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Substitution of Isoleucine L177 by Histidine Affects the Pigment Composition and Properties of the Reaction Center of the Purple Bacterium *Rhodobacter sphaeroides*

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Abstract—Using site-directed mutagenesis, we obtained the mutant of the purple bacterium *Rhodobacter sphaeroides* with Ile to His substitution at position 177 in the L-subunit of the photosynthetic reaction center (RC). The mutant strain forms stable and photochemically active RC complexes. Relative to the wild type RCs, the spectral and photochemical properties of the mutant RC differ significantly in the absorption regions corresponding to the primary donor P and the monomer bacteriochlorophyll (BChl) absorption. It is shown that the RC I(L177)H contains only three BChl molecules compared to four BChl molecules in the wild type RC. Considering the fact that the properties of both isolated and membrane-associated mutant RCs are similar, we conclude that the loss of a BChl molecule from the mutant RC is caused by the introduced mutation but not by the protein purification procedure. The new mutant missing one BChl molecule but still able to perform light-induced reactions forming the charge-separated state $P^+Q_A^-$ appears to be an interesting object to study the mechanisms of the first steps of the primary electron transfer in photosynthesis.

Key words: bacterial photosynthesis, reaction centers, photochemical charge separation, site-directed mutagenesis, *Rhodobacter sphaeroides*

The photosynthetic apparatus of purple bacteria comprises reaction center (RC) and two types of light-harvesting antenna complexes. RC of *Rhodobacter sphaeroides* is a membrane pigment–protein complex that is responsible for the first steps of light energy conversion into the energy of charge separated states. The RC consists of three protein subunits L, M, and H, two strongly interacting bacteriochlorophyll *a* (BChl *a*) molecules that form a dimer of the so-called "special pair" (P), two BChl *a* monomers (B), two bacteriopheophytin *a* (BPhe *a*) molecules, two ubiquinones (Q), a carotenoid molecule, and a non-heme iron atom [1]. X-Ray diffraction studies demonstrated that BChl *a*, BPhe *a*, and quinone molecules are arranged in two trans-membrane branches—active and non-active branches of electron transfer (A and B, respectively) [2]. According to spectroscopic data, upon photon absorption by RC, electron transfer proceeds from the excited state of the primary electron donor P* to Q_A molecule through BChl *a* monomer and BPhe *a* in the active branch A [3]. The efficiency of the photochemical charge separation in RC depends on the properties of electron transfer cofactors, their positions, and interactions with each other and with the surrounding protein environment. Determination of three-dimensional structures of RCs from purple bacteria by X-ray diffraction studies opened up possibilities to generate controlled alterations in the structure of the RC protein in close proximity to its chromophores. Such alterations introduced by site-directed mutagenesis allow investigation of how the protein environment of chromophores influences the efficiency and directionality of the primary charge separation and stabilization in bacterial photosynthesis [4]. In our previous paper [5], it was shown that after the substitution of Ile M206 by His the pigment composition of RC was altered. Namely, in the mutant RC some structural changes in one out of four BChl molecules were suggested.

In this paper, we present experiments on the mutant RC from *Rb. sphaeroides*, in which Ile L177, symmetrically related to Ile M206, was substituted by His.

Abbreviations: RC) reaction center; BChl) bacteriochlorophyll; BPhe *a*) bacteriopheophytin *a*.

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METHODS OF INVESTIGATION

The methods of genetic engineering and cell growth conditions used in this study were described earlier [6]. Bacterial strains and plasmids used in this work are listed in the table. Mutagenesis was performed by PCR using the method of overlapping DNA fragments [11]. The modified genetic system for the RC site-directed mutagenesis described in [6] was employed. Recombinant strains synthesizing no light-harvesting antenna and containing RC as the only photosynthetic complex were constructed as follows. The *puf*$^-$*puc*$^-$ *Rb. sphaeroides* strain DD13 devoid of RC and both light-harvesting antennas (kindly provided by M. Jones) [9] was complemented by conjugation with a plasmid carrying *puf*-operon. The 4 kb *Eco*RI-*Sph*I DNA fragment of *Rb. sphaeroides* strain RV *puf*-operon [6] was used for the complementation. This DNA fragment includes several unique restriction sites useful for isolating and cloning of different parts of the *puf-L* and *puf-M* genes for mutagenesis (Fig. 1a). In particular, *Kpn*1 and *Sal*1 sites were used to introduce I(L177)H mutation into the *puf*-operon. *Puf-BA* genes encoding protein subunits of the light-harvesting antenna-1 were deleted from the *Eco*RI-*Sph*I fragment. The deletion was made in a similar way as described by Jones [9] taking into account the existing differences in the restriction maps of the corresponding DNA fragments (Fig. 1b). The resulting *puf*-DNA fragment with *puf-BA* genes deleted was cloned into pRK-415 and used for DD13 strain complementation (table). For cultivation of the antenna-less strains tetracycline (1 μg/ml), kanamycin (5 μg/ml), and streptomycin (5 μg/ml) were added to the growth medium.

Chromatophores were prepared by sonication of the cell suspension with subsequent separation from cell debris by centrifugation (60 min, 4°C, 20,000*g*, r_m 10 cm).

For RC isolation, we used the previously described recombinant strains containing light-harvesting antenna [6]. RCs were isolated by the standard method followed by purification on a DEAE ion-exchange column [12]. RC pigments were extracted with acetone–methanol mixture (80 : 20) according to [13]. RC 3D structural data were downloaded from the data bank [Protein Data Bank, 1OGV.pdb], and the protein matrix energy was mini-

Bacterial strains and plasmids

Strain/plasmid	Genotype/description	Source/reference
E. coli		
DH5	*supE*44, *Δlac* U169 (*Φ80 lacZ* Δ M15), *hstR17*, *recA1, endA1*, *gyr A96*, *thi-1*, RelA1	[7]
S17-1	*Pro-Res-Mod-recA*, integrated plasmid RP4-Tc:Mu-Km::Tn7	[8]
Rb. sphaeroides		
RV	wild type	[6]
DD13	genomic deletions of *pufBALMX* and *pucBA*, insertions of Km^R and Sm^R genes, respectively	[9]
RCO	strain DD13 complemented *in trans* by pREH-D2; Km^R, Sm^R, Tc^R	this work
RCO-I(L177)H	strain DD13 complemented *in trans* by pREHD-I(L177)H; Km^R, Sm^R, Tc^R	–"–
Plasmids:		
pUC-119	Amp^R	[7]
pRK-415	Tc^R	[10]
pUC-4D	Amp^R, derivative of pUC-119, contains *puf*-operon with *puf-BA* genes deleted	this work
pREH-D2	Tc^R, *Eco*RI-*Hind*III fragment containing *puf*-operon with *puf-BA* genes deleted was cloned from pUC-4D into pRK-415	–"–
pREHD-I(L177)H	Tc^R, pREH-D2, carries I(L177)H mutation inside of *puf*-operon	–"–

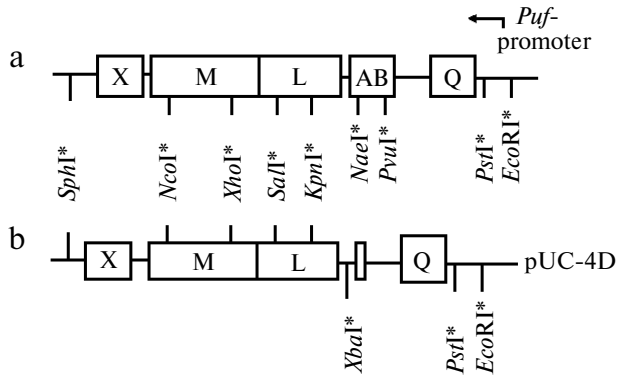

Fig. 1. Scheme and genetic map of *Eco*RI-*Sph*I DNA fragment containing *Rb. sphaeroides* RV *puf*-operon before (a) and after (b) *puf-BA* genes deletion. Asterisks denote unique restriction sites.

mized using the Swiss-PDB-Viewer program (www.genebee.msu.su (Moscow State University)). Ground state optical spectra of the RCs were measured at room temperature with a Shimadzu UV-1601PC spectrophotometer (Shimadzu, Japan). Light minus dark difference spectra were obtained upon sample illumination with continuous blue light using an incandescent lamp and crossed glass filters SZS-22 and KS-15. Light-induced absorption difference kinetics and ΔA spectra in the millisecond range were measured with a single-beam spectrophotometer with a phosphoroscopic set up. Weak monochromatic light was used as the measuring light. Absorption changes were induced by actinic excitation at $\lambda = 874$ nm.

RESULTS AND DISCUSSION

A new photosynthetic mutant of the purple bacterium *Rb. sphaeroides* was constructed with Ile177 substituted by His in the L subunit of the RC. According to X-ray diffraction data for the wild type RC structure, Ile L177 is located between the primary electron donor P molecule and the monomer BChl B_B (non active branch of the electron transfer, Fig 2a). Virtual replacement of Ile177 by His performed by the computer program Swiss-PDB-Viewer showed the most probable disposition of the His residue in the vicinity of P_L and B_B (Fig. 2b). The size of a His molecule is considerably larger than that of an Ile molecule. In the RC structure, Ile M206 is located symmetrically to Ile L177 on the other side of P (not shown).

Figure 3 presents the absorption spectra of isolated wild type and mutant RCs normalized at 532 nm, which corresponds to the Q_X absorption band of BPhe *a*. The most prominent alterations in the mutant RC spectrum were noted in the BChl absorption region. In this spectrum, we observed a decrease in the monomer BChl *a* absorption at 800 nm, a blue shift of the P Q_Y absorbance band (between 870 and 850 nm), and a considerable decrease in the dipole strength of this band (by a factor of ~2). We suppose that the blue shift of the P absorption

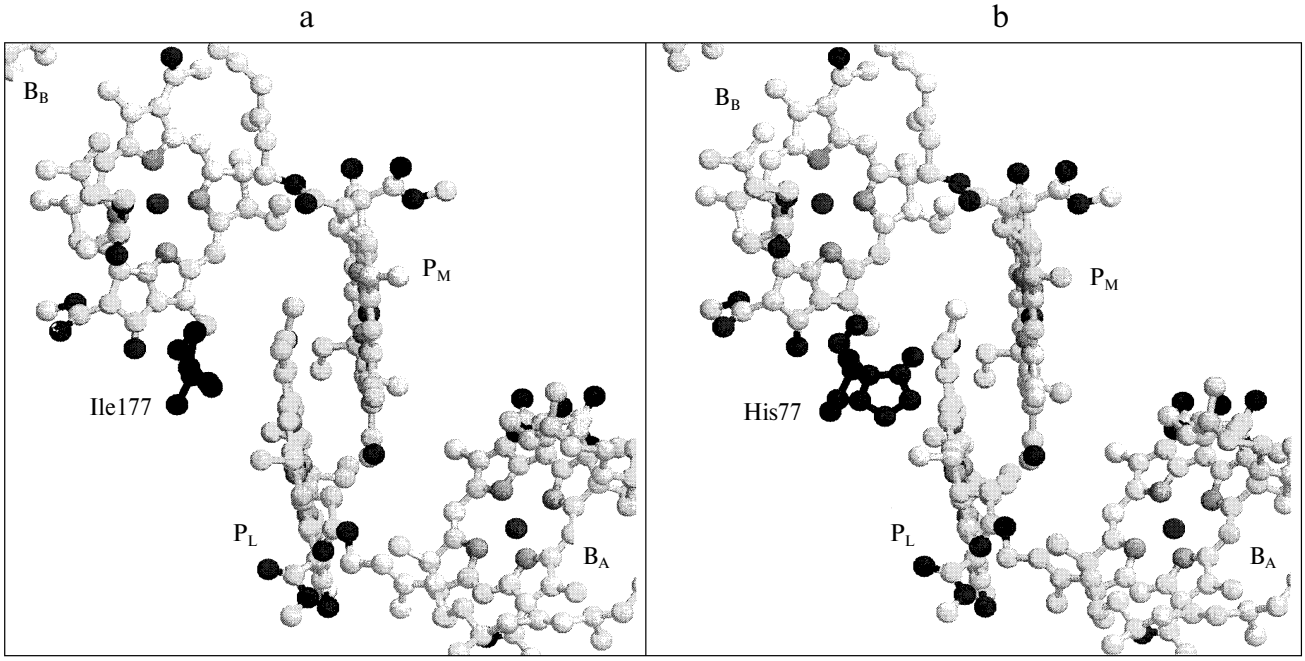

Fig. 2. Location of isoleucine (a) and histidine (b) at position L177 in the vicinity of chromophores in *Rb. sphaeroides* RC structure (Protein Data Bank, 1OGV.pdb).

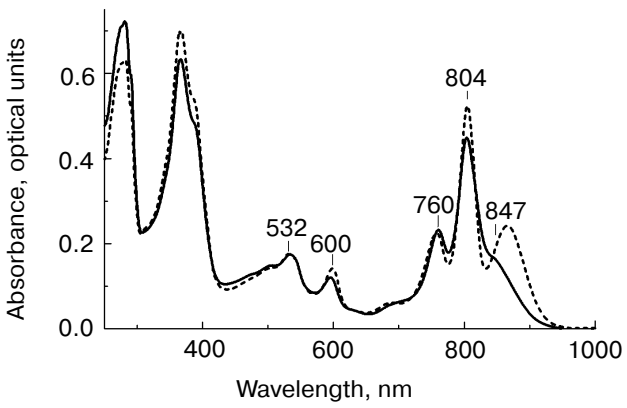

Fig. 3. Room temperature absorption spectra of isolated RCs from *Rb. sphaeroides* wild type (dashed line) and I(L177)H mutant (solid line). Spectra were normalized at 532 nm, which corresponds to the Q_X transition of BPhe.

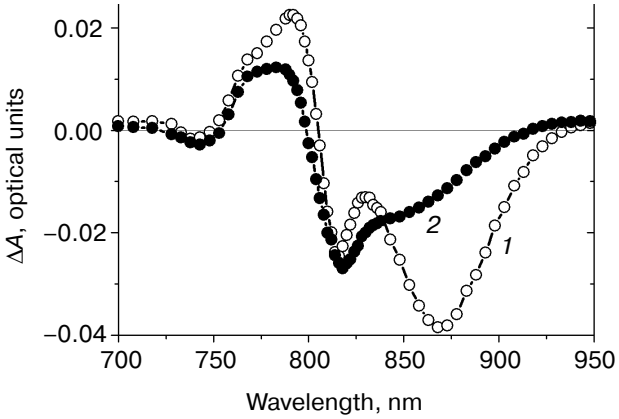

Fig. 4. Room temperature difference (light minus dark) absorbance spectra of isolated RCs of the wild type (curve *1*) and the mutant I(L177)H (curve *2*). The spectra were measured using a spectrophotometer with phosphoroscopic set up. Excitation light with $\lambda = 874$ nm was applied.

band may result from the decrease in the intradimer resonance interaction, while the decrease of the dipole strength may reflect the decrease in the number of absorbing BChls (see below).

Figure 4 shows the difference (light minus dark) absorption spectra corresponding to $P^+Q_A^-$ formation in the mutant and wild type isolated RCs. In the wild type RC light-induced formation of the charge separated state $P870^+Q_A^-$ results in the bleaching of the long-wavelength P band at 870 nm, in the short-wavelength shift of the monomer BChl *a* band at 800 nm, and in the long-wavelength shift of the 760 nm band. In the difference absorption spectrum of the mutant RC, we also observed the light-induced bleaching of the long-wavelength absorption band of the primary electron donor with the absorption maximum at 850 nm, together with other spectral changes characteristic of the $P^+Q_A^-$ state formation. The shape of the light-induced spectral changes in the mutant RC, and the similarity of the charge separation quantum yields in both RCs (not shown) demonstrate that under illumination the mutant RC is able to perform the primary electron transfer leading to the $P^+Q_A^-$ state formation. However, in the ΔA spectrum of the mutant RC the dipole strength of the functionally active primary donor band decreases by a factor of ~2, and its maximum is blue shifted to 850 nm compared to the corresponding band in the ΔA spectrum of the wild type RC. Several factors could possibly affect the dipole strength of the P band and cause its blue shift: structural alterations in the protein environment in the proximity of P, changes in the relative positions of the two BChls in the dimer, structural changes in a BChl *a* molecule or its loss from the dimer.

To find out why the spectral and photochemical properties of the primary donor have changed, we compared pigment composition of the RCs isolated from the mutant and wild type strains. Figure 5 shows the room temperature absorption spectra of the pigments extracted from the wild type RC (curve *1*) and mutant RC (curve *2*) and their difference (curve *3*). The data show the ratio of the absorption maxima of carotenoid at 485 nm and the Q_X band of BPhe *a* at 523 nm to be identical for the spectra *1* and *2*. Moreover, the absolute values of the optical densities in this region in both extracts are also equal. Hence, we conclude that in the mutant RC the concentration of carotenoid and BPhe *a* remains unchanged, and the content of these pigments in the both RC types is similar. In contrast, considerable differences are observed in the BChl a Q_X region at 600 nm and near the long-wavelength BChl absorption band. The difference spectrum (curve *3*) obtained by subtraction of spectrum *2* from spectrum *1* shows a band at 771 nm, which is attrib-

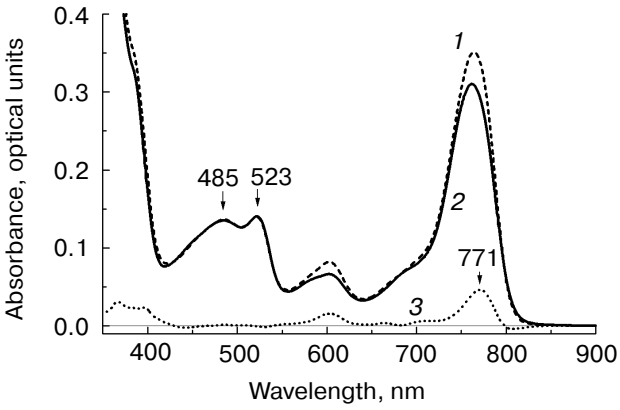

Fig. 5. Room temperature absorption spectra of pigment extracts from the wild type RCs (*1*) and mutant RCs (*2*). Curve *3*, the difference between spectrum *1* and spectrum *2*.

uted to BChl *a* absorption. Thus, the BChl *a* absorbance and consequently its concentration in the mutant RC extract averages about 3/4 of that in the pigment extract from the wild type RC.

We also determined the BChl/BPhe ratio in the RCs by calculating the pigment concentrations using the molar extinction coefficients of BChl *a* and BPhe *a* and the absorbance values at 771 and 747 nm of the pigment extracts [3]. In the extracts from the wild type RC, the concentration of BPhe *a* was determined to be $2.327 \cdot 10^{-3}$ M whereas the concentration of BChl *a* was $4.779 \cdot 10^{-3}$ M. The molar ratio BChl/BPhe in the wild type RC was estimated as 2.05 : 1 indicating that this RC contains four BChl molecules per two molecules of BPhe. In the pigment extracts from the mutant RC the BPhe *a* concentration was determined to be $2.445 \cdot 10^{-3}$ M, and the concentration of BChl *a* was $3.961 \cdot 10^{-3}$ M. The molar ratio BChl/BPhe in the mutant RC was 1.62 : 1 which corresponds to 3.2 BChl molecules per two molecules of BPhe. These measurements and calculations also confirm that the main part of the isolated mutant RCs I(L177)H contains not four but only three BChls and two BPhes.

The data suggest that the isolated mutant RC I(L177)H lacks one BChl *a* molecule. This missing BChl *a* either could not be incorporated in the RC complex due to local structural and electrostatic alterations caused by the mutation, or due to the mutation its bonding to the protein interior became weaker so that this BChl *a* molecule might be easily dissociated from the complex during RC isolation and purification. To exclude possible effects of the RC purification process on its spectral properties, we studied membrane-bound RCs without their isolation from chromatophores. For this purpose we used antenna-less strains of *Rb. sphaeroides* the chromatophores of which contain RC (wild type or the mutant I(L177)H) as the sole photosynthetic complex.

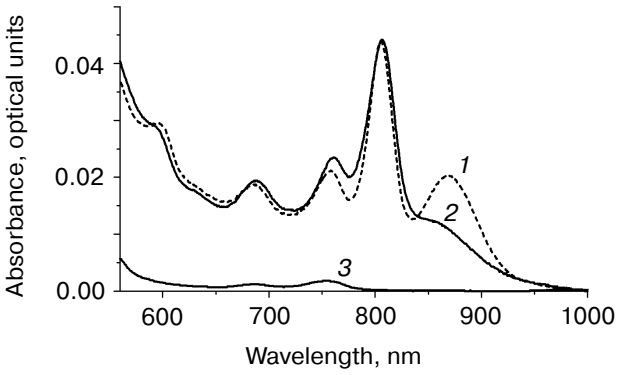

Fig. 6. Room temperature absorption spectra of antenna-less chromatophores of the wild type (*1*) and I(L177)H mutant (*2*). Curve *3*, chromatophores of the DD13 strain containing no photosynthetic complexes. Spectra were normalized at 800 nm, which corresponds to the monomer BChl absorption.

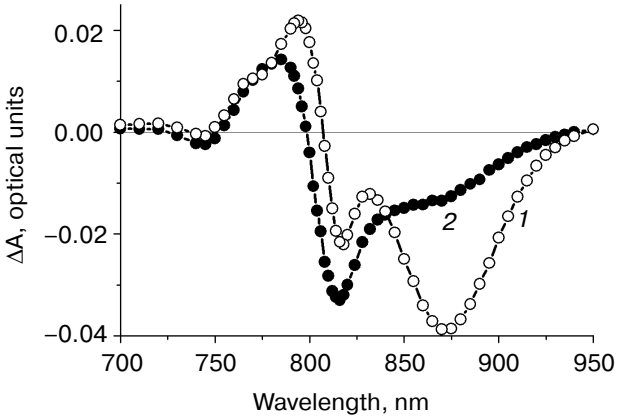

Fig. 7. Room temperature difference (light minus dark) absorption spectra of antenna-less chromatophores of the wild type (*1*) and mutant I(L177)H (*2*).

Figure 6 shows the absorption spectra of chromatophores of the *Rb. sphaeroides* antenna-less strains containing unmodified (RCO strain, curve *1*) or mutant RCs (RCO-I(L177)H strain, curve *2*). To decrease light scattering, the spectra were measured using thin samples with optical path of a few dozens of microns. As can be seen, in both samples the spectral changes in the region of the long-wavelength absorption of the primary donor are similar to those we have observed in the corresponding spectra of isolated RCs. The spectra of the mutant chromatophores also demonstrate a decrease (~2 times) in the dipole strength of the P absorption band and the blue shift of its maximum.

Figure 7 presents the spectra of light-induced absorbance changes ΔA corresponding to the formation of the charge-separated state $P^+Q_A^-$ in antenna-less chromatophores containing the wild type RC or the mutant I(L177)H RC. Similar to what was observed in the spectra of isolated RCs, the ΔA spectrum of the mutant chromatophores shows the decrease in the dipole strength of the primary electron donor band and its blue shift compared to the ΔA spectrum of the wild type chromatophores.

These results demonstrate that the isolated and the membrane-bound mutant RCs display similar properties. We thus conclude that the loss of a BChl molecule happens in the course of assembling of the mutant pigment–protein complex in the membrane, i.e., it is an effect of the introduced mutation rather than the result of RC purification. Presumably, the missing molecule is one of the BChls of the primary donor dimer, P_L, which is located close to the mutation locus L177 (Fig. 2).

There are a number of investigations describing chemically modified or mutated bacterial RCs with altered pigment composition. Among them, one can find mutant RCs with alterations in the P composition and

structure. In particular, it was shown that when one of the P dimer BChls was replaced by BPhe in the mutant RCs H(M202)L (so-called heterodimer) or H(L173)L RC (reverse heterodimer), radically different primary donor absorption and alterations of the RC photochemical properties were observed [14, 15]. Two *Rb. sphaeroides* mutants were described that possessed RCs with the special pair missing altogether [16, 17]. Neither the P absorption band nor charge separation was detected in those mutant RCs.

Recently we presented data on the new *Rb. sphaeroides* RC mutant having Ile M206 substituted by His in the vicinity of the special pair BChl [5]. This mutation lead to drastic changes of the RC spectral and photochemical properties, and the quantum yield of the charge separation was significantly decreased. It was shown that one BChl molecule in the mutant RC had undergone some structural changes caused by the mutation. The origin of these changes still remains unclear. We assumed another bacteriochlorin molecule, probably one of the BChl *a* biosynthesis intermediates, to be incorporated in the RC I(M206)H instead of the special pair BChl, P_M. In this work we present the new *Rb. sphaeroides* mutant RC I(L177)H with similar Ile–His substitution in the position symmetrically related to Ile M206. Previously Williams and coauthors reported the replacement of Ile L177 by Asp undertaken in order to introduce a hydrogen bond to 9-keto carbonyl of B_B in the *Rb. sphaeroides* RC [17]. In the absorption spectrum of that mutant RC the authors observed a 10 nm short-wavelength shift of the P band with no change in its dipole strength. According to our data, the substitution of Ile L177 by a large positively charged His residue leads to BChl *a* loss from the mutant RC. Yet, this RC remains stable and photochemically active both within the photosynthetic membranes and in the isolated state. Further study should provide information on how the absence of a BChl molecule in the special pair may affect the rate and efficiency of the primary electron transfer in RC.

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