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Involvement of two groups in reversal of the bathochromic shift of *pharaonis* phoborhodopsin by chloride at low pH

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Abstract

Pharaonis phoborhodopsin (*p*pR; or *pharaonis* sensory rhodopsin II, *p*sRII) is a photophobic receptor of the halobacterium *Natronobacterium pharaonis*. Its λ_{max} is at 496 nm, but upon acidification in the absence of chloride, λ_{max} shifted to 522 nm. This bathochromic shift is thought to be caused by the protonation of Asp75, which corresponds to Asp85 of bacteriorhodopsin (bR). The D75N mutant, in which Asp75 was replaced by Asn, had its λ_{max} at approximately 520 nm, supporting this mechanism for the bathochromic shift. A titration of the shift yielded a pK_a of 3.5 for Asp75. In the presence of chloride, the spectral shifts were different: with a decrease in pH, a bathochromic shift was first observed, followed by a hypsochromic shift on further acidification. This was interpreted as: the disappearance of a negative charge by the protonation of Asp75 was compensated by the binding of chloride, but it is worthy to note that the binding requires the protonation of another proton-associable group other than Asp75. This is supported by the observation that in the presence of chloride, upon acidification, the λ_{max} of D75N even showed a blue shift, showing that the protonation of a proton-associable group (pK_a = 1.2) leads to the chloride binding that gives rise to a blue shift.

Keywords: Acid-induced red shift; Reversal of red shift by Cl; Cl binding; *Pharaonis* phoborhodopsin (*p*pR); Sensory rhodopsin II (sRII)

Abbreviations: DM, *n*-dodecyl β-D-maltoside; D75N, a mutant protein whose Asp75 is replaced by Asn; pR, phoborhodopsin; *p*pR, *pharaonis* phoborhodopsin, pR-like pigment protein in *Natronobacterium pharaonis*; *p*sRII, sensory rhodopsin II in *Natronobacterium pharaonis*, which is the same as *p*pR; sRI, sensory rhodopsin I; sRII, sensory rhodopsin II, which is the same as pR; λ_{max}, absorption maximum

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1. Introduction

The archaeal species of *Halobacterium salinarum* has a phototactic signal transduction system [1,2]. Two photoreceptors have been identified: one is sensory rhodopsin (sR) or sensory rhodopsin I (sRI) [3,4], and the other is phoborhodopsin (pR) or sensory rhodopsin II (sRII) [5–9]. Both have retinal as their chromophore. In addition to these two, the cell has two other retinal proteins, bacteriorhodopsin (bR) [10] and halorhodopsin (hR) [11–13]. These two are energy transducing retinal proteins; bR acts as a light-driven proton pump, while hR acts as a Cl pump. These four retinal proteins are closely related structurally to each other. Retinal is bound to the seventh membrane helix of the protein via a protonated Schiff base. On light excitation, a characteristic photoreaction cycle for each of these four retinal proteins is observed, which is coupled to each pigment's respective physiological function.

The extent of investigations on pR (or sRII) is less than bR, hR or sR (or sRI), especially than bR [14–16]. This is because the amount of pR expressed in the *salinarum* cell membrane is very small, and because pR is not stable in its solubilized state. On the other hand, we [17] and Engelhard and his colleagues [18] have succeeded in purifying a pR-like protein from *Natronobacterium pharaonis*, a halophilic alkaliphilic bacterium, and have begun to characterize it in detail. We termed the pigment *pharaonis* phoborhodopsin (*p*pR; also called *pharaonis* sensory rhodopsin II, *p*sRII). Furthermore, we [19] succeeded in the functionally expressing *p*pR in *Escherichia coli*.

The maximum wavelength (λ_{max}) of pR or *p*pR is at approximately 500 nm, differing from the other three retinal pigments whose λ_{max} is 560–590 nm. In *N. pharaonis*, *p*pR acts as a photoreceptor, which mediates the negative phototaxis induced by green–blue light [20], similar to pR (sRII) in *H. salinarum*. At low pH the λ_{max} of bR is red-shifted (bathochromic shift) from 568 to 605 nm (acid form) concomitant with the cessation of light-induced proton transport [21,22]. This bathochromic shift is due to the protonation of Asp85, which accepts a proton from the protonated Schiff base upon photo-excitation. Protonation of Asp85 weakens the interaction with the protonated Schiff base, thereby causing the bathochromic shift. The addition of Cl converts the acid-blue species into the acid-purple [23], which is caused by Cl-binding near the Schiff base to create a negative charge.

The same color changes are also observed in other bacterial rhodopsins; *pharaonis* halorhodopsin (*p*hR) is an another example in which the negative charge of a Cl^- near the Schiff base leads to a blue shift: *p*hR absorbs normally at 570 nm and is converted to a blue pigment (λ_{max} = 600 nm) by extracting Cl, which binds near the Schiff base and acts as a counterion of the base [24]. For *salinarum* pR (*s*pR) [25,26] and *p*pR [27], a similar acid-induced bathochromic shift has been reported. High concentration of Cl can reverse the bathochromic shift in *p*pR at low pH, as shown in [27]. Spudich and his co-workers [26] showed that D73N mutant of *s*pR has an absorption maximum at approximately 520 nm, which is almost the same as that of the acid-form of *s*pR. Asp73 of *s*pR corresponds to Asp85 of bR, or to Asp75 of *p*pR. In spite of these reports, a detailed analysis on the effect of pH and Cl has not yet been reported; using *p*pR expressed in *E. coli* membrane [19], we found that Cl caused a blue shift of the acid-induced red-shifted *p*pR, and that the protonation of two groups was required for the Cl effect.

2. Materials and methods

2.1. Sample preparations

The expression of the recombinant *p*pR in *E. coli* [strain BL21(DE3)] and its purification is described elsewhere [28,29]. The Kunkel method [30] was used to prepare D75N and D201N mutants. The buffer was exchanged by ultrafiltration (UK-50 membrane, Advantech, Tokyo).

2.2. Spectroscopic titrations

Absorption spectra were taken by a Model

V-560 spectrophotometer (Jasco, Tokyo). The temperature was kept at 20°C. For spectroscopic titration in the absence of Cl, the *p*pR sample ($OD_{500} \sim 0.1$) was first suspended in a medium containing 0.1% dodecyl maltoside (DM) and 100 mM sodium citrate. For wild-type *p*pR, the pH was first adjusted to almost 0 with 1 M H_2SO_4. The pH was then adjusted to the desired value by addition of very small amounts of conc. NaOH, and the absorbance spectrum measured. Before and after each spectrum, the pH of the sample was measured to ensure that the variation was less than 0.05 pH units. The concentration changes in *p*pR caused by the addition of the NaOH solution were very small. For experiments with the D75N mutant, the titration was carried out from neutral to acidic pH by adding conc. H_2SO_4, because the mutant showed gradual denaturation below pH 1; under acidic conditions the measurements were carried out as quickly as possible, and we confirmed that the same absorption maximum was obtained when a back-titration was done. For spectral measurements in the presence of Cl, the above procedure was carried out after adding an appropriate concentration of NaCl.

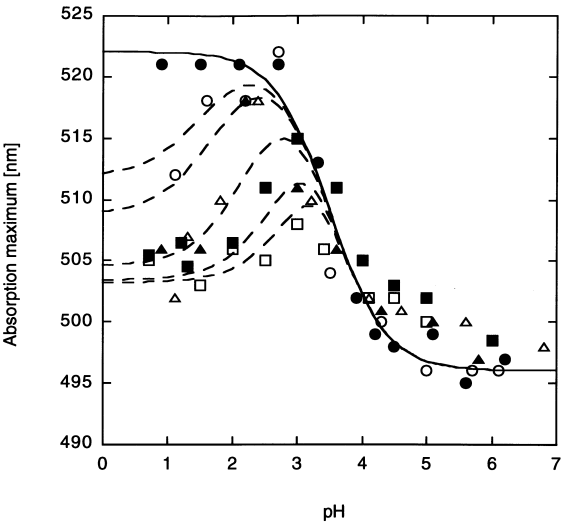

Fig. 1. A plot of the absorption maximum against pH in the presence of varying concentrations of NaCl. NaCl concentrations are: 0 mM, ●; 50 mM, ○; 100 mM, ▲; 500 mM, △; 2 M, ■; 4 M, □. The solid line is the simulated curve using the Henderson–Hasselbach equation, assuming a single pK_a, which was estimated to be 3.5. The broken lines represent the simulated curves in accordance with Scheme 1. The values of the parameters are given in the text. Composition of the medium is referred to in Section 2, and temperature was 20°C.

2.3. Curve fitting of absorbance maximum in varying pH

Data in the absence of Cl was analyzed by the Henderson-Hasselbach equation with one pK_a value. Data in the presence of Cl were fitted in accordance with Scheme 1 (see Section 3). The equation is as follows:

$$\lambda_{max} = \frac{[A^-B^-]}{[p\mathrm{pR}_{total}]}\lambda_{max}^{A^-B^-} + \frac{[p\mathrm{pR}]_{long}}{[p\mathrm{pR}_{total}]}\lambda_{max}^{p\mathrm{pR}_{long}} + \frac{[\mathrm{AHBHCl}^-]}{[p\mathrm{pR}_{total}]} \times \lambda_{max}^{AHB}$$

Here, $p\mathrm{pR}_{tot}$ represents the total concentration of *p*pR, $[A^-B^-] + [AHB^-] + [AHBH] + [AHBHCl^-]$; $p\mathrm{pR}_{long}$, $[AHB^-] + [AHBH]$ where the subscript $_{long}$ signifies the species having an absorption maximum of long wavelength. The concentrations of these species are calculated according to Scheme 1 (see Section 3) using the mass action law. The absorption maxima of A^-B^-, the long-wavelength absorbing species ($p\mathrm{pR}_{long}$) and $AHBHCl^-$ are represented by λ_{max}^{AB}, $\lambda_{max}^{p\mathrm{pR}_{long}}$ and $\lambda_{max}^{AHBHCl^-}$, respectively, assuming that the absorption maxima of AHB^- and AHBH are equal to $\lambda_{max}^{p\mathrm{pR}_{long}}$ (see Section 3). It should be noted that all data (λ_{max} vs. pH data of 5 Cl concentrations of 50, 100, and 500 mM, 2 and 4 M shown in Fig. 1) were simultaneously fitted using Origin (Microcal Software) to estimate parameters, which are pK_{aA}, pK_{aB} and K_{Cl} (see Scheme 1 for the notation), as well as three absorption maxima.

3. Results and discussion

As mentioned in Section 1, two papers have reported the acid-induced bathochromic shift of

*s*pR or *p*pR: Using *s*pR expressed in *H. salinarum*, Zhu et al. [26] observed the acid-induced bathochromic shift, and Chizhov et al. [27] also observed the shift using *p*pR expressed in *H. salinarum* and the reversal of λ_{max} by addition of NaCl (final conc. of 3 M). We also confirmed their data (data not shown).

The present paper further examined the λ_{max} shift in the presence of varying NaCl concentrations, data are shown in Fig. 1. The Cl concentrations employed were 0 mM (●), 50 mM (○), 100 mM (▲), 500 mM (△), 2 M (■) and 4 M (□). In the absence of Cl, no spectral changes were observed below pH 2.7. The data were well fitted with a monoprotic titration with $pK_a = 3.5$. As described above, this pK_a may be that of Asp75. Chizhov et al. [27] reported that pK_a of this bathochromic shift is 5.6 for *p*sRII reconstituted with purple membrane lipids, and when solubilized in dodecyl maltoside, the pK_a is decreased ($pK_a < 3.5$). Zhu et al. [26] determined that the pK_a of Asp73 in *s*sRII is 3.0. Here, *s*sRII-Asp73 corresponds to *p*pR (*p*sRII)-Asp75 and to bR-Asp85. The value of the pK_a of the reconstituted *p*pR (*p*sRII) seems high. Negative surface charges originating from the lipids could be a reason for this high pK_a. It has been established that the surface potential greatly influences the apparent pK_a of bR-Asp85, which is estimated from the purple to blue transition [31–34], although that of bR-Asp85 is reported to be 2.2 (in high salt concentration) or 3.2 (in low salt concentration) [33,35]. Negative surface potential increases the local surface proton concentration, which increases the apparent pK_a.

The bathochromic shift of λ_{max} of *p*pR is reversed by adding NaCl, as shown in Fig. 1; in the presence of NaCl a blue shift was observed, even with a decrease in pH. It is very interesting that the pH where the λ_{max} is the longest depends on the NaCl concentration (see later). Fig. 1 also indicates that the λ_{max} at pH 0.5 and 4 M NaCl is 503 nm, which is still bathochromic-shifted from λ_{max} at the neutral conditions, suggesting that the binding of a Cl anion cannot compensate completely for the disappearance of the negative charge of Asp75.

Fig. 2 shows λ_{max} vs. pH in the absence of Cl;

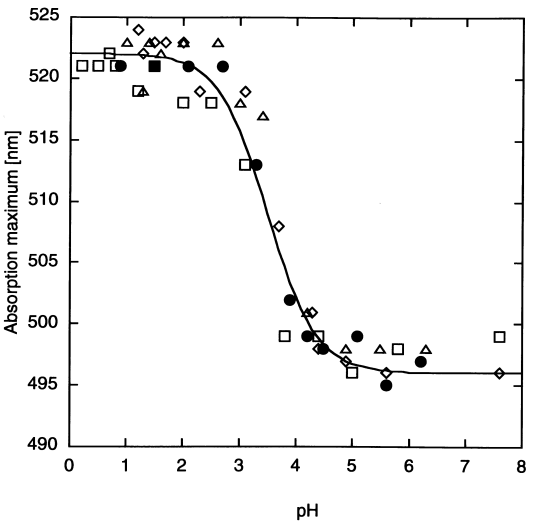

Fig. 2. Plots of the absorption maximum against pH in varying concentrations of Na_2SO_4. The concentrations are: 0 mM, ●; 50 mM, △; 500 mM, □; 1 M, ◇. The solid line represents the simulated curve using the Henderson–Hasselbach equation with a single pK_a, and is the same as the solid line of Fig. 1.

*p*pR suspended in Na_2SO_4 of varying concentrations was titrated with H_2SO_4. Obviously, no blue shift is observed, unlike in the Cl-containing solution (Fig. 1). This clearly means that Cl (maybe binding of Cl near the Schiff base) induces the blue shift under the condition of protonated Asp75, a counterion of the Schiff base, as is similar to bR. This figure indicates 3.5 for the pK_a value of Asp75, as is the same of the data (closed circle) in Fig. 1. Another point is that the acid-induced bathochromic shift of the present sample is independent of the ionic strength. The reason is not clear at present, although the simplest explanation might be the very small surface charge of the present sample. As described above, the purple-to-blue transition of bR depends strongly on the ionic strength in the medium.

Again, we shall examine Fig. 1; the characteristic feature is that in the presence of Cl, the λ_{max} shifts to the red, followed by a reverse shift with a decrease in pH. At certain pH values the λ_{max} reaches maximum value (the peak in Fig. 1). Such pH values (the pH at the peak) shift to higher pH with an increase in the Cl concentration. One

$$A^-B^- \underset{pK_{a1}}{\rightleftarrows} AHB^- \underset{pK_{a2}}{\rightleftarrows} AHBH \underset{K_{Cl}}{\rightleftarrows} AHBHCl^-$$

(496 nm) (522 nm) (522 nm) (503 nm)

(Scheme 1)

Scheme 1.

could simply assume that the protonation of Asp75 leads to Cl binding, which compensates for the loss of the negative charge to restore λ_{max}. This assumption, however, cannot explain the blue shift at more acidic pH values than that of the peak. This observation implies that another proton-associable group other than Asp75 may be involved in the Cl binding, and the pK_a of this group must be smaller than 3.5, the pK_a of Asp75. In other words, Cl does not bind until these two groups are neutralized. Therefore, we propose the following scheme:where AH stands for Asp75 whose pK_a is pK_{aA}, and BH is another proton-dissociable group whose pK_a is pK_{aB}. The dissociation constant of Cl binding is denoted as K_{Cl}. Numbers in parentheses represent λ_{max} of the respective species, and were obtained from the fitting of the data shown in Fig. 1. According to Scheme 1, upon acidification, Asp75 (presented by A^-) is first protonated, which raises the λ_{max} to 522 nm. Further acidification induces the neutralization of B^-, but this disappearance of the negative charge does not change the λ_{max}, the reason for which is not clear. Thereafter, Cl binds near the Schiff base, which leads to the blue shift of the λ_{max}. In this scheme the synergistic effect between the protonation and Cl-binding is not taken into consideration, although this effect was proved in gecko P521 cone-type visual pigment [36].

The fitting was performed simultaneously for all the data of five Cl concentrations in Fig. 1. The best fit was obtained as $pK_{aA} = 3.5$, $pK_{aB} = 1.2$ and $K_{Cl} = 0.023\ mM^{-1}$ ($1/K_{Cl} = 43.5$ mM)($\chi^2 = 7.386$). The value of pK_{aA} ($= 3.5$) is equal to that estimated from the data shown by the filled circles in Fig. 1 and the solid curve in Fig. 2. The broken lines in Fig. 1 represent the simulated curves using the values of $pK_{aA} = 3.5$, $pK_{aB} = 1.2$ and $K_{Cl} = 0.023\ mM^{-1}$ ($1/K_{Cl} = 43.5$ mM). Although deviations from the data points are observed, the characteristic feature is well expressed.

Fig. 3 shows the λ_{max} of the *p*pR D75N mutant vs. pH. Data shown by open circles indicate λ_{max} in the absence of Cl. It is noted that even at neutral pH, the λ_{max} is at approximately 520 nm, meaning that neutralization of Asp75 induces a bathochromic shift, which was obtained by previous authors [26,27]. The reason for the small blue shift, whose pK_a seems to be approximately 2, is not clear. Closed circles show the λ_{max} vs. pH in the presence of 2 M NaCl, and reveals a blue shift with a decrease in pH. This clearly indicates that another proton-associable group other than Asp75, B^- in the above scheme, is involved in the blue shift, i.e. the Cl binding. From this figure, the apparent pK_a of BH seems to be approximately 2, which differs somewhat from the value fitted from Fig. 1, i.e. 1.2. The reason for this discrepancy will require further study; for example, Fig. 2 reveals the independence of the ionic strength from the pK_a of Asp75 but its independence from the pK_a of the other proton-dissociable group is not known.

If the proton-associable group (B^-) having the low pK_a is assumed to be a carboxyl group, which

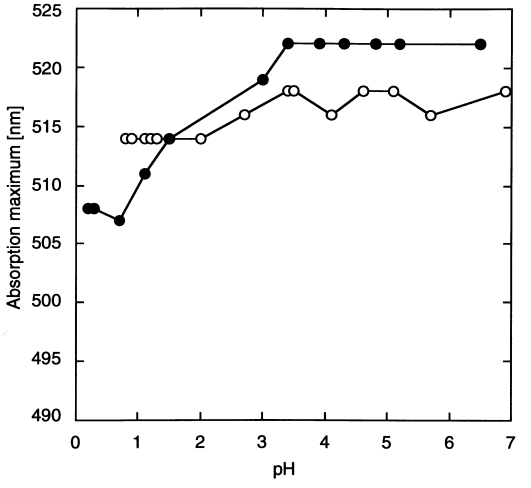

Fig. 3. Plots of the absorption maximum of the D75N mutant against pH in the absence (open circle) and presence (closed circles) of 2 M NaCl.

carboxyl group is BH? Alignment of the *p*pR amino acid sequence and structural analogy with bR suggest that BH is Asp201 (Asp212 in bR). Therefore, we tried to prepare *p*pR D201N mutant, but unfortunately we were unsuccessful in getting it to express in *E. coli*. There is another possibility that this proton-associable group may be a phosphate of the lipid.

The present paper shows that in acidic media, protonation of at least two groups (Asp75 and an unknown group) is required for the Cl binding. The pK_a values are estimated, but detailed analysis and further precise experiments are necessary to determine the values. In bR, the Cl-bound acid form, which is purple in color, acts as a Cl ion pump when irradiated [23]. One of interesting questions for further study is to elucidate whether the Cl-bound acid form of *p*pR is capable of photo-induced Cl transport.

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