Journal of Photoscience (2002), Vol. 9(2), pp. 302–304

Importance of The Location of The Negative-charged Counter-ion against The Protonated Schiff Base on The Chromophore Configuration of *pharaonis* Phoborhodopsin

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pharaonis phoborhodopsin (*p*pR), a photophobic sensor of haloalkaliphilic bacteria, *Natronobacterium pharaonis*, has retinal as a chromophore covalently bound to Lys in G-helix *via* a protonated Schiff base (PSB), as is the same as bacteriorhodopsin (bR). For *p*pR, the corresponding counter-ion is Asp residue (Asp75) located in C-helix. Here we investigated the influence of the protonated state of this counter-ion and its location on the chromophore configuration. Under alkaline condition, the chromophore configuration of D75E mutant was analyzed by HPLC. D75E had a much larger content of 13-*cis* isomer: the ratio of 13-*cis* to all-*trans* was 6:4 while the wild-type had this ratio of 1:9. On the other hand, under acidic condition where Glu was associated, D75E had no 13-*cis* retinal isomer. Mutants whose Asp75 was replaced by neutral amino acids (D75N and D75Q) did not contain 13-*cis* retinal. Furthermore, retinal isomer compositions and the change in the visible absorption spectra (indicating the dissociation state of Glu75) were measured under varying pH, and these were almost the same dependencies. These results indicate that an important factor determining the 13-*cis* isomer content is the presence of negative charge of the counter-ion against PSB, but not the size of this residue. Comparison between the wild-type and D75E in alkaline solutions indicates the influence of the location of the counter-ion.

Key words: photosensor, archaeal retinal protein, retinal isomer, counter-ion, protonated Schiff base

INTRODUCTION

Haloarchaea, *Halobacterium salinarum* has at least four retinal proteins: bacteriorhodopsin (bR) [1], halorhodopsin (hR) [2], sensoryrhodopsin (sR or sRI) [3] and phoborhodopsin (pR also called sensory rhodopsin II, sRII) [4]. The former two proteins work as light-driven ion pumps. The latter two work as photosensor and control the motion of this bacteria against light. It is covalently bound to a retinal chromophore at a conserved lysine residue on G helix *via* a protonated Schiff base (PSB) bond. In the dark, light-driven ion pumps (bR and hR) have all-*trans* and 13-*cis* retinal, whereas photosensors (sR and pR) only all-*trans* [5]. This difference of retinal isomer composition may be related to the difference of function (pump or sensor).

pR-like protein (*pharaonis* phoborhodopsin, *p*pR) from haloalkaliphilic bacterium, *Natronobacterium pharaonis*, is stable under low ionic strength condition and in detergent micelle [6]. Furthermore, we succeeded in the functional expression of *p*pR in the *Escherichia coli* membrane [7]. These features are suitable on the study of photosensor proteins. As is previous Fourier transform Infrared (FT-IR) study shown, in M-intermediate of *p*pR, Asp75 (corresponding to Asp85 of bR) is proton acceptor from PSB (Y. Furutani, unpubilished and ref. 8). Furthermore, D75N mutant has large red shifted absorption spectrum, but D201N spectrum has no alternation from the wild-type (data not shown). Therefore, Asp75 acts as a counter-ion for PSB. Recently, two groups reported the crystallographic structure of *p*pR [9,10]. The structure around chromophore in *p*pR is similar to that in bR. However, the hydrogen bond between PSB and counter-ion or internal water molecule in *p*pR is stronger than that in bR according to low-temperature FT-IR spectroscopy [11]. We previously constructed a multiple mutant of *p*pR (BR/*p*pR) whose all amino acid residues of the retinal binding pocket were replaced simultaneously with those of bR, and reported that the hydrogen bond of PSB in BR/*p*pR remained strong from FT-IR study [12,13]. These facts may indicate that the environment around PSB in *p*pR slightly differs from that in bR.

Here we report that influence of the size and electric charged state of the counter-ion on the chromophore configuration of *p*pR.

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MATERIALS AND METHODS

Sample preparation. The genes for the single replacements were prepared by QuickChange Site-Directed Mutagenesis Kit (STRATAGENE), and then pET/*p*pRHis was used as template [11]. The expression of histidine-tagged recombinant ppRs in *E.coli* BL21(DE3) and its purification were described elsewhere [11].

Spectroscopic titrations. For spectroscopic titration in the absence of Cl, the *p*pR sample was suspended in 6 mix buffer (citric acid, MES, HEPES, MOPS, CHES and CAPS whose concentrations were 10 mM each) and 0.1 % n-dodecyl-β-D-maltoside (DM). The pH was first adjusted to almost 9 with 10 N NaOH. The titration was carried out from alkaline to acidic pH by adding 1 N H_2SO_4.

HPLC analysis. A high performance liquid chromatograph (HPLC) was applied as described previously [12]. A sample was suspended in 6 mix buffer and 0.1 % DM adjusted appropriate pH.

RESULTS AND DISCUSSION

pH dependency of the pigment spectrum.

The spectrum of D75E was greatly pH-dependent because pKa of Glu-75 was raised to 6.2 while that of wild-type Asp-75 was 3.6 (Fig. 2 and ref. 15). For bR, the replacement of Asp-85 to Glu also raises pKa of the counterion, but the shift is larger than that of *p*pR [16]. This may indicate the difference of circumstances around the chromophore between bR and *p*pR; there exists the "pentagon structure" involving PSB, Asp85 and water molecules in the wild-type bR and *p*pR. In the *p*pR, this pentagon structure is perturbed relatively to that in bR [9,10,17]. Replacement with Glu might weaken this structure, which raises pKa. Therefore, Glu-replacement of *p*pR raises pKa by relatively small value.

Retinal configuration of Asp75 mutant.

Figure 1 shows HPLC elution pattern of the chromophore extracted in the dark from D75E mutant. Glu75 is almost dissociated at pH 9 (Fig. 2). At this condition, D75E mutant had a much larger content of 13-*cis* isomer than the wild-type: ratio of 13-*cis* to all-*trans* retinal is 6:4 for D75E, 1:9 for wild-type (Fig. 1A). Next, we investigated whether this existence of the stable 13-*cis* isomer is originated from the size or the charge of the introduced glutamate residue. In acidic condition where Glu is protonated, the 13-*cis* content decreases (Fig. 1B). Furthermore, we analyzed the retinal configuration of mutants whose Asp-75 was replaced by neutral amino acids (D75N and D75Q). D75N and D75Q does not have 13-*cis* isomer (data not shown). These observations suggest that the charged state of the counterion (Glu75) determines the composition of the retinal isomer; under the condition of Glu75 being deprotonated, the

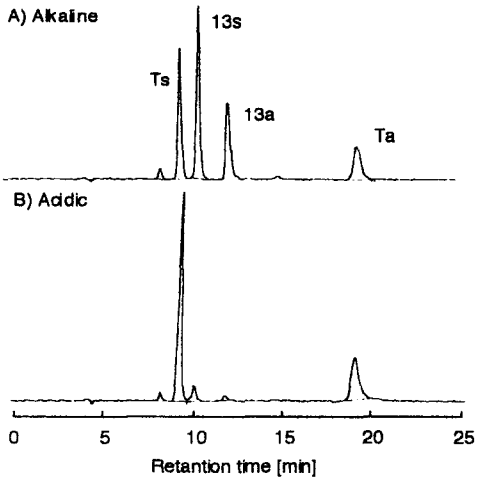

Figure 1. HPLC elution pattern of the chromophores extracted in dark from D75E *p*pR mutants in the alkaline (A) and acidic condition (B). The detection beam was set at 360 nm. The assignment of each peaks was done by comparison with HPLC pattern of the chromophores extracted from bR. The pH of a sample medium was adjusted 9 (A) or 5 (B). Ts, Ta, 13s and 13a stand for all-*trans* 15-syn retinal oxime, all-*trans* 15-anti retinal oxime, 13-*cis* 15-syn retinal oxime and 13-*cis* 15-anti retinal oxime, respectively.

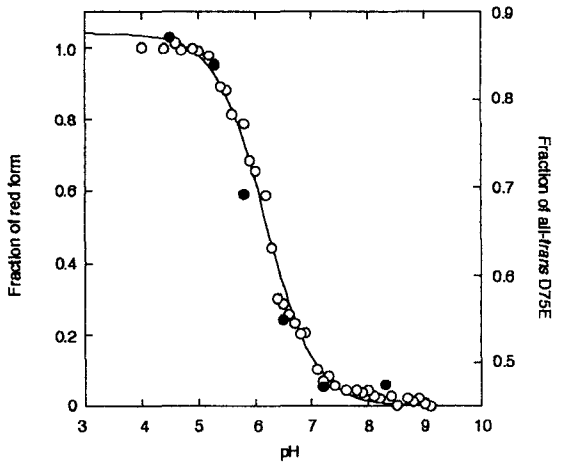

Figure 2. pH dependencies of absorption spectrum (open circle) and the ratio of all-*trans* isomer determined by HPLC (closed circle) in D75E. For determining the fraction of red form, the increase in absorbance at 542 nm was plotted as the fraction of its maximum value for the preparation as a function of pH. The curves correspond to the equation for a monoprotic titration with pKa values determined by a fit to the data.

pigment has 13-*cis* retinal while the 13-*cis* content decreases when the position of Glu75 become neutralized.

To confirm this hypothesis, we investigated the pH dependency of the retinal isomer composition of D75E mutant under varying pH. The pH dependency of retinal isomer composition was almost the same to that of the absorption spectrum which indicates the degree of protonated state of Glu75 (Fig. 2). This indicates that the negative charge of counter-ion is necessary for the existence of stable 13-*cis* chromophore. The distance between PSB and the counter-ion depends on the retinal isomer; for 13-*cis* isomer, the distance is shorter than that for all-*trans* isomer. When the counter-ion is deprotonated, the larger electrical interaction between this negative charge and the positive PSB stabilizes more 13-*cis* conformation than all-*trans* isomer. Therefore, when the counter-ion is protonated, PSB is not stable, and the 13-*cis* form is not stable. This is consistent that in the case of bR, the all-*trans* isomer composition is increased under acidic condition [18].

Since the wild-type *pp*R has all-*trans* retinal with Asp75, the counter-ion being deprotonated, this rule seems not to hold for *pp*R. However, careful examination shows that the 13-*cis* content seems to increase where Asp75 is deprotonated (data not shown). The reason why the effect of the charged state is small in the wild-type might be the longer distance between PSB and the counter-ion of the wild-type than D75E by one methylene chain.

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