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Effect of Asp193 on Proton Affinity of the Schiff Base in *pharaonis* phoborhodopsin

Masayuki Iwamoto[1]*, Yuji Furutani[2], Yuki Sudo[1], Kazumi Shimono[1], Hideki Kandori[3] and Naoki Kamo[1]

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

[2]Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

[3]Department of Applied Chemistry, Nagoya Institute of Technology, Nagoya 466-8555, Japan

Spectroscopic titration of D193N and D193E mutants of *pharaonis* phoborhodopsin (*p*pR) were performed to evaluate the pK_a of the Schiff base. Asp193 corresponds to Glu204 of bacteriorhodopsin (bR). The pK_a of the Schiff base (SBH^+) of D193N was 10.1~10.0 (at XH^+) and 11.4~11.6 (at X) depending on the protonation state of a certain residue (designated by X) and independent on Cl^-, while those of the wild-type and D193E were >12. pK_a of XH^+ were; 11.8~11.2 at the state of SB, 10.5 at SBH^+ state in the presence of Cl^-, and 9.6 at SBH^+ without Cl^-. These imply the presence of a long-range interaction in the extracellular channel.

Key words : hydrogen bonding network, pK_a of the Schiff base, spectroscopic titration, sensory rhodopsin II, archaeal rhodopsin

INTRODUCTION

Pharaonis phoborhodopsin (*p*pR; also *pharaonis* sensory rhodopsin II, psRII) is a receptor of the negative phototaxis of *Natronobacterium pharaonis*. The amino acid sequence of *p*pR is homologous to that of bacteriorhodopsin (bR), a well known light-driven proton pump. Important residues in the extracellular channel (EC) of bR (Asp85, Asp212, Arg82) are all conserved in *p*pR as Asp75, Asp201 and Arg72, respectively, with the exception of Glu194 that is replaced by Pro183 in *p*pR. Asp75 of *p*pR serves as a counterion of the protonated Schiff base (PSB or SBH^+) [1,2]. Upon absorption of a photon, *p*pR undergoes a photo-reaction cycle [3-5] similar to bR. Proton uptake and release during the photocycle of *p*pR have been detected [6], and the trans-membranous proton transport from inside to outside was detected [7-9], although this activity was weak. Many studies of bR have revealed the existence of a complex linkage from Glu204 to the Schiff base via its counterion Asp85 in EC ([10] for review). During the photocycle, this linkage cooperatively regulates the protonated state of these residues to achieve the prompt proton release. Therefore, it is an important question whether an intra-molecular linkage between the Schiff base and the outer surface of the protein exists in *p*pR as in bR.

In this study, we examined this problem. To this end, the pK_a of the PSB of Asp193 mutants, corresponding residue to Glu204 in bR, were determined. The presence of a negative charge at the 193-position increases the pK_a of the PSB by more than 2 units, indicating the existence of a long-range interaction. In addition, existence of another protonable residue (XH) affecting pK_a of the PSB, and Cl^- effects on the pK_a of XH are also suggested.

MATERIALS AND METHODS

Sample preparations. The expression of the histidine-tagged *p*pR in *E. coli* BL21(DE3) and its purification were described elsewhere [11]. QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, CA) was used to prepare D193N and D193E mutant.

Spectroscopic analysis. The absorption spectra were taken using a Model V-560 spectrophotometer (Jasco, Tokyo). The temperature was kept at 20 °C. During the titration experiments for the absorption spectra of D193N, the pH was initially adjusted to 7.0 using a mixture of six buffers

*To whom correspondence should be addressed.
E-mail : iwamoto@pharm.hokudai.ac.jp

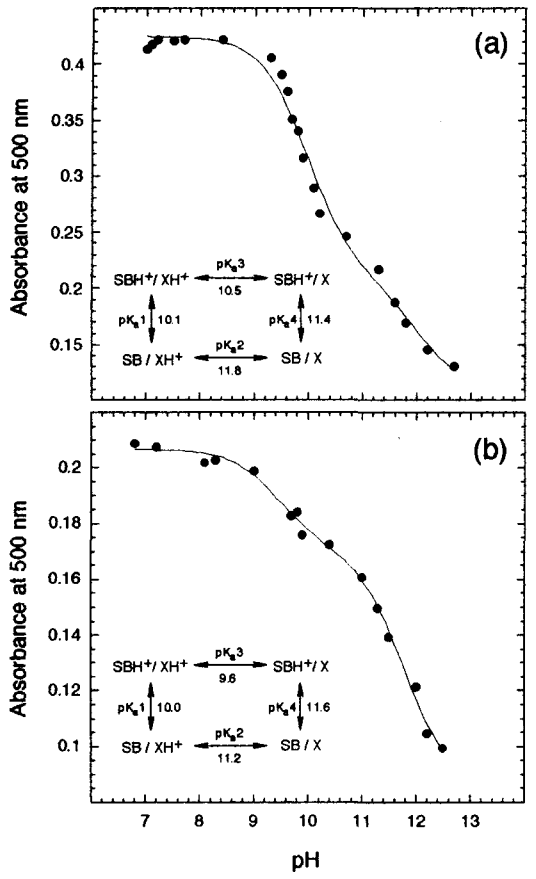

Figure 1. pH titration curves of the Schiff base in D193N. (a) in the presence of 200 mM Cl^-. (b) in a Cl^-free medium (containing 67 mM Na_2SO_4 for keeping the ionic strength of 200 mM NaCl). Fitted curves were obtained with a model of two interacting residues, which is shown in the panel. Residue X is an unknown protonatable residue whose protonation state affects the pK_a of the Schiff base.

(containing citric acid, Mes, Hepes, Mops, Ches and Caps, all concentrations of which were 10 mM each) and 0.1% DDM (n-dodecyl-b-D-maltoside). The pH titration of the PSB started from 7.0. The pH was adjusted to the desired value by the addition of concentrated NaOH, and the absorption spectra were then measured. Data fitting was done using Microcal Origin™ software (Microcal Software, MA).

RESULTS

The spectroscopic titration of D193N was conducted. With an increase in the pH values, the absorption band at 500 nm decreased accompanying with increase at 360 nm due to the deprotonated Schiff base. Figure 1 shows the titration curve of the PSB in D193N in the presence (a) and absence (b) of 200 mM NaCl. In the Cl^- free experiment, 67 mM Na_2SO_4 was added so as to keep the ionic strength constant. Deprotonation of the PSB in D193N exhibited a complex titration curve, indicative of the interaction by another protonatable residue. Thus, we fitted these titration curves with a model of two interacting residues [12] (whose model is depicted in Fig. 1), and estimated pK_a's are listed in Table 1. The pK_{a1} and pK_{a4} values are pK_a of the PSB when another protonatable residue (X) is protonated and deprotonated, respectively. The pK_{a2} and pK_{a3} values are pK_a of the X residue when the Schiff base is deprotonated and protonated, respectively. The pK_a of the PSB in the wild-type *p*pR was 12.4 (Balashov et al., in preparation) and no remarkable pK_a change in the PSB of D193E was observed (data not shown). On the other hand, that of D193N was lowered to 10.1 or 11.4, depending on whether residue X was protonated or deprotonated, respectively. Interestingly, the pK_a value of residue X was affected by Cl^- when the Schiff base is protonated (see pK_{a3} in Table 1), suggesting that the Cl^--binding site of D193N may locate near the X residue and that the negative charge of Cl^- may increase the pK_a of the X residue.

Table 1. pK_a values for titration of the Schiff base

	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
Wild-type	>12	–	–	–
D193N	10.1	11.8	10.5	11.4
D193N (Cl^- free)	10.0	11.2	9.6	11.6
D193E	>12	–	–	–

DISCUSSION

The pK_a of the Schiff base in D193N was estimated to be ~10 while that of the wild-type or D193E was ~12, suggesting that the negative charge at the 193-position increases the proton affinity of the Schiff base in the wild-type *p*pR. Referring to the recent crystal structure of *p*pR [13,14], the distance between the Schiff base and the side chain of Asp193 is ca 14 Å. Therefore, the existence of the long-range interaction, like the hydrogen bonding network between the Schiff base and the extracellular surface of the protein revealed in bR, is expected. If this interaction exists, the pK_a shifts of the Schiff base and Asp75 (the counter-ion of the PSB) might be observed in the Arg72 *p*pR mutant as is similar to the R82A bR mutant. The effect of the Arg-residue on these pK_a value is now in progress.

Another interesting point is the existence of a protonatable residue whose protonation states affect the

pK_a of the Schiff base. A possible candidate for this protonatable residue is Arg72 judging from the location within the EC channel and from its pK_a value; Arg72 guanidinium of *p*pR locates ca 11 Å from the Schiff base and ca 5 Å from Asp193 carboxyl, and the estimated pK_a of residue X (see Fig. 1) resembles that of Arg (11.8 or 10.5 when the Schiff base is deprotonated or protonated, respectively) although the pK_a value is somewhat smaller than the usual value. In addition, our results show that Cl^- mainly affects the pK_a not of the Schiff base (pK_{a1} and pK_{a4}) but of residue X when the Schiff base is protonated. The change in the pK_a is due presumably to the electrostatic interaction (pK_{a2} and pK_{a3}, see Fig. 1 and Table 1). This might indicate that in the D193N mutant, the Cl^--binding site is close to residue X, presumably Arg72. Royant et al. [14] proposed the existence of the Cl^--binding site near Arg72 in the wild-type *p*pR, and then it is probable that this site might be the Cl^--binding site which is proved in the D193N mutant in the present paper. The conclusion whether the Cl-binding site exists in the wild-type must await further studies. In any case, it may be certain for the D193N mutant that bound Cl^- has a role in the regulation of the pK_a's.

The present investigation revealed the existence of a long-range interaction that extended from the extracellular surface to the Schiff base in *p*pR. Water molecules may be involved in this interaction as is proved in bR. Thus, cooperative linkages among the amino acid residues and water molecules in the EC channel via hydrogen bonding would be a common feature of the archaeal retinal proteins.

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