_B^-$ state free energy level is above that of P* (by 240 meV in RC from *Blc. viridis* [196], by 100 meV in RC from *Rba. capsulatus* [203], and by ~50 meV in RC from *Rba. sphaeroides*), and the level of

$P^+B_A^-$ is somewhat below the level of P* [73]. This difference in free energy levels is believed to be one of the main factors determining the functional inactivity of the B-branch [43]. Mutations that promote B-branch electron transfer and limit A-branch transfer are discussed in detail in reviews [4, 172]; we briefly mention only a few of them.

Kirmaier and coworkers have described the RC from *Rba. capsulatus* with L(M212)H substitutions that led to the replacement of the BPheo molecule H_A by BChl β_A, and, consequently, to a significant increase in free energy of the $P^+\beta_A^-$ state compared to that of $P^+H_A^-$ (Fig. 3). Another substitution G(M201)D near the ring V of the B_A molecule caused an increase of the $P^+B_A^-$ state free energy, slowing electron transfer through the A-branch by several times [204]. Both formation of the $P^+H_B^-$ state with a quantum yield of 15% and drastic acceleration of the recombination processes in the A-branch were observed in the double mutant G(M201)D+L(M212)H RC from *Rba. capsulatus*. Addition of a third mutation S(L178)K near the ring V of B_B BChl led to a decrease of the $P^+B_B^-$ state energy level and to an increase in the electron transfer quantum yield through the B-branch up to 23% [204]. A similar result is achieved by the F(L121)D mutation, which leads to significant increase of $P^+H_A^-$ energy. Reaction center of *Rba. capsulatus* with a triple substitution F(L97)V+F(L121)D+L(M212)H has $P^+H_B^-$ state formed with a quantum yield of 12%, while the addition of a fourth mutation G(M201)D increases it to 18% [203]. The quantum yield of $P^+H_B^-$ formation in photochemically active RC fraction of recently obtained *Rba. capsulatus* triple mutant F(L181)Y+Y(M208)F+L(M212)H reaches 30%, and further electron transfer to Q_B is registered with an overall quantum yield of 13% [205, 206]. The highest quantum yield of $P^+H_B^-$ state formation (80%) was registered in the photochemically active RC fraction of D_{LL}-FYLFM mutant of *Rba. capsulatus*, and the electron transfer through the B-branch appeared to be activationless process such as through the A-branch [207]. Electron transfer to H_B through the B-branch was also observed in heterodimer RC from *Rba. capsulatus* that had the additional mutation L(M212)H [153].

Although the rates of electron transfer in RC from *Rba. sphaeroides* and *Rba. capsulatus* are approximately equal, it was somewhat more difficult to direct electron transfer through the B-branch in RC of *Rba. sphaeroides* as compared to *Rba. capsulatus* [208]. Katilius et al. have shown that substitution of His M182 by Leu in RC from *Rba. sphaeroides* leads to the replacement of the BChl molecule B_B by a BPheo molecule (Φ_B) [161]. Such mutant complexes have rate of electron transfer through the B-branch comparable to the rate of electron transfer through the A-branch in wild type RCs. However, the electron transfer in H(M182)L RC occurs only from P* to $P^+\Phi_B^-$ and not to $P^+H_B^-$, as the free energy level of the $P^+\Phi_B^-$ state is lower than that of P* by 160 meV, while the corresponding difference for $P^+H_B^-$ is only 70 meV (Fig. 3) [209]. Additional amino acid substitutions M(L174)D, V(M175)D, and T(M133)D, designed to increase the energy of $P^+\Phi_B^-$, did not increase the electron flow through the inactive branch [210].

Highly efficient electron transfer through the B-branch in RC of *Rba. sphaeroides* has been described in a recent paper by Khatypov et al. [142, 143]. The double mutation H(L153)Y+H(M182)L led both to the replacement of BChl molecule B_B by BPheo molecule Φ_B and to the loss of monomeric BChl B_A. The absorption spectrum of these RC has no Q_Y band of monomeric BChl in the 800-nm region and has an additional band with a maximum at 785 nm corresponding to the absorption of BPheo Φ_B. The results of high time resolution spectroscopy revealed that electron transfer in membrane-bound RC H(L153)Y+H(M182)L only occurs through the B-branch with the formation of charge-separated state $P^+\Phi_B^-$ within 2 psec and subsequent recombination to the ground state with a time constant of ~180 psec [142, 143]. These data are consistent with the results obtained in [161] on H(M182)L RC, but while the quantum yield of radical pair $P^+\Phi_B^-$ formation in the double mutant RC is close to 100% with subsequent recombination to the ground state [142], the corresponding value in the H(M182)L RC is around 35%, and the other 65% are accounted for by $P^+H_A^-$ radical pair formation [161]. Further electron transfer through the A- or B-branch of the H(L153)Y+H(M182)L RC is blocked, so this mutant strain of *Rba. sphaeroides* is incapable of phototrophic growth.

Using differential femtosecond spectroscopy Yakovlev and coworkers showed that the electron transfer through the B-branch of cofactors is activated, and the electron transfer through the A-branch is inhibited in RC of the triple mutant S(L178)K+G(M203)D+L(M214)H of *Rba. sphaeroides* [211]. As a result, charge separation processes are registered for both cofactor branches, and the $P^+H_B^-$ state is formed ~50 fsec earlier than the $P^+B_A^-/P^+\beta_A^-$ state.

Modification of reaction center–cytochrome *c* interaction region. Electron transfer from cytochrome *c* to oxidized primary donor P^+ is one of the important stages of the photochemical charge separation. In 2002, Axelrod et al. determined the structure of the combined cyt c_2–RC complex from *Rba. sphaeroides* using X-ray crystallography, and the rate of electron transfer from cyt c_2 to the primary donor P both in crystals and in solution was shown to be similar [212]. The cyt c_2 interacts with the center of the RC periplasmic surface, and the cytochrome heme is turned straight to the P dimer in the process, just like in the RC of *Blc. viridis* in which cyt *c* is the fourth subunit of the complex [13]. There are two important domains notable in the area of contact between cyt c_2 and RC from *Rba. sphaeroides*. The first is the central hydrophobic domain consisting of residues involved in both van der Waals and hydrophobic interactions. The second is the

peripheral region of the contact with amino acid residues carrying electrostatic charge. Surfaces of the cyt c_2 and RC provide positive and negative charges, correspondingly [45].

The molecular basis of the binding of two complexes and electron transfer between them has been studied using methods of chemical [213] and genetic modification [214, 215]. Site-directed mutagenesis was used to investigate the role of hydrophobic residues as well as residues forming hydrogen bonds [187, 216, 217]. It was found that mutations of amino acid residues located at the contact surface of the RC and cyt c_2 can influence the association–dissociation of the complexes, as well as electron transfer between them. Substitutions of charged residues in this region mainly influence the binding of complexes and do not significantly change the rate of electron transfer [214]. These results suggest that close interactions between the complexes, responsible for electron tunneling, occur through the hydrophobic domain in the RC and cyt c_2 contact area. Oppositely charged residues on the contact surface of the two complexes are separated by solvent and therefore have no noticeable effect on electron tunneling [218]. In turn, substitutions of hydrophobic residues in the area of contact show that hydrophobic interactions are important both for binding and for the electron transfer from cyt c_2 to primary donor P [187, 216].

Mutations affecting water molecules. The structure of *Rba. sphaeroides* RC with resolution of 1.87 Å [219] has more than 400 water molecules, which are concentrated mainly at the periplasmic or cytoplasmic side of the membrane, but are also found near the BChl dimer and quinones and, in rare cases, in the central membrane part of the RC [12, 21, 219]. As is generally known, water molecule is a polar one capable of forming hydrogen bonds and being a proton donor, so water appearance in the hydrophobic part of the membrane complex or its disappearance from the complex as a result of mutation can severely affect properties and functioning of the RC, although prediction of such effects is often impossible [54]. For example, while substitution of Ser L223 with Ala or Asp produces RCs with non-functional Q_B site, the substitution of this Ser with Gly does not disturb the functionality of the Q_B site [220]. Similarly the substitution of Glu L212 with Gln disrupts Q_B functioning, and the replacement of this Glu with Ala does not [221]. It has been suggested that substitutions of Ser and Glu with residues of smaller molecular volume might leave a space in the protein pocket that can be occupied by a water molecule capable of performing the functions of natural serine or glutamic acid [220, 221].

Despite the fact that glycine cannot serve as a ligand for the Mg atom of BChl, H(L173)G and H(M202)G RC mutants have primary donor consisting of a pair of BChl molecules [222]. The authors of that paper suggested that a water molecule could be integrated in the protein environment of BChl, together with a small Gly molecule, which replaced His, and this water molecule could serve as the fifth ligand of the magnesium atom. A similar suggestion was proposed by Heller et al. [167] to explain the substitution of H_A BPheo with BChl in the RC of double mutant F(L97)V+F(L121)H, in which the location of the newly introduced His L121 was not optimal for ligation of the BChl. Since it is very difficult to predict the appearance or disappearance of water molecules in the mutant RC structure theoretically, it is appropriate to combine spectral measurements and X-ray crystal diffraction studies to elucidate the effects of mutations if possible [54].

The G(M203)L RC from *Rba. sphaeroides* can be noted as an example how the mutation affects a specific water molecule. In this RC glycine M203, located between the dimer P and the monomeric BChl of the A-branch, was replaced by leucine, and this mutation resulted in a fourfold slowing of the primary electron transfer rate from P* to B_A at 90 K [22]. X-Ray analysis of the mutant RC showed that the water molecule located in wild type RC at the distance of a hydrogen bond from the keto-group of BChl B_A and His M202 was displaced by a Leu residue [21]. Although the detailed reasons for the slowing of electron transport in G(M203)L RC remain unclear, it was suggested that the removal of this water from the RC of the G(M203)L mutant arrests electron transfer from P* to B_A along the most efficient way through the chain of polar atoms N-Mg(P_B)-N-C-N(His M202)-HOH55-O-(B_A) [22].

Mutations for study of the evolutionary relationship between bacterial reaction centers and photosystem 2. Structural comparison of the two complexes, RCs from purple bacteria and photosystem 2 of cyanobacteria and plants, that belong to the same type (quinone-type) of reaction centers, began after the first X-ray crystal structure of the bacterial reaction center was resolved [223] and continued with the appearance of data on the three-dimensional structure of PS2 [6, 172]. The main difference between the PS2 complex and the bacterial RC is that the former is able to accept electrons directly from water molecules, whereas the bacterial reaction center uses cytochrome molecules (with a significantly lower potential) as electron donors. The oxidation of water to oxygen in PS2 is provided by a number of factors: the primary electron donor, Chl dimer, has a very high redox potential; a Tyr residue serves as a secondary electron donor; a cluster containing four manganese ions is able to accept four electrons for water oxidation. None of these factors are present in reaction centers of bacteria.

In Allen and Williams' laboratory a number of bacterial RC modifications were introduced to study their ability to photooxidize tyrosine, as well as to bind and oxidize manganese. With directed substitutions of Leu L131, Leu M160, and Phe M197 with His and of Tyr M210 with Trp, RC of *Rba. sphaeroides* with mid-point redox potential of P/P^+ near +800 mV was obtained,

which is 300 mV higher than the special pair potential of wild type RC [224]. Evidence of tyrosine oxidation in the mutant RC was obtained by EPR spectroscopy after the introduction of additional Tyr residues at M164, L135, or L167 positions [224, 225]. Additional His residues were also placed near each of the newly introduced Tyr residues, as well as near natural Tyr L162 and M193, to provide an acceptor for the proton formed and released during photooxidation of tyrosine [226]. Thus the possibility of each of the five tyrosines to be photooxidized in the mutant RCs was shown, as well as dependence of this process on pH and on the presence of proton acceptor. The RCs capable of binding and photooxidizing manganese were obtained on the basis of the mutant strain L(L131)H+L(M160)H+F(M197)H+R(M164)Y with high E_m P/P^+ and an additional tyrosine in the primary donor environment [183, 227]. For this purpose, additional replacements of Met M168 and Val M192 with Glu and Gly M288 with Asp have been introduced in different combinations. All these amino acid residues are located close to Glu M173, which is a structural analog of Asp170 in PS2 D1 polypeptide, that plays a key role in the binding of the manganese cluster. Three of the four mutant RCs were able to bind manganese, and one of them displayed electron transfer from manganese to photooxidized primary donor P [183]. It was found that the binding of manganese in the mutant RCs was dependent on pH, and release of two protons was observed at pH 8.0 [228]. Thus, using a small number of amino acid substitutions, bacterial RCs can be modified in such a way that they acquire the ability to photooxidize tyrosine, as well as to bind and oxidize redox-active manganese, which indicates the dominant role of the protein environment of cofactors in the evolution of photosynthetic complexes. It should be noted that global analysis of protein sequences of bacterial RCs and PS2 of cyanobacteria showed that the mechanisms regulating the redox properties of the cofactors and direction of the electron transport in PS2 may be different from those operating in bacterial reaction centers [6, 191].

The BChl dimer P in RC of a photochemically active *Rba. capsulatus* mutant called D1-ILMH was similar to the Chl dimer of plant PS2 due to changes in the protein environment [229]. A segment of 11 amino acid residues was formed near the P dimer after substitution of four amino acids in the L-subunit (L167-L170). It was similar to the analogous segment of the D1-subunit of PS2. According to EPR, the electronic properties of the BChl dimer changed so significantly that it became functionally more "monomer-like", while maintaining a low oxidation potential of P/P^+ and orientation in the membrane characteristic of the bacterial RC.

Lin et al. described a magnesium chelatase mutant strain (*bchD*) of *Rba. sphaeroides* in whose RC the special pair and the monomeric BChl are represented by Zn-BChl instead of Mg-BChl, and Zn-BChl is also detected in the BPheo binding site. Despite significant changes in pigment composition, these RC displayed high efficiency of electron transport, more than 95% of that in wild type RC. The authors consider that the difference between energy levels, which define the kinetics and direction of the electron transport, in Zn-BChl-containing RC, is determined solely by the protein environment of six identical bacteriochlorophyll cofactors, as in plant PS1 [170].

Unexpected effects of directed mutations. Despite the fact that bacterial reaction centers are fairly well studied, these complexes still keep many secrets, as indicated in the literature referred to unexpected results of some amino acid substitutions. Photochemically inactive reaction center Q_AQ_A of a *Rba. capsulatus* mutant was mentioned in the section "Symmetry of the Reaction Center Complex and Mutations for Its Recovery" [132], in which the Q_B protein binding site was replaced with Q_A binding site, resulting in the loss of the secondary quinone acceptor affinity to the protein. When incubated under light, this mutant regains the ability for phototrophic growth by two spontaneous reverse mutations – M(M144)I and A(M145)S, which were found away from the Q_B site, next to H_B BPheo. The resulting mutant RC, the so-called A_6D_1-complex, was relatively stable, and the affinity of quinone Q_B to its binding site increased to 33% of the initial value of wild type RC as a result of the compensatory mutations, although the rate of electron transfer from Q_A to Q_B remained slow (500 times slower than in wild type RC) [51]. The lack of structural data on the A_6D_1-complex makes it difficult to explain this surprising phenomenon, but other authors who have studied mutant RCs with substitutions of key residues in the Q_B site, Glu L212 and Asp L213, also noted that photosynthetic activity of mutants was often restored by reverse mutations remote from the site of the original amino acid substitution [52, 230]. Since many of these substitutions either introduced negatively charged residues or removed positively charged ones, it is assumed that they generally had a compensating effect on electrostatic interactions between the protein and the cofactor [52]. However, a considerable remoteness of these reverse mutations from the Q_B site suggests that this effect may be due to more significant structural changes, which include substitutions of several amino acid residues [50, 231, 232].

An unexpected result of a single amino acid substitution is described for I(L177)H mutant RC of *Rba. sphaeroides* [233]. Isoleucine L177, located in the vicinity of two BChl molecules P_A and B_B, is relatively conservative in the structure of *Rba. sphaeroides* RC. Earlier Williams et al. described substitution of Ile L177 with Asp, which caused reduced rate of $P^+Q_A^-$ state recombination [179]. Another substitution, Ile L177 by His, affected the spectral properties of the P_A and B_B BChl. The pigment analysis suggested the absence of a BChl mole-

cule in the mutant reaction center I(L177)H [234]. However, later it was shown that the mutation I(L177)H resulted in covalent attachment of BChl P_A to the L-subunit; therefore, one BChl cannot be extracted from RC by organic solvents [233, 235]. The BChl–protein bond is stable in the presence of 8 M urea, 5% SDS, as well as at pH 3.0, during denaturing electrophoresis, and when heated. Unusually stable coordination of Mg atom in the protein-bound BChl was detected [233, 235]. It is interesting to note that the substitution of Ile by His in symmetrically placed position M206 did not cause the formation of a covalent bond between the protein and the cofactor, although the changes in the absorption spectra of I(M206)H and I(L177)H RCs were similar [236].

To study the nature of this covalent bond, as well as to investigate a reason of stable ligation of covalently bound BChl, RC with double amino acid substitutions I(L177)H+H(M182)L and I(L177)H+H(L173)L were designed. Each of these two additional mutations leads to the removal of native ligands of bacteriochlorophylls B_B and P_A, respectively, which are expected to result in appearance of BPheos in their places. However, none of the RCs with double mutations demonstrated this. It was shown that both a covalent bond of P_A BChl with the L-subunit, and coordination of B_B BChl were retained in I(L177)H+H(M182)L RC. Changes in the spectral properties of BChl in RC of this double mutant indicate that ligation of BChl B_B can occur from the β-side of the macrocycle, similar to that shown for the RC of the F(L181)R+H(M182)L mutant [237]. It is suggested that a water molecule may be involved in ligation of the BChl B_B magnesium atom in this mutant RC, as it is described for F(L181)R RC [237], although experimental confirmation of this has not been obtained yet [238]. According to the spectral data, the primary electron donor of the double mutant RC I(L177)H+H(L173)L is a BChl homodimer, despite the substitution of the native ligand His L173 by Leu. Besides, the absorbance band of the dimer P undergoes an unprecedented blue shift of 45 nm [239]. The His L177 apparently plays the role of the P_A ligand in these RCs, although its modeled location in the RC appears not to be optimal for the coordination. The covalent BChl–protein bond is not formed in the I(L177)H+H(L173)L mutant RC, assuming possible involvement of His L173 in the formation of this bond [239]. The origin of the strong covalent bond between BChl P_A and the L-subunit in the RC with mutations I(L177)H and I(L177)H+H(M182)L still remains unknown.

Therefore, the method of site-directed mutagenesis allows changing the structure of the reaction center and cofactor composition, providing new opportunities for study of the primary electron transfer process. This method was applied to demonstrate the significance of the protein asymmetry in the reaction center both for the primary act of charge separation and for the subsequent electron transfer to quinone Q_B. Furthermore, the role of individual cofactors, amino acid residues, and even single internal water molecules in the RC functioning was established.

Relatively simply organized and well-studied bacterial RCs are used as a convenient model for alteration of photosynthetic electron transport parameters, studies of separate stages of this process, and general principles of RC structure, functions, and evolution. Spectral methods in combination with structural analysis and biomolecular and biochemical approaches provide complementary information and make it possible to understand mechanisms that ensure high efficiency of photosynthetic reaction center functioning. There is no doubt that the RC protein is not only a backbone that holds electron transport cofactors at optimal distance from each other and in optimal orientation, but also it plays an important and perhaps crucial role in regulation of the rate and the direction of photosynthetic electron transport.

Numerous studies of directed mutagenesis of RCs from purple bacteria indicate extraordinary plasticity of these complexes and their steadiness to local changes in protein structure. It is noted that only in rare cases a single amino acid substitution can significantly influence the process of primary electron transfer in RC. Relatively high stability of RC complexes after their isolation from membranes is also remarkable. Factors, which define this feature of RCs, are not sufficiently studied. This question deserves attention because of the important role of membrane proteins in functioning of live organisms. Additionally, the search for conditions of stable long-term bacterial RC functioning is of interest in connection with the future trends of using these complexes in biotechnological projects, such as creation of light energy converters. There is still a high topic fundamental problem concerning the physical mechanisms that guarantee the highest efficiency of initial steps of electron transfer in RC.

This study was conducted with financial support of the Federal Program "Scientific and Scientific-Pedagogical Personnel of Innovative Russia" of 2009-2013 (No. 02.740.11.0293), "Molecular and Cellular Biology" program, Russian President Grant (No. NSh 3201.2010.4-), and the Russian Foundation for Basic Research (Grant 09-04-00109).

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