



HOKKAIDO UNIVERSITY

Title	Tyr-199 and charged residues of pharaonis Phoborhodopsin are important for the interaction with its transducer.
Author(s)	Sudo, Yuki; Iwamoto, Masayuki; Shimono, Kazumi et al.
Citation	Biophysical Journal, 83(1), 427-432
Issue Date	2002-07
Doc URL	https://hdl.handle.net/2115/16002
Type	journal article
File Information	BJ83-1.pdf



**Tyr-199 and charged residues of *pharaonis* phoborhodopsin are
important for the interaction with its transducer**

By

Yuki SUDO, Masayuki IWAMOTO, Kazumi SHIMONO and Naoki KAMO*

Laboratory of Biophysical Chemistry, Graduate School of
Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812,
Japan

***Corresponding author:** N. Kamo; fax +81-11-706-4984, E-mail
nkamo@pharm.hokudai.ac.jp

Key words: Sensory rhodopsin, protein-protein interaction, M-
intermediate, *Halobacterium salinarum*, *Natronobacterium pharaonis*

Running title: Binding site of ppR with pHtrII

Abbreviations: bR, bacteriorhodopsin; DM, n-dodecyl- β -D-
maltoside; hR, halorhodopsin; pHtrII, *pharaonis* halobacterial
transducer of ppR; ppR, *pharaonis* phoborhodopsin; pR,
phoborhodopsin (sensory rhodopsin II); t-Htr, truncated pHtrII;
D75N, Y199F and Y199V, ppR mutants in which Asp75 or Tyr199 are
substituted by Asn, Phe and Val, respectively.

ABSTRACT

pharaonis phoborhodopsin (ppR; also *pharaonis* sensory rhodopsin II, psRII) is a retinal protein in *Natronobacterium pharaonis* and is a receptor of negative phototaxis. It forms a complex with its transducer, pHtrII, in membranes and transmits light signals by protein-protein interaction. Tyr-199 is conserved completely in phoborhodopsins among a variety of archaea, but it is replaced by Val (for bacteriorhodopsin) and Phe (for sensory rhodopsin I). Previously, we (submitted to J. Photochem. Photobiol.) showed that analysis of flash-photolysis data of a complex between D75N and the truncated pHtrII (t-Htr) gives a good estimate of the dissociation constant K_D in the dark. To investigate the importance of Tyr-199, K_D of double mutants of D75N/Y199F or D75N/Y199V with t-Htr was estimated by flash-photolysis and was about 10-fold larger than that of D75N, showing the significant contribution of Tyr-199 to binding. The K_D of D75N/t-Htr complex increased with decreasing pH, and the data fitted well with the Henderson-Hasselbach equation with a single pKa of 3.86 ± 0.02 . This suggests that certain deprotonated carboxyls at the surface of the transducer (possibly Asp-102, Asp-104 and Asp-106) are needed for the binding.

INTRODUCTION

Retinal proteins have retinal as a chromophore and exist in various organisms: archaeal (Haupt et al., 1998), eubacteria (Beja et al., 2000) and eukaryotes (Bieszke et al., 1999; Brown et al., 2001). Functionally, these proteins are distinctly different. Bacteriorhodopsin (bR; Haupt et al., 1999; Lanyi and Luecke, 2001) and halorhodopsin (hR; Váro, 2000) are light-driven ion pumps; the former functions as an outward proton pump and the latter functions as an inward chloride pump. Sensory rhodopsin (sR or sRI; Hoff et al., 1997) and phoborhodopsin (pR, also called sensory rhodopsin II, sRII; Takahashi et al. 1985; Sasaki and Spudich, 2000) work as light sensitive photoreceptors, and they form a signaling complex in archaeal membranes with their cognate transducer proteins, HtrI and HtrII, respectively (Hoff et al., 1997; Sasaki and Spudich, 2000). These transducer proteins activate phosphorylation cascades that modulate flagella motors. By using these signaling systems, these bacteria move toward useful light where bR and hR can function, while they avoid harmful near-UV light.

pharaonis phoborhodopsin (ppR; also called *pharaonis* sensory rhodopsin II, psRII) is a pigment protein of *Natronobacterium pharaonis* and corresponds to pR of *Halobacterium salinarum* (Seidel et al., 1995; Kamo et al. 2001). ppR absorbs maximally 498 nm light (Shimono et al., 2001) and functions as a receptor of negative phototaxis similar to pR. ppR is more stable than pR, and expression systems using *Escherichia coli* cells can provide large amounts of

ppR (Shimono et al., 1997).

Luecke et al. (2001) and Royant et al. (2001) solved the X-ray crystallographic structure of *ppR* and proposed the hypothesis for the binding site of *ppR* to *pHtrII*. The hypotheses they proposed are different from each other. Luecke et al. (2001) proposed that Tyr-199 on face I of *ppR* is a key residue. To date, gene-coded sequences of 23 archaeal rhodopsins have been reported. Figure 1 shows a multiple sequence alignment of these rhodopsins of only helix F and G regions which is assumed a putative transducer-binding surface (Wegener et al., 2000, 2001). Tyr-199 of *ppR* is completely conserved in the *pR*-family. In the crystal structure, this conserved residue locates outward of the protein and possibly toward *pHtrII*. Royant et al. (2001) proposed the importance of a charged surface patch on the cytoplasmic side of *ppR* (Fig.2, circle). This charged surface patch does not exist in other archaeal rhodopsins such as *bR* and *hR*.

We (Sudo et al., 2001) and Engelhard and his colleagues (Wegener et al., 2000) succeeded in expression of a truncated *pHtrII* (*t-Htr*) in *Escherichia coli* cells, where *t-Htr* is a N-terminal sequence of 159 amino acid residues of *pHtrII*. This *t-Htr* can bind with *ppR* (Wegener et al., 2000, 2001; Sudo et al., 2001, 2002 and submitted), and thus *ppR/t-Htr* (complex between *ppR* and *t-Htr*) is a model system of the signal transfer. We found that the M-decay rate of the *ppR/t-Htr* complex was ca. 2-fold slower than that of *ppR* alone. By using this difference the dissociation constant, K_D was estimated

to 15 μM (Sudo et al., 2001). We stress that this value should be of the transducer with the ppR M-intermediate, not the transducer with ppR in the dark.

Spudich et al. (1997) showed that D73N in *H. salinarum* pR, the homologous mutant as D75N in ppR used here, is constitutively active in the dark. We reason that it is therefore possible that a photointermediate of D75N may not transmit the signal. Therefore, it is conceivable that the D75N photo-intermediate may simulate ground state ppR in terms of a resting receptor. The interaction of the intermediate of D75N with the transducer was analyzed by flash-photolysis and K_D was estimated as low as 146 nM (Sudo et al., submitted). A calorimetric method gave a K_D of 100 nM between the ground state ppR (the wild-type) and t-Htr (Wegener, 2000). The 146 nM and 100 nM are close to each other, suggesting that the interaction between the photo-intermediate of D75N and t-Htr is a good estimate of that between the ground state of ppR and the transducer.

In this study, K_D values of the complex of double mutants D75N/Y199F or D75N/Y199V with t-Htr are determined. Data indicate the important contribution of Tyr-199 for binding. In addition, K_D increases with decrease in pH, and the data fit well with the Henderson-Hasselbach equation with a single pKa of 3.86 ± 0.02 . This implies that electrical interaction is also important for the binding of ppR with the cognate transducer.

MATERIALS AND METHODS

Sample preparations

Expression plasmids of D75NHis and t-HtrHis were constructed as previously described (Iwamoto et al., 2001; Sudo et al., 2001). Here His is the ppR or t-Htr tagged with 6 x histidine at the C-terminal. The mutant genes, Y199FHis, Y199VHis, D75N/Y199FHis and D75N/Y199VHis, were constructed by PCR using the DNA shuffling method (Stemmer, 1994). Oligonucleotide primers were designed from the nucleotide sequence in the GenBank data base (accession No. Z35086). DNA was sequenced by using a DNA Sequencing Kit (Applied Biosystems). All constructed plasmids were analyzed by using an automated sequencer (377 DNA sequencer, Applied Biosystems).

The mutant ppRs and t-HtrHis were expressed in *E. coli* BL21 (DE3). The preparation of crude membranes and purification of proteins were as described previously (Shimono et al., 2000b; Kandori et al., 2001). The sample medium was exchanged by ultrafiltration (UK-50, Advantech, Tokyo) and the samples were suspended in the final experimental media.

Flash spectroscopy

The apparatus and procedure were essentially the same as described previously (Miyazaki et al., 1992). A photo-intermediate of mutant ppR (D75N, D75N/Y199F or D75N/Y199V) alone or their complex with t-HtrHis was observed at 570 nm. The M-intermediate of Y199F or Y199V and their complexes with t-HtrHis was observed at 350 nm.

The time courses were analyzed with a single exponential equation to determine the kinetic constant (for details, see Results section). All experiments were done at 20 °C.

Titration of free t-Htr with mutant ppRs and estimation of binding parameters

The t-HtrHis concentration was kept constant at 25 μM , and varying concentrations of mutant ppRs were added to change the molar ratio of t-HtrHis to mutant ppRs. The t-HtrHis concentration was determined using the antibody for the histidine tag; the details were described by Sudo et al. (2001). The kinetic constant of the intermediate was determined by flash spectroscopy as described in the previous section. We estimated the binding parameters (K_D and n , the number of binding sites) from the titration data using the same method described by Sudo et al. (2001 and submitted).

Flash-photolysis at varying pH

The ppR samples were suspended in a medium containing 360 mM NaCl, 0.1% n-dodecyl- β -D-maltoside (DM) and a mixture of seven buffers (citric acid, Tris, Mes, Hepes, Mops, Ches and Caps whose concentrations were 10 mM each), because this buffer composition has the same buffer capacity for a wide range of pH (2 ~ 9) that we used in this study. Before the flash-photolysis experiments, samples were incubated for at least 1 hr in a medium whose pH was adjusted to a required value. The curve was fitted by using the Henderson-Hasselbach equation with a single pKa.

RESULTS

D75N lacks the M-intermediate during the photocycle because Asp-75, the proton acceptor from the protonated Schiff base, is replaced by the neutral Asn (Schmies et al. 2000; Shimono et al., 2000a). In an ms time range, an O-like intermediate (max of 570 nm) is observed. The nature of this intermediate is not clear yet, but in this study, we call it an O-like intermediate due to the red-shifted absorption maximum. max of the intermediate of the double mutants D75N/Y199F, D75N/Y199V and their complexes with t-HtrHis did not change from that of D75N. The rate constants of the O-like intermediate decay of D75N, D75N/Y199F and D75N/Y199V were 15.0, 15.4 and 13.1 s⁻¹, respectively, while those of the complex with t-HtrHis were 56, 66.5 and 60.8 s⁻¹, respectively (data not shown; Table 1). The medium contained 0.1% DM, 400 mM NaCl and 10 mM Tris-Cl (pH 7.0). The values of the complex were about fourfold faster than those of the pigment alone. In this study, ppRs (5 μM) and t-HtrHis were mixed at the molar ratios of 1:10. Further adding t-HtrHis did not change the decay rate, implying that free ppR mutant proteins were not present.

We titrated 25 μM t-HtrHis with D75N/Y199F (Fig. 3A, open circles) or D75N/Y199V (Fig. 3A, closed circles) to measure the decay rate constant of the O-like intermediate. The decay curve comprised two components except after enough pigment was added. This is very natural because this sample may contain free ppR, the

ppR/t-HtrHis complex and free t-HtrHis; the former two are active in flash spectroscopy with different kinetic constants. Eight kinetic traces were obtained under different molar ratios of t-HtrHis to D75N/Y199F or D75N/Y199V. All data fitted well with the equation $\exp(-k_1t) + \exp(-k_2t)$, where k_1 and k_2 are the decay constants of the O-like intermediate of the pigment protein alone and its complex, respectively. The free concentration of the pigment protein ($[ppR]$) was plotted against the complex concentration ($[ppR/t-HtrHis]$) in Fig.3(A). Calculation method of these values was described by Sudo et al. (2001 and submitted). From this curve, K_D values were estimated as $1.9 \pm 0.2 \mu\text{M}$ (D75N/Y199F) and $1.3 \pm 0.2 \mu\text{M}$ (D75N/Y199V), and n were 1.1 ± 0.02 (D75N/Y199F) and 1.0 ± 0.02 (D75N/Y199V). These K_D values are about 10-fold larger than that of the D75N single mutant (150 nM; Sudo et al. and submitted).

The K_D of the complex between the M-intermediate of the wild-type ppR and t-Htr is $15 \mu\text{M}$ (Sudo et al., 2001). In this study, therefore, the K_D value of the complex between the M-intermediate of Y199F or Y199V and t-Htr was estimated. The rate constant of M-decay was $1.70 \pm 0.15 \text{ s}^{-1}$ (Y199F) and $1.57 \pm 0.12 \text{ s}^{-1}$ (Y199V), while that of the complex was $0.84 \pm 0.04 \text{ s}^{-1}$ (Y199F/t-Htr) and $0.80 \pm 0.3 \text{ s}^{-1}$ (Y199V/t-Htr) (data not shown; Table 1) which is almost twofold slower than those of the mutant pigment alone. Using the same method used for the O-like intermediate described above and by Sudo et al. (2001), the K_D values (complex between the M-intermediate and t-Htr) were $14 \pm 1.4 \mu\text{M}$ (Y199F) and $10 \pm 1.2 \mu\text{M}$

(Y199V). The values of n were 1.2 ± 0.08 (Y199F) and 1.1 ± 0.07 (Y199V). In Fig. 3B, the $[ppR]$ is plotted against $[ppR/t-Htr_n]$ for these mutant pigments.

Figure 4 shows the pH-dependent rate constants of the decay of the O-like intermediate of the D75N mutant. The rate constants of the transducer-free D75N decreased markedly at low pH. These pH-dependent changes were reversible. This curve was fitted by a Henderson-Hasselbach equation with a single pK_a , which was estimated as 4.4 ± 0.06 . The decay constant of the D75N/t-Htr complex was not affected by pH 2 ~ 10.

Using these rate constants, we estimated the K_D values of the D75N/t-Htr complex at pH 2 ~ 9 by plotting $[ppR]$ against $[ppR/t-Htr_n]$ (Fig. 5A; open circle, pH 2; closed circles, pH 6). Figure 5B delineates the estimated K_D as a function of pH. This curve was fitted by the Henderson-Hasselbach equation with a single pK_a estimated as 3.86 ± 0.02 .

DISCUSSION

Luecke et al. (2001) and Royant et al. (2001) reported X-ray crystallographic structure of ppR . They proposed different, but not mutually exclusive binding sites of ppR and $pHtrII$: Try-199 on face I of ppR (Luecke et al., 2001; Fig. 2) and a charged surface patch on the cytoplasmic side of ppR (Royant et al., 2001; Fig. 2, circle).

To test the proposal of Luecke et al. (2001), we examined the interaction of the transducer with the photo-intermediate of D75N mutant. The interaction of the photo-intermediate of the D75N mutant with the transducer gives good information on the interaction between the pigment and the transducer in the dark. The photo-intermediate of D75N/Y199F and D75N/Y199V has larger K_D values for the formation with t-Htr than that of the parent D75N (Fig. 3, Table 1), which implies the importance of this residue for binding, due possibly to the hydrogen-bonding propensity of the Tyr-199 hydroxyl.

Interestingly, the K_D values of the interaction of t-Htr with M-intermediates of single mutants of Y199F and Y199V are almost equal to that of the wild-type (Table 1). This may be interpreted that at the M-state, the possible signaling state, the transducer interacts with sites of the pigment other than Tyr-199, while in the dark the transducer interacts with Tyr-199 of the pigment. This is consistent with a recent observation using EPR (electron paramagnetic resonance), which concluded that on illumination helix F of ppR moves toward the transducer to rotate (Wegener et al., 2000, 2001).

To examine the proposal of Royant et al. (2001), the decay rates of the intermediate of D75N and D75N/t-Htr complexes at varying pH were measured (Fig. 4). The rate constants of D75N alone depended strongly on the pH while those of D75N/t-Htr did not. This dependence curve was fitted by the Henderson-Hasselbach equation with a single

pKa estimated as 4.4 ± 0.06 . The rate constants of D75N and D75N/t-Htr were the same above pH 6. This may be interpreted that a carboxyl group exists whose dissociation state affects the decay rate of D75N and that this carboxyl group may interact with t-Htr. An amino acid residue having this carboxyl group might be Asp-214 in helix G of ppR, because this residue is outside ppR toward pHtrII. This, however, should be examined in the future, because the distance between Asp-214 and Asp-75 is far (25 Å) and this Asp is not conserved in the pR (sRII) family.

We used this difference of the decay rate to determine the K_D values for the association between D75N and t-Htr (Fig. 5). K_D is larger in acidic media and pKa was estimated as 3.86 ± 0.02 . It was predicted that positive charges of ppR (Lys-157, Arg-162 and Arg-164) interact with negative charges of the transducer (Asp-102, Asp-104 and Asp-106 that are conserved in various transducer proteins) (Royant et al., 2001). The pKa of 3.86 may be the average value of these Asps of the transducer. These Asps locate very close and their respective pKa values might be very close. If these Asps in the transducer are important for the interaction, we should examine other combinations of sensory rhodopsins and their cognate transducers because these Asps are conserved in all transducers. We might also consider that an interaction between the 6 x histidine tag and these Asp is possible.

From our results, we conclude that at least two conditions influence the association of ppR and its transducer, and are

predicted from the X-ray crystallographic results. The amino acid residue (pKa 3.86) is possibly important for keeping the dimer structure of the transducer (Yang et al., 2001). The identification of this residue is awaited for further investigation.

Acknowledgements

This work was in part supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Technology, Sports and Culture.

References

- Beja, O., L. Aravind, E. V. Koonin, M. T. Suzuki, A. Hadd, L. P. Nguyen, S. B. Jovanovich, C. M. Gates, R. A. Feldman, J. L. Spudich, E. N. Spudich, and E. F. DeLong. 2000. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* 289:1902-1906.
- Bieszke, J. A., E. N. Spudich, K. L. Scott, K. A. Borkovich, and J. L. Spudich. 1999. A eukaryotic protein, NOP-1, binds retinal to form an archaeal rhodopsin-like photochemically reactive pigment. *Biochemistry* 38: 14138-14145.
- Brown, L. S., A. K. Dioumaev, J. K. Lanyi, E. N. Spudich, and J. L. Spudich. 2001. Photochemical reaction cycle and proton transfers in *Neurospora* rhodopsin. *J. Biol. Chem.* 276:32495-32505.
- Haupts, U., J. Tittor, and D. Oesterhelt. 1999. Closing in on bacteriorhodopsin: progress in understanding the molecule. *Annu. Rev. Biophys. Biomol. Struct.* 28:367-399.
- Hoff, W.D., K. H. Jung, and J. L. Spudich. 1997. Molecular mechanism of photosignaling by archaeal sensory rhodopsins. *Annu. Rev. Biophys. Biomol. Struct.* 26:223-258.
- Iwamoto, M., Y. Sudo, K. Shimono, and N. Kamo. 2001. Selective reaction of hydroxylamine with chromophore during the photocycle

of *pharaonis* phoborhodopsin. *Biochim. Biophys. Acta.* 1514:152-158.

Kamo, N., K. Shimono, M. Iwamoto, and Y. Sudo. 2001. Photochemistry and Photoinduced Proton-transfer of *pharaonis* Phoborhodopsin. *Biochemistry (Moscow)* 66:1580-1587.

Kandori, H., K. Shimono, Y. Sudo, M. Iwamoto, Y. Shichida, and N. Kamo. 2001. Structural Changes of *pharaonis* Phoborhodopsin upon Photoisomerization of the Retinal Chromophore: Infrared Spectral Comparison with Bacteriorhodopsin. *Biochemistry* 40:9238-9246.

Lanyi, J. K., and H. Luecke. 2001. Bacteriorhodopsin. *Curr. Opin. Struct. Biol.* 11:415-419.

Luecke, H., B. Schobert, J. K. Lanyi, E. N. Spudich, and J. L. Spudich. 2001. Crystal structure of sensory rhodopsin II at 2.4 angstroms: insights into color tuning and transducer interaction. *Science* 293:1499-1503.

Miyazaki, M., J. Hirayama, M. Hayakawa, and N. Kamo. 1992 Flash photolysis study on *pharaonis* phoborhodopsin from a haloalkaliphilic bacterium (*Natronobacterium pharaonis*). *Biochim. Biophys. Acta.* 1140:22-29.

Oesterhelt, D. 1998. The structure and mechanism of the family of retinal proteins from halophilic archaea. *Curr. Opin. Struct. Biol.* 8:489-500.

Royant, A., P. Nollert, K. Edman, R. Neutze, E. M. Landau, E. Pebay-Peyroula, and J. Navarro. 2001. X-ray structure of sensory rhodopsin II at 2.1-Å resolution. *Proc. Natl. Acad. Sci. U.S.A.* 98: 10131-10136.

Sasaki, J., and J. L. Spudich. 2000. Proton transport by sensory rhodopsins and its modulation by transducer-binding. *Biochim. Biophys. Acta.* 1460:230-239.

Schmies, G., B. Lüttenberg, I. Chizhov, M. Engelhard, A. Becker, and E. Bamberg. 2000. Sensory rhodopsin II from the haloalkaliphilic *Natronobacterium pharaonis*: light-activated proton transfer reactions. *Biophys. J.* 78:967-976.

Seidel, R., B. Scharf, M. Gautel, K. Kleine, D. Oesterhelt, and M. Engelhard. 1995. The primary structure of sensory rhodopsin II: a member of an additional retinal protein subgroup is coexpressed with its transducer, the halobacterial transducer of rhodopsin II. *Proc. Natl. Acad. Sci. U.S.A.* 92:3036-3040.

Shimono, K., M. Iwamoto, M. Sumi, and N. Kamo. 1997. Functional

expression of *pharaonis* phoborhodopsin in *Escherichia coli*. *FEBS Lett.* 420:54-56.

Shimono, K., M. Kitami, M. Iwamoto and N. Kamo. 2000a. Involvement of two groups in reversal of the bathochromic shift of *pharaonis* phoborhodopsin by chloride at low pH. *Biophys.Chem.* 87:225-230

Shimono, K., M. Iwamoto, M. Sumi, and N. Kamo. 2000b. Effects of three characteristic amino acid residues of *pharaonis* phoborhodopsin on the absorption maximum. *Photochem. Photobiol.* 72:141-145.

Shimono, K., Y. Ikeura, Y. Sudo, M. Iwamoto, and N. Kamo. 2001. Environment around chromophore in *pharaonis* phoborhodopsin: Mutant analysis of the retinal binding site. *Biochim. Biophys. Acta* 1515:92-100.

Spudich, E.N., W. Zhang, M. Alam, and J. L. Spudich. 1997. Constitutive signaling by the phototaxis receptor sensory rhodopsin II from disruption of its protonated Schiff base-Asp-73 interhelical salt bridge. *Proc. Natl. Acad. Sci. U.S.A.* 94:4960-4965.

Stemmer, W. P. 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc.*

Natl. Acad. Sci. U.S.A. 91:10747-10751.

Sudo, Y., M. Iwamoto, K. Shimono, and N. Kamo. 2001. *Pharaonis* phoborhodopsin binds to its cognate truncated transducer even in the presence of a detergent with a 1:1 stoichiometry. *Photochem. Photobiol.* 74:489-494.

Sudo, Y., M. Iwamoto, K. Shimono and N. Kamo. Association between a M-lacking mutant D75N of *pharaonis* phoborhodopsin and its cognate transducer is about 100-fold stronger than the complex of the wild-type pigment: Implication of the signal transduction. Submitted to *J. Photochem. Photobiol.*

Takahashi, T., H. Tomioka, N. Kamo and Y. Kobatake, 1985. A photosystem other than PS370 also mediates the negative phototaxis of *Halobacterium halobium*. *FEMS Microbiol. Lett.* 28:161-164

Wegener, A.A. 2000. Untersuchungen zur wechselwirkung des archaebakteriellen lichtrezeptors pSRII mit seinem transducerprotein pHtrII. A doctoral dissertation to Univ. Dortmund.

Wegener, A.A., I. Chizhov, M. Engelhard, and H. J. Steinhoff. 2000. Time-resolved detection of transient movement of helix F in spin-labelled *pharaonis* sensory rhodopsin II. *J. Mol. Biol.* 301:881-891.

Wegener, A. A., J. P. Klare, M. Engelhard, and H. J. Steinhoff. 2001. Structural insights into the early steps of receptor-transducer signal transfer in archaeal phototaxis. *EMBO J.* 20:5312-5319.

Yang C. S. and J. L. Spudich. 2001. Light-Induced Structural Changes Occur in the Transmembrane Helices of the *Natronobacterium pharaonis* HtrII Transducer. *Biochemistry* 40:14207-14214

Table 1

Dissociation constants (K_D), number of binding sites (n) of the complex between pPR mutants and t-Htr and rate constants of intermediates

	K_D [μM]	n	Decay rate constant (s^{-1})	
			pigment alone	complex
Wild-type (in the dark) ^a	0.10	1.0	-	-
D75N (O-1like intermediate) ^b	0.15	1.0	15.0	56
D75N/Y199F (O-1like intermediate)	1.9	1.1	15.4	66.5
D75N/Y199V (O-1like intermediate)	1.3	1.0	13.1	60.8
Wild-type (M-intermediate) ^c	15	1.2	1.66	0.82
Y199F (M-intermediate)	14	1.2	1.70	0.84
Y199V (M-intermediate)	10	1.1	1.57	0.80

a) The values of the wild-type pPR and t-Htr in the dark were taken from Wegener et al. (2000)

b) The values estimated from the decay of the O-1like intermediate of D75N pPR.

c) The values estimated from the decay of the M-intermediate of the wild-type pPR. b) and c) are from Sudo et al. (2001).

Figure Legends

Figure 1. Alignment of putative amino acid sequences in helices F and G of 23 archaeal rhodopsins reported so far. Helix F and G locate near and toward the transducer protein. Amino acid residues marked by a star in helix F and G are the site of attention in this study.

Figure 2. X-ray crystallographic structure of *ppR* (Royant et al., 2001; 1H68 of PDB code). The surface patch (circle) on the cytoplasmic side of *ppR* is a putative binding site suggested by Royant et al. (2001). Tyr-199 on face I is another putative binding site of *ppR* (Luecke et al., 2001) and is in the middle of the transmembrane helix of G.

Figure 3. Concentrations of the free *ppR*, [*ppR*] plotted against concentrations of the *ppR*/t-Htr_n complex, [*ppR*/t-Htr_n] during the titration. Details were described by Sudo et al. (2001 and submitted). A): plots from the decay-rates of the O-like intermediate of the double mutants of Asp-75 and Tyr-199. Open circle, D75N/Y199F and closed circle, D75N/Y199V. The circles are data points; the lines (solid and gray) are regression curves from using non-linear regression software (Origin, Micalcal, Northampton, MA). The broken line shows the interaction between the O-like intermediate of D75N and t-HtrHis (data from Sudo et

al., submitted). The t-Htr concentration was 25 μM , and the medium contained 0.1% DM, 400 mM NaCl, 10 mM Tris-Cl at pH 7.0 and 20 °C. B): Plots from the decay-rates of the M-like intermediate of Tyr-199 mutants. Open circle, Y199F and closed circle, Y199V. The circles are the data points; the lines (solid and gray) are regression curves. The broken line shows the interaction between the M-intermediate of the wild-type and t-Htr (data from Sudo et al., 2001). The t-Htr concentration was 25 μM , and the medium composition and temperature were the same as for A). The curves of A) are steeper than those of B), because Asp-75 mutants have a greater affinity (smaller K_D) for the transducer. Table 1 lists estimated K_D and n .

Figure 4. Rate constants of the O-like intermediate decay of D75N alone and D75N/t-Htr complex at varying pH. Open circles are the data of the D75N/t-Htr complex using the right ordinate. Closed circles are the D75N alone using the left ordinate. The solid line is a fitted curve by using the Henderson-Hasselbach equation with a single pKa estimated as 4.4 ± 0.06 . The medium contained a mixture of seven buffers (see Materials and Methods), 0.1% DM and 360 mM NaCl. Temperature was 20 °C.

Figure 5. K_D values of the D75N/t-Htr complex at varying pH. A): the titration of t-Htr (25 μM) with ppR at pH 2 (open circles) and pH 6 (closed circles). Data were fitted by the same method as described for Fig. 2. B): K_D values estimated are plotted against

pH. The solid curve is a fitted curve by using the Henderson-Hasselach equation with pKa of 3.86 ± 0.02 . The experiment conditions were the same as for Fig. 3.

Figure 1 (sudo et al)

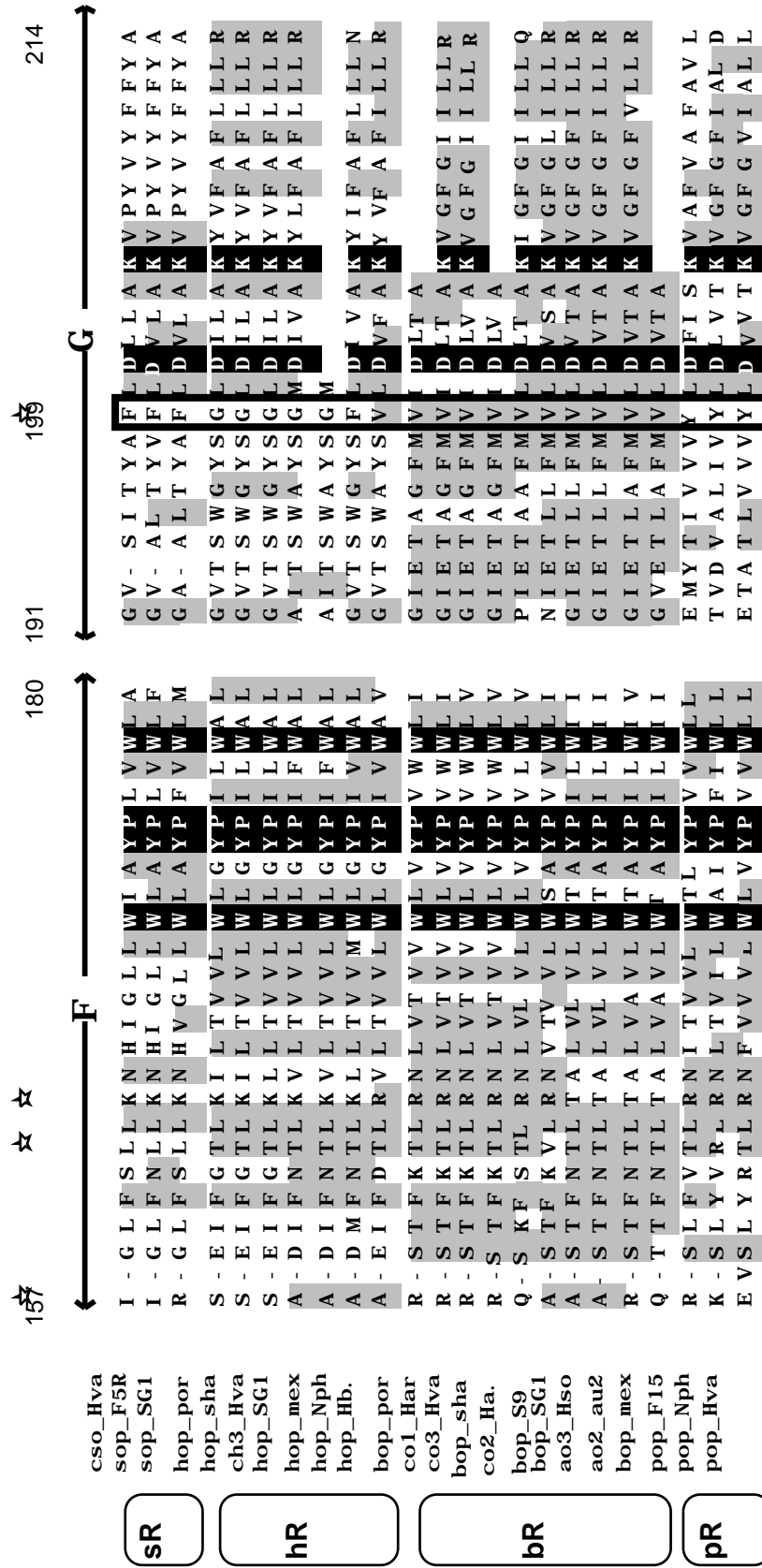
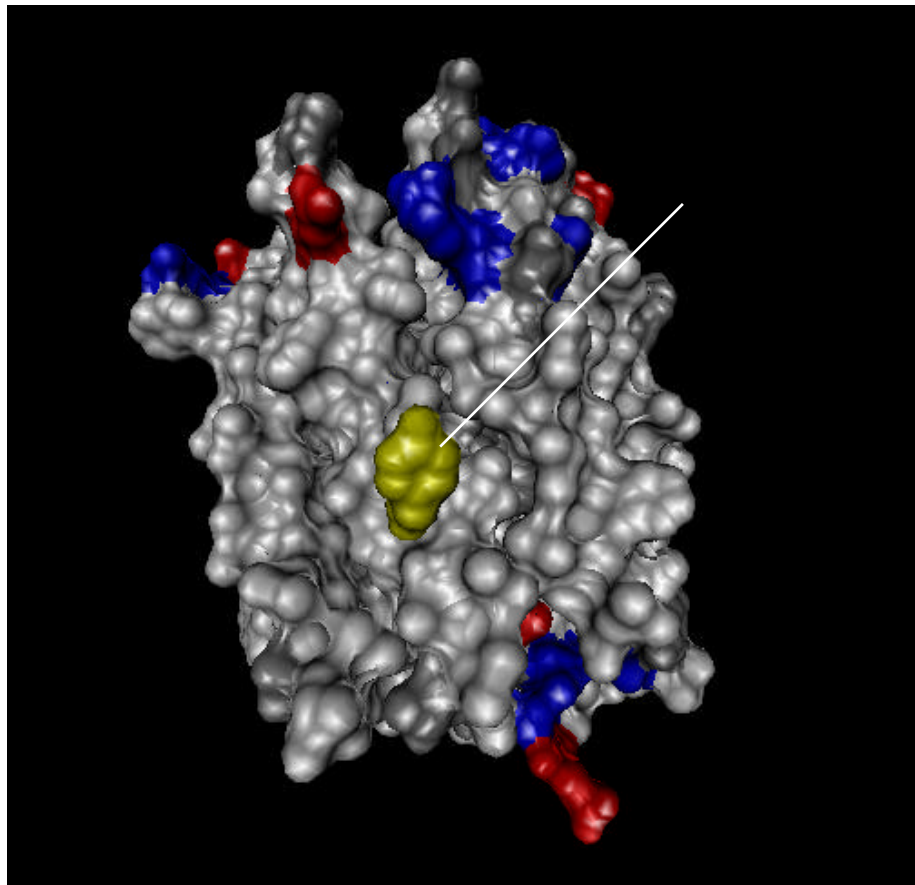


Fig.2



Sudo et al.

Fig.3

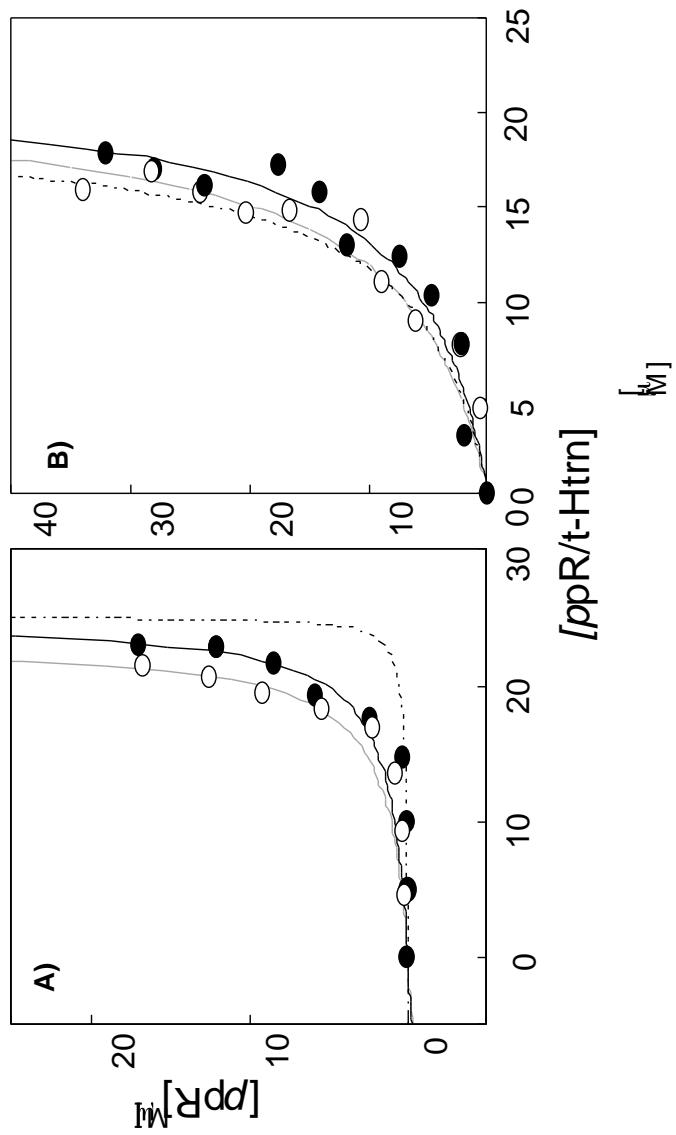
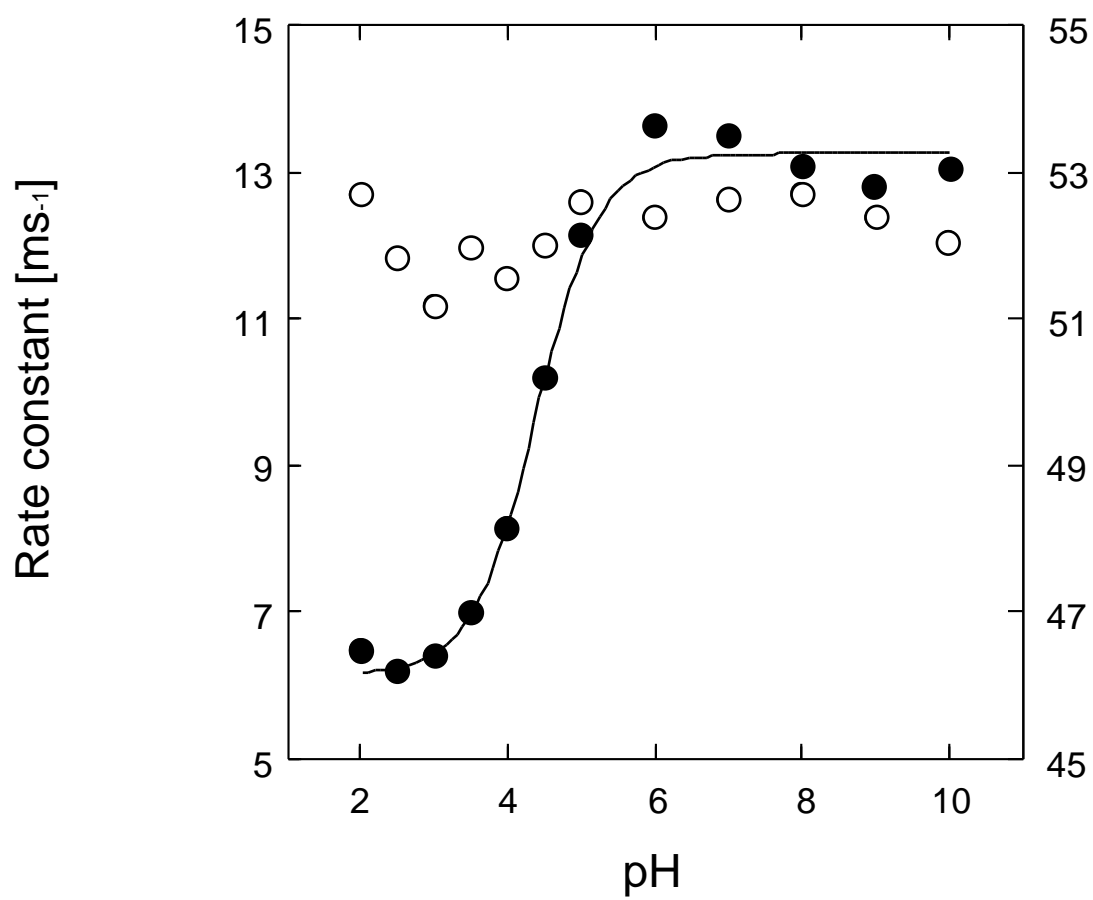


Fig.4



Sudo et al.

Fig.5

