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The site-directed mutation I(L177)H in *Rhodobacter sphaeroides* reaction center affects coordination of P_A and B_B bacteriochlorophylls ☆

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ABSTRACT

To explore the influence of the I(L177)H single mutation on the properties of the nearest bacteriochlorophylls (BChls), three reaction centers (RCs) bearing double mutations were constructed in the photosynthetic purple bacterium *Rhodobacter sphaeroides*, and their properties and pigment content were compared with those of the correspondent single mutant RCs. Each pair of the mutations comprised the amino acid substitution I(L177)H and another mutation altering histidine ligand of BChl P_A or BChl B_B. Contrary to expectations, the double mutation I(L177)H + H(L173)L does not bring about a heterodimer RC but causes a 46 nm blue shift of the long-wavelength P absorbance band. The histidine L177 or a water molecule were suggested as putative ligands for P_A in the RC I(L177)H + H(L173)L although this would imply a reorientation of the His backbone and additional rearrangements in the primary donor environment or even a repositioning of the BChl dimer. The crystal structure of the mutant I(L177)H reaction center determined to a resolution of 2.9 Å shows changes at the interface region between the BChl P_A and the monomeric BChl B_B. Spectral and pigment analysis provided evidence for β-coordination of the BChl B_B in the double mutant RC I(L177)H + H(M182)L and for its hexacoordination in the mutant reaction center I(L177)H. Computer modeling suggests involvement of two water molecules in the β-coordination of the BChl B_B. Possible structural consequences of the L177 mutation affecting the coordination of the two BChls P_A and B_B are discussed. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

1. Introduction

The early processes of photosynthesis are carried out by pigment-containing integral membrane protein complexes. The utilization of solar energy is most often associated with chlorophyll pigments that take part in the light-harvesting process and light energy conversion. The photosynthetic reaction center (RC) of the purple bacterium *Rhodobacter* (*Rba.*) *sphaeroides* consists of three protein subunits and 10 noncovalently bound cofactors that are organized in two membrane-spanning branches, A and B. Cofactors are represented by two bacteriochlorophylls (BChls) P_A and P_B combined into a special pair P that serves as a primary electron donor, two monomer BChls B_A and B_B, two bacteriopheophytins (BPhe) H_A and H_B, two quinones, a carotenoid and a non-heme iron atom [1]. X-ray crystal structures of bacterial RCs also show substantial amounts of bound water molecules, including those that are enclosed into hydrophobic core of the complexes (for review, see [2]). Some of the conservative water molecules not only contribute to stability of the protein structure but appear to be directly involved in the photosynthetic electron transfer [3,4].

The axial ligands, hydrogen bonds and nearby residues that constitute the BChls microenvironment can greatly affect their photophysical and redox properties and can be altered as a result of site-directed mutations. Fig. 1 shows four BChl cofactors in the *Rba. sphaeroides* RC with their central Mg atoms pentacoordinated. In addition to the four in-plane bonds with tetrapyrrol nitrogens there is a fifth coordination bond with histidine residue from the nearest protein side (Fig. 1). When either of these histidine ligands is substituted by an apolar amino acid residue, BPhe integrates into the same protein pocket instead of the native BChl [2]. Incorporation of a BPhe into the BChl dimer P brings about the heterodimer mutant with the value of E_m P/P^+ increased by approximately 130 mV due to the higher potential of BPhe [5]. In general, a histidine residue is the most frequently observed ligand donor, but other residues (for example, Met, Tyr, Asn/Gln and Asp/Glu in Photosystem (PS) I and Asn/Gln in PS II) as well as other groups and

Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; P, primary electron donor; P_A and P_B, BChls constituting P; B_A and B_B, monomer BChls; H_A and H_B, bacteriopheophytins; RC, reaction center; WT, wild type; LH, light-harvesting; LDAO, lauryldimethylamine oxide; PDB, protein data bank

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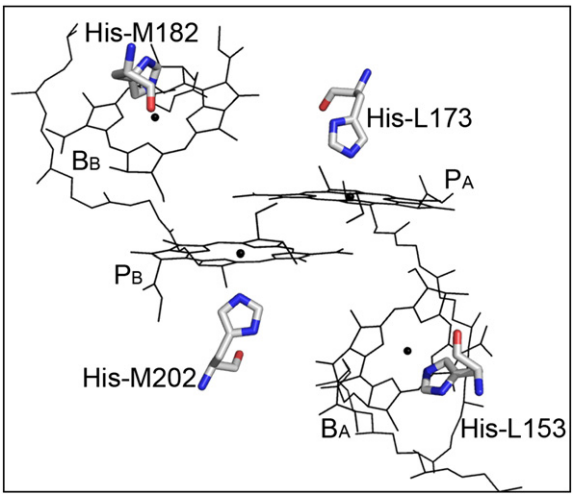

Fig. 1. Bacteriochlorophylls arrangement in *Rba. sphaeroides* RC structure. BChls are shown as lines and histidine ligands are shown as sticks.

water molecules are also able to play this role as can be seen in X-ray crystal structures of (B)Chl-containing complexes [6–8].

An axial ligand can either be located on the same side of the BChl plane as the 17-propionic acid phytol chain (β-side) or on the opposite (α-side) [9]. Energetically more favorable α-ligation of (B)Chls is generally more widespread in photosynthetic complexes compared to β-ligation [7,9,10]. As is obvious from the crystal structure of the light-harvesting (LH) complex-2 of *Rba. sphaeroides*, both BChls that make up BChl-B850 are ligated in β-position [11,12]. Fourteen Chls out of 96 in PS1 are β-coordinated, indicating a preference for the α-coordination [13]. The highest possible coordination number of the central Mg is six, but in most solvents and also in the majority of biological systems, it is pentacoordinated. Natural hexacoordination of (B)Chls in photosynthetic complexes appears to be very rare with only two reports on partial hexacoordination in the *Rhodospirillum rubrum* LH1 antenna complex [14] and on a BChl hexacoordination in the Fenna–Matthews–Olson (FMO) light-harvesting protein from *Prosthecochloris aestuarii* [15] being published. Besides, hexacoordination of the BChl B_B was described in *Rba. sphaeroides* and *Rba. capsulatus* mutant RCs with Phe-L181 substituted by Lys or Arg [16–18]. Both in solvents and in the protein complexes a change of the (B)Chl coordination state is usually accompanied by ~30–40 nm long-wavelength shift of the pigment Q_X absorbance band [19–21]. In all known native bacterial RCs the central Mg atoms of BChls are pentacoordinated with histidine axial ligands settled perpendicular to the α-side of the tetrapyrrole's plane [2] (Fig. 1).

In *Rba. sphaeroides* RC the Ile-L177 site is located in the immediate vicinity of the B_B and P_A BChls, and previous studies have shown that substitution of the Ile residue to Asp altered the spectral properties of the P dimer [22]. In a more recent report the mutation of this Ile to His was introduced that brought about considerable changes of the absorption spectrum of the RC I(L177)H [23]. The ratio of BChl:BPhe in the pigment extract from the mutant RCs was 3:2, indicating that one BChl might be missing. However, careful inspection of the RC spectrum after pigment extraction showed that one BChl remained in the protein sediment [24]. The SDS PAGE data and the results of sodium borohydride treatment allowed to conclude that in the mutant RC I(L177)H the BChl P_A was covalently attached to the L-subunit [24,25]. Remarkably stable magnesium coordination of the protein-bound BChl was noticed during SDS PAGE and under denaturing conditions [24]. So far the origin of the BChl-protein putative covalent bond existing in the RC I(L177)H has not been established. The I(L177)H mutation in the vicinity of the B_B and P_A BChls affects absorption properties of both chromophores though a specific influence on transitions of individual BChl is difficult to differentiate because of the overlapping of Q_Y B band with high-energy and low-energy Q_Y transitions belonging to the special pair P.

To further explore the interaction of His-L177 with the nearest BChls, in the present work RC double mutants were constructed and compared to the wild type RC and to the single mutant RCs that were previously described. Each pair of the mutations comprised amino acid substitution I(L177)H and H(L173)L/T or H(M182)L mutations that altered His ligands of P_A or B_B BChls, respectively, in order to substitute these BChls for BPhes. In addition to conducting pigment analysis, redox titrations and spectral measurements, we have also determined the X-ray crystal structure of the I(L177)H mutant RC at a resolution of 2.9 Å and that of the wild type (WT) RC at a resolution of 2.8 Å. Comparison of these crystal structures give information on how protein-cofactor interaction may affect the spectral properties of BChls and assembly of the RC complex.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutations were generated using PCR oligonucleotides as described previously [23]. Nucleotide changes were confirmed by DNA sequencing. Altered *puf*L or *puf*M genes were shuttled into the broad-host range vector, derivative of pRK415, containing a 4.2 kb EcoRI–HindIII restriction fragment that included the *puf*LMX genes [23]. The resulting plasmids were inserted into the *Rba. sphaeroides* strain DD13 through conjugative crossing to give transconjugant strains with RC-only phenotypes [26]. Control strain comprised DD13 complemented with pRK415- derivative plasmid containing a wild type copy of *puf*LMX genes.

2.2. Bacterial growth and reaction center purification

Details of the growth of the mutant bacterial strains under dark semi-aerobic conditions are given elsewhere [23]. Cells were harvested and then broken by ultrasonication, and membranes for reaction center purification were pelleted by ultracentrifugation [23,24]. Reaction centers were solubilized from the membranes using lauryldimethylamine oxide (LDAO) and then were purified by passage through a DE52 anion exchange column, followed by passage through Fractogel EMD DEAE (S) columns (Merck), as described in detail elsewhere [24]. For unstable mutant reaction centers purification, Triton X-100 was used instead of LDAO. The purity of the reaction center at each step was estimated by absorbance spectroscopy measuring the ratio of protein absorbance at 280 nm to bacteriochlorophyll absorbance at 804 nm (A_{280}/A_{804}) [27]. When the value of A_{280}/A_{804} falls below 1.4, the reaction center is sufficiently pure for crystallization.

2.3. Steady-state absorption spectroscopy

Optical absorption spectra of the isolated reaction centers were measured using a Shimadzu UV-1601PC spectrophotometer (Japan). Measurements of the spectra at 90 K were performed using a nitrogen-cooled cryostat of local design. Sodium ascorbate was added at 1 mM final concentration in order to keep the primary donor in reduced state.

2.4. Pigment extraction and analysis

Prior to pigment extraction purified reaction centers were precipitated by addition of 26% of ammonium sulfate, then the supernatant was removed and pigments were extracted by acetone–methanol mixture (7:2). The relative amount of BChl *a* compared with BPhe *a* was determined using published extinction coefficients for BChl and BPhe at 771 and 747 nm [28]. After pigment extraction the remaining RC protein was collected by centrifugation (3 min, 10,000 g) and dissolved in 20 mM Tris–HCl pH 8.0/0.5% Triton X-100/80 mM NaCl/5% SDS, and

room temperature absorption spectra of the protein were measured. Amount of BChl covalently attached to RC protein was determined by comparison of the Q_X BChl absorption bands in the RC absorption spectrum before and after pigment extraction [24].

2.5. Oxidation–reduction titrations

The P/P^+ midpoint potentials of isolated reaction centers were determined by chemical titrations using potassium ferricyanide and sodium ascorbate in 100 mM Tris–HCl, pH 8.0, 0.1% LDAO, and 1 mM EDTA as described in details previously [22]. Pt electrode and Ag–chloride reference electrode, attached to ionalyzer, were submerged into the sample, and the ambient potential was monitored. Prior to starting the titration, the electrode was calibrated using a fresh equimolar (10 mM) solution of potassium ferri- and ferrocyanide [29]. Titrants were added directly to the sample. The redox state of P was monitored by absorbance at 865 nm (wild type RC), 847 nm (RC I(L177)H) or 820 nm (RC I(L177)H + H(L173)L). The data were fitted to the Nernst equation ($n = 1$), and the E_m P/P^+ values were estimated [22].

2.6. Reaction center crystallization and data analysis

Trigonal crystals of the I(L177)H reaction center, space group $P3_121$, were grown using hanging drop vapor diffusion method as described previously [30,31]. Droppings containing 10 mg/ml of the mutant reaction center, 3.5% 1,2,3-heptanetriol, 2% dioxane, 0.1% LDAO and 1 M potassium phosphate, pH 7.4, were equilibrated against a reservoir solution of 1.5 M potassium phosphate, pH 7.4. Crystals appeared within 3–4 weeks and grew as prisms of variable size. X-ray diffraction data were collected using cryo-cooled crystals and an MAR CCD 165 mm detector, on beam-line X13 of the DESY, Germany. Crystals were prepared for cryo-cooling by sequential soaking in mother liquor containing increasing concentrations of ethylene glycol to give a final concentration of 25%. The crystal used for data collection diffracted to a higher resolution limit of 2.9 Å, and diffraction data were processed and scaled using XDS [32]. Diffraction data from crystal of *Rba. sphaeroides* wild type RC were collected to a resolution of 2.8 Å from one crystal on a laboratory X-ray apparatus consisting of a generator with a Proteum X8 rotating anode equipped with a Montel 200 focusing system (Bruker). Molecular replacement was performed using Phaser [33] using the coordinates of the F(M197)R mutant RC from *Rhodobacter sphaeroides* (PDB ID 1E6D) [34] as the search model. Data collection and refinement statistics are given in Table 1. Rigid body refinement was carried out before partial refinement using restrained maximum likelihood refinement in REFMAC 5.0 [35]. Figures were prepared using the program PyMol [36].

3. Results

3.1. Reaction centers purification and stability

Reaction centers with single mutations I(L177)H, H(L173)L and H(M182)L and double mutations I(L177)H + H(L173)L, I(L177)H + H(L173)T and I(L177)H + H(M182)L were constructed as described in "Materials and Methods" section and expressed in a background with no light-harvesting antenna complexes. The RCs with the single mutations H(L173)L and H(M182)L were stable with the high yield of RCs after purification. The yield of I(L177)H RC after purification was similar to that of the WT RC from the same amount of the cell mass, but about half of its population was represented by RCs with P absorbance band at ~850 nm, and another half showed no P band in the absorption spectrum indicating decreased stability of the mutant RC. The yield of the double mutant RC I(L177)H + H(L173)L after purification was ~60–70% compared to that of the WT RC. There was a significant decrease in stability of the double mutant RCs RC I(L177)H + H(M182)L and I(L177)H + H(L173)T detected during their isolation from the membranes, further purification and storage, and the yield of these purified RCs was ~10% of that in the WT RC. The most unstable RCs I(L177)H + H(L173)T could not survive freezing and thawing, so that all measurements had to be completed at the day of the RC purification.

Table 1
Data-processing and refinement statistics.

	Wild type RC	I(L177)H RC
Collection statistics		
Space group	$P3_121$	$P3_121$
Cell dimensions	$a = 139.75$, $b = 139.75$, $c = 185.60$, $\alpha = \beta = 90.00$, $\gamma = 120$	$a = 140.13$, $b = 140.13$, $c = 186.03$, $\alpha = \beta = 90.00$, $\gamma = 120$
Wavelength (Å)	1.54179	0.8123
Resolution range (Å)	26.6–2.8(2.9–2.8) [b]	28.9–2.9 (3.0–2.9)[b]
No. of observations	195,707 (16,119)	210,898 (18,579)
No. of unique reflections	51,787 (5,117)	45,707 (4,081)
Completeness (%)	99.2 (99.5)[b]	96.5 (90.2)[b]
R_{merge} (%)[a]	18.5 (62.6)[b]	9.2 (66.4)[b]
Redundancy	3.75 (3.91)[b]	4.61 (4.55)[b]
Average $I/\delta(I)$	6.11 (1.70)[b]	14.25 (2.30)[b]
Mosaicity (°)	0.27	0.14
Refinement and final model statistics		
Resolution range (Å)	19.9–2.80 (2.85–2.80)	28.9–2.90 (3.0–2.90)
Molecules/asymmetric unit	1	1
No. of reflections	51,655 (2,671) [b]	45,707 (2,440)[b]
R_{work}[c] (%)	24.40 (31.6) [b]	20.39 (35.5)[b]
R_{free}[d] (%)	28.40 (37.2) [b]	25.06 (41.8)[b]
Bond lengths (Å)	0.010	0.011
Bond angles (Å)	1.742	1.720

[a] $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$.
[b] In high-resolution shell.
[c] $R_{cryst} t = \sum_{hkl} ||Fo| - |Fc|| / \sum |Fo|$, where Fo is the observed structure-factor amplitude and Fc is the calculated structure factor amplitude.
[d] R_{free} is the same but calculated based on 5% of reflection not used in the refinement.

3.2. Absorbance spectroscopy

In the absorption spectrum of the WT RC measured at room temperature, the band at 865 nm is attributable to the low-energy exciton component of the Q_Y transition of the P BChls (Q_Y P band), the asymmetric band at 804 nm is attributable to the Q_Y transitions of the monomeric BChls together with the high-energy exciton component of the Q_Y transition of P (Q_Y B band), and the band at 759 nm is attributable to the Q_Y transition of the two reaction center BPhes (Q_Y H band) [37]. In the Q_X region, the BChls and BPhes of the WT RC gave rise to asymmetric absorbance bands with main peaks at 599 and 533 nm, respectively. It has been reported previously that through substitution of His-L173 with Leu the native axial ligand to BChl P_A can be removed resulting in formation of a heterodimer BChl:BPhe primary electron donor [38,39]. The spectrum of H(L173)L RC showed a diminished amplitude of the Q_Y P band near 865 nm and decreased absorption in the Q_X region of the BChls near 600 nm (Fig. 2). In a similar way the appearance of BPhe in the B_B binding pocket was previously achieved by mutation of His-M182 to Leu [40]. In the absorption spectrum of the H(M182)L RC a new band at 792 nm appeared correspondent to a new BPhe at the B_B position (Fig. 2). In the Q_X region the maximum of the 533 nm band was red-shifted to 536 nm in agreement with spectral changes described for this mutant previously [40].

The absorption spectrum of WT RC measured at 90 K displayed better resolution than that measured at room temperature and showed Q_Y P band at 888 nm, Q_Y B band at 804 nm and H Q_Y band at 760 nm (Fig. 3). The main peak at 804 nm is assigned to the Q_Y transition of the B_A BChl, and a shoulder on the red side at approximately 814 nm is assigned to the Q_Y transition of the B_B BChl [37]. The Q_X region of the spectrum had the main peak of BChl Q_X band at 597 nm with the pronounced shoulder on the red side of this band and two peaks correspondent to BPhe Q_X bands at 532 (H_B)

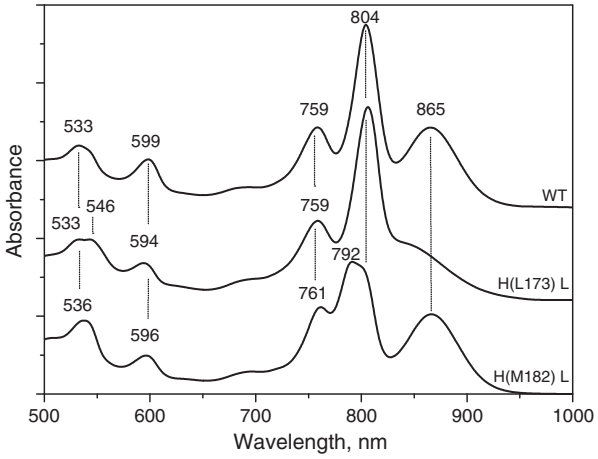

Fig. 2. Room temperature absorption spectra of the wild type reaction center and mutant reaction centers H(L173)L and H(M182)L.

and 544 nm (H_A) (Fig. 3). The absorbance band at 500 nm is attributed to carotenoid absorption.

The main changes found in the spectrum of the RC I(L177)H (Fig. 3) belonged to BChl absorption consistent with previous reports of absorbance spectra at room temperature and 10 K [23,24]. The Q_Y P band was 18 nm blue shifted and the relative intensity of this band was notably decreased. The amplitude of the Q_Y B band was slightly decreased accompanied by some broadening of this band (Fig. 3). The main BChl Q_X absorbance band at 596 nm also became more symmetric with no shoulder resolved on its red side (Fig. 4). Besides, in the Q_X region BChls gave rise to the new broad band at 638 nm, which is resolved in the mutant RC spectrum both at 90 K (Figs. 3 and 4) and 10 K [24]. According to a number of reports such a pronounced red shift of the BChl Q_X absorbance band can indicate that one or more BChls became hexacoordinated [19–21].

Therefore, one of the effects of the I(L177)H mutation was a change of the coordination state of either B_B or P_A BChls located in close vicinity of the His-L177. In order to find out which BChl became hexacoordinated two double mutant RCs were constructed where the I(L177)H mutation was combined with removal of the native axial

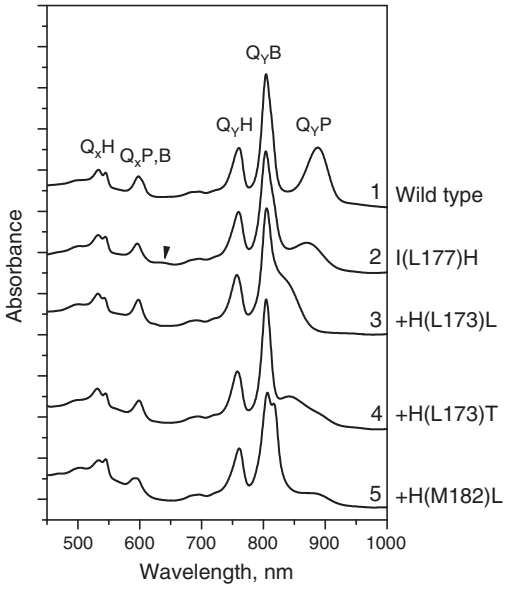

Fig. 3. Absorption spectra of the reaction centers measured at 90 K: 1, wild type RC; 2, RC I(L177)H; 3, RC I(L177)H + H(L173)L; 4, RC I(L177)H + H(L173)T; 5, RC I(L177)H + H(M182)L.

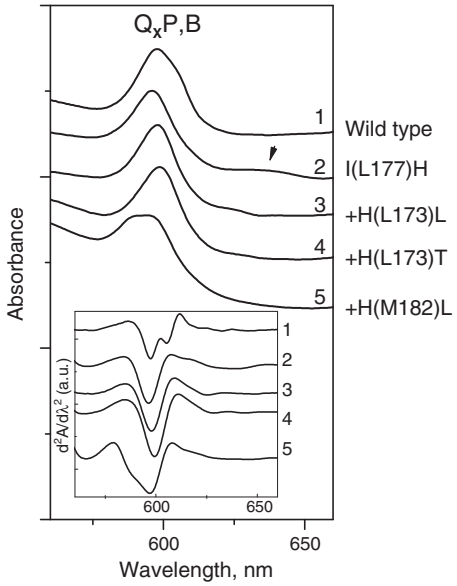

Fig. 4. Q_X region of the absorption spectra from Fig. 3 with second derivative of the spectra shown in the insert. Notations are the same as in Fig. 3.

ligands of B_B or P_A BChls through substitution of His-M182 or His-L173 with Leu. Contrary to expectations the absorption spectra of neither RC I(L177)H + H(M182)L nor RC I(L177)H + H(L173)L displayed changes that could point toward exchange of correspondent BChl for BPhe as it was observed in absorption spectra of RCs with single mutations H(M182)L and H(L173)L. In the absorption spectrum of the RCs I(L177)H + H(M182)L the Q_Y P band peaked at the same position 888 nm as in the WT spectrum, but the dipole strength of this band was even more decreased than in the spectrum of RC I(L177)H (Fig. 3). Broadening of the Q_Y B band was accompanied by splitting of this band in two bands with peaks at 805 nm and 819 nm. The BChl Q_X band with the main peak at 598 nm was also broadened, and the shoulder appeared on the blue side of the band at ~590 nm (Fig. 4). The absorbance band at 638 nm observed in I(L177)H spectrum was absent. Comparison of the RCs I(L177)H + H(M182)L spectrum with those of the single mutants I(L177)H and H(M182)L revealed no signs of a hexacoordinated BChl or an additional BPhe in the double mutant RC. Still, the presence of the I(L177)H mutation was marked as 2 nm red shift of the Q_Y B_B band and 8 nm blue shift of the Q_X B_B band, as was determined by the second derivative of the spectrum (Fig. 4).

In the absorption spectrum of the RC with double mutation I(L177)H + H(L173)L the Q_Y P band was clearly resolved demonstrating 46 nm blue shift to 842 nm with the amplitude of this band similar to that observed in the WT RC spectrum. The position of the Q_Y B band at 804 nm was not changed. BChl Q_X region was represented by one band at 596 nm with its shape similar to that in the I(L177)H RC spectrum, while the absorbance band at 638 nm was absent (Fig. 4). No spectral signatures of a new BPhe were observed when compared with the spectrum of the RC H(L173)L (Fig. 2). One more double mutant RC I(L177)H + H(L173)T was obtained where I(L177)H substitution was combined with exchanging of P_A His ligand to Thr. If compared to the double mutant I(L177)H + H(L173)L the main difference in the absorption spectrum of I(L177)H + H(L173)T RC was associated with the Q_Y P band, which was much broadened and showed two peaks near 895 nm and 843 nm (Fig. 3). Symmetric BChl Q_X absorbance band peaked at 598 nm and was similar to that in the spectra of I(L177)H and I(L177)H + H(L173)L RC (Fig. 4). Thus, the absorption spectra of the two double mutants I(L177)H + H(L173)L and I(L177)H + H(L173)T demonstrated certain similarities except the former was more stable than the latter.

3.3. Pigment analysis and redox potentiometry

In all mutant and WT RCs the bacteriochlorin cofactor composition was determined by performing pigment analysis of acetone/methanol (7:2) extract as well as of the remaining RC protein (Table 2). In the WT RC, I(L177)H + H(L173)L RC and I(L177)H + H(L173)T RC the measured BChl/BPhe ratio was ~2.0 consistent with four BChls and two BPhes in each RC. In line with the spectral data these results confirm that in the I(L177)H + H(L173)L RC and I(L177)H + H(L173)T RC the primary electron donor is a BChl homodimer. In the pigment extract from the mutant RCs I(L177)H and I(L177)H + H(M182)L the BChl/BPhe ratios of 1.55 ± 0.05 and 1.5 ± 0.1, respectively, indicated the presence of three BChls and two BPhes in each RC extract (Table 2). After the extraction procedure was completed, the residual RC protein was dissolved in 20 mM Tris–HCl pH 8.0/0.5% Triton X-100/80 mM NaCl/5% SDS buffer and the absorption spectrum was measured. It was shown that all RC protein sediments except I(L177)H and I(L177)H + H(M182)L were free of pigments. The absorption spectra of the residual RC protein from the latter two mutants retained absorbance bands near 601 nm and 804 nm assigned to the BChl bound to the protein. The amount of this protein-bound BChl, which was found to be 1 BChl per each of these two mutant RCs, was determined by comparison of the Q_X BChl absorption bands in the RC spectrum before and after pigment extraction (see Methods) (Table 2). All features of the BChl bound to the protein, namely positions of the Q_X and Q_Y bands in the absorption spectra and increased stability of the Mg coordination during SDS PAGE electrophoresis, were similar for the I(L177)H RC and the double mutant RC I(L177)H + H(M182)L indicating that the binding of BChl P_A to the L-subunit that was previously assumed for the I(L177)H RC [24] was also preserved in the RC I(L177)H + H(M182)L.

The P/P^+ midpoint potentials were determined for the WT RC and two mutants from consequent oxidative and reductive titrations using the one-electron Nernst equation (Table 2). The value of + 500 mV for the WT RC was in agreement with previous measurements [22]. For the RC I(L177)H the titration data yielded E_m P/P^+ value of + 450 mV. Within the estimated error of ± 5 mV, the P/P^+ midpoint potential of the double mutant RC I(L177)H + H(L173)L was similar to that of the WT RC (Table 2). Similar titrations for the RCs I(L177)H + H(M182)L and I(L177)H + H(L173)T were not possible because of their instability. The E_m value of + 676 mV for the H(L173)L heterodimer has been reported previously [39].

3.4. Crystallization and X-ray crystallography

To investigate the structural consequences of the I(L177)H mutation, the X-ray crystal structure of the RC was determined to a resolution of 2.9 Å (PDB ID: 3V3Z). After refinement, the structural model of the I(L177)H reaction center was compared with the structure of the WT RC, which was also resolved in this work to a resolution of 2.8 Å (PDB ID: 3V3Y). There was a good structural conservation noted in the main body of the protein as well as in cofactor system. Changes in the structure were confined to the side chain of the L177 residue and to the BChl cofactors in the vicinity of this residue. Besides, Thr residue was found in the L178 site (Fig. 5) consistent with the L-subunit amino acid sequence obtained for the RC from *Rba. sphaeroides* strain RV, which was used to design the plasmid for complementation of the *puf*-deficient strain DD13 [23,26]. In known structures of *Rba. sphaeroides* RC [30,31,41], the L178 site is occupied by Ser; however, Thr-L178 is present in many RCs from other related photosynthetic bacteria [42] and is not expected to cause any changes in the microenvironment of the nearest chromophores.

The orientation of the introduced His-L177 was similar to that of the native Ile with the imidazole cycle of the His residue being very close, ~1.9 Å (with the estimated maximal coordinate error of 0.27 Å calculated for this structure by SFCHECK [43]) to the methyl carbon of the ring I of the P_A BChl and directed towards BChl B_B macrocycle (Fig. 5). The torsion angle between C2-acetyl group of BChl P_A and the macrocycle plane was estimated as 4° as compared to 28° in the WT RC structure. Superimposition of the Ile and His shows that the imidazole cycle of the His-L177 in the mutant RC follows orientation of the CG2 methyl group of the Ile-L177 in the WT RC (Fig. 6). In the structure of the I(L177)H mutant RC, 50 fixed water molecules could be traced, with one particular conserved water present in the immediate vicinity of P_A and B_B BChls, which is known in the literature as "water B" [2] (not shown). Fig. 7a shows electron density maps for the region near the His-L177 and the BChl B_B. With the modeled position of His-L177 oriented as shown on the figure the closest approach to the Mg of B_B would be about 6.23 Å, which is too distant for magnesium coordination. The difference electron density map also shows low sigma peaks adjacent to the Mg of B_B suggesting the potential presence of a water molecule. After examining various possibilities, including different conformations of the His imidazole cycle, the best fit of structure to density was achieved by including two water molecules between ND1 nitrogen of His-L177 and the Mg of BChl B_B. One water molecule could be placed within the difference map peak (Fig. 7b, shown in red) and another water molecule (shown in magenta) is modeled between the ND1 nitrogen of His-L177 and the Mg atom with all center-to-center distances of 2.6 Å (Fig. 7b). Fig. 8 shows computer models for the double mutant RCs I(L177)H + H(L173)L and I(L177)H + H(L173)T designed on the basis of the I(L177)H RC structure (Fig. 8a). Together with the spectral data, results of the pigment analysis and redox titration the model suggests that in the structure of these double mutant RCs the His-L177 backbone undergoes reorientation and points towards the central Mg of the P_A macrocycle (Fig. 8b and c). In the model shown in Fig. 8b center-to-center distance between the Mg and the NE2 atom of His-L177 is ~3.42 Å, which is longer than the distances typical for the BChl or Chl axial ligands (2.4–2.7 Å). This implies either repositioning of the P_A macrocycle or possible involvement of a water molecule in the BChl P_A ligation. Fig. 8c shows that the modeled orientation of the hydroxyl group of the Thr-L173 is hardly suitable for ligation with the distance

Table 2
Pigment analysis and E_m P/P^+ of the wild type and mutant reaction centers.

RC	BChl/BPhe ratio [a]	BChl bound to RC protein [b]	E_m P/P^+, mV [c]	References
WT	2.00 ± 0.05	non	+ 500 ± 10	[22], this work
I(L177)H	1.55 ± 0.05	1 BChl/RC	+ 450 ± 10	[24], this work
H(L173)L	0.9	n.d.	+ 676	[39,51]
I(L177)H + H(L173)L	1.95 ± 0.05	non	+ 485 ± 15	this work
I(L177)H + H(L173)T	2.0 ± 0.1	non	n.d.	this work
I(L177)H + H(M182)L	1.5 ± 0.1	1 BChl/RC	n.d.	this work

[a] Measured in acetone–methanol extract.
[b] Determined by comparison of the Q_X BChl absorption bands in the RC spectrum before and after pigment extraction [24].
[c] Following absorption bands attributed to BChl dimer were used to determine the values of E_m P/P^+ − 865 nm (wild type RC), 847 nm (RC I(L177)H), 820 nm (RC I(L177)H + H(L173)L).

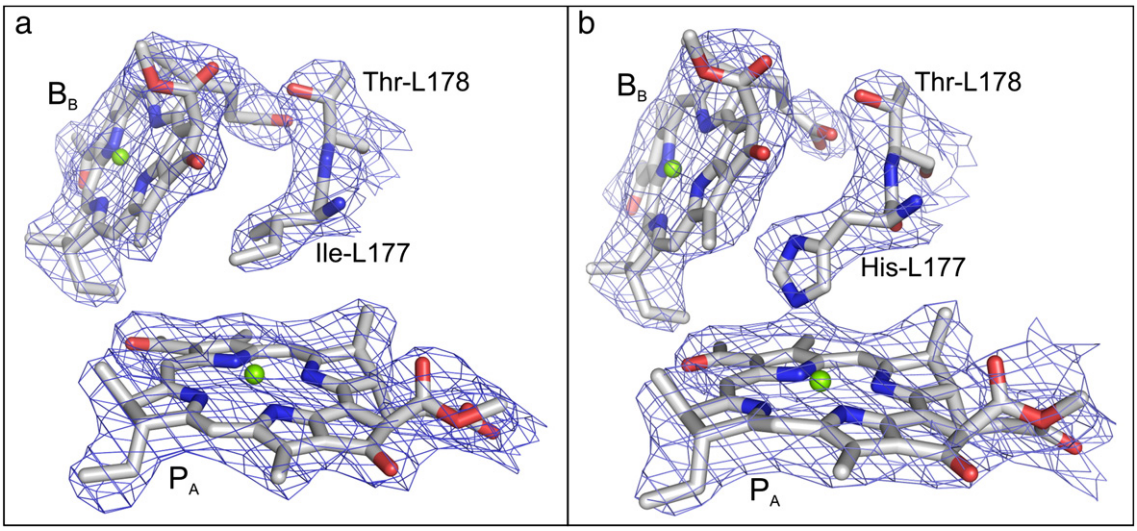

Fig. 5. Electron density maps for wild type RC (a) and mutant RC I(L177)H (b) attributable to the L177 and L178 residues as well as to the P_A and B_B BChls, and the fits of the structural models to the density. Phytol tails of BChls are omitted for clarity.

between this group and Mg (~5.74 Å) being too long for coordination.

4. Discussion

4.1. Effect of the I(L177)H mutation on the optical properties and coordination state of BChl B_B

According to the data presented in the Results section the new absorbance band at 638 nm that indicates a change of the coordination state of either B_B or P_A BChls has appeared in the RC I(L177)H spectrum and the exact assignment of this band could not be straightforwardly interpreted. To resolve this issue the I(L177)H mutation was combined with the substitution of His-M182 residue to Leu in order to change the BChl B_B for a BPhe. The results of absorption spectroscopy and pigment analysis revealed that in the double mutant RC I(L177)H + H(M182)L the B_B binding site is still occupied by a BChl molecule, suggesting that the I(L177)H substitution induced some structural perturbations that provided an alternative for BChl B_B coordination. Previously the data of circular dichroism (CD) spectroscopy have revealed structural changes of the protein environment in the vicinity of B_B and P_A BChls, where the site L177 is located [44]. It was also noticed that in the double mutant RC the coordination of the BChl B_B magnesium atom became more labile compared to that of other RC BChls [45]. Besides, the absorbance band at 638 nm, which is observed in the I(L177)H RC spectrum and is usually assigned to a hexacoordinated BChl, disappears in the spectrum of the double mutant RC. Taken together these observations gave us an idea about potential β-coordination of BChl B_B in the RC I(L177)H + H(M182)L. In line with this suggestion there was a 4 nm red shift of the Q_Y B_B band observed in the absorption spectrum of the RC I(L177)H + H(M182)L at 90 K if compared with the WT RC, which could be interpreted as a small decrease in the energy of the Q_Y B_B transition and may be associated with less energetically favorable β-coordination of this molecule [46]. We are aware that the different influence of mutations on the BChl monomer transitions cannot be always unambiguously interpreted since they also can affect Q_Y P bands. However, CD spectra show that the mutation I(L177)H results in a decrease of both Q_Y P high- and low-energy transitions suggesting that the overlapping of these bands with the BChl Q_Y B band in the RC I(L177)H spectrum is diminished [44]. The dipole strength of the Q_Y P band in the spectrum of the double mutant RC is significantly decreased similar to that in the spectrum of the I(L177)H RC allowing to expect that the absorbance band at 819 nm can be associated mainly with BChl B_B Q_Y transition. The study by Balaban and colleagues predicted that (B)Chls β-ligation can also affect the energy of the Q_X transition [10], and the 8 nm blue shift of the Q_X B_B band observed in the spectrum of the double mutant RC is consistent with this prediction. The appearance of the blue shifted BChl Q_X bands in the spectra of the mutant *Rba. sphaeroides* RCs with various substitutions of His-L153, the axial ligand for BChl B_A, was reported previously [47,48]. However, BChl Q_X transition is also known to be responsive to structural changes other than magnesium coordination as it was shown for RCs with various mutations at M203 site in *Rba. sphaeroides* RC [4], as well as at M200 and M208 sites in *Rba. capsulatus* RC [16,38]. In the absorption spectra of these mutant RCs a 7–17 nm splitting of the BChl Q_X band into two separate bands was detected, although the mutations did not affect the BChls coordination. The splitting of the Q_X band was observed even when the sites of mutations were near quinones, quite distant from the BChls [16].

DiMagno and co-workers reported that in *Rba. capsulatus* RC the mutation of Phe-L181 to Lys resulted in the appearance of the 634 nm absorbance band in the mutant RC spectrum consistent with hexacoordination of the B_B BChl [16]. In the mutant RC from *Rba. sphaeroides* similar effect of the same mutation was supported by X-ray crystal diffraction data and direct β-coordinating bond between the Lys side chain and the Mg atom was suggested [17]. In more recent study of Frolov and colleagues [18], it has been

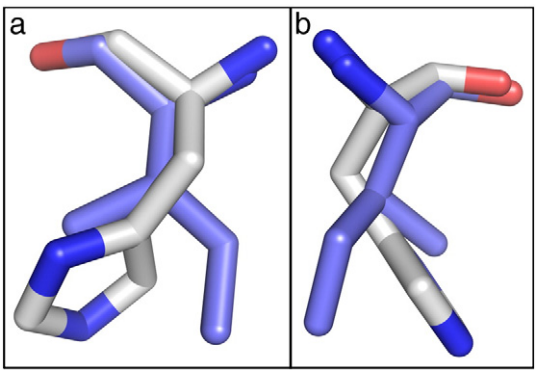

Fig. 6. Superimposition of the Ile-L177 in the wild type reaction center and His-L177 in the mutant I(L177)H reaction center (a) and view of the same pair along the plane of the His ring (b).

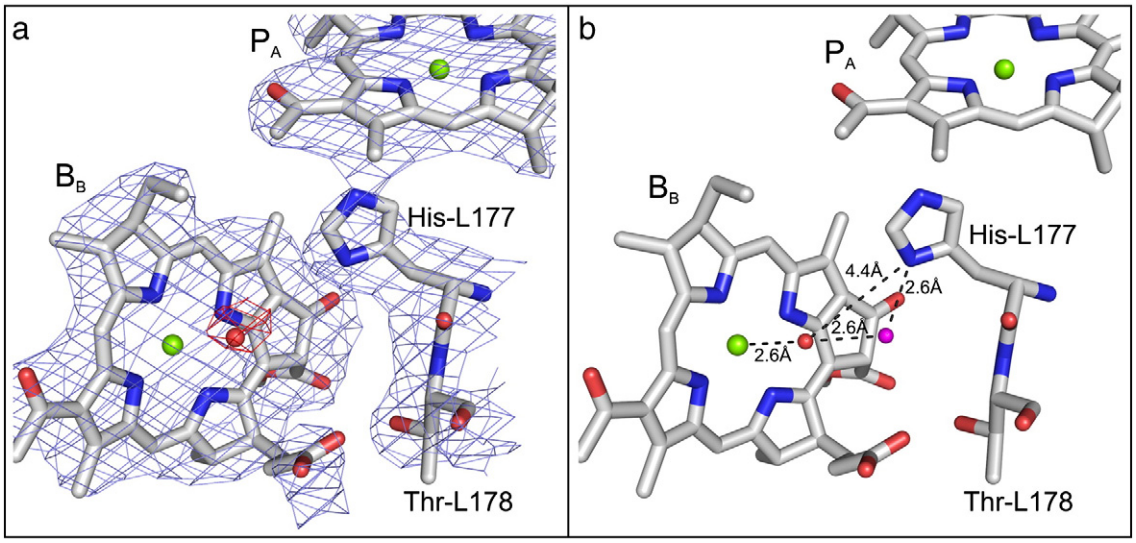

Fig. 7. Electron density map for the region on the β-side of B_B BChl of the I(L177)H RC (a) and the structural model for β-ligation with two waters (shown as red and magenta balls) introduced between Mg and ND1 nitrogen of the His L177 (b).

demonstrated that the mutation of residue Phe-L181 to Arg in *Rba. sphaeroides* RC resulted in hexacoordination of the B_B BChl accompanied by the appearance of the 632 nm band in 77 K absorption spectrum. Introduction of the second mutation H(M182)L produced the double mutant RC F(L181)R + H(M182)L that had pentacoordinated BChl B_B with the axial ligand positioned from the β-side of the macrocycle. In the model proposed on the basis of the X-ray crystal structure of the mutant F(L181)R RC, water molecule adjusted to the Arg-L181 was shown to be the fifth ligand, and solid arguments were listed in support of this model [18]. It should be mentioned that absorption spectrum of the F(L181)K RC exhibited a 10-nm splitting of BChl Q_X band and the blue shifts of both monomers Q_Y bands [16,17], while no obvious signs of Q_X or Q_Y band splitting were noticed in the absorption spectra of both the single mutant RC F(L181)R and the double mutant RC F(L181)R + H(M182)L [18]. Thus, the only reliable evidence of the altered coordination state of BChl appears to be the ~30–40 nm red shift of the Q_X absorbance band, which was observed in the spectra of all complexes containing hexacoordinated (B)Chls with one exception of hexacoordinated BChl in the FMO light-harvesting protein from *Prosthecochloris aestuarii* [15], as was discussed in details elsewhere [18]. Taking into account the following observations, the appearance of the 638 nm absorbance band in the I(L177)H RC spectrum, its disappearance in the spectrum of RC I(L177)H + H(M182)L and the integrity of the BChl B_B ligation in the double mutant RC, we conclude that in the single mutant I(L177)H RC the BChl B_B is hexacoordinated. It is worth to note that comparing to the absorbance bands at 632–634 nm observed in the spectra of the F(L181)R and F(L181)K mutant RCs the band at 638 nm in the I(L177)H RC spectrum was wider and more red-shifted. A possible explanation may be related to a specific structural arrangement of the hexacoordination in the RC I(L177)H, which will be discussed below.

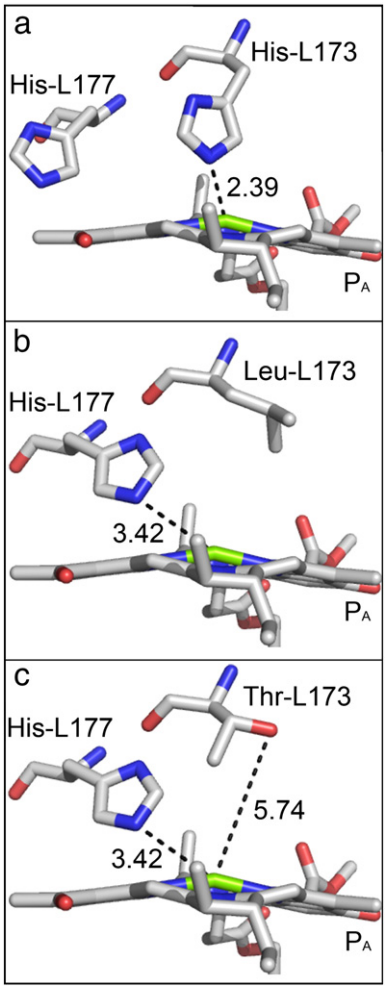

Fig. 8. Orientation of the His-L177 in the single mutant RC I(L177)H (a), and putative structural model for BChl P_A coordination in the double mutant RCs I(L177)H + H(L173)L (b) and I(L177)H + H(L173)T (c).

Thus, considering that the major influence of the I(L177)H mutation on the special pair BChls properties, that is the pronounced decrease in relative intensity of the Q_Y P band and the covalent binding of BChl P_A with the L-subunit, is similar in two RCs with single I(L177)H and double I(L177)H + H(M182)L mutations, we can conclude that the key difference between these mutant RCs is the coordination state of the BChl B_B, which is hexacoordinated in I(L177)H RC and pentacoordinated in the double mutant RC I(L177)H + H(M182)L. Hence, the X-ray crystal structure obtained for the RC I(L177)H can also be applied to model the BChl B_B ligation in the double mutant I(L177)H + H(M182)L RC.

The α-ligation of (B)Chls is generally more widespread in photosynthetic complexes than β-ligation [7,9,10]. Despite the β-ligation is known to be 4 kJ/mol less favorable, it appears to have a

pronounced structural impact on the LH2 antenna from *Rba. sphaeroides* since hydrogen bonds of β-ligated BChls with neighboring helices are known to stabilize the complex [9]. Given that in photosynthetic complexes the hexacoordinated state of (B)Chls is uncommon and strongly avoided as energetically unfavorable, the question arises of why in the L181 mutant RCs mentioned above as well as in the RC I(L177)H the hexacoordination was accepted during mutant RC assembly. Although the exact reasons for these cases remain to be determined, in I(L177)H RC the hexacoordination may be associated with necessity to stabilize protein environment by through bond connection of the polar His residue with Mg of BChl B_B.

4.2. Modeling of the BChl hexacoordination in the RC I(L177)H

Next comes the question of what molecule can serve as a β-side ligand in RC I(L177)H and in the double mutant RC. His residue is the most frequently observed ligand donor for (B)Chls in photosynthetic complexes, but as shown in Fig. 7 the estimated distance from the imidazole cycle to Mg of BChl B_B is more than 6 Å, which is too long for coordination. In the WT RC structure the β-side of BChl B_B is devoid of any potential ligand donors, but the cavity in the interior of the protein, adjacent to the Mg atom could well accommodate one or two water molecules. Therefore in the mutant RC I(L177)H water molecule may be an appropriate ligand donor on the β-side of the BChl B_B, considering the bulk of the protein pocket and the availability of the His-L177 as a potential hydrogen bond donor for water to fix it in a relatively stable position for magnesium coordination. In support of this suggestion, the difference electron density maps show an additional feature in the region adjacent to the Mg of BChl B_B indicating the presence of a small molecule (Fig. 7a). One water shown as a red ball in Fig. 7b could be modeled within this density, and another water (shown as a magenta ball in Fig. 7b) could be modeled between the ND1 nitrogen of His-L177 and the first water. The center-to-center distances of 2.6 Å (Fig.7b) fit well as a hydrogen bond and coordination distances known for bacterial RCs. It is known that insertion of a polar water molecule in the hydrophobic protein interior is energetically expensive, so whenever water appears inside of the mutant RC complexes as a result of amino acid substitution, it is usually fixed by one or two hydrogen bonds with the surrounding residues [2]. In the theoretical study of Heimdal and colleagues [49], it was reported that when His and water were compared as ligand donors to a Chl, His had a longer Mg-ligand distance than H_2O, but a much higher bond dissociation energy. Given that only one hydrogen bond was proposed to fix the putative water–ligand in the position adjusted to Mg (Fig. 7b) the low water–Mg dissociation energy may explain more labile BChl B_B coordination compared to other BChls in the RC. It is also worth to note that His-L177 lies on the interface of L and M subunits, and therefore presumptive hexacoordination of BChl B_B through this residue would organize inter peptide connection in the I(L177)H mutant RC, which may be responsible, at least in part, for the relative instability of the RC I(L177)H as well as for instability of the RC I(L177)H + H(M182)L.

Water is shown as a ligand donor in a number of published structures of photosynthetic complexes such as photosystems I and II [6,8] and in some mutant bacterial RCs as mentioned above [18]. Water molecule was proposed as a ligand to BChl P_B when the native axial ligand His-M202 was replaced by Gly. Since Gly cannot provide ligand to BChl, it was suggested that one or more water molecules could be included in the cavity created by His to Gly mutation, and one of them provided ligand to the BChl P_B [50]. In the work of Katilius and co-authors similar proposal was made when the axial ligand to BChl B_A, His-L153 was substituted to a number of residues that could not provide coordination for BChl Mg atom but none of the mutation resulted in the appearance of BPhe in B_A protein binding site [47].

4.3. Effect of single mutations on optical absorption and redox properties of BChl dimer P

The results presented in this work and published previously [23,24] show that optical and electrochemical properties of the primary donor P are affected by the I(L177)H mutation. In the region of the BChl absorbance, the spectrum of the RC I(L177)H looks quite similar to that of the heterodimer RC H(L173)L with the Q_Y P band strongly blue shifted and amplitude of this band significantly decreased relative to its properties in the WT RC [23,51]. One visible exception is the 638 nm band in the I(L177)H spectrum, which is shown to be assigned to the hexacoordinated BChl B_B absorbance as was discussed above. The results of the pigment analysis give the BChl:BPhe ratio of 3:2 in the acetone-methanol extract from the RC I(L177)H compared to the ratio of 1:1 in the heterodimer H(L173)L mutant indicating that one BChl is missing in the I(L177)H pigment extract rather than it turns into a BPhe. It was reported previously that in the RC I(L177)H one BChl was found non-extracted and presumably covalently bound to the RC protein [24], the issue that will be discussed below. Another point of comparison is the midpoint potential of the dimer P, which is increased in the heterodimer mutant by approximately 130 mV due to the higher potential of bacteriopheophytin [39] and is decreased in the I(L177)H mutant by 50 mV. The reason of the latter decrease is not obvious and one possible explanation may be weakening of the hydrogen bond between the His-L168 and the acetyl carbonyl group of the BChl P_A close to the site of the mutation. This hydrogen bond is known to be sensitive to charge–dipole interactions as shown in ref. [52] and might be altered by the I(L177)H mutation. Indeed, in the structure of I(L177)H RC the torsion angle between the acetyl carbonyl group of BChl P_A and the macrocycle plane was estimated to be 4° as compared to 28° in the WT RC structure, indicating possible disturbance of this hydrogen bond. Therefore, despite the relative spectral similarity of the two mutant RCs I(L177)H and H(L173)L their pigment content and redox properties of the dimer BChl P were found to be dissimilar. However, when these two mutations were combined together in the RC I(L177)H + H(L173)L, the result was quite surprising.

4.3.1. Properties of the RCs I(L177)H + H(L173)L

Previously it has been shown that replacing the axial His-L173 ligand to one of the special pair BChl P_A with a hydrophobic Leu residue, which has no lone pairs of electrons available for Mg coordination is enough to change the tetrapyrrole found at this site [38]. The spectrum of the H(L173)L RC presented in this study is consistent with exchange of the BChl P_A for a BPhe. In the spectrum of the RC I(L177)H + H(L173)L, however, no changes indicating the appearance of the new BPhe were observed in the regions of bacteriopheophytin Q_X and Q_Y transitions, supported by the results on unaltered pigment content and on the value of E_m P/P^+ similar to that of the WT RC. Taken together, these data indicate that, contrary to expectations, in the double mutant RC I(L177)H + H(L173)L the primary donor P remained a homodimer. This double mutation resulted in a 46-nm short-wavelength shift of the Q_Y P band without affecting the dipole strength of this band. This blue shift, the largest among other known band shifts in mutant RCs, completely masked Q_Y P band in the room temperature absorption spectrum by overlapping it with the monomeric BChls absorption band at 804 nm (T. Fufina, unpublished) and a resolved band near 843 nm could be observed only at 90 K. Blue shifts of Q_Y P absorbance band have been reported for a number of RCs with mutations of residues in the vicinity of the primary donor [31,53,54]. The largest reported was the 35 nm blue shift of P absorbance band in the H(L168)F RC from *Blastochloris* (former *Rhodopseudomonas*) *viridis* [55] and in *Rba. sphaeroides* mutant RCs it was in the range of 10–15 nm [52]. A number of suggestions have been made as to the origin of the shift basing both on theoretical studies and structural analysis [56–58]. It was shown that a variety of

different factors could affect the position of the Q_Y P band, among them theoretically predicted orientation of the 2-acetyl carbonyl group, inter-BChl spacing in the P dimer, presence of charged amino acid residues in the P vicinity and other factors as discussed in details elsewhere [58]. The general conclusion was that any alteration of the primary donor environment that disturbed inter dimer interactions might cause the blue shift of the Q_Y P band and/or the decrease of its dipole strength. In agreement with this conclusion preliminary structural data on the RC I(L177)H obtained by the methods of CD and FTIR difference spectroscopy indicated a decrease in the strength of coupling between the P BChls [44].

4.4. Putative ligands for BChl P_A in the double mutant RCs

Here we come to the question of what facilitates the Mg coordination of P_A BChl in the absence of His-L173. Apparently only His-L177 and His-L168 that are present in the relative proximity of the P_A Mg atom can possibly participate in the ligation. More distant His-L168 is known to donate a hydrogen bond to the 2-acetyl carbonyl group of the P_A BChl. Elimination of this hydrogen bond by mutagenesis was shown to result in the decrease of midpoint redox potential of the P/P^+ redox couple by 60–123 mV depending on amino acid replacing His-L168 [52]. In the RC I(L177)H + H(L173)L, the value of E_m P/P^+ was found to be practically unaltered (485 ± 15 mV) suggesting integrity of this hydrogen bond and indicating that His-L168 appears not to be involved in the Mg coordination. His-L177 is another potential ligand, and the availability of the I(L177)H mutant RC structure provides the opportunity to model structural features of the related double mutant RC I(L177)H + H(L173)L. As it is shown in Fig. 8a the imidazole cycle of the His-L177 in the RC I(L177)H lies close to P_A macrocycle (~1.9 Å) but points towards B_B BChl. It appears that in the double mutant RC I(L177)H + H(L173)L the backbone of the His residue undergoes reorientation and is directed towards Mg of P_A (Fig. 8b). There are three interrelated observations that are giving an indication of possible reorientation of the backbone: the recovery of the dipole strength of the Q_Y P band, the recovery of the E_m P/P^+ value and, finally, the covalent binding between the BChl P_A and RC's protein, which is observed in the RC I(L177)H and vanishes in the RC I(L177)H + H(L173)L. All these observations suggest that the inter dimer interactions in the RC I(L177)H and the double mutant RC I(L177)H + H(L173)L are different. According to the model (Fig. 8b), the estimated separation of the NE2 nitrogen of His-L177 from Mg of P_A is about 3.5 Å, which is longer than the distances typical for a BChl axial ligand. On the average such distance for bacterial RCs is 2.3–2.45 Å and the longest known distance is ~2.6 Å [31]. Given the well-known plasticity of bacterial RCs [2], it is plausible to assume a moderate rearrangement of the P dimer microenvironment or repositioning of the P_A and P_B BChls relative to one another that might shorten the N-to-Mg distance between His-L177 and central Mg of BChl P_A. Theoretical calculations and experimental data reported previously [54,56,58], the unprecedented blue shift of the Q_Y P absorbance band observed in the spectrum of the RC I(L177)H + H(L173)L as well as other observations mentioned above are in line with these suggestions. Unaltered shape of the BChl Q_X band indicates that the nature of the BChl ligand remains unchanged, which is also consistent with the proposal of His-L177 as a putative ligand to BChl P_A.

In the modeled structure of the RC I(L177)H + H(L173)L (Fig. 8b), the putative position of the His-L177 imidazole cycle is at acute angle relative to the plane of P_A macrocycle, which seems to be not optimal for Mg coordination. Similar cases of poorly positioned His ligands were described for *Rba. sphaeroides* mutant RCs with mutations resulted in replacement of the native BPhe H_A by a BChl. In the X-ray crystal structure of quintuple mutant RC the backbone of the coordinating His-M214 was non-perpendicular to the plane of the new BChl, though the distance between NE2 nitrogen and Mg was appropriate to donate a ligand (~2.6 Å) [59]. In the double mutant RC F(L97)V + F(L121)H the position of the putative ligand donor, His-L121, was also considered to be unsuitable for Mg coordination and involvement of a water molecule as another possible ligand was discussed [60].

An alternative proposal that also should not be excluded is that a water molecule may be involved in the BChl P_A ligation in the RC I(L177)H + H(L173)L. This assumption, however, is not consistent with the results of McDowell and co-workers who showed that in the RC H(L173)L the substitution of His ligand for a hydrophobic Leu residue prevented a water molecule from approaching Mg and brought about the heterodimer primary electron donor [39]. Moreover, X-ray crystal structure of the RC with symmetry related mutation H(M202)L shows that the Leu side chain expels a nearby conservative water from its binding site [61]. The exact arrangement of the BChl P_A coordination in the RC I(L177)H + H(L173)L remains to be clarified, and such work is under way. It would be also interesting to study how strong change in the optical properties of the primary electron donor observed in the double mutant RC affects the kinetics of electron transfer.

The employment of another double mutant RC I(L177)H + H(L173)T comes in support of our suggestions that were put forward above. In this double mutant RC I(L177)H substitution is combined with exchanging of the P_A His ligand to Thr residue. Theoretically speaking, the hydroxyl group of Thr residue can donate a ligand to a BChl, although no cases of such coordination were described for natural photosynthetic complexes [49]. Fig. 8c shows that the modeled conformation of less bulkier Thr-L173 is poor for ligation with the distance between the hydroxyl group and Mg (~5.74 Å) inappropriate for the coordination. This model partially resembles the microenvironment of the monomer Chl D1 in the photosystem II structure where D1-Thr-179 is adjacent to Mg within ~5 Å. The X-ray crystal structure with 1.9 Å resolution shows that a water molecule positioned within 2 Å to Mg is the actual axial ligand for Chl D1 [8]. The spectral data and pigment analysis indicated that in the very unstable RC I(L177)H + H(L173)T the P_A site is still occupied by a BChl molecule though this double mutation brings about obvious heterogeneity of the Q_Y P band. One possible explanation for this heterogeneity is that there might be a competition between His-L177 and another ligand for magnesium coordination resulting in the splitting of the Q_Y P band into two peaks. Consistent with this explanation the blue shifted peak of the Q_Y P band at 843 nm is similar to that observed in the spectrum of the double mutant RC I(L177)H + H(L173)L, and another maximum at 895 nm is apparently assigned to Q_Y transition of the P dimer that has the BChl P_A coordinated in a different way. Taking into account that Thr residue is less bulkier than His, possible participation of a water molecule as a Mg–ligand should not be excluded. In the context of the assumption, however, Q_X BChl band splitting would also be expected but not observed in the absorption spectrum of the RC I(L177)H + H(L173)T (Fig. 4).

4.5. On the covalent binding caused by I(L177)H mutation

The data presented in this study show that the covalent BChl-protein binding described for the RC I(L177)H [24] is preserved in the double mutant RC I(L177)H + H(M182)L, and the properties of the covalently bound BChl with unusually stable Mg coordination of the BChl remain unaffected. In this double mutant RC the ligation of the BChl B_B is found to be labile while the ligation of the BChl covalently bound to the L-subunit is known to be more stable compared to the other RC BChls [45]. This data is consistent with our previous conclusion that not BChl B_B but BChl P_A is bound to the protein in the RC I(L177)H [25]. Another combination of the mutations I(L177)H + H(L173)L brought about disappearance of the pigment – protein covalent bond together with the recovery of the dipole strength of the Q_Y P band and the value of the midpoint potential E_m P/P^+. It is an indication that these changes of the BChl dimer properties in the RC I(L177)H may be related, at least in part, to the covalent binding of the P_A BChl to the RC protein. Besides, disappearance of the covalent

bond in this double mutant RC indicates that His-L173 may be an important constituent related to the origin of this phenomenon. Computer modeling based on the X-ray crystal structure of the RC I(L177)H suggests that vanishing of the covalent binding in the double mutant RC I(L177)H + H(L173)L may be associated with a reorientation of the His-L177 backbone.

In the X-ray crystal structure of the RC I(L177)H only one orientation of the imidazole cycle fits both the electron density map and the through-bonding model of the BChl B_B β-coordination shown in Fig. 7. According to this orientation ND1 nitrogen of His-L177 is involved in the putative hydrogen bonding with water and NE2 nitrogen points towards the methyl carbon of the ring I of the P_A BChl. One may expect that hydrogen bonding of the ND1 nitrogen with water would increase the dipole moment of the imidazole cycle making it potentially more reactive [62]. In principle, the obtained RC I(L177)H structure is compatible with a new covalent bond, however, the so far limited resolution does not allow to unambiguously indicate the functional groups involved. The integrity of the 17-propionic acid phytol chain of the BChl P_A shown in the RC I(L177)H structure argues against the previous assumption on possible involvement of the 17^3-ester group in that binding [44]. Thus, the nature of the BChl-protein binding found in the RC I(L177)H is still uncertain and is the subject of ongoing investigation.

Of particular interest is the finding that Mg coordination of the BChl covalently bound to the protein in the RC I(L177)H is exceptionally stable during SDS PAGE and under variety of denaturing conditions [24,25]. Given that Mg does not participate in this covalent binding and in acidic environment the resulting BPhe remains to be bound to the L-subunit (T. Fufina, unpublished observation) we suggest that the increased strength of the Mg coordination of the covalently bound BChl may be nonspecific. It is known that in plant light-harvesting complexes the electron density in Chl *b* is pulled outward by H-bonds between the 7-formyl group and other structures thus enhancing Mg coordination [62]. Hence one can expect that stronger interaction such as the putative covalent bond of BChl with protein found in the RC I(L177)H would seriously reinforce the pigment ligation. It remains to be established, however, why stronger Mg coordination had very little effect on the absorbance of the covalently bound BChl in denatured RC protein [24].

5. Conclusion

Mg coordination is known to be an important factor that determines (B)Chl properties and binding in the photosynthetic protein complexes. The results presented in this study demonstrate that the substitution of Ile to His in the L177 site located in the interface of the L and M subunits affects coordination of the two nearest BChls P_A and B_B. Combining the I(L177)H mutation with H(M182)L and H(L173)L mutations as well as modeling studies based on the X-ray structure of the mutant I(L177)H RC obtained with 2.9 Å resolution provided information on the structural consequences of the I(L177)H single amino acid substitution. Study of the absorption spectra and pigment content of the double mutant RC I(L177)H + H(M182)L presented evidence for the hexacoordination of the BChl B_B in the I(L177)H RC and its β-pentacoordination in the double mutant. Computer modeling suggests involvement of two water molecules in the β-coordination of the BChl B_B. It is shown that the presence of the I(L177)H mutation prevents formation of the heterodimer in the RC I(L177)H + H(L173)L causing instead a 46 nm blue shift of the long-wavelength P absorbance band. The data presented in this work suggest that taking into account possible rearrangements of the primary donor environment, His-L177 or a water molecule can be a ligand donor for BChl P_A in the absence of the native ligand His-L173.

Given that BChl hexacoordination in bacterial photosynthetic complexes is uncommon and β-coordination of the pigment is rare, the mutant RCs described in this work seem to be interesting objects to study protein–cofactor interactions, their role in self-assembly and functioning of the RC complex.

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