Tyr-199 and Charged Residues of *pharaonis* Phoborhodopsin Are Important for the Interaction with its Transducer

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ABSTRACT   *pharaonis* Phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, *p*sRII) is a retinal protein in *Natronobacterium pharaonis* and is a receptor of negative phototaxis. It forms a complex with its transducer, *p*HtrII, 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 (Sudo, Y., M. Iwamoto, K. Shimono, and N. Kamo, submitted for publication) showed that analysis of flash-photolysis data of a complex between D75N and the truncated *p*HtrII (t-Htr) give 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 ~10-fold larger than that of D75N, showing the significant contribution of Tyr-199 to binding. The K_D of the D75N/t-Htr complex increased with decreasing pH, and the data fitted well with the Henderson-Hasselbach equation with a single pK_a 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: archaea (Oesterhelt, 1998), eubacteria (Beja et al., 2000), and eukaryotes (Bieszke et al., 1999; Brown et al., 2001). Functionally, these proteins are distinctly different. Bacteriorhodopsin (bR; Haupts et al., 1999; Lanyi and Luecke, 2001) and halorhodopsin (hR; Váró, 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 (*p*pR; also called *pharaonis* sensory rhodopsin II, *p*sRII) is a pigment protein of *Natronobacterium pharaonis* and corresponds to pR of *Halobacterium salinarum* (Seidel et al., 1995; Kamo et al. 2001). *p*pR maximally absorbs 498 nm light (Shimono et al., 2001) and functions as a receptor of negative phototaxis similar to pR. *p*pR is more stable than pR, and expression

systems using *Escherichia coli* cells can provide large amounts of *p*pR (Shimono et al., 1997).

Luecke et al. (2001) and Royant et al. (2001) solved the x-ray crystallographic structure of *p*pR and proposed the hypothesis for the binding site of *p*pR to *p*HtrII (the *pharaonis* halobacterial transducer of *p*pR). The hypotheses they proposed are different from each other. Luecke et al. (2001) proposed that Tyr-199 on face I of *p*pR is a key residue. To date, gene-coded sequences of 23 archaeal rhodopsins have been reported. Fig. 1 shows a multiple sequence alignment of these rhodopsins of only helix F and G regions, which is assumed to be a putative transducer-binding surface (Wegener et al., 2000, 2001). Tyr-199 of *p*pR is completely conserved in the pR family. In the crystal structure, this conserved residue locates outward of the protein and possibly toward *p*HtrII. Royant et al. (2001) proposed the importance of a charged surface patch on the cytoplasmic side of *p*pR (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 expressing a truncated *p*HtrII (t-Htr) in *Escherichia coli* cells, where t-Htr is a N-terminal sequence of 159 amino acid residues of *p*HtrII. This t-Htr can bind with *p*pR (Wegener et al., 2000, 2001; Sudo et al., 2001, submitted for publication), and thus *p*pR/t-Htr (complex between *p*pR and t-Htr) is a model system of the signal transfer. We found that the M-decay rate of the *p*pR/t-Htr complex was about twofold slower than that of *p*pR 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 *p*pR M-intermediate, not the transducer with *p*pR in the dark.

Spudich et al. (1997) showed that D73N in *H. salinarum* pR, the homologous mutant to D75N in *p*pR used here, is constitutively active in the dark. We reason that it is there-
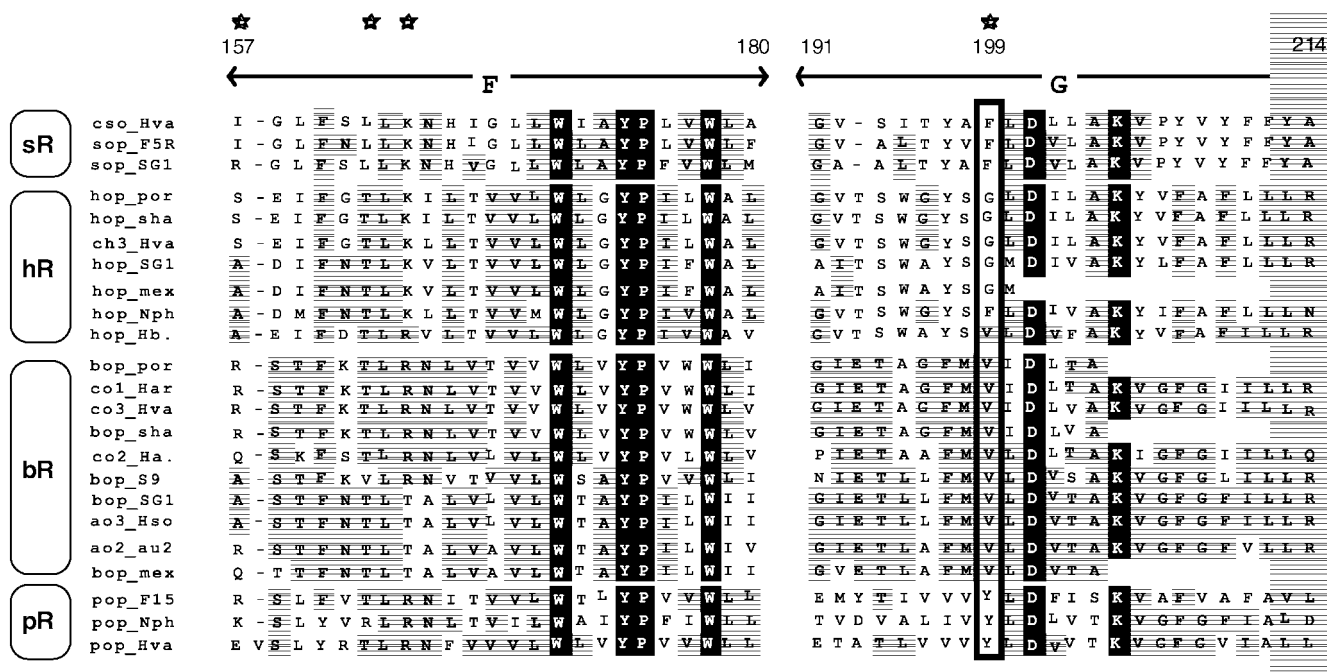
FIGURE 1 Alignment of putative amino acid sequences in helices F and G of 23 archaeal rhodopsins reported so far. Helices F and G are located near and toward the transducer protein. Amino acid residues marked by a star in helices F and G are the site of attention in this study.
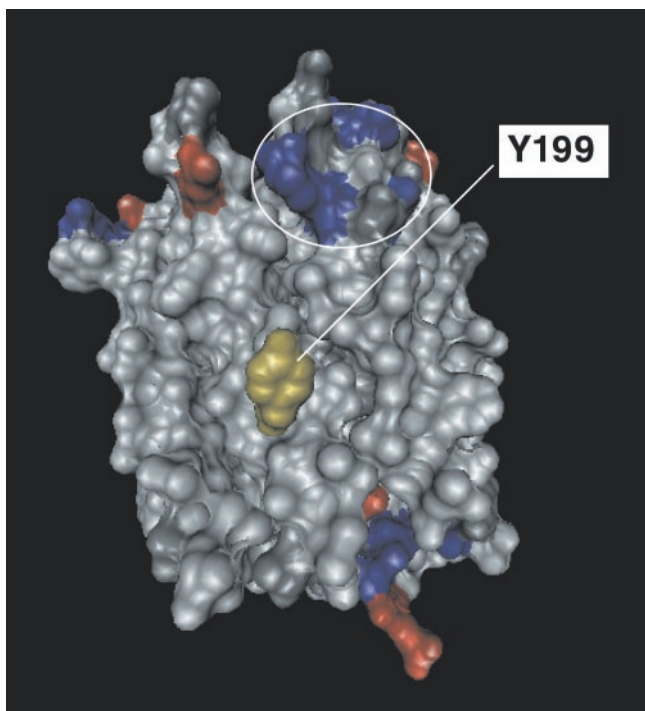

FIGURE 2 X-ray crystallographic structure of *p*pR (Royant et al., 2001; 1H68 of PDB code). The surface patch (*circle*) on the cytoplasmic side of *p*pR is a putative binding site suggested by Royant et al. (2001). Tyr-199 on face I is another putative binding site of *p*pR (Luecke et al., 2001) and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

fore possible that a photointermediate of D75N may not transmit the signal. Therefore, it is conceivable that the D75N photointermediate may simulate ground-state *p*pR in terms of a resting receptor (D75N, Y199F, and Y199V are *p*pR mutants in which Asp-75 or Tyr-199 are substituted by Asn, Phe, and Val, respectively). 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 for publication). A calorimetric method gave a K_D of 100 nM between the ground-state *p*pR (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 photointermediate of D75N and t-Htr is a good estimate of that between the ground state of *p*pR 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 pK_a of 3.86 ± 0.02. This implies that electrical interaction is also important for the binding of *p*pR 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

*p*pR or t-Htr tagged with 6× 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, CA). All constructed plasmids were analyzed by using an automated sequencer (377 DNA sequencer, Applied Biosystems).

The mutant *p*pRs 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 photointermediate of mutant *p*pR (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). All experiments were done at 20°C.

Titration of free t-Htr with mutant *p*pRs and estimation of binding parameters

The t-HtrHis concentration was kept constant at 25 μM, and varying concentrations of mutant *p*pRs were added to change the molar ratio of t-HtrHis to mutant *p*pRs. 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 for publication).

Flash-photolysis at varying pH

The *p*pR 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 values (2–9) that we used in this study. Before the flash-photolysis experiments samples were incubated for at least 1 h in a medium whose pH was adjusted to a required value. The curve was fitted by using the Henderson-Hasselbach equation with a single pK_a.

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 a millisecond time range an O-like intermediate (λ_{max} of 570 nm) is observed. The nature of this intermediate is not clear, but in this study we call it an O-like intermediate due to the red-shifted absorption maximum; λ_{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

TABLE 1 Dissociation constants (K_D) and the number of binding sites (n) of the complex between *p*pR mutants and t-Htr and rate constants of intermediates

| | | | Decay Rate Constant (s^{-1}) | |
	K_D (μM)	n	Pigment Alone	Complex
Wild-type (in the dark)*	0.10	1.0	—	—
D75N (O-like intermediate)[†]	0.15	1.0	15.0	56
D75N/Y199F (O-like intermediate)	1.9	1.1	15.4	66.5
D75N/Y199V (O-like intermediate)	1.3	1.0	13.1	60.8
Wild-type (M-intermediate)[‡]	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

*The values of the wild-type *p*pR and t-Htr in the dark were taken from Wegener et al. (2000).
[†]The values estimated from the decay of the O-like intermediate of D75N *p*pR (from Sudo et al., 2001).
[‡]The values estimated from the decay of the M-intermediate of the wild-type *p*pR (from Sudo et al., 2001).

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, *p*pRs (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 *p*pR mutant proteins were not present.

We titrated 25 μM t-HtrHis with D75N/Y199F (Fig. 3 *A*, *open circles*) or D75N/Y199V (Fig. 3 *A*, *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 *p*pR, the *p*pR/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 $\alpha\exp(-k_1t) + \beta\exp(-k_2t)$, 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 ([*p*pR]) was plotted against the complex concentration ([*p*pR/t-HtrHis]) in Fig. 3 *A*. The method of calculating these values was described by Sudo et al. (2001 and submitted for publication). From this curve, K_D values were estimated as 1.9 $\pm$ 0.2 μM (D75N/Y199F) and 1.3 $\pm$ 0.2 μM (D75N/Y199V), and n were 1.1 $\pm$ 0.02 (D75N/Y199F) and 1.0 $\pm$ 0.02 (D75N/Y199V). These K_D values are ∼10-fold larger than that of the D75N single mutant (150 nM; Sudo et al., 2001, and submitted for publication).
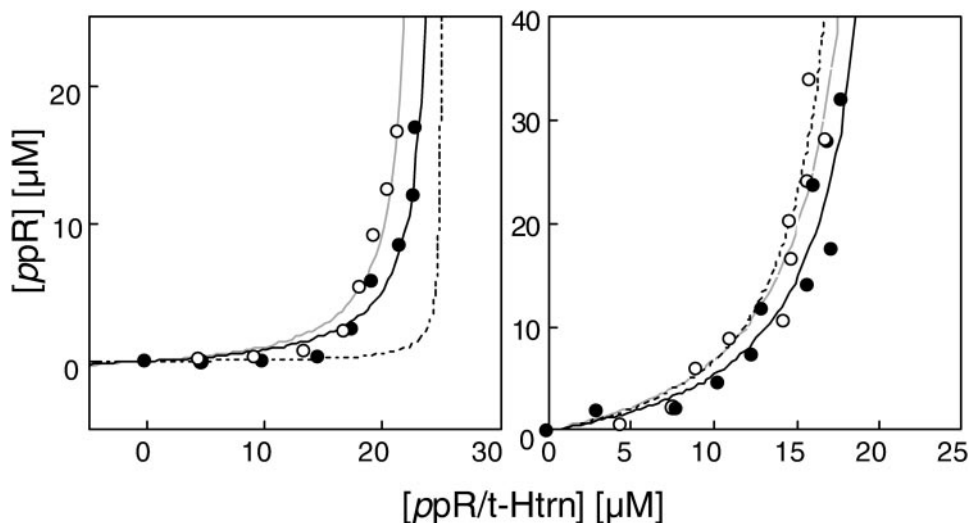
FIGURE 3 Concentrations of the free ppR, [ppR], plotted against concentrations of the ppR/t-Htr$_n$ complex, [ppR/t-Htr$_n$] during the titration. Details were described by Sudo et al. (2001 and submitted for publication). (A) Plots from the decay rates of the O-like intermediate of the double mutants of Asp-75 and Tyr-199. *Open circle*, D75N/Y199F; *closed circle*, D75N/Y199V. The circles are data points; the lines (*solid* and *gray*) are regression curves from using nonlinear 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 for publication). 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; *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.

The K_D of the complex between the M-intermediate of the wild-type ppR and t-Htr is 15 μM (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 ± 0.15 s^{-1} (Y199F) and 1.57 ± 0.12 s^{-1} (Y199V), while that of the complex was 0.84 ± 0.04 s^{-1} (Y199F/t-Htr) and 0.80 ± 0.3 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 ± 1.4 μM (Y199F) and 10 ± 1.2 μM (Y199V). The values of n were 1.2 ± 0.08 (Y199F) and 1.1 ± 0.07 (Y199V). In Fig. 3 B, the [ppR] is plotted against [ppR/t-Htr$_n$] for these mutant pigments.

Fig. 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 $\sim$ 9 by plotting [ppR] against [ppR/t-Htr$_n$] (Fig. 5 A; *open circles*, pH 2; *closed*
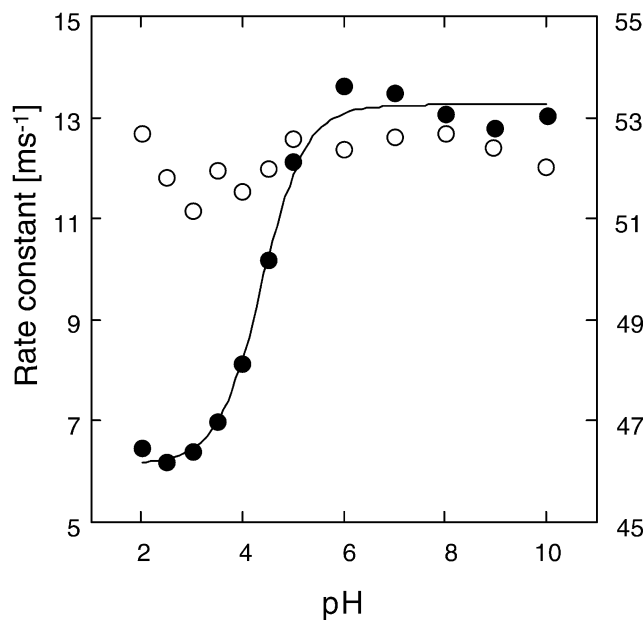

FIGURE 4 Rate constants of the O-like intermediate decay of D75N alone and D75N/t-Htr complex at varying pH. Open circles are the data of the D75N/t-Htr complex using the right ordinate. Closed circles are the D75N alone using the left ordinate. The solid line is a fitted curve by using the Henderson-Hasselbach equation with a single pK_a 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. The 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-Hasselbach equation with pK_a of 3.86 ± 0.02. The experimental conditions were the same as for Fig. 3.
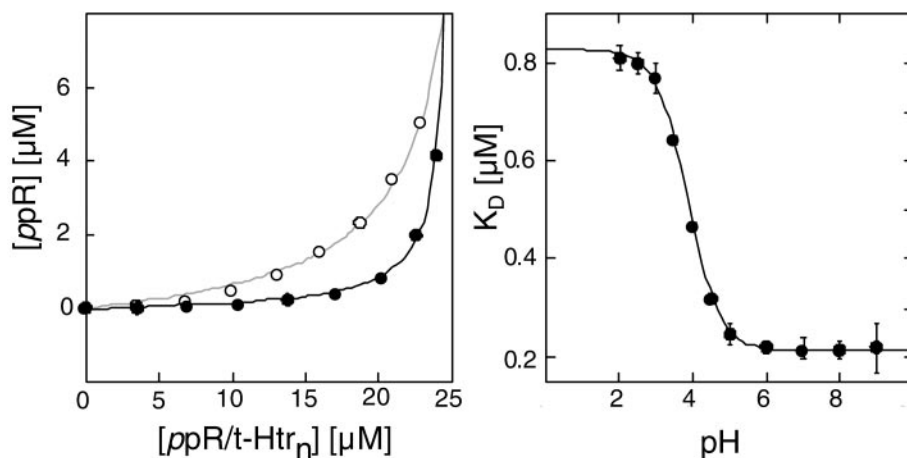
circles, pH 6). Fig. 5 *B* delineates the estimated K_D as a function of pH. This curve was fitted by the Henderson-Hasselbach equation with a single pK_a estimated as 3.86 ± 0.02.

DISCUSSION

Luecke et al. (2001) and Royant et al. (2001) reported x-ray crystallographic structures 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 photointermediate of the D75N mutant. The interaction of the photointermediate of the D75N mutant with the transducer gives good information on the interaction between the pigment and the transducer in the dark. The photointermediate 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 pK_a 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 pK_a 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, which are conserved in various transducer proteins) (Royant et al., 2001). The pK_a of 3.86 may be the average value of these Asps of the transducer. These Asps locate very close and their respective pK_a 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× histidine tag and these Asps 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 (pK_a 3.86) is possibly important for keeping the dimer structure of the transducer (Yang and Spudich, 2001). The identification of this residue is awaited for further investigation.

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