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Examination of Stability of Mutant Photosynthetic Reaction Center of *Rhodobacter sphaeroides* I(L177)H and Determination of Location of Bacteriochlorophyll Covalently Bound to the Protein

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Abstract—We demonstrated earlier that as a result of the I(L177)H mutation in the photosynthetic reaction center (RC) of the bacterium *Rhodobacter sphaeroides*, one of the bacteriochlorophylls (BChl) binds with the L-subunit, simultaneously raising coordination stability of the central magnesium atom of the bacteriochlorophyll associated with the protein. In this study, spectral properties of wild type RC and I(L177)H in the presence of urea and SDS as well as at 48°C were examined. It is shown that the I(L177)H mutation decreases the RC stability. Under denaturing conditions, some changes indicating breakdown of oligomeric structure of the complex and loss of interaction between pigments and their protein environment are observed in I(L177)H RC spectra. In addition, pheophytinization of bacteriochlorophylls occurs in both types of RC in the presence of SDS. However, an 811-nm band is observed in the spectrum of the mutant RC under these conditions, which indicates retention of one of the BChl molecules in the protein binding site and stable coordination of its central magnesium atom. It is shown that in both types of RC, monomeric BChl B_B can be modified by sodium borohydride treatment and then extracted by acetone–methanol mixture. Spectral properties of the BChl covalently bound with the protein in I(L177)H RC do not change. The results demonstrate that BChl P_A is the molecule of BChl tightly bound with the L-subunit in mutant RC as it was supposed earlier.

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In photosynthesizing organisms, transformation of light energy into energy of chemical bonds occurs in pigment–protein complexes connected with membranes called photosynthetic reaction centers (RC). The structure of RC of the purple non-sulfur bacterium *Rhodobacter sphaeroides* comprises three protein subunits and 10 electron transfer cofactors [1]. The cofactors include six bacteriochlorin molecules (four bacteriochlorophylls (BChl) and two bacteriopheophytins (BPheo)), two ubiquinone molecules, a non-heme iron atom, and a carotenoid molecule. Two BChl molecules form a special pair (P) which functions as an electron donor. Electron transfer cofactors form two branches: active (A) and inactive (B) [2]. The RC complex also contains associated water molecules, some of which play a functional role in electron transfer [3, 4].

Basic structural elements of porphyrin molecules (including BChl and BPheo) include an 18-member system of conjugated bonds (tetrapyrrole), a fifth cyclopentanone ring, and phytol. In addition, a central magnesium atom, which significantly influences spectral properties of the tetrapyrrole, is included in the structure of the BChl macrocycle [5]. The BChl molecule easily loses magnesium and converts to BPheo in the absence of a fifth ligand as well as at acidic pH and under denaturing conditions.

In all known photosynthetic membrane complexes, pigments are bound with surrounding protein by relatively weak noncovalent interactions. Connection of pig-

Abbreviations: BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; P, special pair of bacteriochlorophylls; P_A and B_A, bacteriochlorophylls of active electron transport branch; P_B and B_B, bacteriochlorophylls of inactive electron transfer branch; RC, reaction center; SDS, sodium dodecyl sulfate.

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ments with protein in RC has important functional significance since it fixes certain distances between pigment molecules and their mutual orientation, which is required for high efficiency of light absorption and energy migration [5]. Bacteriochlorin cofactors of RC exhibit characteristic spectral properties depending on their protein environment. Study of stability of mutant RC under denaturing conditions is one of the approaches providing information about the influence of mutation on structural and functional properties of the pigment–protein complex. In contrast to water-soluble proteins, data showing the stability of membrane proteins and, especially, bacterial RC are not numerous [6-8].

We described earlier that a mutant RC in which a single I(L177)H mutation near the B_B and P_A chromophores leads to significant spectral changes in a region of bacteriochlorophyll absorption [9]. According to pigment analysis data, BChl/BPheo ratio in pigment extract from I(L177)H RC is 1.5, while in wild type RC it is 2. The original supposition about loss of one BChl from mutant RC proved to be wrong. Further studies revealed that one of the BChl molecules in I(L177)H RC is not extracted by organic solvents since a covalent bond between the pigment molecule and the L-subunit of RC is formed as a result of the mutation [10]. Currently, it is still unclear which of the two bacteriochlorophylls nearest to the mutation site is covalently bound to protein. However, under conditions of denaturing electrophoresis, very tight liganding of a magnesium atom in the BChl molecule bound with the protein was noted [10]. Covalent binding of BChl to protein in photosynthetic membrane complex *in vivo* resulting from the single mutation is the first such case described in the literature. Therefore, elucidation of the reason and mechanisms of such binding is of significant interest.

The goal of the present work was to study the stability of I(L177)H RC under heating and in the presence of sodium dodecyl sulfate (SDS) and urea, as well as to define the BChl molecule covalently bound with the protein of the mutant RC.

MATERIALS AND METHODS

Cells of recombinant *Rhodobacter sphaeroides* strains lacking light-harvesting antenna were cultivated in Huttner medium in the presence of tetracycline (1 μg/ml), kanamycin (5 μg/ml), and streptomycin (5 μg/ml) as described earlier [9]. Reaction centers were isolated according to a method described earlier [10]. Stability of RC in the presence of detergents was examined at 20°C in 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 180 mM NaCl containing 5% SDS or 8 M urea. To examine thermostability at 48°C, 1.5 ml of solution of reaction centers (A_{760} = 0.3) was added to 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 180 mM NaCl, and then sodium ascorbate (1 mM) was added to reduce the primary electron donor. The RC were treated with $NaBH_4$ by a method described earlier [11]. The pigments were extracted by acetone–methanol mixture (7 : 2) by a method from [12]. After pigment extraction, the protein of I(L177)H RC was dissolved in 20 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 180 mM NaCl, and 5% SDS. Absorption spectra were recorded at room temperature using a Shimadzu UV-1601PC spectrophotometer (Japan).

RESULTS

The following pigment absorption bands are present in the absorption spectrum of wild type RC (Fig. 1a, spectrum *1*): long-wave absorption band of the primary donor at 865 nm (Q_Y P), absorption band of monomeric BChl at 805 nm (Q_Y B), absorption band of BPheo molecules at 758 nm (Q_Y H), absorption band of all BChl molecules in RC at 598 nm (Q_X P and Q_X B), short-wavelength absorption band of BPheo at 533 nm (Q_X H), and absorption

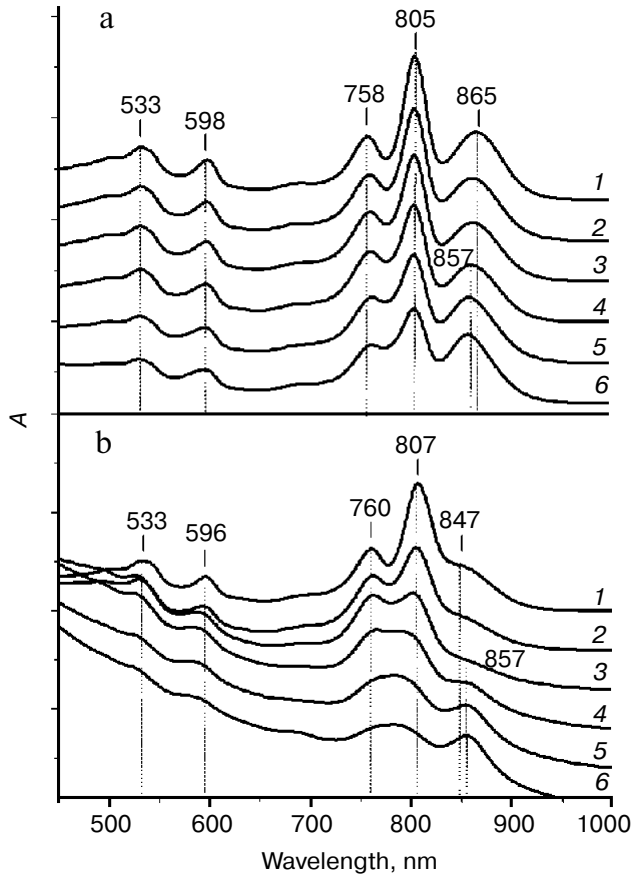

Fig. 1. Optical absorption spectra of wild type RC (a) and I(L177)H mutant (b) at 48°C. Incubation time: *1*) 0; *2*) 10; *3*) 25; *4*) 60; *5*) 120; *6*) 240 min.

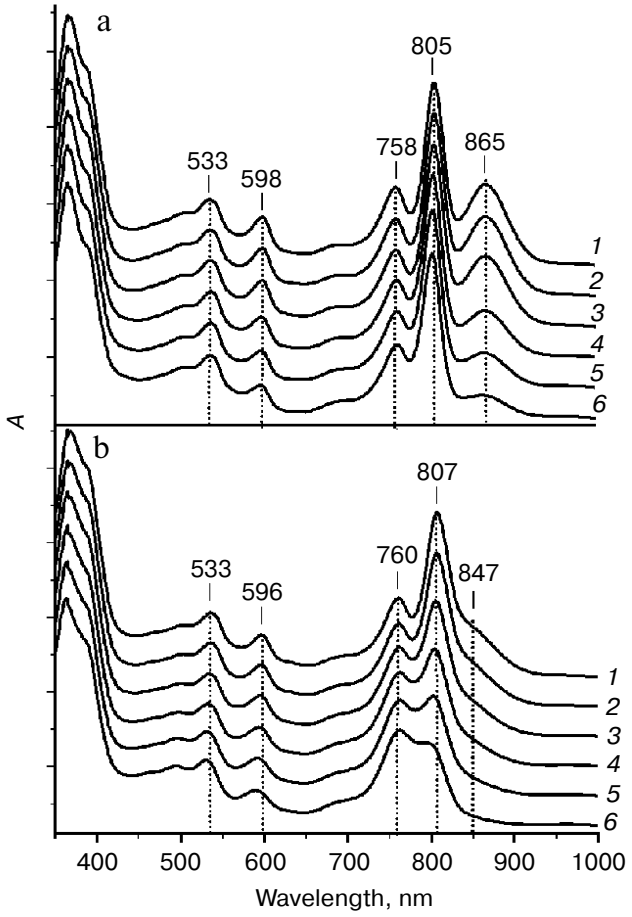

Fig. 2. Optical absorption spectra of wild type RC (a) and I(L177)H mutant (b) in the presence of 8 M urea. Incubation time: *1*) 0; *2*) 10; *3*) 25; *4*) 60; *5*) 120; *6*) 240 min.

bands of carotenoids at about 500 nm. The most evident differences in the absorption spectrum of the mutant RC are observed in the absorption region of BChl molecules (Fig. 1b, spectrum *1*). A shift to shorter wavelength of the absorption band of the primary electron donor to 847 nm, a small shift of a band of monomeric BChl to longer wavelength (807 nm) as well as decrease in amplitude of these bands are obviously a consequence of the I(L177)H mutation [10].

According to the literature, isolated bacterial RCs are less heat-resistant than membrane-bound complexes [13, 14]. To compare thermostability of wild type RC and the mutant with the avoiding disruption of bacteriochlorins [14], these complexes were carefully thermally denatured by incubation at 48°C for 4 h in darkness. Under these conditions in the spectrum of wild type RC, an 8-nm shift of the Q_Y P band to shorter wavelength and decrease in amplitude of the Q_Y bands of bacteriochlorin absorption were observed; this is evidence for conformational changes of the protein environment of the cofactors influencing allocation and binding of the pigments. Under the same conditions, rapid and significant changes in spectral properties of bacteriochlorins in I(L177)H RC were observed: disappearance of the Q_Y P band, blue shift of the Q_Y B band, appearance of a longer wavelength band at 857 nm, and rise in light scattering in the sample (Fig. 1b, spectra *4*-*6*). The absence of changes in the region of the Q_X bands of BChl and BPheo indicated that there was no pheophytinization of bacteriochlorophylls under these experimental conditions. All of the mentioned changes indicate thermal denaturation of the mutant RC protein and release of pigments from the protein environment and subsequent aggregation. After the experiment, denatured protein of I(L177)H RC was easily isolated from the pigment solution by centrifugation. The precipitated protein of mutant RC was green colored, which indicated preservation of covalent coupling between the BChl and protein under these conditions.

Membrane proteins are known to have strong hydrophobic interactions between their α-helixes.

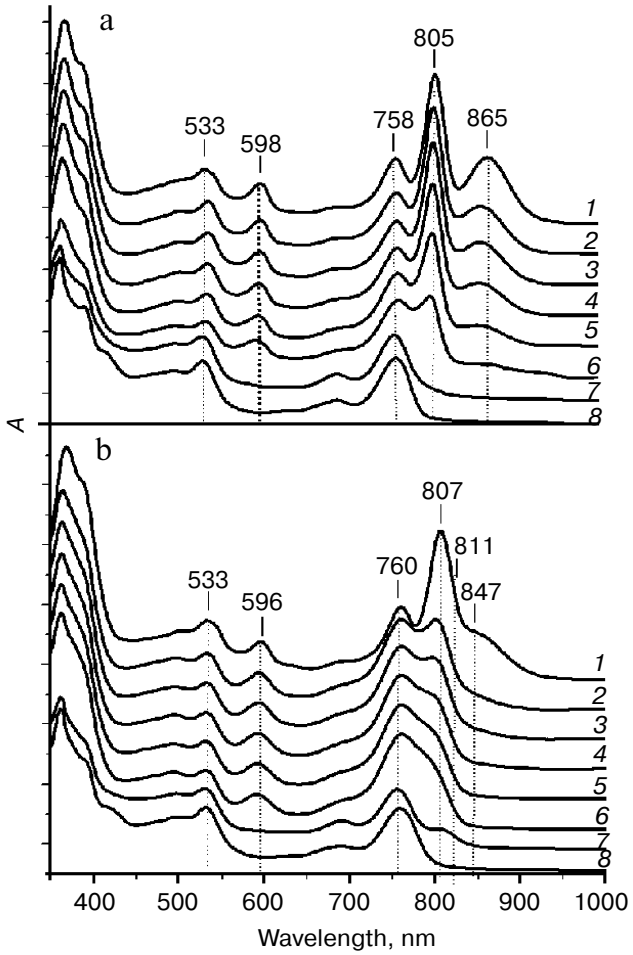

Fig. 3. Optical absorption spectra of wild type RC (a) and I(L177)H mutant (b) in the presence of 5% SDS. Incubation time: *1*) 0; *2*) 10; *3*) 25; *4*) 60; *5*) 120; *6*) 240 min; *7*) 4 days; *8*) 4 days (+HCl).

Therefore, in the presence of the relatively mild detergent urea, only slight denaturation of them is observed [15]. Due to some losses of weak hydrogen bonds, the protein globule "swells" while preserving its contours [16]. As under heating conditions, wild type RC exhibits significant stability in the presence of 8 M urea (Fig. 2a). During 4 h incubation under these conditions, gradual lowering of amplitude of the Q_Y P absorption band without any change in position is observed, which might indicate the breakage of dimer interaction of the BChl molecules resulting from conformational changes of the protein (Fig. 2a, spectrum *6*). More significant and rapid changes occur in the spectrum of I(L177)H RC under the same conditions. As under heating conditions, disappearance of the Q_Y P band and of the Q_Y B band from 807 to 800 nm as well as increase in absorption at about 760 nm are observed, which suggests disruption of a significant part of the pigment–protein interactions in the mutant RC (Fig. 2b, spectrum *6*). The absence of porphyrin aggregates (857 nm band) indicate that the pigments remain in the RC complex.

Compared with urea, SDS is a stronger denaturing agent and leads to disruption of both intermolecular and, particularly, intramolecular interactions. On incubation in the presence of 5% SDS, the Q_Y P, Q_Y B, and Q_X bands of the bacteriochlorophylls gradually disappear, and 758 and 533 nm bands corresponding to BPheo absorption remain in the RC absorption spectrum (Fig. 3a). These spectral changes suggest that RC structure gradually denatures under these conditions with SDS, and the interaction between porphyrins and their protein environment decreases. At the same time, BChl loses its central magnesium atom and converts into BPheo, which seems to be due to the interaction of Mg with polar groups of SDS (Fig. 3a, spectra *1*-*7*). Pheophytinization of BChl occurs fully as indicated by the absence of spectral changes when adding 1/1000 of volume of 0.1 M HCl to the preparation of wild type RC [17] (Fig. 3a, spectrum *8*). In the presence of SDS, similar changes are observed in the absorption spectrum of mutant I(L177)H RC but they occur significantly faster (Fig. 3b, spectra *1*-*7*). The spectra in Figs. 3a and 3b significantly differ from each other by survival of the 811-nm band in the mutant spectrum after 4 day incubation in the presence of SDS (Fig. 3b, spectrum *7*). Only on addition of 1/1000 of volume of 0.1 M HCl this band shifts to shorter wavelength, which indicates pheophytinization of BChl (Fig. 3b, spectrum *8*).

To determine which BChl molecule of RC is covalently bound to the protein as a result of the I(L177)H mutation, RCs were treated with sodium borohydride as described earlier [11]. It is known that during such treatment $NaBH_4$ selectively interacts with monomeric BChl of inactive chain of cofactors reducing the C-3^1-acetyl carbonyl group of the B_B molecule to hydroxyethyl. This reaction is accompanied by spectral changes in the Q_Y B band and appearance of a new band of modified BChl at about 715 nm [11, 18-20]. Absorption spectra of wild type (a) and I(L177)H (b) RC before (*1*) and after (*2*) $NaBH_4$ treatment are presented in Fig. 4. In the absorption spectra of both types of RC, decrease in amplitude and short-wavelength shift of the Q_Y absorption band of bacteriochlorophylls B to 802 nm are observed, and the absorption band of modified pigment appears at 718 nm. At the same time, the Q_Y absorption bands of the special pair and BPheo do not change. In absorption spectra of pigment extracts from wild type RC and the mutant, after $NaBH_4$ treatment a band of modified BChl B_B also occurs at 715 nm (Fig. 5). After extraction of the pigments, absorption bands vanish in the spectrum of the denatured protein of wild type RC, which proves completeness of extraction (Fig. 6, spectrum *1*). In the spectrum of I(L177)H RC under similar conditions, the 802 and 598 nm bands (Fig. 6, spectrum *2*) characteristic, as described earlier [10], for absorption of covalently bound BChl are observed. The results show that $NaBH_4$ treatment of the mutant RC does not influence the location of the maxima of these bands.

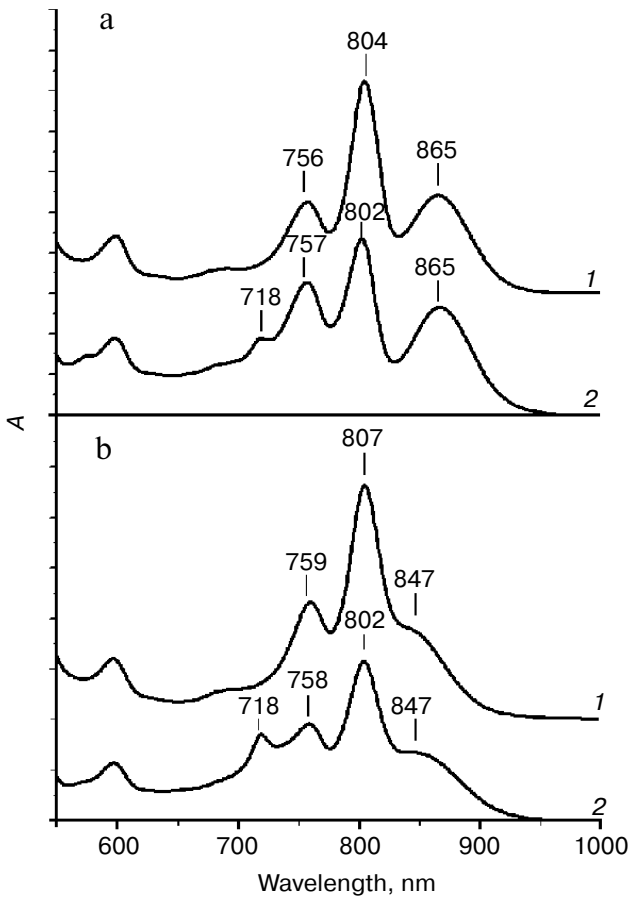

Fig. 4. Optical absorption spectra of wild type RC (a) and I(L177)H mutant (b) before (*1*) and after (*2*) sodium borohydride treatment.

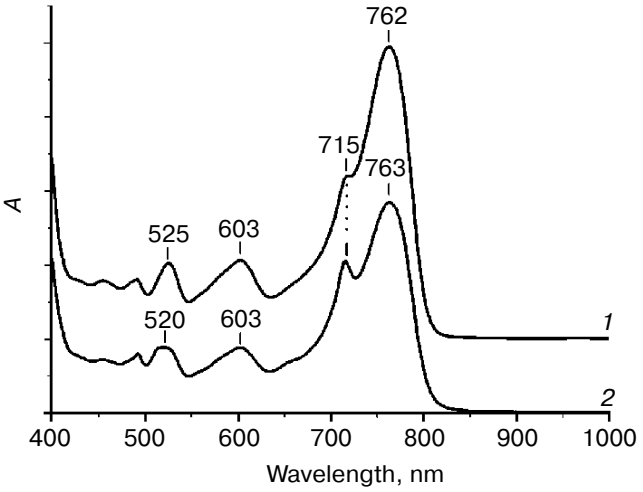

Fig. 5. Optical absorption spectra of pigments extracted from wild type RC (*1*) and I(L177)H mutant (*2*) after sodium borohydride treatment.

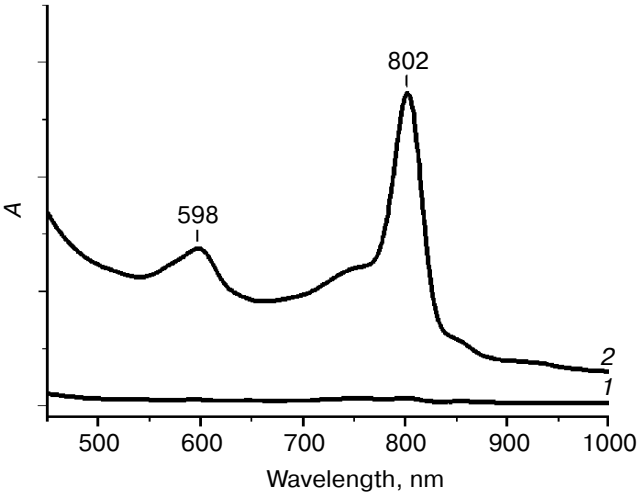

Fig. 6. Optical absorption spectra of denatured protein of wild type RC (*1*) and I(L177)H mutant (*2*) after sodium borohydride treatment.

DISCUSSION

The results provide novel information about properties of I(L177)H RC and confirm the suggestion about localization of the BChl molecule covalently bound with the protein. We demonstrated that in the spectrum of mutant RC after prolonged incubation in the presence of a strong detergent, SDS, the 811-nm absorption band is preserved. This result correlates with data obtained earlier showing stable coordination of the BChl magnesium atom covalently bound with the protein in I(L177)H RC [10]. It was demonstrated earlier that under conditions of denaturing PAGE, liganding of the magnesium atom of the BChl associated with the L-subunit is preserved, while the rest of the bacteriochlorophylls of the RC converts into BPheo under these conditions [10]. In the structure of RC, the L177 mutation site is located near the P_A and B_B BChl molecules. At the protein side, the central magnesium atoms of the bacteriochlorophylls P_A and B_B are coordinated by histidines L173 (L-subunit) and M182 (M-subunit), respectively. Basing on this we suggest that the preservation of magnesium atom coordination indicates that the BChl molecule covalently bound with the L-subunit is P_A. Pigment–protein covalent bond was supposed to tightly fix histidine L173 in optimal coordination distance from P_A that prevents pheophytinization of the BChl molecule under denaturing conditions [10]. The appearance of the 811 nm absorption band in the spectrum of I(L177)H RC in the presence of 5% SDS means that, in the mutant RC, the BChl molecule remains in its protein binding site and does not lose the central magnesium atom, i.e. exhibits properties described earlier for the BChl molecule covalently bound to the protein [10]. However, assignment of this band cannot clearly indicate which of the RC bacteriochlorophylls is covalently bound with the protein. To answer this question, RC was treated with sodium borohydride. Although $NaBH_4$ seems to impose a complex effect on *Rhodobacter sphaeroides* RC including partial proteolysis of the M-subunit [21], it was shown in several works that, owing to structural peculiarities of these RC, sodium borohydride selectively reacts only with BChl B_B [11, 18-20]. According to our data reported here, changes in spectral properties of BChl of both wild type RC and I(L177)H RC were observed in a fraction of pigments extracted by acetone–methanol. Absorption bands of BChl remaining in the pellet of mutant RC protein did not change after sodium borohydride treatment of RC. These results prove that BChl B_B is not the pigment covalently bound to the L-subunit of I(L177)H RC and correlate with our earlier supposition that a covalent bond is formed between the protein and BChl P_A. Establishment of the location of this BChl is important for further determination of the nature of the covalent bond between the pigment and protein in I(L177)H RC.

The results represented in the work indicate that the single I(L177)H mutation leads to significant lowering in stability of *Rhodobacter sphaeroides* RC. We suppose that as a result of isoleucine-to-histidine substitution in L177 position, a certain factor important for stability of the pigment–protein complex is lost. It is known that strong interaction between α-helices is typical for membrane proteins. Therefore, loss in functional activity of these proteins under denaturing conditions is commonly not connected with refolding of protein globule but with separation of the subunits and loss of interaction between separate parts of the membrane complex [13]. Hydrogen bonds are thought to play a much more significant role in providing structural stability of membrane complexes

compared with water-soluble proteins [15]. Appearance of polar molecules inside integral proteins, such as polar amino acids and water, is never accidental, and it is commonly due to their particular function [16]. In the case of isoleucine-to-histidine substitution, there is no reason to expect significant distortions in protein structure near the mutation site since molecular volumes of these amino acid residues are similar. However, it must be taken into account that histidine is positively charged (+1) at neutral pH. So, isoleucine-to-histidine substitution must inevitably lead to change in local charge and change in electrostatic interactions in surroundings of the P_A and B_B chromophores. It is known that in *Rhodobacter sphaeroides* RCs there is a conserved water molecule (so-called "water B") which is located near bacteriochlorophylls P_A and B_B at the interface of the L- and M-subunits [3, 4]. By analogy with water A this water molecule is assumed to be bound with histidine L173 and C-13^1-ketocarbonyl group of BChl B_B by hydrogen bonds [22]. "Water B", in contrast to symmetrically located "water A", obviously does not play a functional role in photosynthetic electron transfer since it is located on the side of inactive chain of cofactors. However, high conservativeness of this water suggests that it is necessary for stabilization of the RC structure. It can be supposed that the I(L177)H mutation causes a change in the electrostatic environment of "water B" in the vicinity of the mutation with subsequent shift in its location and breakage of hydrogen bond connecting molecules of bacteriochlorophylls P_A and B_B at the interface of the L- and M-subunits of the RC. This could be a reason for lowering of I(L177)H RC stability. However, to confirm this suggestion further investigations are necessary.

Thus, in this work the I(L177)H mutation is shown to decrease the stability of *Rhodobacter sphaeroides* RC. Moreover, our results confirm the suggestion that the BChl molecule covalently bound to protein in I(L177)H RC is P_A.

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