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Substitution of isoleucine L177 by histidine in *Rhodobacter sphaeroides* reaction center results in the covalent binding of P_A bacteriochlorophyll to the L subunit

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Abstract In this work, we report the unique case of bacteriochlorophyll (BChl) – protein covalent attachment in a photosynthetic membrane complex caused by a single mutation. The isoleucine L177 was substituted by histidine in the photosynthetic reaction center (RC) of *Rhodobacter sphaeroides*. Pigment analysis revealed that one BChl molecule was missing in the acetone–methanol extract of the I(L177)H RCs. SDS–PAGE demonstrated that this BChl molecule could not be extracted with organic solvents apparently because of its stable covalent attachment to the mutant RC L-subunit. Our data indicate that the attached bacteriochlorophyll is one of the special pair BChls, P_A. The chemical nature of this covalent interaction remains to be identified.
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Keywords: Photosynthetic reaction center; Bacteriochlorophyll; Site-directed mutagenesis, pigment–protein binding; *Rhodobacter sphaeroides*

1. Introduction

Photosynthesis in bacteria and plants is carried out through a network of (bacterio)chlorophyll–protein complexes that absorb solar energy and convert it into a chemically useful form. In the majority of known photosynthetic complexes pigments interact with each other and with the protein environment by relatively weak contacts and normally can be easily extracted with organic solvents. One of the structurally most well studied photosynthetic complexes is the bacterial reaction center (RC), an integral membrane pigment–protein complex where the first steps of light energy conversion take place. In purple bacteria *Rhodobacter* (Rb.) *sphaeroides* the RC consists of three protein subunits (L, M, and H) and 10 non-covalently bound cofactors – four bacteriochlorophylls (BChl), two bacteriopheophytins (BPhe), two quinones, one iron atom and a carotenoid. Pigments are arranged in two almost symmetrical branches labeled A and B. Each branch starts at the primary electron donor (P, a dimer of strongly interacting bacteriochlorophyll molecules), proceeds via a monomeric BChl molecule (B_A or B_B) and a BPhe molecule (H_A or H_B) and terminates with a quinone acceptor (Q_A or Q_B) [1]. According to the current knowledge, the mutual distances and orientations of the pigments are specifically controlled by the surrounding protein [2]. For the last decades a good deal of data on protein-cofactor interactions in photosynthetic RCs was accumulated by means of site-directed mutagenesis [3].

Recently a new mutant RC of *Rb. sphaeroides* was described that had Ile in the L177 position substituted by His [4]. This residue is located in the close vicinity of B_B and P_A molecules (Fig. 1). The RC I(L177)H was found to be active in charge separation with the formation of the $P^+Q_A^-$ state. According to the data obtained, the mutation led to considerable changes in spectral properties of the RC. In particular, in the room temperature absorption spectrum of I(L177)H RC a strong blue shift of the long-wavelength P band was accompanied by a substantial decrease of its dipole strength. Besides, it was found that in acetone–methanol (7:2, v/v) extract of the I(L177)H RCs the BChl:BPhe ratio was dramatically altered [4]. In the present work, we report new data on the I(L177)H RC which suggest that Ile-His substitution in this position cause covalent attachment of a BChl molecule, namely P_A, to the L-subunit.

2. Materials and methods

Cell growth, preparation of intracytoplasmic membranes and construction of the antenna deficient strains of *Rb. sphaeroides* containing reaction centers with I(L177)H mutation were described previously [4]. For all experiments, wild-type (WT) reaction centers were those isolated from the *puf*-deficient strain DD13 [5] complemented with the wild type genes.

RCs were prepared as described earlier [6] with minor modifications. Chromatophores were treated with 0.1% (60 min) and 0.7% (30 min) lauryldimethylamine N-oxide (LDAO) followed by centrifugation after every treatment. The RCs were then purified by anion exchange chromatography on DEAE cellulose DE52 and on Fractogel EMD DEAE (S) cellulose and suspended in 20 mM Tris–HCl, pH 8.0/0.1% LDAO/180 mM NaCl buffer. The A804/A848 ratio of ~2.7 was found to be typical for purified I(L177)H RCs.

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Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; B_A and B_B, monomer BChls of the A- and B- cofactor branches, respectively; P, primary electron donor; P_A and P_B, BChls constituting P; RC, reaction center; WT, wild-type; LDAO, lauryldimethylamine N-oxide; DMSO, dimethylsulfoxide; DMFA, dimethylformamide

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RC three-dimensional structural data were downloaded from the Protein Data Bank (1OGV, [7]), and the modelling of the amino acid substitution was carried out using SwissPDB Viewer software.

Room temperature absorption spectra were recorded on Shimadzu UV-1601PC spectrophotometer. Low temperature (10 K) absorption spectra were recorded on a QE 65000 Scientific grade spectrophotometer (Ocean Optics). Sodium ascorbate was added at 1 mM final concentration in order to keep the primary donor in reduced state.

The amount of BChl and BPhe was determined by analyzing the near – infrared pigment absorption bands in acetone–methanol (7:2, v/v) [8] or acetone [9] extracts of RCs. In order to avoid the presence of water and/or detergent in the extracts the RCs (10–20 nmol) were collected at 4 °C on Microcon micro concentrators (Millipore) with the pore size 30 kD. The filters with precipitated RCs were washed 3 times with 20 mM Tris–HCl buffer pH 8.0, dried by short centrifugation and then placed into new tubes with organic solvents for pigment extraction. RC proteins were separated from pigment extracts 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. In some experiments prior to pigment extraction RCs were precipitated by addition of 26% of ammonium sulfate. When needed, 10 N HCl (1/1000 of the total volume) was added to pigment extracts for turning all bacteriochlorophylls into bacteriopheophytins [9].

Effect of pH on the BChl–RC protein binding was studied by incubation of equal amounts of wild-type and mutant RCs during 2 h at room temperature under darkness with continuous stirring in 50 mM Tris–HCl buffer containing 0.1% Triton X-100 and 8 M of urea at pH 8.0 or 3.0.

SDS–PAGE was done according to the method of [10]. Approximately 10 nmol of RCs were applied on the gel. Prior and after the coomassie brilliant blue staining the gels were scanned with a digital scanner. For absorption spectra measurements SDS–PAGE slices in 20 mM Tris–HCl buffer pH 8.0 were placed in quartz cuvette of 2 mm path length.

3. Results and discussion

In our recent paper, we showed that in the acetone–methanol (7:2) extract of I(L177)H RCs the ratio of BChl:BPhe was close to 3:2 (4:2 in the WT RCs) [4]. Originally it was suggested that the mutation I(L177)H could affect BChl content in the RC. Considerable changes in the room temperature absorption spectrum of the mutant RC, namely the blue shift and the significantly decreased oscillator strength of the Q_y P band seemed to be in line with this assumption [4]. However, this interpretation is challenged by detection of Q_y absorption bands for all four RC BChls (two monomer BChls and two BChls in the dimer P) in the low temperature absorption spectrum of I(L177)H RCs (Fig. 2). The 803 nm absorption band is attributed to Q_y transition of B_A and the shoulder on the red

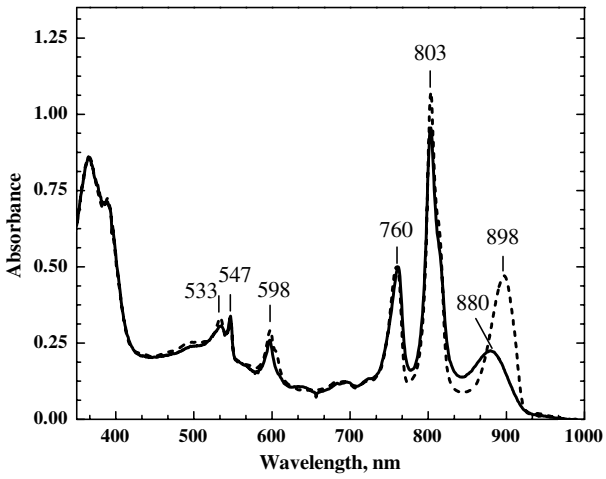

Fig. 2. Low temperature (10 K) absorption spectra of the wild-type (dashed line) and I(L177)H (solid line) RCs normalized at 760 nm.

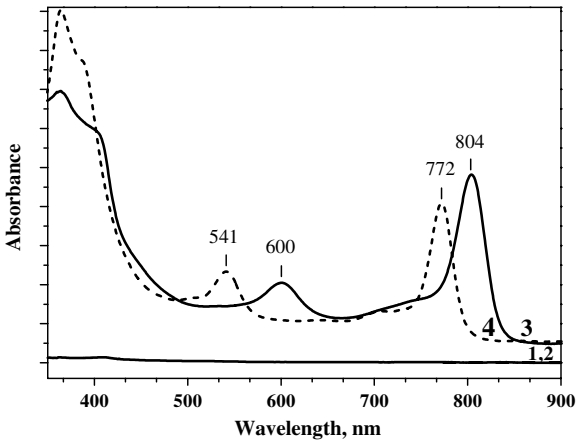

Fig. 3. Room temperature absorption spectra of the RC proteins after pigment extraction. 1, 2 - WT RC, 3, 4 - I(L177)H RC, 2 , 4 – HCl was added to the samples.

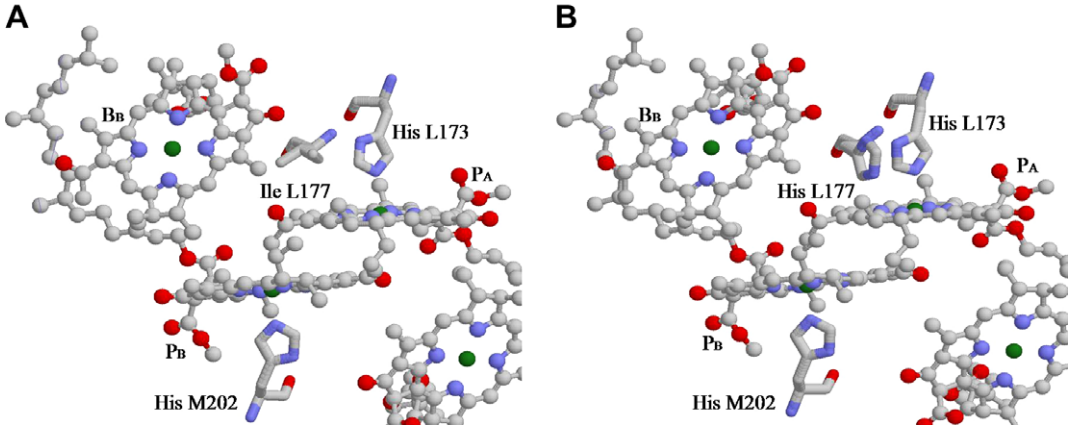

Fig. 1. Location of isoleucine (A) and histidine (B) in the position L177 in *Rb. sphaeroides* RC structure. (B) is a model based on the structure of the WT RC (A) [7].

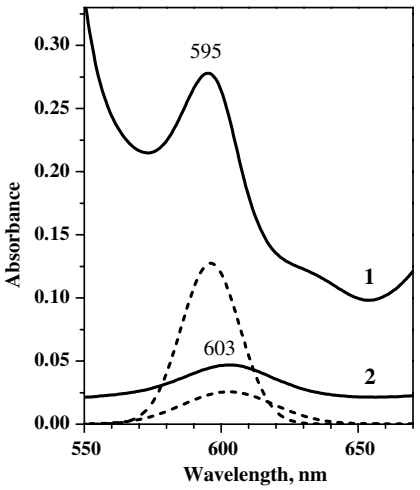

Fig. 4. Absorption spectra of the I(L177)H RCs taken for pigment analysis (1) and of the same RC protein after pigment extraction (2). Gaussian decomposition of the spectra is shown by dashed lines.

side of this band is attributed to Q_y transition of B_B. Despite the substantial differences in the absorption spectra of WT and I(L177)H RCs in the region of P Q_y transition that probably reflect some structural changes in/around the primary donor molecule, the strong red shift of this band observed upon lowering temperature from 293 K (847 nm, [4]) to 10 K (880 nm, Fig. 2) indicates that this band can be attributed to the dimer P (P_A and P_B molecules) absorption [11]. It was thus decided to reexamine the pigment content of I(L177)H RCs using Microcon filters instead of DEAE columns [4] for RC concentration prior to pigment extraction. This approach provided an easy way to collect RC protein after pigment extraction. The amount of total pigments/RC in the acetone–methanol extracts was shown to be 6.0 ± 0.2 for the WT and 5.0 ± 0.2 for I(L177)H. Pigment extraction yielded BChl/BPhe ratios of 2.0 ± 0.05 for WT and 1.5 ± 0.1 for I(L177)H consistent with four BChls and two BPhes in wild-type RCs and three BChls and two BPhes in the mutant RCs. It was noticed that after pigment extraction the pellet of WT RC protein had white color while under the same conditions the pellet of the mutant RC protein had bright green color. None of the strong organic solvents (acetone, DMSO or DMFA) could extract pigments from the mutant RCs completely. Fig. 3 shows absorption spectra of the dissolved RC proteins after pigment extraction. No absorbance bands were observed in WT RC protein verifying that all pigments were extracted (spectra 1 and 2, showing material without and with acidification, respectively). The absorption spectrum of I(L177)H RC after pigment extraction shows a Soret band at around 360 nm and two bands at 804 nm (Q_y) and 600 nm (Q_x) that presumably correspond to monomeric BChl *a* absorbance in *Rb. sphaeroides* RCs (spectrum 3). After addition of HCl the 804 nm and 600 nm bands were blue shifted to 772 and 541 nm, respectively (spectrum 4), consistent with the conversion of BChl into BPhe. These data allow us to conclude that the pigment which can not be extracted from I(L177)H RC and remains attached to RC protein is BChl *a*. It is notable that the Q_y transition band of this BChl at 804 nm (Fig. 3, spectrum 3) is red-shifted with respect to that of BChl in acetone–methanol (771 nm) [8], and the Q_y transition band of BPhe at 772 nm (Fig. 3, spectrum 4) is red-shifted with respect to Q_y of BPhe at 760 nm in the I(L177)H RC absorption spectra (Fig. 2 and [4]) corroborating the fact of specific pigment-protein attachment. The quantity of the protein-attached BChl was estimated by comparison of the 595 nm BChl Q_x band amplitude in the absorption spectrum of the I(L177)H RCs taken for analysis and 605 nm band in the absorption spectrum of the same RC sample after pigment extraction (Fig. 4). Within the limits of the approach it was shown that approximately one BChl molecule (0.8 ± 0.1) per one I(L177)H RC remains attached to the RC protein.

In order to examine the stability of the pigment-protein binding the mutant I(L177)H RCs were incubated at pH 3.0 and pH 8.0 in the presence of 8 M urea for soft protein denaturing. It was found that the pigment-protein attachment in I(L177)H RC was stable at both pH, and the observed conversion of BChl into BPhe at pH 3.0 did not affect the attachment (data not shown). To check further the strength of the BChl–protein binding the RC samples were analyzed by PAGE and SDS–PAGE. In polyacrylamide gel RC pigments and protein migrated in the gel as one broad spot (not shown). During 18% SDS–PAGE due to disruption of weak intermolecular interactions forming tertiary structure of the RC complex the

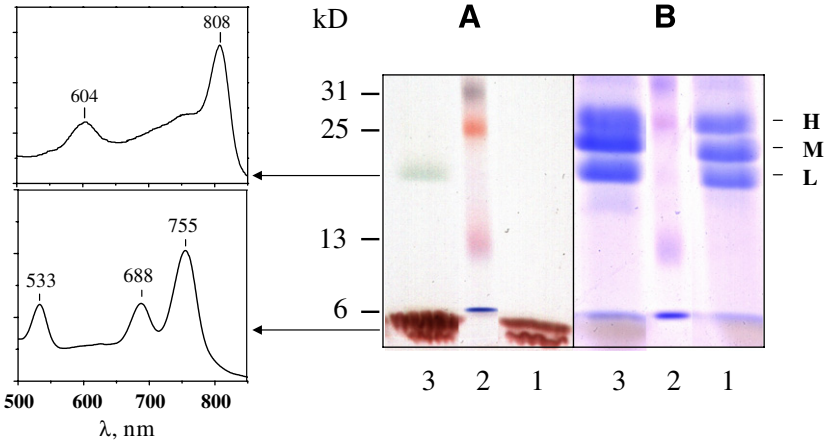

Fig. 5. 18% SDS–PAGE of isolated *Rb. sphaeroides* RCs. Line assignments: (A) No gel staining; 1 – WT RCs, 2 – protein marker, 3 – I(L177)H RCs; (B) the same gel, coomassie blue staining, numbering as in (A). Absorption spectra of indicated pigment lines are shown on the left.

protein subunits and pigments migrated through the gel separately as shown in Fig. 5. Tracks 1 and 3 (Fig. 5B) show three main protein bands (H, M, L) in the samples of the wild-type and mutant RCs, respectively. One can see that prior to gel staining (Fig. 5A) the brown-colored spots of pigments (BPhes and carotenoids) in both samples migrate through the gel on the level of the 6 kD marker. In the absorption spectrum of these pigments measured directly in the gel, absorption bands at 755 nm and 533 nm presumably of the free BPhes were observed. The absorption band at 688 nm can be ascribed to degraded bacteriochlorines. Track A3 (Fig. 5) shows that in the sample of I(L177)H RCs besides the main brown pigment spot there is an additional green band that migrates between 13 kD and 25 kD protein markers. Gel staining reveals that this green band is superimposed with the RC L-polypeptide (Fig. 5B, track 3). In the absorption spectrum of the green band, measured directly in the gel, absorption bands at 808 nm and 604 nm apparently attributed to BChl were detected. This data shows that in the mutant I(L177)H RC a BChl molecule is tightly, presumably covalently linked to the L-subunit.

The question that arises next – what is the origin of the BChl molecule which is strongly attached to the L-polypeptide of the I(L177)H RC protein? As mentioned above, L177 position is located in the immediate vicinity of B_B and P_A molecules, and so presumably one of these two chromophores could be involved in that linkage. The results obtained seem to indicate that this is the P_A molecule. First of all, in the absorption spectra and in the photo-induced differential spectrum of the I(L177)H RC the most dramatic changes were observed in the region of P bands (Fig. 2 and [4]). Then, it is noteworthy that under denaturing conditions of SDS–PAGE the BChl molecule associated with the RC L-subunit remains green colored while all other RC bacteriochlorophylls lose Mg atoms and turn into bacteriopheophytins (Fig. 5A). This observation leads to the conclusion that during SDS–PAGE an axial ligand for the central Mg^{2+} of that protein-attached BChl molecule remains located in the position that guarantees its stable coordinating contact with the Mg atom. Therefore this ligand must be sited in the L-subunit of RC. According to the bacterial RC structure the L-subunit contains His L173 which serves as a ligand for P_A BChl, and this histidine is located close to the locus of the mutation [1]. As for the axial ligand of the B_B molecule, His M182, it is positioned in M subunit of RC. Thus, most likely the BChl molecule that is covalently attached to the I(L177)H RC protein is P_A. Apparently the binding of P_A to the L-subunit provides increased stability of Mg ligation during SDS–PAGE comparing to the other BChl molecules in the mutant RC.

In BChl molecule a few side groups were shown to be reactive and were used to obtain BChl derivatives [12]. However, hitherto there is no data on a chromophore–protein covalent binding in photosynthetic membrane pigment–protein complexes. Only in phycobiliproteins, non-membrane light-harvesting complexes of cyanobacteria and red alga, linear tetrapyrrole phycobilins are known to be covalently attached to conserved cysteines [13]. Generally, examples of covalent binding of chromophores to apoproteins are very rare among the wide variety of pigment–protein complexes. Covalent bonds between side vinyl groups of hems and thiol groups of cysteines are present in the structure of *c*-type cytochromes. In rhodopsin and bacteriorhodopsin the chromophore retinal is covalently bound to the protein via a protonated Shiff base [14]. Shiff bases are normally hydrolyzed at pH below 5. In our experiments the BChl–protein binding in I(L177)H RCs remained stable both at pH 3.0 and pH 8.0, demonstrating sizeable strength of the found covalent binding.

To summarize, in this work it was shown for the first time that a single site-directed mutation in the RC of *Rb. sphaeroides* caused tight, presumably covalent attachment of BChl molecule to the RC protein. The chemical nature of this attachment remains to be identified and its consequences for the RC function are currently under investigation. The RC I(L177)H seems to be a promising example for the study of protein-cofactor interactions in photosynthetic complexes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.11.032.

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