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An Attempt to Increase Thermostability of the Mutant Photosynthetic Reaction Center of *Cereibacter sphaeroides* Using Disulfide Bonds

Tatiana Yu. Fufina[1] and Lyudmila G. Vasilieva[1,a*]

[1]*Institute of Basic Biological Problems, Russian Academy of Sciences,*
Pushchino Scientific Center for Biological Research, Russian Academy of Sciences,
142290 Pushchino, Moscow Region, Russia
[a]*e-mail: vsyulya20@yandex.ru*

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Abstract—Methods of site-directed mutagenesis are successfully used in structural and functional studies of photosynthetic reaction centers (RCs). It has been noted that many mutations near electron transfer cofactors reduce temperature stability of the *Cereibacter sphaeroides* RCs and affect amount of RCs in the membranes. We previously reported [Selikhanov et al. (2023) *Membranes*, **25**, 154] that introduction of inter-subunit disulfide bridges on the periplasmic or cytoplasmic surface of the complex promotes increase in thermal stability of the *C. sphaeroides* RCs. In this work, an attempt was made to increase thermal stability of the mutant RC with the Ile M206 – Gln substitution by introducing inter-subunit disulfide bonds. This RC is of considerable interest for studying mechanisms of early electron transfer processes in RCs. The effect of mutations on the amount of RCs in chromatophores was analyzed and it was found that the I(M206)Q mutation leads to twofold decrease in the RC content in chromatophores, introduction of disulfide bonds on the cytoplasmic or periplasmic sides of the complex reduces the amount of RCs in membranes by one third, the triple substitution I(M206)Q/G(M19)C/T(L214)C reduces the amount of RCs in membranes almost 4-fold, and the substitutions I(M206)Q/V(M84)C/G(L278)C lead to disruption of RC assembly in the membrane. It was shown that introduction of the inter-subunit S-S bond on the cytoplasmic surface of the complex did not have a significant effect on thermal stability of the I(M206)Q RC. Our own and literature data on the factors influencing assembly processes and ensuring stability of the structure of integral membrane complexes are discussed.

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Keywords: photosynthetic reaction center, *Cereibacter sphaeroides*, protein thermal stability, absorption spectra, disulfide bridges

INTRODUCTION

Photosynthetic apparatus of the purple bacterium *Cereibacter sphaeroides* includes two light-harvesting complexes (LHC-1 and LHC-2) and a reaction center (RC) [1]. The RC of *C. sphaeroides* is one of the best studied bacterial pigment–protein complexes; it is used to investigate mechanisms of the primary processes of photosynthesis and is a model for studying integral membrane proteins. This RC comprises three protein subunits (L, M, and H) and ten cofactors arranged in two branches, active (A) and inactive (B), for electron transfer (Fig. 1). Cofactors are represented by four molecules of bacteriochlorophyll (BChl) *a*, two molecules of bacteriopheophytin (BPheo) *a*, two molecules of ubiquinone (Q), a non-heme iron atom, and a carotenoid molecule [2]. After decoding spatial structure of this RC, quite a lot of works have been devoted to studying the role of protein environment of the cofactors in providing high quantum efficiency of the photochemical reaction [3]. One of the methods used in such studies is site-directed mutagenesis. The RC of purple bacteria is considered to be a relatively stable membrane complex [4];

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* To whom correspondence should be addressed.

however, there are cases described in the literature where point mutations resulted in the loss of cofactors, partially or completely impaired assembly of the RC in membrane [5, 6]. Taking into account that the photosynthetic RCs are of significant interest for biotechnology as promising components of artificial systems for solar energy conversion [7], it is relevant to identify amino acid residues or polypeptide regions in the complex structure, which are critical for its stability, as well as to search for approaches to stabilize structure of the genetically modified RCs. The methods used for this purpose in some works included selection of optimal detergent for RC solubilization from the membranes as well as addition to the buffer for RC purification and storage [8], as well as introduction of H-bonds between the cofactors and the protein [9-11]. Previously we have reported that introduction of inter-subunit disulfide bridges on the periplasmic or cytoplasmic surface of the complex noticeably increases thermostability of the *C. sphaeroides* RC [12]. In the present work, an attempt has been made to similarly stabilize the structure of the mutant RC with substitution of M206 Ile for Gln, which influenced stability of the complex to heat denaturation. RC I(M206)Q is of significant interest for studying the mechanisms of initial stages of photochemical charge separation, because this mutation close to bacteriochlorophylls of the active electron transfer chain (Fig. 1) led to the substantial decrease in the quantum yield of formation of charge-separated state $P^+Q_A^-$ [13].

In the present work we attempted to increase thermostability of the I(M206)Q RC by introduction of inter-subunit disulfide bonds on the cytoplasmic and periplasmic sides of the mutant membrane complex. In addition, the effects of amino acid substitutions on the amount of RCs in photosynthetic membranes were investigated. Previously, in 2022, Fufina et al. [13] showed that the properties of *C. sphaeroides* RCs with the I(M206)Q substitution were significantly different from the properties of mutant RCs of the closely related bacterium *Rhodobacter capsulatus* with the similar I(M204)Q substitution. It was hypothesized that the different consequences of the same mutation could be due to the different interactions between these two RCs and the membrane environment; however, at the time of publication, spatial structure of the *Rba. capsulatus* RC necessary for such comparison was not available yet. The present work involves analysis of the conserved lipid binding sites on the periplasmic side of LHC-1–RC complexes from *C. sphaeroides* and *Rba. capsulatus*.

MATERIALS AND METHODS

A DNA fragment with I(M206)Q mutation in the *puf*-M gene of *C. sphaeroides* was cloned at the NcoI/XhoI restriction sites in the *puf*-operon with double mutations G(M19)C/T(L214)C resulting in formation of disulfide bridges on the cytoplasmic side of RC [12, 13]. In a similar way, this fragment was cloned in the *puf*-operon with V(M84)C/G(L278)C mutations resulting in formation of S–S bridges on the periplasmic side of the wild type RC [12]. Locations of mutation sites in the RC structure is shown in Fig. 1. Next the *puf*-operons with the corresponding

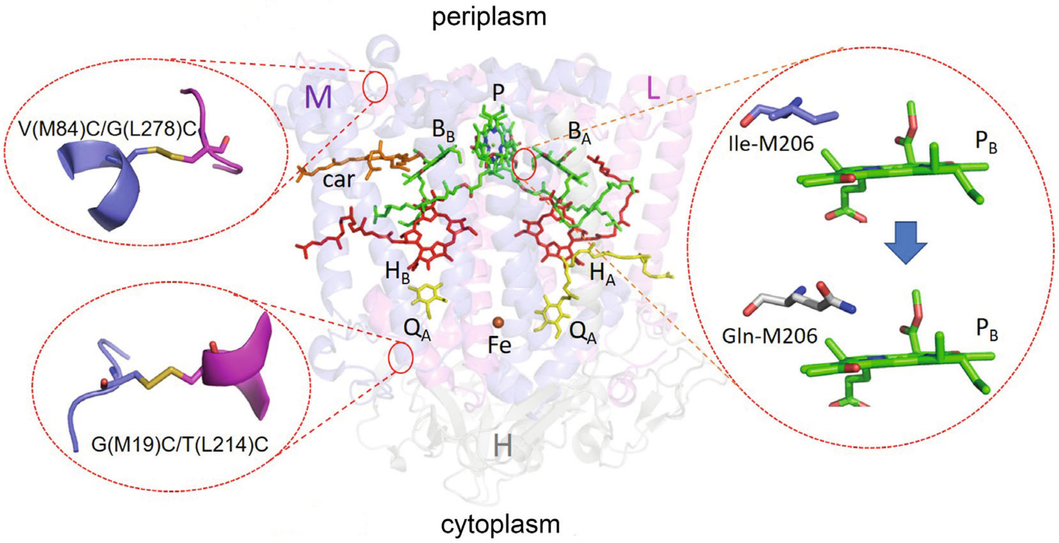

Fig. 1. Structure of *C. sphaeroides* RC (PDB ID: 3V3Y). Inserts on the left show the sites of mutations and disulfide bridges formed as a result of amino acid substitutions V(M84)C/G(L278)C (PDB ID: 8C7C) and G(M19)C/T(L214)C (PDB ID: 8C88) [12]. Insert on the right shows the site of mutation and the model of amino acid substitution I(M206)Q constructed with PyMol. P, bacteriochlorophyll dimer; P_B, one of bacteriochlorophylls of the dimer; B_A and B_B, monomeric bacteriochlorophylls; H_A and H_B, bacteriopheophytins; Q_A and Q_B, ubiquinones.

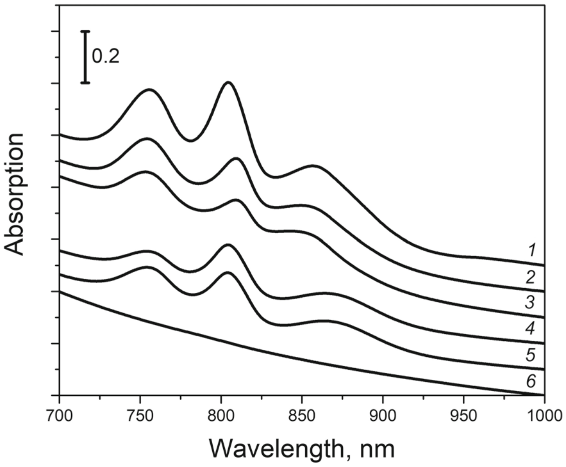

Fig. 2. Absorption spectra of chromatophores of the wild type *C. sphaeroides* (*1*); with mutation I(M206)Q (*2*); with triple mutation I(M206)Q/G(M19)C/T(L214)C (*3*); with a disulfide bond on the periplasmic side of the membrane, double mutation V(M84)C/G(L278)C (*4*); with a disulfide bond on the cytoplasmic side of the membrane, double mutation G(M19)C/T(L214)C (*5*); with triple mutation I(M206)Q/V(M84)C/G(L278)C (*6*).

triple substitutions I(M206)Q/G(M19)C/T(L214)C and I(M206)Q/V(M84)C/G(L278)C were cloned in a shuttle vector pRK-415 as described by Khatypov et al. [14]. The resultant plasmids were transferred by conjugation into an antenna-free strain *C. sphaeroides* DD13 [15]; as a result, recombinant strains *C. sphaeroides* DD13//I(M206)Q/V(M84)C/G(L278)C and DD13//I(M206)Q/G(M19)C/T(L214)C were obtained. Reaction centers used as a wild (pseudo-wild) type reaction center were isolated from the strain *C. sphaeroides* DD13 containing the pRK-415 derivative, which carried unmodified copies of the *puf-LMX* genes [14]. Recombinant *C. sphaeroides* strains were grown under semi-aerobic conditions in the dark on a Hutner's medium [16] in the presence of tetracycline (1 μg/mL) and kanamycin (5 μg/mL). Inoculum density, time and conditions of bacterial growth were the same for all used strains; absorption of a culture suspension measured at 600 nm in a cuvette with an optical path length of 1 cm after cultivation was 1.65-1.7. RC content in the membranes was evaluated by absorption at 800 nm in chromatophores suspension prepared from 1 L of cell cultures grown under identical conditions and dissolved in a fixed volume of 20 mM Tris-HCl (pH 8.0). Reaction centers were purified using affinity chromatography as described previously [17] with subsequent purification by ion exchange chromatography [18]. Membrane solubilization of the complexes was performed with a lauryldimethylamine oxide (LDAO) detergent. Purified RCs were dissolved in a TL buffer (20 mM Tris-HCl (pH 8.0) and 0.1% LDAO). Thermostability of isolated RCs was studied by heating them at 48°C for 60 min and recording thermodependent changes in the amplitude of the Q_Y band of monomeric bacteriochlorophylls as described previously [8]. Thermostability of RCs within the membranes was studied at 70°C by recording for 60 min spectral changes caused by elevated temperature [9]. Electronic absorption spectra were measured at room temperature with a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Japan). Sodium ascorbate (1 mM) was added to the samples to maintain the primary electron donor in a reduced state.

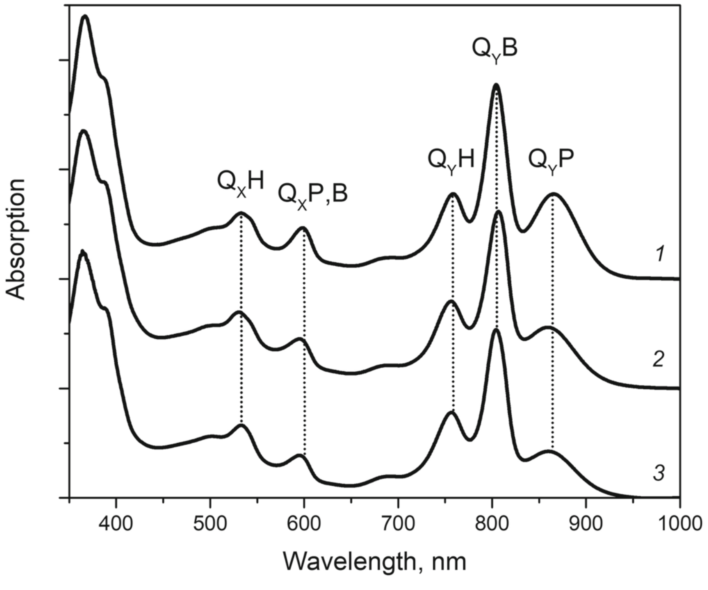

Fig. 3. Absorption spectra of wild type RC (*1*), RC with mutations I(M206)Q (*2*) and I(M206)Q/G(M19)C/T(L214)C (*3*) normalized to the Q_YH band. The bands of low-energy exciton transition of the dimeric primary electron donor (Q_YP), monomeric molecules of BChl (Q_YB), monomeric molecules of BPheo (Q_YH), as well as the band of Q_X transitions of four molecules of BChl (Q_XP,B) and two molecules of BPheo (Q_XH), are shown.

Electrophoresis of RC under denaturing conditions in 18% polyacrylamide gel was performed as described previously [12]. Visualization of the reaction center structure presented in Fig. 1 was carried out using PyMol [19].

RESULTS

In the present work, as a result of combination of amino acid substitutions in the *puf*-operon, a new mutant RCs with triple substitution I(M206)Q/G(M19)C/T(L214)C have been obtained. Absorption spectra of chromatophores of the recombinant strain with this triple mutation are shown in Fig. 2 (spectrum *3*) together with the spectra of chromatophores from other strains used in the work.

Combination of mutations I(M206)Q/V(M84)C/G(L278)C aimed at introducing an inter-subunit disulfide bond on the periplasmic side of the mutant RC I(M206)Q, instead of the expected stabilization of RC structure, seemed to result in disruption of the complex assembly in the membrane, because absorption bands typical of the BChl and BPheo RCs were absent in the spectrum of chromatophores of this mutant (Fig. 2; spectrum *6*).

Figure 3 shows electronic absorption spectra of the wild type *C. sphaeroides* RC and the mutant RCs with single I(M206)Q and triple I(M206)Q/G(M19)C/T(L214)C substitutions.

Absorption spectrum of the wild type RC (Fig. 3; curve *1*) corresponds to the literature data, including presence in the spectral region of the Q_Y band of low-energy exciton transition of the dimeric primary electron donor (Q_YP) at 865 nm, absorption band of monomeric BChls (Q_YB) at 804 nm, and of BPheo molecules (Q_YH) at 758 nm. In the Q_X region of the spectrum the band near 600 nm is associated with Q_X transitions of four BChl molecules. There are the maxima of absorption bands of BPheo molecules of the inactive (533 nm) and active (545 nm) electron transfer chains, as well as a shoulder in the region of 500 nm associated with absorption of the carotenoid molecule. Detailed discussion of the electronic absorption spectrum of RC I(M206)Q is given in the work of Fufina et al. [13]; its main difference from the wild type RC spectrum is a minor short-wave shift of the Q_YP band and decrease in the amplitude of this band. Significant differences between the absorption spectra of the mutant RC I(M206)Q and RC I(M206)Q/G(M19)C/T(L214)C have not been revealed (Fig. 3).

To compare the levels of RCs in the chromatophores of different *C. sphaeroides* strains, the cells were grown under identical conditions (see Materials and Methods). The results presented in Table 1 show that introduction of mutations affects the RCs content in the photosynthetic membranes. In particular, substitution of the Ile M206 with Gln results in 2-fold reduction in the RC content in chromatophores. Introduction of disulfide bonds on the cytoplasmic or periplasmic sides of RCs decreases their amount in the membranes by about one third (Table 1). Combination of amino acid substitutions I(M206)Q/G(M19)C/T(L214)C led to almost 4-fold decrease in the RCs content in chromatophores, while mutations I(M206)Q/V(M84)C/G(L278)C resulted in disruption of the RC assembly in the photosynthetic membrane.

Table 1. Content of RC in chromatophores of recombinant strains of *C. sphaeroides*

Strain/mutation	RC content in chromatophores, OD_{800}
Wild type	19 ± 4
I(M206)Q	9 ± 1
V(M84)C/G(L278)C	12 ± 2
G(M19)C/T(L214)C	13 ± 2
I(M206)Q/G(M19)C/T(L214)C	5 ± 1
I(M206)Q/V(M84)C/G(L278)C	0

Note. OD_{800}, absorption at 800 nm.

The results of electrophoresis under denaturing conditions in 18% polyacrylamide gel show formation of an inter-subunit S–S bond in the RC of the triple mutant I(M206)Q/G(M19)C/T(L214)C, similar to the RC with double mutation G(M19)C/T(L214)C [13], demonstrated by disappearance of the L- and M-subunits bands (21 and 24 kDa, respectively) and appearance of the band between 37 and 50 kDa corresponding to the combined mass of L- and M-subunits of about 45 kDa (Fig. S1 in the Online Resource 1).

Changes in the RC absorption spectrum at increased temperature were used as an indicator of stability of the RC's structure [8]. Figure 4 shows kinetics of thermodependent changes of the Q_YB band amplitude for the wild type RC, RC I(M206)Q, and RC I(M206)Q/G(M19)C/T(L214)C measured during 60-min incubation at 70°C for the membrane preparations (Fig. 4a) and at 48°C for the isolated RCs (Fig. 4b). The results show that the I(M206)Q mutation causes significant decrease in stability of both membrane-bound and isolated RCs, while additional introduction of a disulfide bond on the cytoplasmic side of the complex (G(M19)C/T(L214)C mutations) to the RC I(M206)Q has no effect on thermostability of the RC (Fig. 4b). It should be noted that the stabilizing effect of inter-unit disulfide bonds on thermostability of the

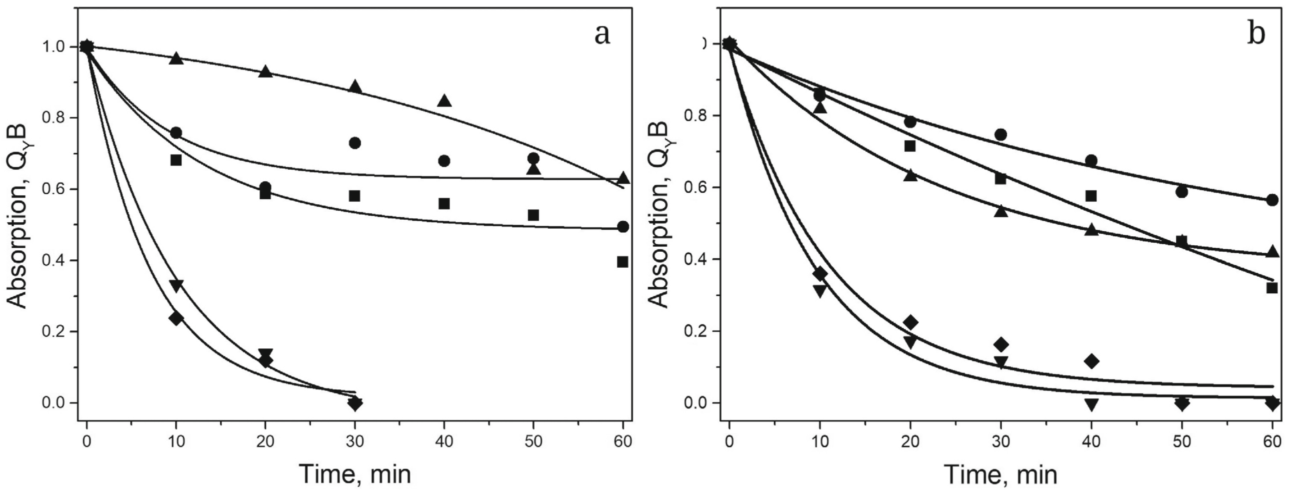

Fig. 4. Changes in the amplitude of the Q_YB band in the spectra of membrane-bound (a) and isolated (b) wild type *C. sphaeroides* RC (■), RC with the disulfide bond on the cytoplasmic side of the complex, G(M19)C/T(L214)C mutations (●), RC with the disulfide bond on the periplasmic side of the complex, V(M84)C/G(L278)C mutations (▲), RC with mutation I(M206)Q (♦), RC with triple mutation I(M206)Q/G(M19)C/T(L214)C (▼) measured in TL buffer at 70°C (a) or at 48°C (b).

wild type RC reported by Selikhanov et al. [12] was more pronounced than in the present work. This is probably associated with the method of purification of the wild type RC: Selikhanov et al. [12] used the longer method of ion exchange chromatography, while in the present work all RCs were purified by more rapid affinity chromatography, which prevents destabilization of the complex.

DISCUSSION

The mutant *C. sphaeroides* RC with substitution of Ile M206 with Gln is of interest for studying mechanisms of the initial stages of photochemical charge separation; however, the attempts to crystallize this complex and to decode its spatial structure have not been successful so far [13]. One of the possible causes could be destabilizing effect of mutation on the RC protein structure, which manifests itself as a decrease in resistance of the mutant RC to elevated temperatures. In the literature, several possible approaches to increase stability of the membrane proteins have been described. In particular, replacement of LDAO with sodium cholate in the buffer for RC dissolution significantly slowed down RC denaturation at elevated temperatures [8], while introduction of H-bonds between 2-acetyl group of the BChl P_B and the protein increased resistance of RC to temperature and elevated pressure [9, 10, 20]. However, the latter approach increases redox potential of the primary electron donor P and thereby affects the photochemical process; therefore, it cannot always be used in the structural and functional studies of RCs. The method for stabilization of local protein structure via introduction of S–S bridges is used mainly for globular proteins, but there are also few known examples of using this approach for membrane complexes [21, 22]. Recently it has been reported that introduction of inter-subunit disulfide bonds on the periplasmic or cytoplasmic surfaces of RC from *C. sphaeroides* noticeably increases its thermostability [13]. In the present work we have shown that this approach, namely, formation of a disulfide bridge between the α-helices of L- and M-subunits on the cytoplasmic side of the mutant RC I(M206)Q, had no effect on resistance of the complex to heat denaturation both in membranes and in solution. The findings suggest that substitution of Ile M206 with Gln not only destabilizes local structure of the RC close to bacteriochlorophylls of the active electron transfer chain, but also impairs interaction between the complex and other membrane components. Dezi et al. [23] have shown that the RC–LHC-1 core complex from *C. sphaeroides* contains more than 150 molecules of lipids: cardiolipin (50%), phosphatidylglycerol (24%), phosphatidylethanolamine (12%), and phosphatidylcholine (14%). Lipids are assumed to stabilize interactions between the RC and LHC-1, as well as between the RC–LHC-1 complex and the membrane bilayer into which it is incorporated. It seems that violation in the RC conformation and disruption of RC's interactions with the membrane environment caused by the single substitution I(M206)Q cannot be compensated by introduction of a disulfide bond.

Previously it has been shown that properties of the *C. sphaeroides* RCs with I(M206)Q substitution were significantly different from properties of the mutant RCs of the closely related bacterium *Rba. capsulatus* with analogous substitution I(M204)Q, where this mutation resulted in the loss of BChl B_A

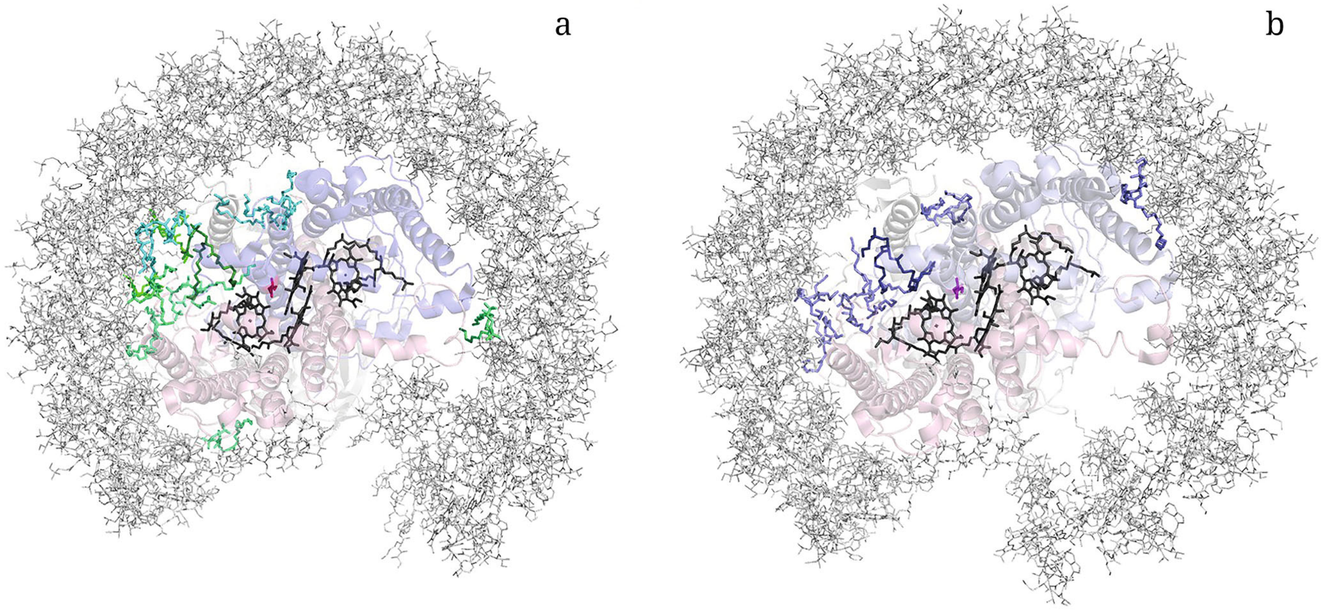

Fig. 5. Structures of LHC-1–RC complexes from *C. sphaeroides* (PDB ID: 7VNY) (a) and *Rba. capsulatus* (PDB ID: 8B64) (b), view from the periplasmic side of the membrane. LHC-1 are gray; L-subunit of RC is pale pink; M-subunit is light blue. BChl dimer P and monomeric CHhl B_A and $-B_B$ are dark gray. Isoleucine M206 (*C. sphaeroides*) and M204 (*Rba. capsulatus*) are bright pink. Cardiolipin is blue; 1,2-diacyl-sn-glycero-3-phosphocholine is green; 1,2-distearoyl-sn-glycerophosphoethanolamine is violet.

from the RC structure [5, 13]. Two structures of RC–LHC-1 from *Rba. capsulatus* obtained by the method of cryoelectron microscopy were published in 2023 [24, 25], making it possible to compare membrane environments of these complexes in the two bacteria. Figure 5 shows the RC–LHC-1 structures from *C. sphaeroides* and *Rba. capsulatus*, view from the periplasmic side of the membrane. It can be noticed that the regions of tight binding of the conserved lipids in the structures of both bacteria are localized mainly on the side of the active chain of cofactors (Fig. 5). Qualitative and quantitative lipid compositions in the two bacteria are noticeably different. Close to the site of mutation, M206, in *C. sphaeroides* there is 1,2-diacyl-sn-glycero-3-phosphocholine; in the structure of *Rba. capsulatus*, there is 1,2-distearoyl-sn-glycerophosphoethanoamine (Fig. 5). These structural data are in agreement with the assumption that interactions between the two RCs and the membrane environment are significantly different, which could be a potential cause of different consequences of the same substitution Ile → Gln [5, 13].

In the present work we have demonstrated that introduction of mutations could have effect not only on thermostability of RC but also on the amount of these complexes in photosynthetic membranes. It has been shown that the double mutations resulting in formation of inter-subunit disulfide bonds on the periplasmic or cytoplasmic surface of RC lead, simultaneously with the increase in thermostability of RC, to the decrease in the membrane level of RC by one third. The substitution of Ile M206 with Gln leads to 2-fold reduction of the RC level in chromatophores; addition of the disulfide bridge on the cytoplasmic side of RC I(M206)Q leads to 4-fold decrease in the level of RC in the membranes, while introduction of the disulfide bond on the periplasmic side completely disrupts assembly of the RC in the photosynthetic membrane. The findings indicate that point substitutions of amino acid could affect the processes of incorporation of the complex into the membrane.

In view of the above, theoretical studies of the factors that determine stability of α-helices in the protein are of interest. Calculations show that deformation energy, which counteracts protein folding, arises when position of the side-chain amino acid group introduced as a result of site-directed mutation does not coincide with the most stable conformation of this group in a free α-helix [26, 27]. This factor seems to cause disruption of the assembly of the mutant RCs and, as a consequence, decrease in their quantity in the membranes.

At present, the general concept of protein transport and assembly in the cytoplasmic membrane of bacteria has been formed in the literature (https://simbac.gatech.edu/translocon/). During the synthesis on ribosome, the proteins that must pass through or be embedded into the membrane enter the translocon, a heterotrimeric molecular complex with the shape of a channel. The translocon “analyzes” hydrophobicity of the polypeptide passing through it and, upon reaching a certain hydrophobicity threshold, directs the polypeptide not through the channel to the extracellular space but through the regulated cavity in the channel wall to the membrane lipid bilayer. There is a large amount of literature data on formation, sizes, and functional models of the membrane vesicles of purple bacteria; spatial structures and interrelationships between the pigment–protein complexes involved in light energy absorption and conversion are also well-known [28]. In *C. sphaeroides*, membranes are formed both in the presence of light and in the dark under low aeration conditions [29]. The number of mature chromatophores per cell may vary depending on cultivation conditions and light intensity, but composition of a single vesicle is relatively constant. The mature chromatophore of *C. sphaeroides* has a photosynthetic apparatus containing, on the average, 63-67 peripheral light-harvesting complexes LHC-2, 11 dimers of core complexes RC–LHC-1–PufX, 2 monomeric complexes RC–LHC-1–PufX, 4 dimers of cytochrome *bc1* complex, and 2 complexes of ATP synthase [30, 31].

Although photosynthetic membranes of purple bacteria are well characterized, little is known about the details of the assembly of photosynthetic complexes. This is a complex multistage process, which includes polypeptide translocation and incorporation into the membrane, posttranslational modifications and protein folding, and cofactor attachment [28]. According to the recent data, the LhaA protein plays an important role in the RC–LHC-1 assembly; the gene of this protein, *lhaA*, is localized in the photosynthetic gene cluster along with most of the genes related to biosynthesis, assembly, regulation, and function of RC–LHC-1 complexes in phototrophic bacteria [32]. It has been shown that the LhaA protein forms oligomers at the sites of initiation of membrane invagination during chromatophore formation and that it interacts with RC, BChl synthase (BchG), protein YajC translocase subunit, and membrane protein YidC insertase [33]. It has been suggested that LhaA is part of the membrane nanodomain, where close proximity of the components of biosynthesis and membrane translocation contributes to coordinated delivery of the pigments (cofactors), co-translational insertion of polypeptides, their folding and assembly for formation of photosynthetic complexes [28].

Swainsbury et al. [28] drew some general conclusions about the sequence of events that form RC and then surround it with LHC-1 subunits. It is believed that RC–LHC-1 complexes are built from the inside out: first L- and M-subunits are attached to the H-polypeptide, which is permanently present in the membrane, thereby forming RC. Next, the first subunit of LHC-1 binds to the specific region of RC and, beginning from this point of initiation, a ring of LHC-1 subunits begins to form [34]. Taking into account that our work was carried out with the genetically modified strains of *C. sphaeroides* with chromatophores containing RCs without light-harvesting antenna complexes, it could be suggested that the mutations mentioned in the work could influence the processes such as translocation and insertion of L- and M-subunits into the membrane, protein folding, as well as interaction between the RC proteins and membrane lipids. Further research is needed to draw more detailed conclusions.

CONCLUSIONS

It was shown in the present work that introduction of the mutations resulting in formation of an inter-subunit disulfide bridge on the periplasmic surface of mutant RC with substitution of Ile M206 with Gln leads to disruption of RC assembly in the membrane. Formation of the disulfide bond on the cytoplasmic side of RC I(M206)Q does not promote increase in thermostability of the mutant complex both in membranes and in solution. In addition, introduction of the I(M206)Q, V(M84)C/G(L278)C, G(M19)C/T(L214)C, I(M206)Q/G(M19)C/T(L214)C, and I(M206)Q/V(M84)C/G(L278)C mutations significantly reduces the amount of RCs in chromatophores to varying degrees, apparently affecting assembly of an integral membrane complex. Thus, in spite of the fact that introduction of disulfide bonds increases thermostability of the wild type RC, this method is not suitable for stabilization of the structure of the mutant RC with I(M206)Q amino acid substitution close to bacteriochlorophylls of the active chain cofactors.

Abbreviations. BPheo, bacteriopheophytin; BChl, bacteriochlorophyll; RC, reaction center; LHC, light-harvesting complex; B_A and B_B, monomeric bacteriochlorophylls; P, dimer of bacteriochlorophylls P_A and P_B; Q, ubiquinone.

Supplementary information. The online version contains supplementary material available at https://doi.org/10.1134/S0006297925600978.

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Contributions. T. Yu. Fufina and L. G. Vasilyeva – experimental part, discussion and editing of the manuscript; T. Yu. Fufina – preparation of figures; L. G. Vasilyeva – writing the manuscript.

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Ethics approval and consent to participate. This work does not contain any studies involving human and animal subjects.

Conflict of interest. The authors of this work declare that they have no conflicts of interest.

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