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| Title            | Importance of specific hydrogen bonds of archaeal rhodopsins for the binding to the transducer protein †   |
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| Citation         | JOURNAL OF MOLECULAR BIOLOGY, 357(4), 1274-1282<br><a href="https://doi.org/10.1016/j.jmb.2006.01.061">https://doi.org/10.1016/j.jmb.2006.01.061</a> |
| Issue Date       | 2006-04-07   |
| Doc URL          | <a href="http://hdl.handle.net/2115/10532">http://hdl.handle.net/2115/10532</a>  |
| Type             | article (author version)   |
| File Information | JMB01687_2.pdf   |



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(JMB revised; D-05-01687)

## **Importance of specific hydrogen bonds of archaeal rhodopsins for the binding to the transducer protein<sup>†</sup>**

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Running Title: A transducer binding to four archaeal rhodopsins

Key words: photo-sensor, protein-protein interaction, ion pump, sensory  
rhodopsin, phoborhodopsin

<sup>†</sup>This work was supported by grants from the Japanese Ministry of Education,  
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#### Footnotes

<sup>1</sup>Abbreviations: *N. pharaonis*, *Natronomonas pharaonis*; *H. salinarum*, *Halobacterium salinarum*; bR, bacteriorhodopsin from *H. salinarum*; hR, halorhodopsin from *N. pharaonis*; sR, sensory rhodopsin from *H. salinarum*; pR, phoborhodopsin from *N. pharaonis*; HtrII, halobacterial transducer protein II from *N. pharaonis*; ITC, isothermal titration calorimetry;  $K_D$ , dissociation constant; DDM, n-dodecyl- $\beta$ -D-maltoside

## ABSTRACT

Four rhodopsins, bacteriorhodopsin (bR), halorhodopsin (hR), sensory rhodopsin (sR) and phoborhodopsin (pR) exist in archaeal membranes. bR and hR work as a light-driven ion pump. sR and pR work as a photo-sensor of phototaxis, and form signaling complexes in membranes with their respective cognate transducer proteins HtrI (with sR) and HtrII (with pR), through which light signals are transmitted to cytoplasm. What is the determining factor(s) of the specific binding to form the complex? Binding of the wild or mutated rhodopsins with HtrII was measured by Isothermal titration calorimetric analysis (ITC). bR and hR could not bind with HtrII. On the other hand, sR could bind to HtrII, although the dissociation constant ( $K_D$ ) was about 100-times larger than that of pR. X-ray crystallographic structure of the pR/HtrII complex (Gordeliy et al. Nature 419, 484-487) revealed the formation of two specific hydrogen bonds whose pairs are Tyr199<sup>pR</sup>/Asn74<sup>HtrII</sup> and Thr189<sup>pR</sup>/Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup>. To investigate the importance of these hydrogen bonds, the  $K_D$  for the binding of various mutants of bR, hR, sR and pR with HtrII was estimated by ITC. The  $K_D$  value of T189V<sup>pR</sup>/Y199F<sup>pR</sup>, double mutant/HtrII complex, was about 100-fold larger than that of the wild-type pR whose  $K_D$  value was 0.16  $\mu$ M. On the other hand, bR and hR double mutants, P200T<sup>bR</sup>/V210Y<sup>bR</sup> and P240T<sup>hR</sup>/F250Y<sup>hR</sup>, were able to bind with HtrII. The  $K_D$  value of these complexes was estimated to be  $60.1 \pm 10.7$   $\mu$ M for bR and to be  $29.1 \pm 6.1$   $\mu$ M for hR while the wild bR and hR did not bind with HtrII. We concluded that these two specific hydrogen bonds played important roles in the binding between the rhodopsins and transducer protein.

## INTRODUCTION

Many organisms utilize light as energy source and as a signal. Retinal proteins (rhodopsins) have retinal as a chromophore and exist in various organisms; *i.e.*, archaea, eubacteria, and eukaryotes (1-4). The archaeon *Halobacterium salinarum*, halophilic prokaryote, contains four retinal proteins (rhodopsins), *i.e.*, bacteriorhodopsin (bR) (5), halorhodopsin (hR) (6), sensory rhodopsin (sR, also called sensory rhodopsin I, sRI) (7) and phoborhodopsin (pR, also called sensory rhodopsin II, sRII) (8) (see Fig. 1). Functionally, these proteins are distinctly different. The function of bR and hR is a light-driven outward proton pump and a light-driven inward chloride pump, respectively. sR and pR work as light-sensitive photo-receptors, and they form a signaling complex in archaeal membranes with their cognate transducer proteins, HtrI and HtrII, respectively (7-9) (Fig. 1). These complexes transmit the light signal to a cytoplasmic part of the transducer to activate the phosphorylation cascades that modulate the flagellum motors (10). The transducer (HtrI or HtrII) is a two-transmembrane helical protein that belongs to a family of methyl-accepting chemotaxis proteins (MCPs) (11,12). MCP exists as a homodimer composed of a 50-60 kDa subunit and forms a ternary complex with CheA and CheW. Using this signaling system, the bacterium can avoid harmful near-UV light (negative phototaxis) or can be attracted to longer wavelength light where bR and hR can utilize light energy (positive phototaxis) (7-9).

Spudich and co-workers obtained evidence showing that the signal transfer between the receptor (sR or pR) and its respective cognate Htr (HtrI or HtrII) was proceeded by the trans-membranous region; they made a variety of chimeras of Htr's which were composed of the trans-membranous and cytoplasmic part of two Htr's (9). Only when the correct pair of receptor and its cognate trans-membranous helix was formed, the phototaxis was observed.

What is the specificity of the interaction between the rhodopsin receptors and transducers? Previously, we examined amino acid residues that may be important to the binding of the pR with HtrII whose complex catalyzes the negative phototaxis from blue-green light (~ 500 nm), and the importance of Asp193<sup>pR</sup>, Thr204<sup>pR</sup> and the linker region of HtrII have been shown (13-15). However, the contribution of these residues to the binding was low, and therefore another factor(s) is important for the specific binding between the rhodopsin and the transducer. The x-ray crystallographic structure of the pR/HtrII complex reveals the formation of two hydrogen bonds whose pairs are Tyr199<sup>pR</sup>/Asn74<sup>HtrII</sup> and Thr189<sup>pR</sup>/Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup> (16) (Fig. 2). In this paper, we demonstrate that these two hydrogen bonds play important roles in the binding between the rhodopsins and transducer protein. In addition, it is shown that bR and hR that originally cannot bind with HtrII become able to bind when hydrogen-bond forming amino acid residues are introduced at the proper positions of rhodopsins.

## **MATERIALS AND METHODS**

### ***Protein expression and purification***

Expression plasmids for bR, hR (from *Natronomonas pharaonis*), pR (from *N. pharaonis*) and HtrII (from *N. pharaonis*) were constructed as previously described (17-20). The plasmid including a full-length *sop* (the gene of sensory rhodopsin) with a fusing histidine tag was prepared by PCR using genomic DNA from the *H. salinarum* strain R1, as described in Ref 21. PCR was carried out using two oligonucleotide primers introducing a 5'-*Nde*I (sense primer) and 3'-*Xho*I (antisense primer) restriction site. The stop codon was deleted during amplification. A PCR product containing the *sop* gene was obtained, purified, and subcloned into a plasmid vector pGEM-T Easy (Promega, Madison, WI).

The construction of the expression plasmid is essentially the same as that previously described (20, 22).

All mutant genes were constructed by PCR using the QuickChange site-directed mutagenesis method. The oligonucleotide primers were designed from nucleotide sequences in the Genbank database. The DNA obtained was sequenced using a DNA sequencing kit (Applied Biosystems, Foster City, CA). All the constructed plasmids were analyzed using an automated sequencer (377 DNA sequencer, Applied Biosystems).

All proteins including mutant proteins were expressed in *E. coli* BL21 (DE3) cells (17, 20). The preparation of crude membranes and purification of the proteins were performed using essentially the same method as previously described (17, 22). The sample was concentrated by ultrafiltration (UK-50, Adantech, Tokyo, Japan) and completely exchanged by dialysis (15) against media. Its composition was typically 300 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 0.05 % n-dodecyl- $\beta$ -D-maltoside (DDM), but for sR, 4 M NaCl was used instead of 300 mM NaCl.

### ***Analysis of HtrII-induced thermal stabilization***

Absorption spectra were taken using a Model V-560 spectrometer (Jasco, Tokyo, Japan). The temperature was kept at the desired value by circulating thermostated water, and the temperature inside the cuvette was directly measured with a thermocouple. The activities of various rhodopsins (bR, hR, sR and pR) after incubation at high temperature were estimated from the absorbance of  $\lambda_{max}$  (23). During incubation, the suspension became turbid, maybe due to the aggregation by denatured proteins. Therefore, before the spectral measurement, the sample was briefly centrifuged at 15,000 g for 1 min to remove the aggregated protein. This method is easy to examine whether

rhodopsins can bind with the transducer, but does not give  $K_D$  values. Hence, this method was used in this paper as an auxiliary method to confirm the ability of binding or no binding deduced by ITC.

### ***Isothermal titration calorimetry (ITC) measurement***

For ITC experiments, the rhodopsins (bR, hR, sR and pR) and HtrII solutions were completely exchanged by dialysis against the buffer solution described above for 1 week using a 3-kDa cutoff dialysis cassette. All ITC experiments were performed using a VP-ITC Micro Calorimeter (Microcal Inc., Studio City, CA). For the control experiments, DDM-buffer solutions (0.05%) without proteins were used to ensure no effect caused by the detergent. The binding parameters were estimated using the following binding scheme:



where  $n$  represents the number of HtrII molecules required for the formation of the complex with rhodopsin. Data were evaluated by employing an Origin-ITC software package.

## **RESULTS**

### ***Binding between the wild-type rhodopsin (bR, hR, sR or pR) and HtrII***

In an effort to determine whether HtrII binds with four retinal proteins, bR, hR, sR and pR, we performed ITC measurements (see the Materials and Methods and ref. 15). Previously, Engelhard and co-workers reported that the dissociation constant of pR/HtrII complex was quantitatively estimated using ITC (24). Figure 3 shows titration curves of the wild-type bR, hR, sR and pR with HtrII. In these experiments, bR, hR and pR and sR were maintained at 318 or 308 K, and these rhodopsin solutions were added to the HtrII solution in increments of 10  $\mu\text{L}$  using a syringe. Measurement for sR was performed at 308

K and in the presence of a high salt concentration (4 M NaCl) because they aggregated at temperatures over 313 K and in a lower salt concentration (1 M NaCl <) (25). The effect of temperature on the dissociation constants was neglected for the case of either 318 or 308 K, because of the small (3%) difference in the temperatures. Other thermodynamic quantities were calculated under the consideration of the temperature (see Table 1). The dissociation constants of the sR/HtrII and pR/HtrII complexes were estimated as  $12.6 \pm 1.8$  and  $0.16 \pm 0.06$   $\mu\text{M}$ , respectively, whereas bR and hR showed neither a release nor uptake of heat (Fig. 3), suggesting no binding activity with HtrII.

At high temperature, the time-dependent color loss of pR, denaturation, was observed, and we reported that HtrII retarded the denaturation rate of pR in a previous paper (23). It was shown that the increase in the thermal stability was a good estimation method for the binding activity in spite of being semi-quantitative. The increase in the HtrII-induced thermal stability of sR was detected, whereas the increases were not observed for bR and hR (data not shown). The results of both ITC and the thermal stability clearly show that bR and hR cannot bind with HtrII whereas the receptor proteins, sR and pR, can bind with HtrII. Note that the binding of sR with HtrII is weak by the factor of about 2 orders, because HtrII is not a primary partner.

### ***Importance of the Thr189 and Tyr199 position of receptors (sR and pR) for the binding with HtrII***

The x-ray crystallographic structure of the pR/HtrII complex reveals the formation of two specific hydrogen bonds between Tyr199<sup>pR</sup> and Asn74<sup>HtrII</sup> and between Thr189<sup>pR</sup> and Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup> (Fig. 2) (16). Figure 4 (right panels) shows the titration curves of T189V<sup>pR</sup>/Y199F<sup>pR</sup> mutant pR with HtrII. Note that in this mutant, hydrogen-bonding amino acid residues were replaced. The

dissociation constant ( $K_D$ ) of this complex increased by nearly two orders (9.9 from 0.16  $\mu\text{M}$ ) and was almost identical to that of the signaling complex, *i.e.* the complex between M-intermediate of pR ( $pR_M$ ) and HtrI (15  $\mu\text{M}$ ) (20). This result clearly suggests that these two hydrogen bonds are very important for the binding between pR and HtrII. The  $K_D$  values of a T189V<sup>pR</sup> and a Y199F<sup>pR</sup> single mutant were estimated as  $0.73 \pm 0.17$  and  $0.35 \pm 0.10$   $\mu\text{M}$ , respectively. In these experiments, temperature was maintained at 318K.

The similar experiment using sR was carried out.  $K_D$  values of F199Y<sup>sR</sup> (corresponding to Tyr199<sup>pR</sup>) and T189V<sup>sR</sup> (corresponding to Thr189<sup>pR</sup>) single mutants as well as T189V<sup>sR</sup>/F199Y<sup>sR</sup> double mutant were estimated as  $10.1 \pm 3.8$ ,  $22.3 \pm 3.4$  and  $56.6 \pm 10.7$   $\mu\text{M}$ , respectively (left panels in Fig. 4 and summarized in Fig. 6). As described above, a  $K_D$  value of the wild-type sR is 12.6  $\mu\text{M}$ , which is larger than that of F199Y<sup>sR</sup>, because an amino acid residue of the wild-type sR corresponding to Thr189<sup>pR</sup> is Thr, and hence F199Y<sup>sR</sup> has two hydrogen bond-forming amino acid residues. The  $K_D$  values of the various mutants and the number of hydrogen bonds putatively formed are well correlated (Fig. 6).

### ***Binding of mutant bR and hR in which two hydrogen-bonding amino acid residues are introduced***

We further tried to reveal the importance of these two hydrogen bonds. As described above, bR neither hR can bind to HtrII (or the  $K_D$  values are too large to be determined by ITC). What happened when the hydrogen bonding amino acid residues were introduced into these ion-pumping rhodopsins? Figure 5(a) shows the titration curves of P200T<sup>bR</sup> (corresponding to Thr189<sup>pR</sup>), V210Y<sup>bR</sup> (corresponding to Tyr199<sup>pR</sup>), P240T<sup>hR</sup> (corresponding to Thr189<sup>pR</sup>) and F250Y<sup>hR</sup> (corresponding to Tyr199<sup>pR</sup>) mutants with HtrII. These four

mutants of bR and hR do not show any heat flow (Fig. 5 (a)), suggesting no binding activity with HtrII. No binding was also detected by the HtrII-induced thermal stabilization (data not shown). The  $K_D$  values of these mutants may be greater than  $>300 \mu\text{M}$  because the detectable limitation of the heat changes is about  $>300 \mu\text{M}$ .

Of interest, the binding of mutants having two hydrogen bonding residues is observed:  $K_D$  values of P200T<sup>bR</sup>/V210Y<sup>bR</sup> and P240T<sup>hR</sup>/F250Y<sup>hR</sup> mutants were estimated as  $60.1 \pm 10.7$  and  $29.1 \pm 6.1 \mu\text{M}$ , respectively (Fig. 5 (b)). Consistently, the HtrII-induced thermal stability changes of the P200T<sup>bR</sup>/V210Y<sup>bR</sup> and P240T<sup>hR</sup>/F250Y<sup>hR</sup> mutants were detected (data not shown).

## DISCUSSION

The X-ray structure (16) shows that three hydrogen bonds between pR and HtrII are formed at two amino acid residues of pR, whose pairs are Tyr199<sup>pR</sup>/Ans74<sup>HtrII</sup>, Thr189<sup>pR</sup>/Glu43<sup>HtrII</sup> and Thr189<sup>pR</sup>/Ser62<sup>HtrII</sup>. Using mutants of pR at the 199<sup>th</sup> and/or the 189<sup>th</sup> position, this paper proved this experimentally. Hippler-Mreyen et al. (24) reported that the hydrogen bond formed between the phenolic group of Tyr199<sup>pR</sup> and Asn74<sup>HtrII</sup> has only a minor contribution to the binding, because  $K_D$  value of Y199F<sup>pR</sup>/HtrII complex ( $0.3 \mu\text{M}$ ) is similar to the value of the wild-type pR/HtrII complex ( $0.16 \mu\text{M}$ ). They assumed that the phenolic ring – phenolic ring interaction between Tyr199<sup>pR</sup> and Phe28<sup>HtrII</sup> is important for the interaction between pR and HtrII. The existence of this phenolic ring – phenolic ring ( $\pi - \pi$ ) interaction was also suggested in our previous paper (23). The present result argues against the primary importance of this phenolic ring – phenolic ring ( $\pi - \pi$ ) interaction for the binding: If this interaction exerts primarily to bind, no significant increase in  $K_D$  should be

observed for a combination of a Y199F<sup>pR</sup>/T189V<sup>pR</sup> double mutant and HtrII, because the phenolic ring – phenolic ring interaction is still alive. As is seen from pR data in Fig. 6, the large increase in  $K_D$  values is observed only when pR protein is mutated simultaneously at both sites. We further did the similar experiment using a HtrII mutant, S62A<sup>HtrII</sup>. The  $K_D$  value of 9.5  $\mu\text{M}$  for a combination of a Y199F<sup>pR</sup>/T189V<sup>pR</sup> double mutant and S62A<sup>HtrII</sup> was obtained while  $K_D$  value for a combination of a T189V<sup>pR</sup> single mutant and S62A<sup>HtrII</sup> was 0.7  $\mu\text{M}$  in which the hydrogen bond at Tyr199 was possibly formed (compare 0.16  $\mu\text{M}$  of the wild-type combination). This indicates the importance of the hydrogen bond at the Tyr199 position of pR. From these observations together with the results of Hippler-Mreyen et al. (24), we assume as follows: In the case where the hydrogen bond forms between Thr189<sup>pR</sup> and Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup>, the phenolic ring (Tyr199<sup>pR</sup>) – phenolic ring (Phe28<sup>HtrII</sup>) interaction works instead of the hydrogen bonds although the ring-ring interaction may be weak in comparison with the hydrogen bond. On the other hand, in the case where the hydrogen bond breaks between Thr189<sup>pR</sup> and Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup>, the hydrogen bond between Tyr199<sup>pH</sup> and Asn74<sup>HtrII</sup> becomes important instead of the ring-ring interaction. In this sense, the hydrogen bond between Thr189<sup>pR</sup> and Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup> might be more important than that between Tyr199<sup>pH</sup> and Asn74<sup>HtrII</sup>, which may be stemmed from the formation of two hydrogen bonds.

The similar story might be accepted for sR: the  $K_D$  value of sR (Thr189, Tyr199), 10.1  $\mu\text{M}$ , was changed to a small increase to 12.6  $\mu\text{M}$  due to the mutation of the 199<sup>th</sup> position, while the mutation at the 189<sup>th</sup> position induced larger increase in the  $K_D$  value (from 10.1 to 22.3  $\mu\text{M}$ ) as shown in Fig. 6. On the other hand, for bR and hR, the simultaneous formation of the hydrogen bonds at the two positions seems indispensable.

Are not the hydrophobic and/or van der Waals interactions important?

Even the hydrogen-bond-less mutant (T189V<sup>pR</sup>/Y199F<sup>pR</sup>) of pR has a binding activity with HtrII (Fig. 4 and 6) although the binding is about 100-times weaker. Of interest, bR and hR mutants (P200T<sup>bR</sup>/V210Y<sup>bR</sup> and P240T<sup>hR</sup>/F250Y<sup>hR</sup>) can bind with HtrII in spite of the weaker binding than that of the wild-type pR/HtrII complex (Fig. 6). These results suggest that the hydrophobic and/or van der Waals interaction is also important for the binding between rhodopsins and transducers. Note that the X-ray structure (16, 26) shows intimate contact between pR and HtrII; The helix 2 of HtrII is nested in the groove formed by helices F and G of pR (see later discussion).

Table 1 lists the thermodynamic values of the binding. Hippler-Mreyen et al. obtained the thermodynamic data of the binding between pR and HtrII (24). Their values are  $\Delta G = -41.35 \text{ kJ mol}^{-1}$ ,  $\Delta H = -17.9 \text{ kJ mol}^{-1}$  and  $\Delta S = 73.74 \text{ J mol}^{-1}\text{K}^{-1}$ . In comparison with Table 1,  $\Delta G$  values of both are almost the same while  $\Delta H$  of our data is larger by about  $11 \text{ kJ mol}^{-1}$  (absolute value) and hence the value of the entropy change is different each other. The reason for this difference is not known at present. Table 1 indicates that values of  $\Delta G$  for bR, hR, sR and their mutants are contributed almost from  $\Delta H$  changes, suggesting that the formation of the hydrogen bonds is the main origin of the binding. On the other hand, the data of pR show large entropy changes and less contribution of  $\Delta H$  to  $\Delta G$  than those of other rhodopsins. The exact reason is not known at present. However, a possible reason is: Formation of the hydrogen bonds put pR and HtrII together. Since both the interface surfaces of these proteins match geometrically each other (26), the bound lipids or detergent molecules may be squeezed out from the binding interface, which leads to an increase in  $\Delta S$  and a change in  $\Delta H$ . This intimate contact will result in the strong binding.

During the photocycling of pR, how do these hydrogen bonds change?

This is an interesting and important question to be investigated. Recently, we applied Fourier transform infrared (FTIR) spectroscopy to the active M intermediate of pR in the absence and presence of HtrII (27, 28). We found that the hydrogen bond of Asn74<sup>HtrII</sup> is strengthened in M, because of the change in the interaction with Tyr199<sup>pR</sup>. However, Bergo et al. reported the disappearance of the hydrogen bond in the M and O photointermediates (29). Thus, the conclusion on this point is controversial yet, but it is certain that the hydrogen bonding between rhodopsins and HtrII is important not only for the binding at the ground state but also for the signal transfer reaction from pR to HtrII. Previously, we reported that the signal transduction from pR to HtrII accompanies a weakened binding in the M-state of pR (30,31). As pointed out above, Tyr199<sup>pR</sup> forms hydrogen bonds with Asn74<sup>HtrII</sup> not only in the ground state, but also in the active M-state (20, 30) (although the opposite observation (29) was reported). Hence, the hydrogen bonds between Thr189<sup>pR</sup> and Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup> should be broken at M-state or weakened so that the interaction between the receptor and the transducer becomes weak. Thus, two hydrogen bonds may play different roles for the signal transduction through the photocycling. This point should be awaited for a further detailed investigation.

In this study, we show that bR, hR and sR can also potentially bind with HtrII if the proper amino acid residues are introduced to form hydrogen bonds with HtrII. We previously reported that pR has a proton pumping activity, whereas the pR/HtrII complex could not pump (30). In the case of sR, it was reported that the removal of HtrI allows electrogenic proton transfer by sR (31, 32). Therefore, following questions arise: Do the bR (double mutant)/HtrII and hR (double mutant)/HtrII complexes have the ion-pumping activity? Do the sR/HtrII and pR (double mutant)/HtrII complex have a photo-signal transfer activity? Can we make a protein that has both pumping activity and photo-signal

transfer activity? These are our next focus. In addition, we should examine whether these two hydrogen bonds may change during photocycling and if it is the case, how these changes would contribute the signal transduction.

#### **ACKNOWLEDGMENT**

We thank Dr. Harumi Fukada for her expert technical assistance with the ITC measurements and Dr. Seiji Miyauchi for his stimulus discussions.

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**Table 1.** Thermodynamic parameters for the binding of various wild-type or mutants of rhodopsins with HtrII

|                 | $K_D$<br>( $\mu\text{M}$ ) | $\Delta G$<br>( $\text{kJ mol}^{-1}$ ) | $\Delta H$<br>( $\text{kJ mol}^{-1}$ ) | $-T\Delta S$<br>( $\text{kJ mol}^{-1}$ ) |
|-----------------|----------------------------|--|--|--|
| bR              | >300                       | ND                                     | ND                                     | ND                                       |
| bR(P200T)       | >300                       | ND                                     | ND                                     | ND                                       |
| bR(V210Y)       | >300                       | ND                                     | ND                                     | ND                                       |
| bR(P200T/V210Y) | 60.1                       | -25.7                                  | -24.2                                  | -1.5                                     |
| hR              | >300                       | ND                                     | ND                                     | ND                                       |
| hR(P240T)       | >300                       | ND                                     | ND                                     | ND                                       |
| hR(P250Y)       | >300                       | ND                                     | ND                                     | ND                                       |
| hR(P240T/P250Y) | 29.1                       | -27.6                                  | -23.4                                  | -4.2                                     |
| sR              | 12.6                       | -28.9                                  | -28.4                                  | -0.5                                     |
| sR(T189V)       | 56.6                       | -25.1                                  | -26.3                                  | +1.2                                     |
| sR(F199Y)       | 10.1                       | -29.5                                  | -29.7                                  | +0.2                                     |
| sR(T189V/F199Y) | 22.3                       | -27.4                                  | -27.2                                  | -0.2                                     |
| pR              | 0.16                       | -41.4                                  | -29.3                                  | -12.1                                    |
| pR(T189V)       | 0.73                       | -37.4                                  | -28.4                                  | -9.0                                     |
| pR(Y199F)       | 0.35                       | -39.3                                  | -28.4                                  | -10.9                                    |
| pR(T189V/Y199F) | 9.9                        | -30.5                                  | -25.9                                  | -4.1                                     |

The temperature was 318 K for bR, hR, pR and their mutants, and was 308 K for sR and its mutants. The medium contained 300 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 0.05 % n-dodecyl- $\beta$ -D-maltoside (DDM), but for sR, 4 M NaCl was used instead of 300 mM NaCl. ND means not detectable due to the very weak binding.

## FIGURE LEGENDS

### FIGURE 1

Four retinal proteins in membranes of *Halobacterium salinarum*. The membrane contains rhodopsins (retinal proteins) such as bacteriorhodopsin (bR), halorhodopsin (hR), sensory rhodopsin (sR) and phoborhodopsin (pR), and two transducer proteins, HtrI and HtrII. Here, bR and hR work as a light-driven proton pump and a halogen ion pump, respectively, while sR and pR work as a photo-sensors, and form signaling complex with its cognate transducer protein, HtrI and HtrII, respectively. These photo-receptors transmit light signals to HtrI or HtrII in the membrane. HtrI or HtrII that forms a ternary complex with CheA and CheW activates phosphorylation cascade that modulate flagella motors. By using these signaling systems, this bacterium cells move toward longer wavelength light ( $\lambda > 520$  nm) where bR and hR work, while they avoid shorter wavelength light ( $\lambda < 520$  nm), which contains harmful near-UV light.

### FIGURE 2

X-ray crystallographic structure of the binding interface of pR/HtrII complex. The structure was obtained from the Protein Data Bank (PDB code 1H2S) (16), and this figure reveals the formation of two specific hydrogen bonds between Tyr199<sup>pR</sup> and Asn74<sup>HtrII</sup> (enclosed by a purple box) and between Thr189<sup>pR</sup> and Glu43<sup>HtrII</sup>/Ser62<sup>HtrII</sup> (enclosed by a yellow box). The numbers in smaller font are the length between respective amino acid residues. Previous results showed that Asp193<sup>pR</sup>, Th204<sup>pR</sup> and linker region of HtrII is important for the binding between pR and HtrII. The membrane normal is roughly in the vertical plane of this figure, and the top and bottom regions correspond to the extracellular and cytoplasmic sides, respectively.

### FIGURE 3

Isothermal titration calorimetry data of binding of the wild-type bR, hR, sR and pR with HtrII. The upper panels represent raw data. The lower panels represent the enthalpy changes per mole plotted as a function of the molar ratio of rhodopsins to HtrII. The solid lines represent best-fit curves. The determined binding parameters are listed in Table 1 and Figure 6. Published data of the wild type pR are reproduced from Ref. 15. Experiments were done three times and the average was taken. Details in the experimental conditions are in Materials and Methods

### FIGURE 4

Isothermal titration calorimetry data for binding of F199Y<sup>sR</sup> (correspond to Tyr199<sup>pR</sup>) and T189V<sup>pR</sup>/Y199F<sup>pR</sup> mutant with HtrII. The upper panels represent raw data. The lower panels represent the enthalpy changes per mole plotted as a function of the molar ratio of photo-receptor rhodopsins to HtrII. The solid lines represent best-fit curves. The determined binding parameters are listed in Table 1 and Figure 6. Experiments were done three times and the average was taken. Details in the experimental conditions are in Materials and Methods

### FIGURE 5

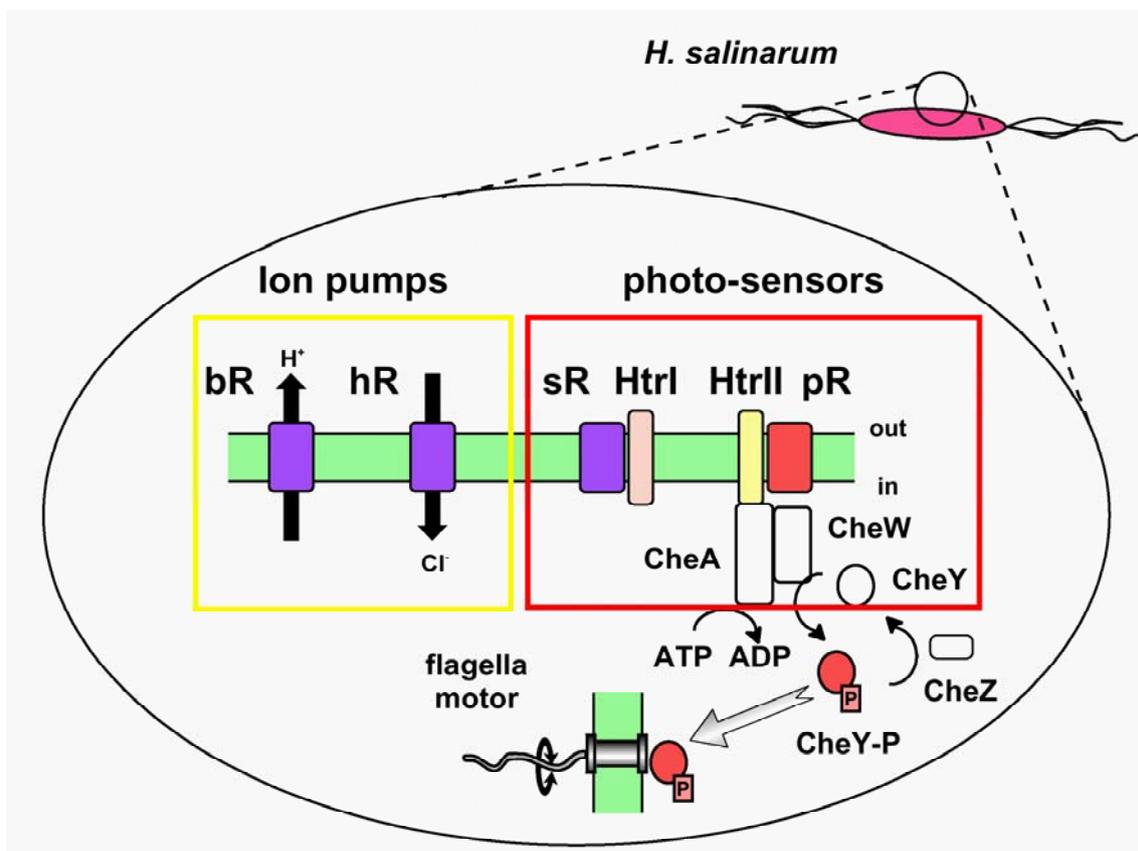
Isothermal titration calorimetry data for binding of a) P200T<sup>bR</sup> (corresponding to Thr189<sup>pR</sup>), V210Y<sup>bR</sup> (corresponding to Tyr199<sup>pR</sup>), P240T<sup>hR</sup> (corresponding to Thr189<sup>pR</sup>) and F250Y<sup>hR</sup> (corresponding to Tyr199<sup>pR</sup>) mutants with HtrII, and of b) P200T<sup>bR</sup>/V210Y<sup>bR</sup> and P240T<sup>hR</sup>/F250Y<sup>hR</sup> mutants with HtrII. The upper panels of a) and b) respectively represent raw data. The lower panels of a) and b) represent the enthalpy changes per mole plotted as a function of the molar ratio of rhodopsins to HtrII. The solid lines represent best-fit curves.

Experiments were done three times and the average was taken. Details in the experimental conditions are in Materials and Methods. The determined binding parameters are listed in Fig. 6.

### **FIGURE 6**

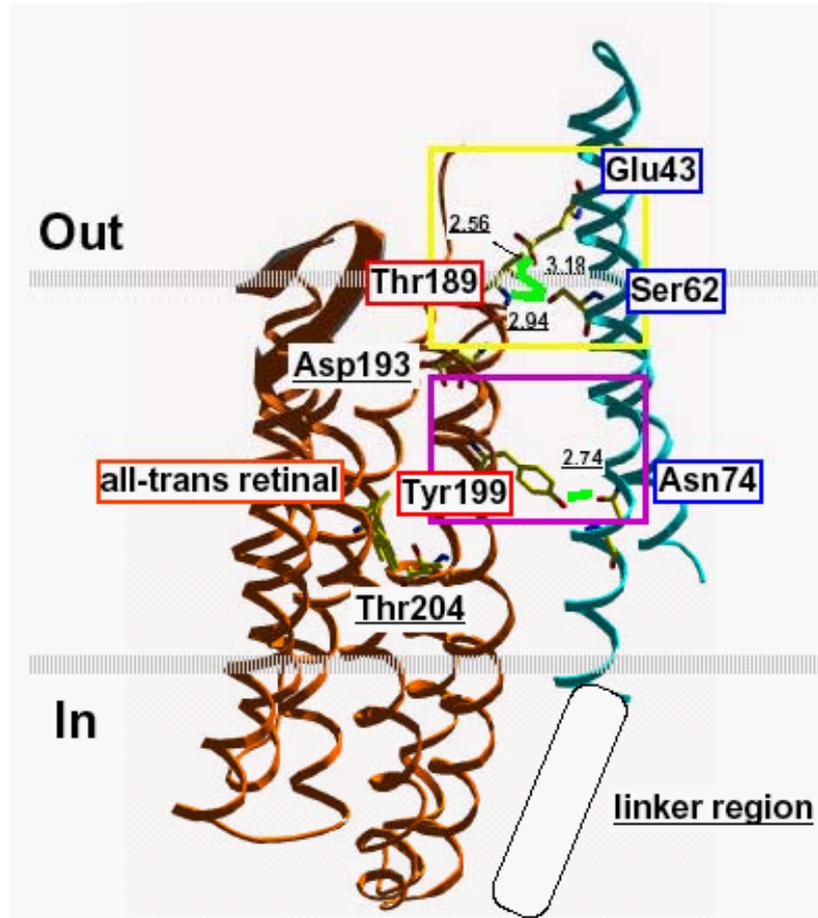
The dissociation constants of the binding of bR, hR, sR pR and their mutants with HtrII. From the left to right on the abscissa, the points stand for (1) rhodopsins that have Thr and Tyr residues at the positions corresponding to 189<sup>pR</sup> and 199<sup>pR</sup>, respectively, (2) rhodopsins that have Thr at the position corresponding to 189<sup>pR</sup>, (3) the rhodopsins that have Tyr at the position corresponding to 199<sup>pR</sup> and (4) the rhodopsins that do not have both Thr and Tyr at the positions corresponding to 189<sup>pR</sup> and 199<sup>pR</sup>, respectively.

**FIGURE 1**

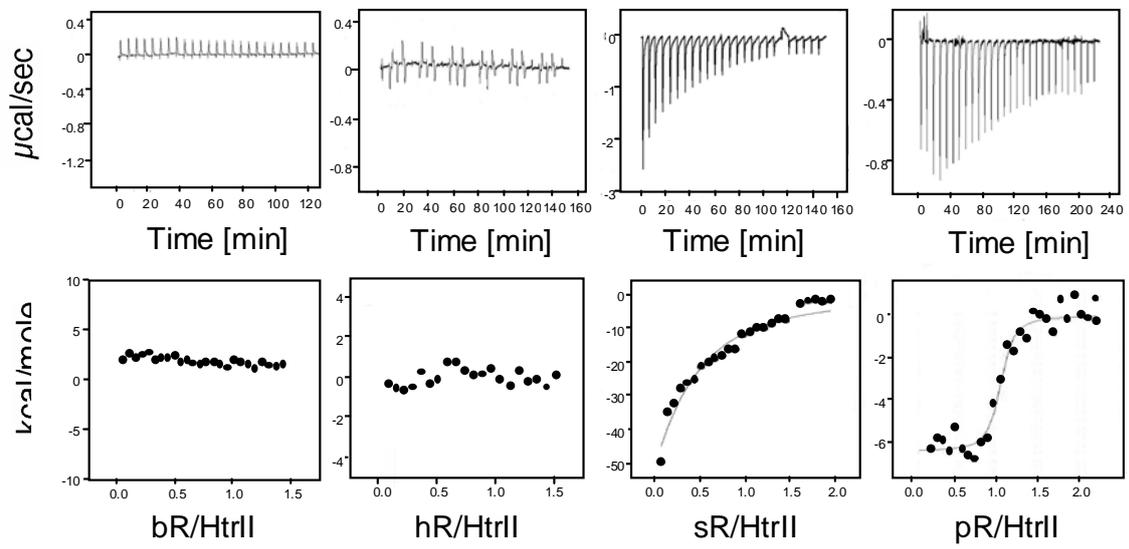


**Sudo et al.**

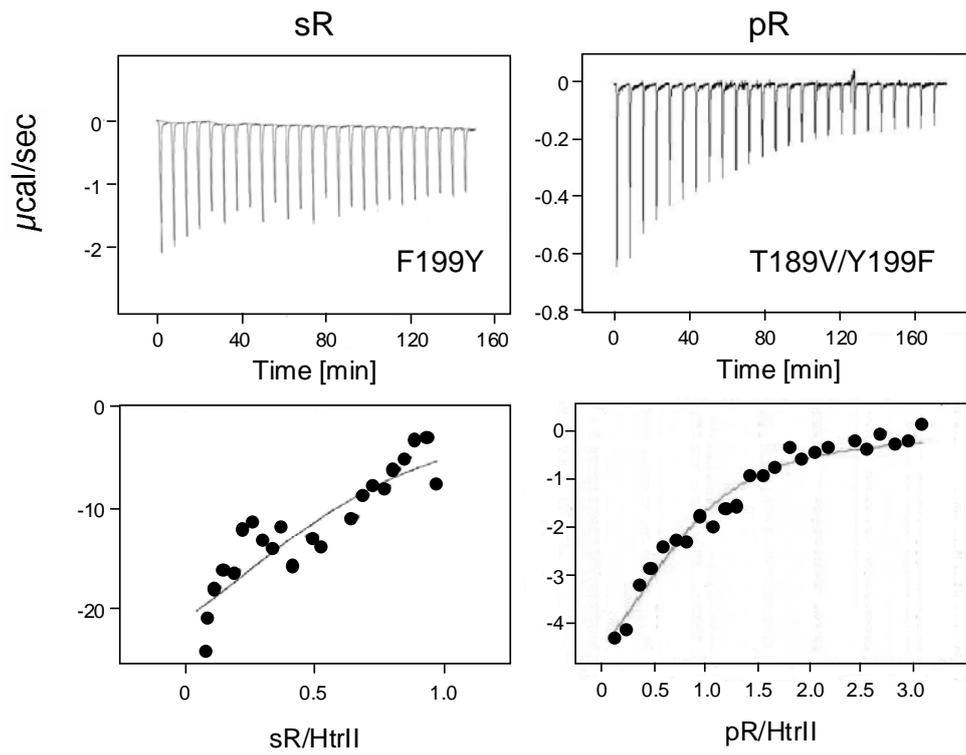
**FIGURE 2**



**FIGURE 3**



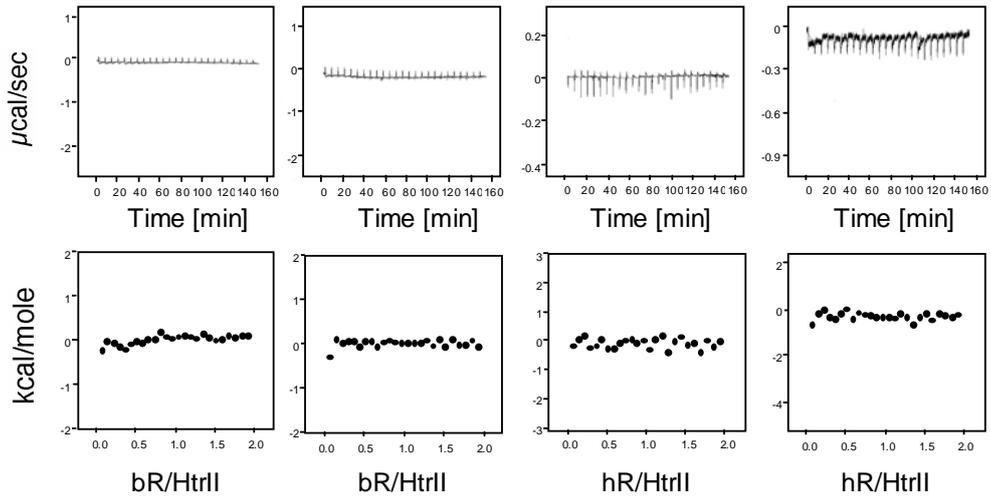
**FIGURE 4**



**Sudo et al.**

# FIGURE 5

a)



b)

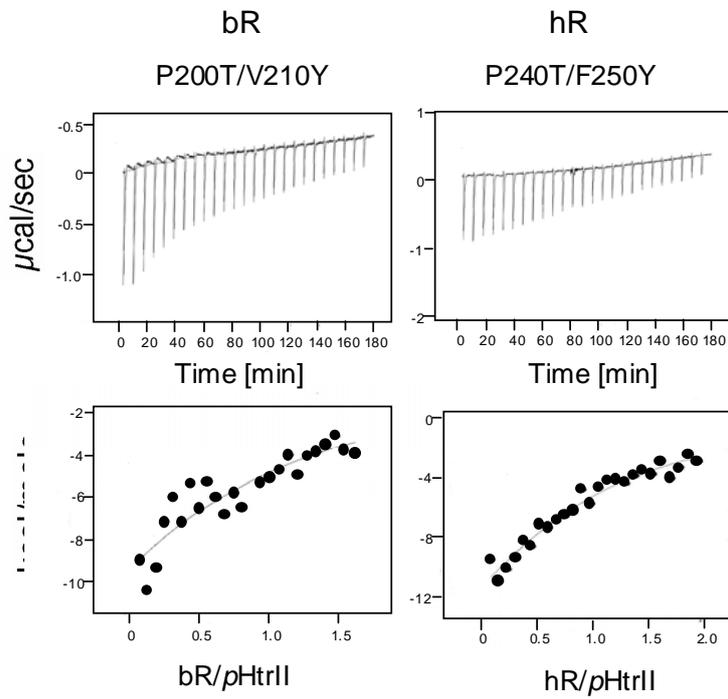


FIGURE 6

