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Properties of *Rhodobacter sphaeroides* Photosynthetic Reaction Center with Double Amino Acid Substitution I(L177)H+H(M182)L

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Abstract—Histidine M182 in the reaction center (RC) of *Rhodobacter sphaeroides* serves as the fifth ligand of the bacteriochlorophyll (BChl) B_B Mg atom. When this His is substituted by an amino acid that is not able to coordinate Mg, bacteriopheophytin appears in the B_B binding site instead of BChl (Katilius, E., et al. (1999) *J. Phys. Chem. B*, **103**, 7386-7389). We have shown that in the presence of the additional mutation I(L177)H the coordination of the BChl B_B Mg atom in the double mutant I(L177)H+H(M182)L RC still remains. Changes in the double mutant RC absorption spectrum attributed to BChl absorption suggest that BChl B_B Mg atom axial ligation might be realized not from the usual α-side of the BChl macrocycle, but from the opposite, β-side. Weaker coordination of BChl B_B Mg atom compared to the other mutant RC BChl molecules suggests that not an amino acid residue but a water molecule might be a possible ligand. The results are discussed in the light of the structural changes that occurred in the RC upon Ile/His substitution in the L177 position.

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Key words: bacterial photosynthesis, *Rhodobacter sphaeroides*, photosynthetic reaction center, site-directed mutagenesis, bacteriochlorophyll, Mg coordination

Photosynthetic organisms utilize light energy by means of special pigments, and one of the most widespread types is bacteriochlorophylls (BChl). Being included in light harvesting complexes and photosynthetic reaction centers (RC), these pigments directly participate in the process of light energy transduction. The RC of the purple non-sulfur bacterium *Rhodobacter* (*Rba.*) *sphaeroides* consists of three protein subunits and ten cofactors that form two branches of electron transfer, A and B. Cofactors are represented by four BChl molecules, with two of them combined into a special pair (dimer) that serves as a primary electron donor. RC also contains two bacteriopheophytin (BPhe) molecules, two ubiquinones, a carotenoid molecule, and a non-heme iron atom [1]. Besides, a considerable number of bound water molecules were found in the RC structure. At least some of these play an important role in the photosynthetic electron transfer and also are essential for stability of the pigment–protein complexes [2-4].

BChl structure includes a central magnesium atom, which considerably affects spectral properties of the tetrapyrroles [5]. The highest possible coordination number of the central Mg is six, but often in biological systems it is pentacoordinated. In addition to the chelation by tetrapyrrolic nitrogens it has one axial ligand from the nearest protein side. The axial ligand can be situated from either of the two sides of the BChl macrocycle. If the amino acid ligand and the 17-propionic acid phytol ester of BChl are positioned on the same side of the BChl plane this is ligation of β-type, and if they are on opposite sides this is ligation of α-type [6]. In photosynthetic complexes α-ligated (B)Chls are generally much more abundant compared to β-ligated (B)Chls [6-8]. In all known bacterial RCs the central Mg atoms of BChls are always α-coordinated by histidines.

Previously a new mutant RC of *Rba. sphaeroides* was obtained with the single mutation I(L177)H that resulted

Abbreviations: B_A and B_B, monomer bacteriochlorophylls; BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; LDAO, lauryldimethylamine N-oxide; P, special pair of bacteriochlorophylls; P_A and P_B, bacteriochlorophylls of the special pair; RC, reaction center.

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in considerable changes of the RC absorption spectrum [9] and also led to covalent binding of BChl P_A and L-subunit of RC [10, 11]. In the RC structure isoleucine L177 is located in the immediate vicinity of the B_B and P_A molecules, and obviously its substitution for histidine affects properties of both chromophores. However, the overlap of the monomer and dimer BChl absorption bands in the long-wavelength part of the RC spectrum presents problems for its detailed interpretation. One possible solution is the replacement of BChl by BPhe in the B_B protein-binding pocket that should result in short-wavelength shift of the pigment absorption band from 812 to 785 nm. It is known that such replacement can be achieved by substitution of histidine M182 (the ligand of the Mg atom of BChl B_B) by leucine [12]. We expect that study of the double mutant RC H(M182)L+I(L177)H can bring new information concerning dramatic influence of the single mutation I(L177)H on the properties of BChl B_B.

MATERIALS AND METHODS

Rhodobacter sphaeroides recombinant strains were grown on Huttner medium in the presence of tetracycline (1 μg/ml), kanamycin (5 μg/ml), and streptomycin (5 μg/ml) as described earlier [9]. The procedure of chromatophore preparation and RC purification from "no-antenna" strains of *Rba. sphaeroides* was described previously [9, 10] except that nonionic detergent Triton X-100 was used instead of zwitterionic detergent lauryldimethylamine N-oxide (LDAO) for RC purification by ion-exchange chromatography. Electrophoresis in polyacrylamide gel in the presence of SDS and urea was performed as described earlier [13]. Pigments were extracted from reaction centers by acetone–methanol mixture (7 : 2) according to a published method [14]. After pigment extraction the remaining RC protein was dissolved in 20 mM Tris-HCl buffer, pH 8.0, containing 0.05% Triton X-100, 80 mM NaCl, 5% SDS. Ground state absorption spectra of the RCs were measured at room temperature with a Shimadzu UV-1601PC spectrophotometer (Japan). Light-minus-dark difference spectra were obtained upon illumination of the sample with continuous light as described earlier [9].

RESULTS

Figure 1 presents absorption spectra of *Rba. sphaeroides* chromatophores containing wild type (Fig. 1a) or H(M182)L+I(L177)H mutant RCs (Fig. 1b) in the absence of antenna pigment–protein complexes. The absorption band at 865 nm is attributed to the primary donor P, the band at 804 nm is attributed to the monomer BChl absorbance, and the absorption band at 760 nm corresponds to BPhe.

The absorption spectrum of H(M182)L+I(L177)H chromatophores shows a blue shift of the P absorption band from 865 to 855 nm, which probably results from the overlap of this band with the absorption band of aggregated free BChls present in the "no-antenna" membranes (T. Fufina, unpublished results). In this spectrum the monomer BChl absorption is represented by two maxima at 804 and 817 nm. Increased absorbance at 817 nm may be due to higher transition probability to the upper excitonic level in the BChl dimer P. In the mutant spectra,

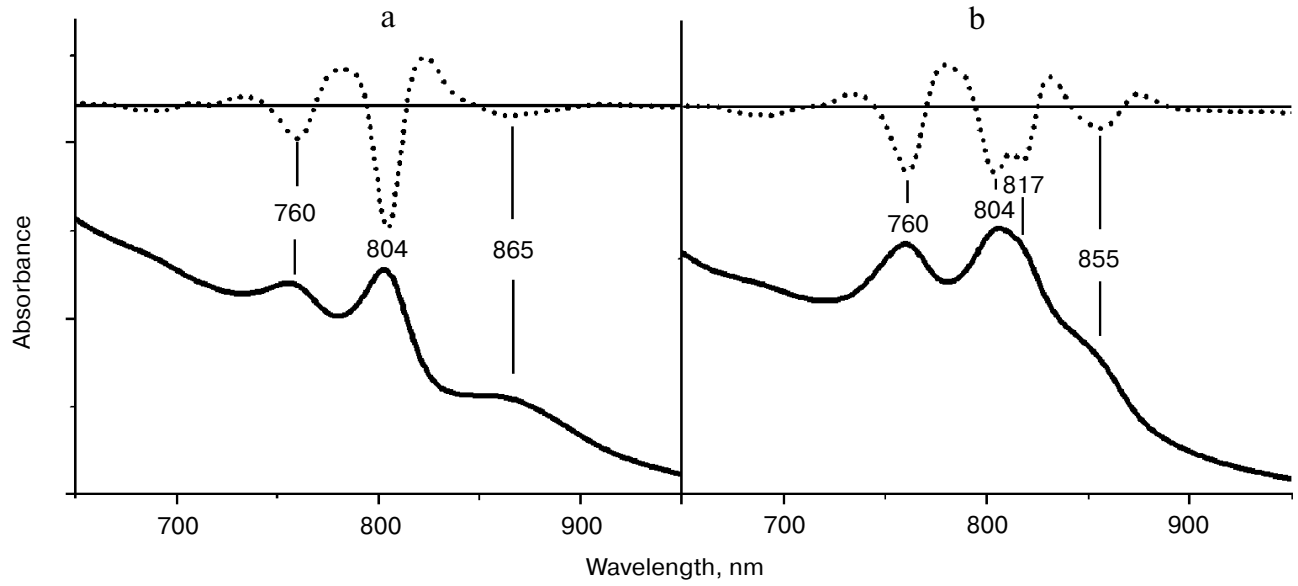

Fig. 1. Absorption spectra of chromatophores from *Rba. sphaeroides* recombinant strains containing wild type (a) or mutant reaction center H(M182)L+I(L177)H (b) and no light-harvesting antennas. Second derivatives of the spectra are shown as dashed lines.

neither change in the BPhe absorption band position nor appearance of a new BPhe absorption band was detected.

It was noted that during the procedure of isolation and purification the double mutant RCs were less stable compared to the wild type and I(L177)H RCs. Besides, it was shown that the quality of the final absorption spectrum of the purified double mutant RCs could depend on the detergent used for RC purification. For example, when detergent LDAO was in use, some part of the monomer BChl B_B in the double mutant RCs was transformed into BPhe, indicating weaker BChl B_B Mg atom coordination in comparison with the other BChls of this RC. Because of this, after RC isolation from the membranes the detergent LDAO was replaced by Triton X-100 (see "Materials and Methods").

In the long-wavelength part of the wild type RC absorption spectrum, Q_Y-bands of the primary electron donor P, monomer BChls, and BPhes were observed at 865, 805, and 759 nm, correspondingly (Fig. 2, spectrum *1*). This spectrum also shows an absorption band at 599 nm, which is attributed to the Q_X-transition of BChl molecules, an absorption band at 532 nm corresponding to Q_X-transition of BPhe molecules, and a carotenoid absorption band near 500 nm. In the spectrum of RC H(M182)L+I(L177)H the position of the long-wavelength band of the primary donor P at 865 nm is not changing, but the amplitude of this band is considerably decreased compared to that in the spectrum of the wild type RC (Fig. 2, spectrum *2*). Broadening of the monomer BChl absorption band accompanied by the decrease of its amplitude and by splitting of this band into two bands with maxima at 807 and 814 nm was also detected. In the short-wavelength part of the mutant spectrum the BChl Q_X-band was broadened and blue-shifted from 599 to 593 nm. Similar to the spectra of chromatophores, none of the new BPhe absorbance

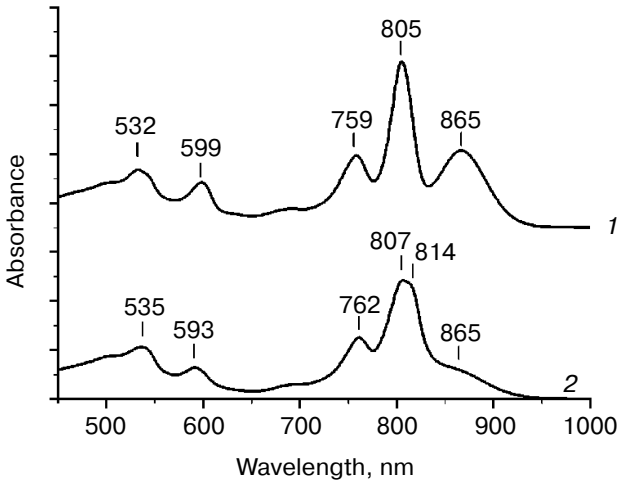

Fig. 2. Absorption spectra of wild type (*1*) and mutant I(L177)H+H(M182)L (*2*) reaction centers.

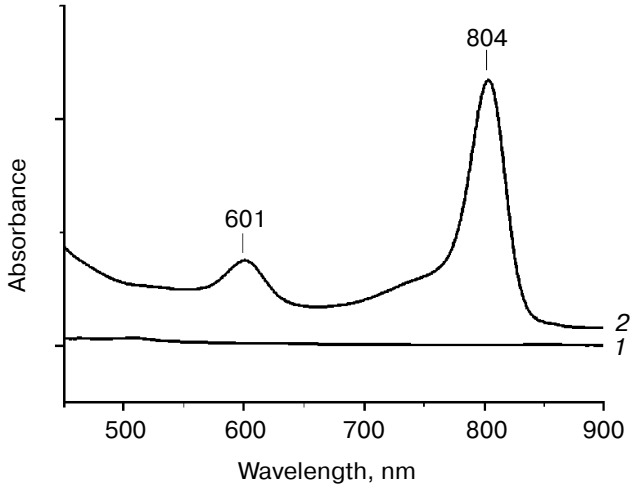

Fig. 3. Absorption spectra of wild type (*1*) and mutant I(L177)H+H(M182)L (*2*) reaction centers measured after pigment extraction.

bands were detected in the mutant RC spectrum though 3-nm long-wavelength shifts of BPhe Q_Y- and Q_X-transitions were observed.

In the difference (light-minus-dark) absorption spectra corresponding to $P^+Q_A^-$ state formation, the light-induced process of charge separation results in the bleaching of the long-wavelength P band, in the short-wavelength shift of the monomer BChl *a* band, and in the long-wavelength shift of the 760 nm band both in the wild type and the mutant RCs (data not shown). Thus, we conclude that under illumination the mutant RC is able to perform the primary electron transfer reactions leading to $P^+Q_A^-$ state formation.

Pigment analysis of the acetone–methanol (7 : 2) extracts of RCs revealed that the wild type RC contained four BChl molecules and two BPhe molecules, while the mutant RC H(M182)L+I(L177)H contained only three BChls and two BPhes. Missing of one BChl per RC in the acetone–methanol extract related to the strong attachment of a BChl to the protein was demonstrated earlier in RC mutant I(L177)H [9]. To explore the reason of the decreased BChl content in the pigment extract from the double mutant RC, the absorption spectra of RCs after pigment extraction were measured (Fig. 3). They showed that no absorption bands were present in the spectrum of the wild type RC, verifying that all pigments were extracted from the protein (Fig. 3, spectrum *1*). In the related spectrum of the double mutant RC two bands at 804 and 601 nm characteristic for protein-attached monomer BChl were detected (Fig. 3, spectrum *2*). By comparison of the Q_X BChl absorption bands in the spectrum of the mutant H(M182)L+I(L177)H RC before (Fig. 4, spectrum *1*) and after (Fig. 4, spectrum *2*) pigment extraction, it was estimated that one BChl molecule per mutant RC was strongly attached to the protein. Analogous data were

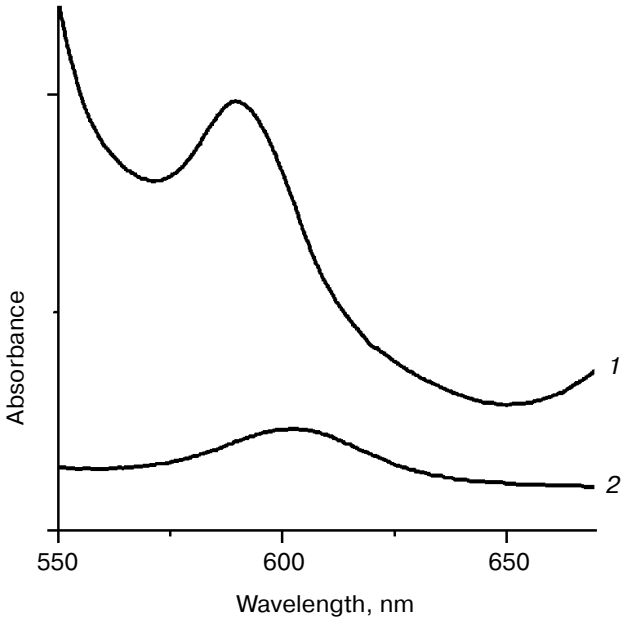

Fig. 4. Absorption spectra of reaction center I(L177)H+H(M182)L before (*1*) and after (*2*) pigment extraction.

previously obtained for RC mutant I(L177)H [9]. During electrophoresis of the double mutant RC in 18% polyacrylamide gel in the presence of SDS and urea, a green band of BChl that moved in the gel together with the L-polypeptide could be detected (data not shown) similar to what has been observed in the RC I(L177)H [9]. These results show that the covalent binding of the BChl P_A with L-subunit occurs both in RC I(L177)H and in RC H(M182)L+I(L177)H.

DISCUSSION

In this work the properties of the new mutant *Rba. sphaeroides* RCs with double amino acid substitution H(M182)L+I(L177)H are described. It is shown that the mutant RC is photochemically active, and that it is less stable during isolation and purification if compared to the wild type and I(L177)H RCs. Spectral and pigment analysis has revealed that in the H(M182)L+I(L177)H RC upon substitution of the histidine M182 by leucine the expected appearance of BPhe in the BChl B_B binding pocket is not observed, though the ligation of the BChl B_B Mg atom becomes weaker compared to that of the other RC BChls. It is known that leucine is not able to coordinate the BChl Mg atom. Having similar molecular volume with histidine, hydrophobic leucine does not permit another amino acid or a water molecule to approach the Mg atom of BChl B_B from the α-side. This is the reason of BChl/BPhe interchange in the B_B binding site of the RC with the single mutation H(M182)L [12]. However, in the presence of the additional mutation I(L177)H an alternative possibility of BChl B_B Mg atom coordination seems to arise. Previously we have shown that the amino acid substitution I(L177)H considerably affects properties of the *Rba. sphaeroides* RC. In particular, the dipole strength of the dimer BChl absorption band was dramatically decreased. In addition, this mutation resulted in the covalent binding of the BChl P_A with the L-subunit [9, 10]. CD spectra have revealed structural changes of the protein environment in the vicinity of P_A and B_B BChls [15]. According to the results obtained in this study, the serious influence of the I(L177)H mutation also remains in the double mutant RC H(M182)L+I(L177)H. Amino acid residue L177 is located between P_A and B_B molecules closer to the β-side of the BChl B_B plane (Fig. 5), which suggests protein structural changes in this particular site. So we suggest that coordination of the BChl B_B Mg atom might be realized not from the usual α-side but from the opposite β-side. Although *Rba. sphaeroides* RC structure shows that there are no appropriate amino acid residues for Mg coordination in the protein environment on the β-side of BChl B_B (Fig. 5) [16], it is known that such coordination could become possible after altering this environment. Morris and coauthors [17] showed the structure of RC F(L181)K where lysine L181 ligated the BChl B_B Mg atom from the β-side. Frolov and others [18] described another RC F(L181)R+H(M182)L structure, in which the fifth BChl B_B coordination was put into effect from the β-side through a water molecule.

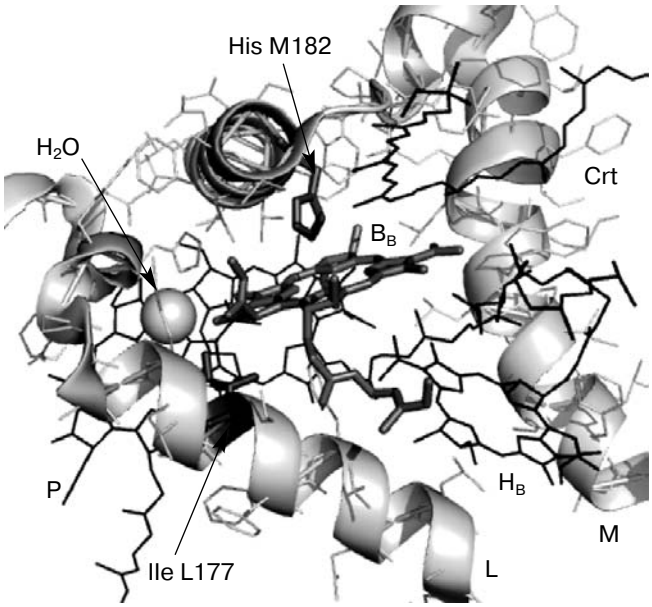

Fig. 5. Structure of *Rba. sphaeroides* RC (Protein Data Bank, 1M3X [16]). Bacteriochlorophyll B_B and dimer BChl P, carotenoid (Crt), bacteriopheophytin of B branch (H_B), histidine M182, isoleucine L177, and a water molecule as well as truncated polypeptides of L (L164-185) and M (M145-190) subunits are shown.

Some changes in absorption spectrum of RC H(M182)L+I(L177)H, namely a 6 nm short-wavelength shift of the BChl Q_X band and a 4 nm long-wavelength shift of the B_B Q_Y band support the proposal that the ligand of the BChl B_B Mg atom is situated on the β-side of the macrocycle. Short-wavelength shifts of Q_X bands (4-5 nm) in β-coordinated BChls were predicted by theoretical calculations [6], and according to Oba and Tamiaki [19] a long-wavelength shift of the Q_Y B_B band can be related to energy difference between the two ligation states since the ligation from the β-side is 4 kJ/mol less favored than ligation from the α-side. So far the origin of the axial ligand for BChl B_B in the double mutant RC is not clear. Figure 5 shows a protein pocket on the β-side of BChl B_B in *Rba. sphaeroides* RC structure. This pocket is spacious enough to harbor, for example, a water molecule, which is often found as a BChl Mg ligand in mutant RCs [2, 18]. In the wild type RC structure this pocket is empty since attaching of a water molecule inside of the hydrophobic protein demands additional hydrogen bonds with peptide surroundings [20]. For example, in the structure of mutant RC F(L181)R+H(M182)L there is a NH-group of arginine L181 in close vicinity of a water molecule on the β-side of BChl B_B, and the nitrogen atom of this group appears to form a hydrogen bond with the hydrogen atom of the water molecule, therefore stabilizing this β-coordination [18]. Previously we have suggested that as a result of the mutation I(L177)H the conservative water, which is located in the RC structure close to BChls P_A and B_B and which is important for RC complex stability may be shifted. Noticeable decrease of the RC I(L177)H stability supports for this suggestion [11]. So far we can only speculate about the direction of this shift and about a possibility for this water molecule to be a ligand for the BChl B_B Mg atom. Only detailed structure of the RC H(M182)L+I(L177)H close to the mutation site can provide reliable information. Nevertheless, the relative instability of the BChl B_B Mg atom coordination compared to the other BChls in the double mutant RC indicates that not an amino acid residue but a water molecule serves as a β-ligand in this case.

The results presented in this work help to explain the appearance of the 638 nm band in the absorption spectrum of the RC I(L177)H [10]. Apparently this band is attributed to BChl B_B Q_X-transition, and 40-nm long-wavelength shift of this band is evidence of hexacoordination of the BChl B_B Mg atom. Such shifts when coordination number of Mg atom is increased are observed both in solutions [21] and in photosynthetic complexes [18]. Thus, by means of RC with double amino acid substitution (M182)L+I(L177)H it was shown that the single mutation I(L177)H affected coordination number of the BChl B_B Mg atom.

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