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Mutant Reaction Centers of *Rhodobacter sphaeroides* I(L177)H with Strongly Bound Bacteriochlorophyll *a*: Structural Properties and Pigment–Protein Interactions

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Abstract—Methods of photoinduced Fourier transform infrared (FTIR) difference spectroscopy and circular dichroism were employed for studying features of pigment–protein interactions caused by replacement of isoleucine L177 by histidine in the reaction center (RC) of the site-directed mutant I(L177)H of *Rhodobacter sphaeroides*. A functional state of pigments in the photochemically active cofactor branch was evaluated with the method of photo-accumulation of reduced bacteriopheophytin H_A^-. The results are compared with those obtained for wild-type RCs. It was shown that the dimeric nature of the radical cation of the primary electron donor P was preserved in the mutant RCs, with an asymmetric charge distribution between the bacteriochlorophylls P_A and P_B in the P^+ state. However, the dimers P in the wild-type and mutant RCs are not structurally identical due probably to molecular rearrangements of the P_A and P_B macrocycles and/or alterations in their nearest amino acid environment induced by the mutation. Analysis of the electronic absorption and FTIR difference P^+Q^-/PQ spectra suggests the 17^3-ester group of the bacteriochlorophyll P_A to be involved in covalent interaction with the I(L177)H RC protein. Incorporation of histidine into the L177 position does not modify the interaction between the primary electron acceptor bacteriochlorophyll B_A and the bacteriopheophytin H_A. Structural changes are observed in the monomer bacteriochlorophyll B_B binding site in the inactive chromophore branch of the mutant RCs.

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Key words: *Rhodobacter sphaeroides*, mutant reaction centers, bacteriochlorophyll *a*, trans-esterification reaction, covalent bond

Reaction center (RC) of purple bacteria is a comprehensively studied photosynthetic pigment–protein complex [1]. RC of *Rhodobacter sphaeroides* contains three protein subunits: L, M, and H. Electron transfer cofactors are bound to the subunits. The cofactors are arranged as two quasi-symmetric branches A and B [2]. The cofactors are: exciton-coupled dimer of bacteriochlorophyll *a* molecules (BChl; P_A and P_B) (the bacteriochlorophyll molecules produce a special pair, which is the primary electron donor, P); two bacteriochlorophyll monomers (B_A and B_B); two molecules of bacteriopheophytin *a* (BPheo; H_A and H_B); two ubiquinone molecules (Q_A and Q_B); carotenoid molecule; and a non-heme iron atom. Although two potentially active chains of electron transfer exist, charge separation predominantly occurs in chain A. The absorption of a light quantum induces electron transfer from the lowest singlet excited state of the primary electron donor P* to the molecule of the secondary quinone acceptor Q_B via molecules B_A, H_A, and Q_A. The total quantum yield of the electron transfer is 100% [1].

Noncovalent binding of (bacterio)chlorin molecules to protein moiety is a specific feature of RC and other photosynthetic complexes; treatment with organic solvents eliminates relatively weak pigment–protein (and pigment–pigment) bonds, thereby facilitating pigment escape to solution [3, 4]. However, it was shown recently in our laboratory that a bacteriochlorophyll molecule (perhaps, P_A) in isolated RC of the site-directed mutant *R. sphaeroides* I(L177)H is tightly (perhaps covalently)

Abbreviations: BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; CD, circular dichroism; FTIR, Fourier transform infrared spectroscopy; LDAO, N,N-dimethyldodecylamine-N-oxide; P, primary electron donor; RC, reaction center.

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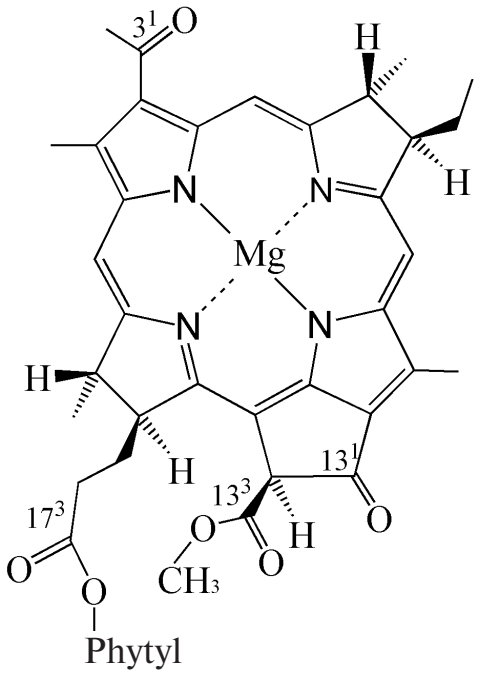

Fig. 1. Molecular structure of bacteriochlorophyll *a*. Atoms are numerated according to the IUPAC recommendations.

bound to the protein L-subunit and failed to be extracted using the organic solvents usually used for extracting BChl and BPheo from the wild-type RC [5]. Although the possibility of the covalent interaction of BChl molecules (Fig. 1), chlorophylls, and their derivatives with amino acids or small synthetic and natural polypeptides has been reported in the literature [6, 7], strong covalent binding of BChl to protein in RC I(L177)H seems unexpected and intriguing, particularly as a result of single site-directed mutation. Comparative analysis of the RC I(L177)H and wild-type RC could provide a new insight into specificity of the pigment–protein interaction and its role in adaptation of spectral and redox properties of pigments to the main RC function (stable charge separation).

Lack of crystallographic data about the structure of the mutant RC I(L177)H is the main problem hampering elucidation of spectral and functional properties of bacteriochlorophyll cofactor in the mutant [5, 8]. The nature of the primary electron donor P in the RC I(L177)H remains uncertain [5, 8]. Spectral properties of P could be interpreted in favor of monomeric [8] or dimeric [5] structure. There is also no information about the BChl functional group involved in hypothetical covalent interaction with protein.

In this work structural information about RC I(L177)H was obtained using the methods of circular dichroism (CD) and photoinduced Fourier transform infrared (FTIR) difference spectroscopy. The sensitivity of the CD spectroscopy to protein conformation and intermolecular interaction is higher than sensitivity of absorption spectroscopy to the same factors [9]. Analysis of photoinduced FTIR difference spectra of primary electron donor photooxidation provides information about molecular and electronic structure of P^+ and its interaction with protein matrix [10]. Structural integrity of the photochemically active chain of chromophores in RC I(L177)H was assessed in this work using the method of photo-accumulation of RC with reduced bacteriopheophytin H_A^-. A possible mechanism of formation of the covalent bond between P_A molecule and mutant RC L-subunit postulated in [5] is also discussed in this work.

MATERIALS AND METHODS

The antenna-free strain of *R. sphaeroides* with amino acid substitution Ile by His in position L177 was constructed as described earlier [8]. RC preparations isolated from the *puf*-deficient strain transformed by a plasmid containing the *puf*-operon [8, 11] were used as the wild-type RC. Both the wild-type RC and the mutant RC contained threonine in L178.

RC preparations were isolated using a standard procedure with detergent N,N-dimethyldodecylamine-N-oxide (LDAO) and purification on DEAE-cellulose [5]. Mutant preparations were found to be spectrally heterogeneous containing two RC populations. The RC populations differ from one another by amplitude of long-wavelength absorption band of the primary electron donor. The absorption spectrum of the main population (more than 80%) contained the P absorption band at ~850 nm, whereas the absorption band of the minor population at this wavelength was negligibly. To minimize the contribution of the RC population containing no P absorption band, mutant RC preparations were subjected to repeated chromatographic purification. Detergent LDAO was replaced by detergent Triton X-100 or *n*-dodecyl-β-D-maltoside using a membrane (30 kD; Millipore, USA) and an ultrafiltration cell under argon gas pressure.

All measurements were performed at room temperature. Absorption spectra were measured in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1% *n*-dodecyl-β-D-maltoside using a Shimadzu UV-1601 PC spectrophotometer (Japan). CD spectra were measured as described in [12] using a spectrophotometer similar to that described in [13]. Difference spectra H_A^-/H_A corresponding to accumulation of reduced photochemically active bacteriopheophytin H_A were measured as described in [14]. The reaction medium contained RC preparations solubilized in 20 mM Tris-HCl buffer (pH 8.0), 0.1% Triton X-100, potassium indigotetrasulfonate (0.1 mM), neutral red (0.1 mM), and sodium dithionite (2 mM). Indigotetrasulfonate and neutral red were used as redox mediators; sodium dithionite was used as reductant [15]. Photo-accumulation reaction was induced by RC expo-

sure to actinic light (1100 nm > λ > 720 nm; ~120 mW/cm^2) for 2 min. Photoinduced FTIR difference spectra P^+Q^-/PQ were recorded using a Bruker IFS66v/S Fourier-transform spectrophotometer (Germany) with DTGS photodetector and KBr beam-splitter. RC sample (10 μl, ~85 μM) solubilized in 20 mM Tris-HCl buffer (pH 8.0) containing 0.18% *n*-dodecyl-β-D-maltoside was applied to a CaF_2 plate, partially dehydrated using an argon gas jet, and covered with another CaF_2 plate. FTIR difference spectra were recorded under conditions of exposure to continuous light (1100 nm > λ > 720 nm; ~2 mW/cm^2). Spectral resolution was 4 cm^{-1}. To obtain satisfactory signal/noise ratio, illumination cycles were repeated hundreds of times.

RESULTS

Figure 2 (solid lines) shows the electronic absorption spectra of the RC *R. sphaeroides* wild-type and mutant I(L177)H within the spectral range 500-1000 nm. The spectra were measured using RC preparations normalized to BPheo absorption band at 760 nm. This normalization band was chosen on the basis of assumption that Ile L177 → His substitution has virtually no effect on the RC bacteriopheophytin molecule electronic structure and optical properties of the molecules, because the mutation locus is at large distance from the H_A and H_B binding sites. It follows from Fig. 2 (see also [8]) that although molecular volumes of isoleucine and histidine are close to each other (124 and 118 Å^3, respectively [16]), histidine incorporation into protein structure significantly modifies the RC absorption spectrum. Absorption spectral changes are particularly pronounced in the range of Q_y optical transitions: mutation induces 10-15 nm short-wavelength shift of the absorption band of the primary electron donor P at 865 nm (to ~850 nm). Mutation also induces a substantial decrease in the dipole strength of the absorption band. There is an slight decrease in the absorption band of monomeric bacteriochlorophylls $B_{A,B}$ at 800 nm. The absorption band is also slightly (1-2 nm) shifted toward longer wavelength. These findings are consistent with I(L177)H mutation location near the molecules P_A and B_B [5, 8].

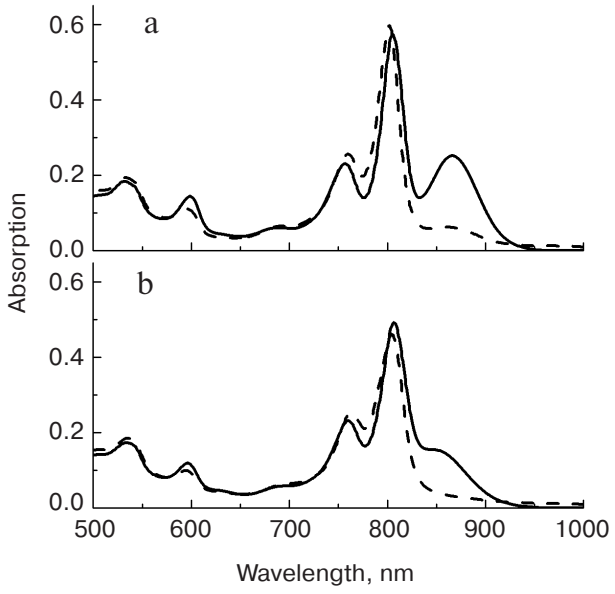

Fig. 2. Absorption spectra of the RC *R. sphaeroides* wild-type (a) and mutant I(L177)H (b) within the spectral range 500-1000 nm as measured before (solid lines) or after (dashed) potassium ferricyanide-induced (2 mM) oxidation of the primary electron donor P.

Spectra in Fig. 2 (dashed lines) show that in the presence of the exogenous electron acceptor (potassium ferricyanide, 2 mM) P absorption in the Q_y band in the wild-type RC and in mutant RC is at least 90% decreased as a result of P oxidation to P^+. In this case spectral changes also include well-known blue shift of the monomer BChl absorption band at 800 nm and minor absorption changes presumably related to red shift of BPheo absorption band at 760 nm. Further addition of sodium ascorbate (1 mM) caused virtually complete reduction of P in the RC preparations of the two types, thereby indicating reversibility of the observed oxidation. Therefore, isoleucine–histidine substitution does not induce significant modification of the redox potential of the primary electron donor (495 mV in the wild-type RC [17]).

The CD spectra of the wild-type RC are consistent with similar CD spectra reported in the literature [15, 18-21]. Within the absorption band of the BPheo molecules, in the initial state of the mutant RC there is a negative CD band at 750 nm. The amplitude and shape of the negative CD band are similar to those in the wild-type RC band at 748 nm. Within the Q_y bands of bacteriochlorophylls $B_{A,B}$ and P (780-950 nm) there are significant changes in the CD spectrum of the mutant RC (Fig. 3a). In general, these spectral changes are similar to the changes in electronic absorption spectra induced by Ile L177 → His mutation (Fig. 2) [8]. A broad positive CD spectral band observed in the wild-type RC at 862 nm corresponds to the low-energy exciton transition of dimer P [22]. Mutation induces shift of this broad CD spectral band to 846 nm and a decrease in the intensity of the band. Mutation also induces a significant decrease in the intensities of two intense opposite CD bands in wild-type RC spectrum at 811 and 796 nm. The negative band at 811 nm is mainly attributed to the high-energy exciton transition of P (corresponding absorption band has low dipole strength and is not manifested in the RC absorption spectrum at room temperature) [20, 21, 23]. The origin of the positive band at 796 nm is rather uncertain. Some authors believe that this band mainly represents specificity of structural properties of one or the two monomer BChl molecules. The structural properties are

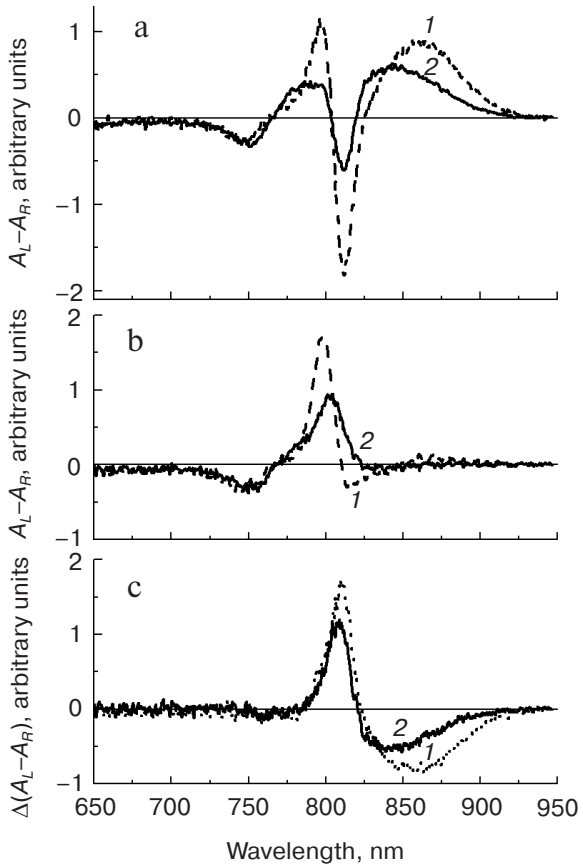

Fig. 3. a, b) CD spectra of the RC *R. sphaeroides* wild-type (*1*) and mutant I(L177)H (*2*) within the spectral range 650-950 nm as measured before (a) or after (b) potassium ferricyanide-induced (2 mM) oxidation of the primary electron donor P. CD spectra were measured using RC preparations with absorption spectra shown in Fig. 2. c) Difference CD spectra (oxidized minus reduced) of the wild-type RC (*1*) and mutant I(L177)H (*2*) calculated from the CD spectra shown in Fig. 3 (a and b).

due to out-of-plane deformations of the chlorine macrocycles and interaction with protein environment [21, 23]. It is probable that the exciton interactions of the $B_{A,B}$ molecules with other RC chromophores also contribute to the CD spectrum in this spectral region.

Upon chemical oxidation with potassium ferricyanide, the exciton bands of P in the CD spectrum disappear virtually completely in the RC preparations of the two types (Fig. 3b). The positive band at 796 nm persists under these conditions. Upon chemical oxidation, there is an slight increase in the amplitude of the positive band at 796 nm, and this band is red-shifted to 798 nm in the wild-type RC or to 804 nm in the mutant RC. The addition of potassium ferricyanide has no effect on the CD signals of the bacteriopheophytin molecules H_A and H_B within the spectral region at 750 nm (Fig. 3b). As a result, the difference CD spectra (oxidized-minus-reduced) shown in Fig. 3c contain mainly two components corresponding to two exciton transitions of the primary electron donor. The similarity between the two spectra indicates that the exciton interactions in the dimer P are conserved in mutant RC.

Difference (light-minus-dark) H_A^-/H_A spectra representing photoinduced accumulation of reduced bacteriopheophytin H_A^- at low medium redox potentials in the wild-type RC *R. sphaeroides* (*1*) and mutant I(L177)H RC (*2*) are shown in Fig. 4. The absorption changes were fully reversible, except the band at 560 nm, presumably attributed to a carotenoid molecule (long-term exposure of RC to light induced disappearance of this band). The difference spectra measured in these experiments are in good agreement with the spectra of H_A^-/H_A reported earlier for RC *R. sphaeroides* R-26 [14, 15]. The spectra are characterized by bleaching of H_A absorption bands at 762 and 542 nm, blue shift of the B band at 800 nm, and appearance of a broad absorption band of the anion radical H_A^- at 660 nm; there are also typical bands at 842-849, 909-912, and 963 nm. The fact that the spectra of the wild-type RC and mutant RC have virtually identical shape and amplitude can be regarded as evidence that amino acid substitution Ile L177 → His does not disturb the sequence of reactions resulting in photoinduced accumulation of H_A^- and interaction between chromophores involved in this process.

The photoinduced FTIR difference spectra P^+Q^-/PQ of the wild-type RC and mutant I(L177)H RC are shown in Fig. 5. Positive and negative peaks in the spectra represent absorption in the states P^+Q^- and PQ, respectively. The difference spectrum of the wild-type RC is consistent with the spectra described in the literature [24]. It follows from Fig. 5 that the shape of the difference spectrum of the mutant RC in general is similar to the

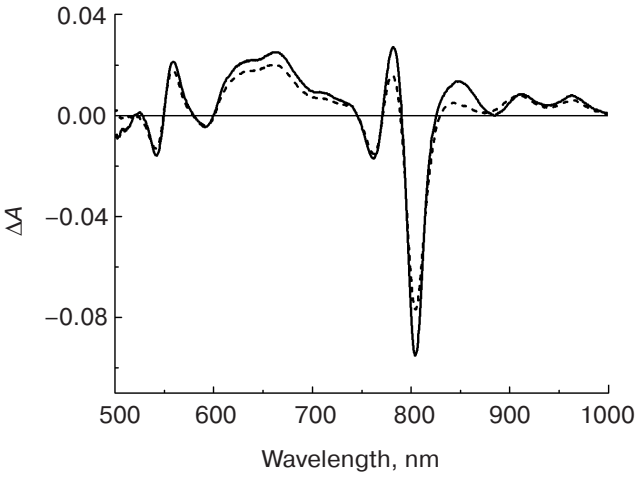

Fig. 4. Difference (light-minus-dark) spectra of photoinduced accumulation of reduced bacteriopheophytin H_A^- in the wild-type RC *R. sphaeroides* (solid line) and mutant I(L177)H RC (dashed). Spectra were recorded using RC preparations with equal (0.2 absorption unit) optical density at 760 nm.

wild-type RC spectrum shape. The RC spectra of the two types contain a broad hole-transfer band with maximum at ~2700 cm^{-1} and three peaks at ~1550, 1480, and 1290 cm^{-1} representing phase-phonon bands corresponding to deformation of porphyrin macrocycles of the P_A and P_B molecules [25]. The intensity of the spectral bands in the mutant RC was one-half to one-third that value in the wild-type RC.

Certain difference between the FTIR spectra of RC of the two types is observed at the region of vibrational stretching modes of keto-carbonyl groups at ~1650-1710 cm^{-1} (Fig. 6). Perhaps, in the FTIR spectrum of the mutant RC the positive band at 1702 cm^{-1} (stretching mode of 13^1-keto-group of P_B in the state P^+ [26]) is slightly shifted toward lower frequency and is partially compensated by a negative band of the stretching mode of 13^1-keto-group of P_A in the state P. The negative band of the 13^1-keto-group of P_A is at 1692 cm^{-1} [26]. (In the P^+Q^-/PQ FTIR spectrum of the wild-type RC *R. sphaeroides* measured at room temperature there is no spectral band at 1692 cm^{-1}. However, this band is manifested as a shoulder in similar spectra measured at cryogenic temperatures [26].) The positive band at 1713-1714 cm^{-1} is attributed to vibrations of 13^1-keto-group of P_A in the state P^+. The difference spectrum of the RC I(L177)H also contains well-pronounced spectral changes in the amid I band of vibrations of the protein carbonyl groups: in the mutant RC spectrum, there is a decrease in the amplitude of the peak corresponding to the wild-type RC spectral maximum at 1674 cm^{-1}. In addition, this peak in the mutant RC is shifted to 1668 cm^{-1}. The amino acid substitution has virtually no effect on the differential signal shape within the 1710-1750 cm^{-1} range, which is attributed to 13^3-ester groups of the primary electron donor bacteriochlorophylls [26].

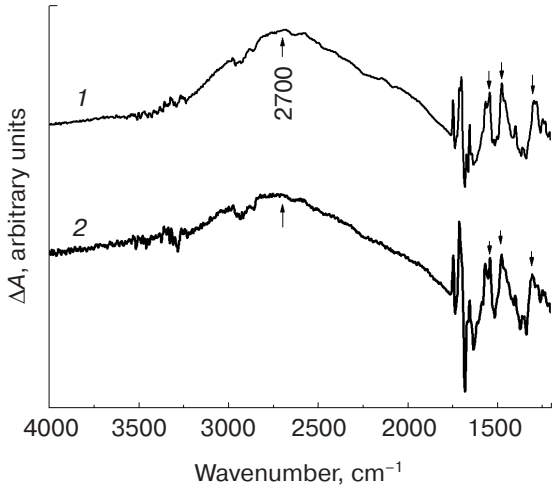

Fig. 5. Photoinduced FTIR difference spectra P^+Q^-/PQ of the wild-type RC *R. sphaeroides* (*1*) and mutant I(L177)H RC (*2*). Arrows indicate maximums of hole-transfer band (↑) and phase-phonon bands (↓). The spectra are normalized to the amplitude of the band at 2700 cm^{-1}. Spectral resolution is 4 cm^{-1}. Spectra *1* and *2* were obtained as a result of averaging of 2048 and 4096 interferograms, respectively.

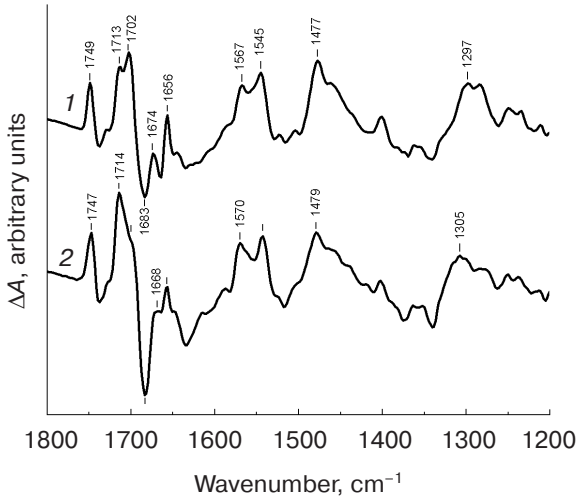

Fig. 6. Photoinduced FTIR difference spectra P^+Q^-/PQ of the wild-type RC *R. sphaeroides* (*1*) and mutant I(L177)H RC (*2*). Expanded-scale low-frequency spectral component of data shown in Fig. 5. The positions of the extrema are determined accurate to ±1 cm^{-1}.

DISCUSSION

The photoinduced FTIR difference spectra P^+Q^-/PQ obtained in this work provide direct evidence of dimeric structure of the of the primary electron donor P in the RC I(L177)H (Figs. 5 and 6). The hole-transfer band in the wild-type RC spectrum at 2700 cm^{-1} is attributed to the electronic transition $P_A^+P_B \rightarrow P_AP_B^+$ and reflects the resonance interaction between bacteriochlorophyll molecules in the oxidized dimer P^+ [24]. The intensity of the band depends on the electronic coupling degree between molecules P_A and P_B [24, 25]. The phase-phonon bands at ~1550, 1480, and 1290 cm^{-1} are spectral markers of the dimer cation-radical of the primary electron donor P^+ [25]. The presence of the band at 2700 cm^{-1} and phase-phonon bands in the photoinduced FTIR difference spectrum of the RC I(L177)H (Fig. 5) suggests that both in the wild-type RC [24, 25] and in the mutant RC the primary electron donor is a dimer with positive charge asymmetrically distributed between molecules P_A and P_B in the state P^+. The dimeric structure of P in the mutant RC is also supported by existence of the excitonic bands at 846 and 811 nm in the CD spectrum (Fig. 3). These findings are consistent with the data of low-temperature absorption spectroscopy [5].

The decrease in the intensity of the hole-transfer band and phase-phonon bands in the FTIR difference

spectrum of the mutant RC I(L177)H can be interpreted in terms of enhancement of the degree of localization of positive charge in one of the halves of the dimer cation-radical P^+ [25]. This effect can be expected in case of weakening of the electronic coupling between molecules P_A and P_B caused by modification of the dimer P structure and/or its nearest amino acid environment. This suggestion is confirmed by the low-frequency shift of the band at 1674 cm^{-1} in the mutant RC spectrum within the protein mode region (amid I). In addition, mutation-induced minor molecular rearrangement in the BChl macrocycles is evidenced by band-shifts of 13^1-keto-groups of P (Fig. 6). The stretching mode frequencies of these groups is sensitive to local amino acid environment and to charge density in the ring V of the BChl molecule [27]. The short-wavelength shift of the Q_y electronic transition of P to ~850 nm is consistent with the decrease in the electronic coupling degree in the dimer P of mutant RC (Fig. 2b). Structural modification or electrostatic interactions were earlier suggested for the site-directed mutant RC I(L177)D *R. sphaeroides*, in which isoleucine L177 is substituted by aspartic acid [17]. However, it should be expected that weakening of coupling could be associated with changes in the hole-transfer band maximum position. In contrast to this suggestion, this is not observed in the experiment. Therefore, other factors may contribute to the decrease in the intensity of the FTIR spectrum of the mutant RC I(L177)H. In particular, the presence of the P-deficient RC population (incapable of charge separation) in the mutant RC preparations would decrease the amplitude of the photoinduced IR signal. However, this effect should be relatively small, because this RC population is minor. It is obvious that a significantly larger role in this process belongs to the substantial decrease in the steady-state concentration of P^+ caused by the P^+Q^- state formation quantum yield decrease in mutant RC (57% of the wild-type RC quantum yield) [28].

There is a significant decrease in the amplitude of intense positive monomer BChl band of the CD spectrum of the mutant RC I(L177)H. In the wild-type RC, this CD band is at 796 nm (Fig. 3). Perhaps, this effect is thought to be due to disturbed interaction of monomer BChl B_B with protein and/or with other RC chromophores. Such disturbance is due to structural modification induced by the mutation Ile L177 → His.

The results of the comparative analysis of difference spectra of photo-accumulation of RC in the state with reduced bacteriopheophytin H_A^- (Fig. 4) revealed that photochemically active chromophore chain and interaction between molecules B_A and H_A were conserved in the mutant RC.

Analysis of FTIR difference spectra (Fig. 6) revealed that within the spectral region 1680-1750 cm^{-1} the wild-type RC and mutant RC I(L177)H spectra were virtually identical to each other in terms of frequency and profile of vibrational bands (except minor spectral shifts). This indicates that mutation does not induce substantial modification of the chemical structure of the 13^1-keto- and 13^3-ester groups of P_A and P_B molecules (Fig. 1) contributing to the IR spectrum. These groups are presumably not involved in formation of the hypothetical covalent bond. Unfortunately, the FTIR difference spectra (Fig. 6) do not provide direct information about the state and possible modification of the other two functional molecular groups of P_A and P_B (3^1-acetyl and 17^3-ester groups) (Fig. 1). There is overlap of IR bands of the 3^1-C=O acetyl groups with protein and quinone mode bands [29]; vibrational modes of 17^3-C=O ester groups do not contribute to the photoinduced FTIR difference spectrum [30]. However, it is well known that modification of 3^1-acetyl group of BChl *a* usually causes strong (tens of nanometers) short-wavelength shift of the Q_y-band in the electronic absorption spectrum [31]. The short-wavelength shift typical of modification of this group is not observed in the absorption bands of strongly bound BChl in the mutant I(L177)H [5]. Therefore, 17^3-ester group of one of the BChl molecules of special pair P is seemingly the most probable candidate for formation of the covalent bond in mutant RC. Given the chemical properties of the ester groups of the BChl molecules [6] and crystallographic structure of the wild-type RC *R. sphaeroides* (1pcr; Protein Data Bank) [2], the covalent bond in the mutant RC is presumably formed in the trans-esterification reaction of the 17^3-ester group of the P_A molecule with the primary alcohol group of serine L244 with contribution from imidazole of histidine L177 as a catalytic group. (It is interesting to note that this mechanism is observed in the chlorophyllase enzyme, whose active center contains a serine residue performing the function of nucleophilic agent attacking the carbonyl carbon atom of the ester group of (bacterio)chlorophyll in 17^3 position and histidine as a proton-acceptor group [32, 33]. In addition to serine and histidine, the active center of chlorophyllase contains aspartic acid, which provides stabilization of the histidine residue [32, 33]. Chlorophyllase catalyzes both the (bacterio)chlorophyll hydrolysis reaction to corresponding (bacterio)chlorophyllides and the reaction of trans-esterification with various alcohols and compounds containing primary alcohol groups [6, 34].) Alternatively, the 17^3-ester group of the B_B molecule and threonine L178 could be involved in the reaction. This could be observed during the assembly of the mutant RC provided that the reacting groups are brought close to each other. Another, perhaps less probable, interpretation implies that a covalent bond is formed during pigment extraction with organic solvents in the RC preparation. An approach to verification of this suggestion is research into the mutant RC I(L177)H with additional site-directed serine L244 substitution or threonine L178 substitution by amino acid residues free of alcohol groups. This research is presently being carried out in our laboratory.

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