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# Environment around the chromophore in *pharaonis* phoborhodopsin: mutation analysis of the retinal binding site

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**Abstract**

Phoborhodopsin (pR or sensory rhodopsin II, sRII) and *pharaonis* phoborhodopsin (*p*pR or *pharaonis* sRII, *p*sRII) have a unique absorption maximum ($\lambda_{max}$) compared with three other archaeal rhodopsins: $\lambda_{max}$ of pR and *p*pR is approx. 500 nm and of others (e.g. bacteriorhodopsin, bR) is 560–590 nm. To determine the residue contributing to the opsin shift from *p*pR to bR, we constructed various *p*pR mutants, in which a single residue was substituted for a residue corresponding to that of bR. The residues mutated were those which differ from that of bR and locate within 5 Å from the conjugated polyene chain of the chromophore or any methyl group of the polyene chain. The shifts of $\lambda_{max}$ of all mutants were small, however. We constructed a mutant in which all residues which differ from those of bR in the retinal binding site were simultaneously substituted for those of bR, but the shift was only from 499 to 509 nm. Next, we constructed a mutant in which 10 residues located within 5 Å from the polyene as described above were simultaneously substituted. Only 44% of the opsin shift ($\lambda_{max}$ of 524 nm) from *p*pR to bR was obtained even when all amino acids around the chromophore were replaced by the same residues as bR. We therefore conclude that the structural factor is more important in accounting for the difference of $\lambda_{max}$ between *p*pR and bR rather than amino acid substitutions. The possible structural factors are discussed. 

*Keywords:* *Pharaonis* sensory rhodopsin II; Site-directed mutagenesis; Absorption maximum; Opsin shift; *Natronobacterium pharaonis*

## 1. Introduction

Retinal pigments are found in various organisms as evolutionally distant as archae, algae and mammals [1]. These retinal pigments react to light of a specific wavelength. Humans have four retinal pigments in the retina: one detects light and the other three sense color. The maximum wavelengths of their color pigments differ in spite of having the same chromophore. From the archae, *Halobacterium salinarum* also has at least four retinal pigments: bacteriorhodopsin (bR) [2,3], halorhodopsin (hR) [4], sensory rhodopsin (sR or sRI) [5] and phoborhodopsin (pR, also called sRII) [6]. The first two work as ion

Abbreviations: bR, bacteriorhodopsin; BR, light-adapted bacteriorhodopsin; DM, *n*-dodecyl-β-D-maltoside; hR, halorhodopsin; IPTG, isopropyl-1-thio-β-galactoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; *p*pR, *pharaonis* phoborhodopsin (*pharaonis* sensory rhodopsin II); BR/*p*ppR′, *p*pR mutant whose seven amino acid residues composing the retinal binding site are substituted by corresponding residues of bR; BR/*p*pR, *p*pR mutant of which both seven residues of BR/ppR′ and three amino acid residues located within 5 Å from the conjugated polyene chain of the chromophore or any methyl group of the polyene chain are substituted by corresponding residues of bR; sR, sensory rhodopsin; $\lambda_{max}$, absorption maximum

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pumps and the latter two as sensors of this bacterium. These four proteins all share the same basic structure. The protein consists of an ~25 kDa polypeptide domain folded into seven helical segments [7,8]. It is covalently bound to an all-*trans* retinal chromophore at a conserved lysine residue on G helix via a protonated Schiff base bond. In $\lambda_{max}$ values, however, pR is markedly different from the other three: bR, hR and sR have their $\lambda_{max}$ at 560–590 nm while that of pR is blue-shifted to ~500 nm [9]. What is the molecular mechanism for $\lambda_{max}$ of pR being different, although all archaeal rhodopsins are highly similar in their primary structure, especially in the chromophore binding site?

A protonated Schiff base of all-*trans* retinal shows $\lambda_{max}$ at approx. 400 nm in methanol [10]. On the other hand, when all-*trans* retinal binds to an apoprotein (called an opsin) to form a protonated Schiff base, a large spectral red shift ('opsin shift' [11]) is observed. The term 'opsin shift' is also used to refer

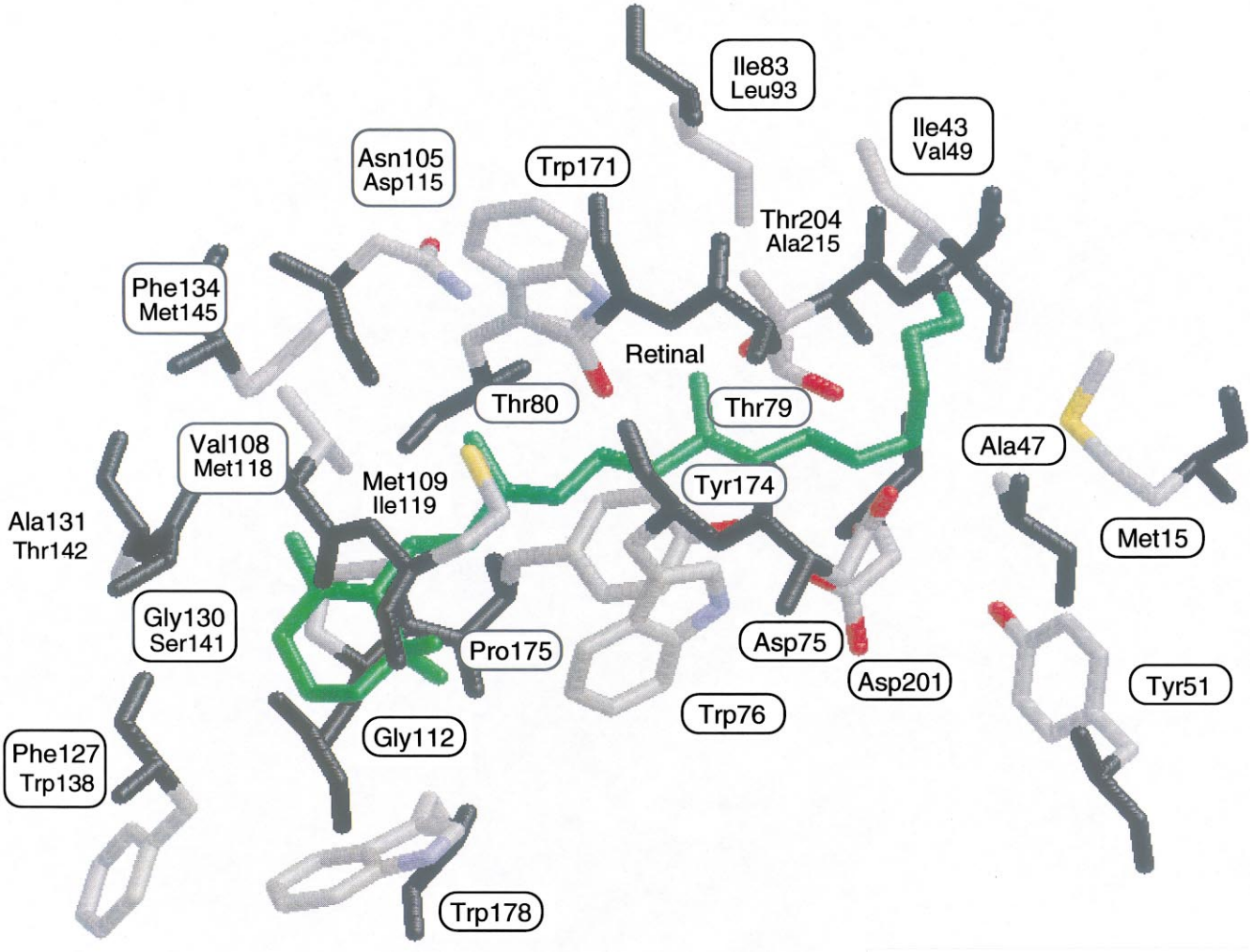


Fig. 1. Computer graphics model of retinal binding site of *pharaonis* phoborhodopsin. The model was generated using X-ray structural data (PDB code, 2BRD): amino acid residues of bR in transmembrane helices were replaced by corresponding residues of *p*pR, and energy was minimized. For details see [21]. Depicted amino acid residues are composing the retinal binding site in addition of three amino acid residues located within 5 Å from the polyene chain or any methyl group of the chromophore. A residue accompanied by two amino acids indicates a residue differing between *p*pR and bR; upper amino acids are of *p*pR and lower amino acids of bR. The enclosed residues are those composed of the 'retinal pocket' [31], and not enclosed are the three additional residues described above. The mutant genotypes are as follows: BR/*p*pR′, I43V/I83L/N105D/V108M/F127W/G130S/F134M; BR/*p*pR, I43V/I83L/N105D/V108M/M109I/F127W/G130S/A131T/F134M/T204A.

to the difference in $\lambda_{max}$ among pigments. The absorption maximum of the chromophore corresponds to its lowest π–π* excitation energy. In the ground state, a positive charge is localized mainly on the Schiff base nitrogen, and upon excitation, it shifts toward the β-ionone ring. Accordingly, any factors lead to change the energy gap between the ground and excited states. Empirical and theoretical studies have suggested several mechanisms by which the opsin shift occurs. They include the following [12–18]: (1) a change in the strength of the electrostatic interaction between protonated Schiff base and its counterion or hydrogen-bond acceptor; (2) a placement of full or partial charges along the polyene chain; (3) an alteration in the polarity or polarizability of the environment of the chromophore-binding site caused by the arrangement of polar or aromatic residues; (4) an isomerization around the 6-s bond connecting the polyene chain to the β-ionon ring.

*Pharaonis* phoborhodopsin (*p*pR, or *pharaonis* sensory rhodopsin II, *p*sRII) is a sensor pigment of *Natronobacterium pharaonis* [19]. It is a corresponding protein of pR in *Halobacterium salinarum*, and thus its $\lambda_{max}$ is 498 nm [20]. We [21] recently reported the effect of the replacement of three characteristic amino acid residues of *p*pR (Val-108, Gly-130 and Thr-204); these are completely conserved among bR, hR and sR groups (whose $\lambda_{max}$ is 560–590 nm) but are replaced by other residues in pR groups (whose $\lambda_{max}$ is $\sim$500 nm). Even for a triple mutant (V108M/G130S/T204A) $\lambda_{max}$ was 515 nm; the opsin shift was as small as 623 $cm^{-1}$ which is only $\sim$30% of the opsin shift observed between bR and *p*pR [21]. This result implied that there might be other amino acid residues determining the color regulation because it is quite conceivable that the change of the environment around the chromophore is one of the most influential factors regulating color. Sakmar, Mathies and their co-workers [22,23] succeeded in the significant blue shift of rhodopsin (500 nm) to 438 nm by simultaneous substitution of nine sites of rhodopsin, whose shift amounts to 80% of the opsin shift between rhodopsin and the blue cone pigment.

In this paper, therefore, we focus on amino acid residues around the chromophore. According to the amino acid sequences of bR and *p*pR [21,24], retinal binding pockets among these proteins differ at only seven positions (Ile-43, Ile-83, Asn-105, Val-108, Phe-127, Gly-130 and Phe-134 for ppR and Val-49, Leu-93, Asp-115, Met-118, Trp-138, Ser-141 and Met-145 for bR, respectively; see Fig. 1). Furthermore, there exist three additional positions different from those of bR when the distance of a residue from the conjugated polyene chain of the chromophore or from any methyl group of the chain extends to within 5 Å (Met-109, Ala-131, and Thr-204 for *p*pR and Ile-119, Thr-142 and Ala-215 for bR, respectively; see Fig. 1). In the present paper, to determine the key residues contributing to the opsin shift between *p*pR and bR, we construct *p*pR mutants in which each of the specific residues of the above sites is replaced by a corresponding residue of bR. In addition, seven- and 10-residue substituted mutants are constructed which are expected to have the same amino acid residues as bR with respect to the retinal binding site. In spite of the amino acid arrangement around the chromophore being the same as that of bR, the blue shift is not large ($\lambda_{max}$ = 524 nm for the 10-substituted mutant). This suggests that the shape of the retinal binding site in *p*pR differs from that in bR, and that the structural factor is more important for the difference between their $\lambda_{max}$ values than the amino acid substitutions.

## 2. Materials and methods

### 2.1. Construction of expression plasmids

The *pharaonis* phobo-opsin single mutant genes containing the replacement (I43V, I83L, N105D, M109I, F127W, A131T and F134M) were constructed by the Kunkel method [25]. Oligonucleotide primers were designed based on the nucleotide sequence in the GenBank data base (accession No. Z35086), and an appropriate restriction enzyme site was added. As a template, pGEM-T Easy including a full-length *psop*II was used [26]. The plasmid including a full-length *psop*II fused histidine tag was prepared by PCR [27]. To fuse the histidine tag to the replacements, *Nde*I and *Sal*I fragments from the single replacement plasmids were ligated to *Nde*I and *Sal*I sites of pET/ppRHis [27]. The mutant genes containing the multiple replacements were constructed by PCR using the DNA shuffling method

[28]. DNA sequencing was carried out using a DNA Sequencing Kit (Applied Biosystems). All constructed plasmids were analyzed using an automated sequencer (377 DNA sequencer, Applied Biosystems).

### 2.2. Protein expression and purification

The wild-type and mutant *p*pRs were expressed in *Escherichia coli* BL21(DE3); induction was done by addition of 1 mM IPTG and 10 μM all-*trans* retinal [26]. Preparation of crude membranes was described previously [26]. The purification of the wild-type and mutant *p*pRs is essentially the same as in [27,29]. Crude membranes were resuspended in buffer S (300 mM NaCl, 50 mM MES, 5 mM imidazole, pH 6.5) containing 1.5% *n*-dodecyl-β-D-maltoside (DM) for 12 h at 4°C. After centrifugation of the solubilized membranes (100 000×*g*) for 1.5 h at 4°C, the supernatant was incubated with Ni-NTA agarose (Qiagen) for 1 h at 20°C with gentle stirring. The Ni-NTA resin was put into a chromatography column and washed extensively with buffer W (0.1% DM, 300 mM NaCl, 50 mM MES, 50 mM imidazole, pH 6.5) to remove nonspecifically bound proteins. The histidine tagged proteins were eluted with buffer E (0.1% DM, 300 mM NaCl, 50 mM Tris–Cl, 150 mM imidazole, pH 7.0).

### 2.3. Absorption spectroscopy

Spectra were obtained by a V-560 spectrophotometer (Japan Spectroscopic). A sample was suspended in buffer E.

### 2.4. HPLC analysis

A high performance liquid chromatograph was equipped with a silica column (6×150 mm, YMC-0123, Yamamura). The solvent was composed of 12% (v/v) ethyl acetate and 0.12% (v/v) ethanol in hexane, and the flow rate was 1.0 ml/min. Extraction of retinal oxime from the sample was carried out by hexane after denaturation by methanol and 500 mM hydroxylamine [30]. The molar compositions of the retinal isomers were calculated from the areas of the peaks in the HPLC patterns. Assignment of the peaks was performed by comparing them with the HPLC pattern from retinal oximes extracted from bR in a dark state by the same method.

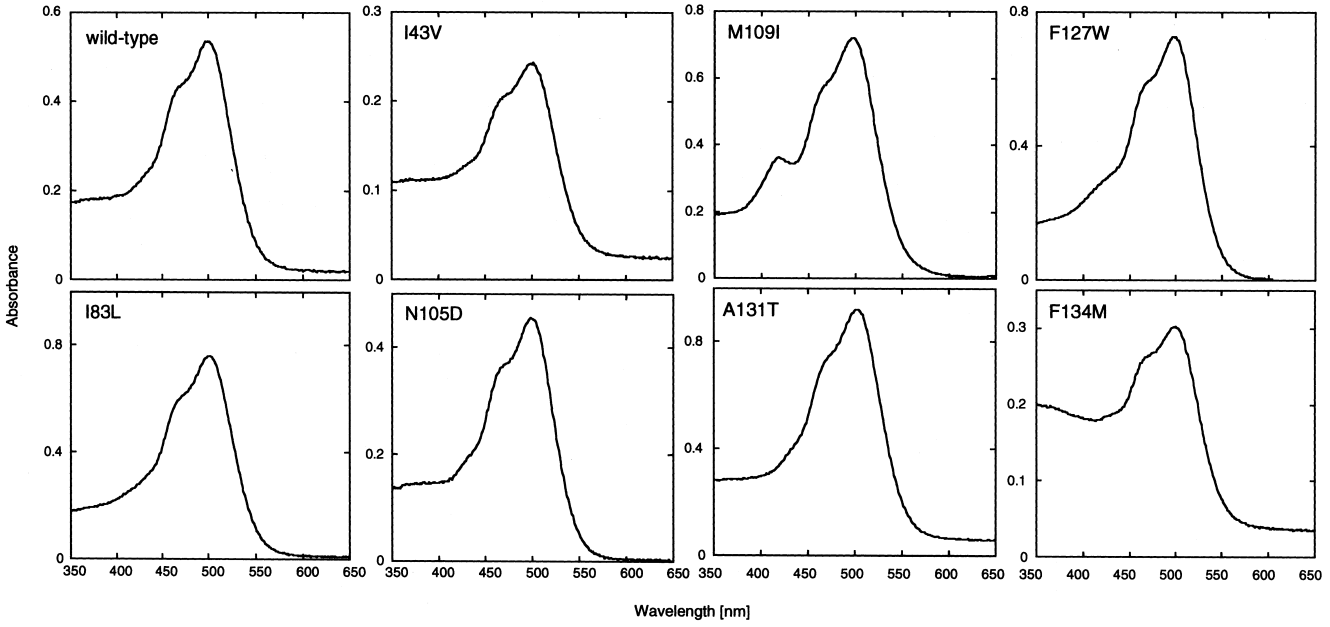


Fig. 2. Visible absorption spectra of mutant *p*pR with a single substitution. Opsin types are indicated in each panel. The solutions contained 300 mM NaCl, 150 mM imidazole and 0.1% DM whose pH was adjusted to 7.0 with 50 mM Tris–HCl.

## 3. Results and discussion

### 3.1. Spectra of mutants with a single substitution

In this work, wild-type and all mutant *p*pRs were fused by a histidine tag at the C-terminal, and the spectrum of the fused wild-type *p*pR was the same as that of a native *p*pR from *N. pharaonis* [19,20] and of wild-type *p*pR expressed in *E. coli* [24]. This implies that the fused tag does not change $\lambda_{max}$. The removal of NaCl or imidazole or both did not change any of the absorption spectra (data not shown). We, hence, deduced no influence of the composition of the suspending medium on at least the visible absorption spectrum of the ground state. Fig. 2 shows visual absorption spectra obtained for the wild-type and various mutants in which a single amino acid residue was replaced by a residue corresponding to bR (I43V, I83L, N105D, M109I, F127W, A131T and F134M) at pH 7.0. The shape of the spectra around 400 nm differs slightly from one spectrum to another. This may be a result of the instability of some mutant proteins (e.g. release of retinal due to denaturation leads to an increase in 360 nm absorption). To avoid denaturation, the spectra shown in Fig. 2 were measured immediately after purification. The spectrum of *p*pR has a characteristic shoulder at around 470 nm, and note that this is not from the contamination. $\lambda_{max}$ values (main absorption) of these mutants are listed in Table 1.

For mutants of which a hydrophobic group was replaced by another non-polar amino acid (I43V, I83L, M109I, F127W and F134M), essentially no or only a small shift of $\lambda_{max}$ was observed. The size of these sites may have almost no effect on the difference of $\lambda_{max}$ between bR and *p*pR.

Ile-83 and Phe-134 in *p*pR correspond to Leu-93 and Met-145 in bR, respectively. In bR, the substitutions of these positions (L93A and M145A mutants) led to a large blue shift [31,32]. In addition, these substitutions affected the re-isomerization rate of retinal during photocycling [31,33]. These results indicate that these sites in bR may interact strongly with the chromophore. Actually, the tertiary structure of X-ray crystallography [34], Leu-93 and Met-145 of bR are close to the $C_{13}$ methyl and the $C_9$ methyl group, respectively. The present results of I83L and F134M mutants of *p*pR suggest that the distance of Ile-83 or Phe-134 from the chromophore may be larger than that of the corresponding residues in bR (Leu-93 or Met-145).

Table 1 shows that if an amino acid residue having a hydroxyl group (Ser or Thr) is replaced or removed, relatively large shifts are observed, which may result from the alteration of the electrostatic environment around the chromophore. In other

Table 1
Absorption maximum, opsin shift and retinal configuration of wild-type and various *p*pR mutants

| Opsin type | $\lambda_{max}$ (nm) | $\Delta\nu$ ($cm^{-1}$) | Retinal isomer composition | | |
|---|---|---|---|---|---|
| | | | all-*trans* | 13-*cis* | others |
| Wild-type | 499 | – | 94.1 | 4.1 | 1.8 |
| I43V | 502 | 120 | 97.8 | 2.2 | 0 |
| I83L | 501 | 80 | 99.1 | 0.9 | 0 |
| N105D | 499 | 0 | 95.1 | 4.0 | 0.9 |
| V108M[a] | 502 | 120 | 91.7 | 7.0 | 1.3 |
| M109I | 497 | 80 | 97.7 | 2.3 | 0 |
| F127W | 498 | 40 | 97.1 | 2.9 | 0 |
| G130S[a] | 503 | 159 | 95.2 | 3.9 | 0.9 |
| A131T | 502 | 120 | 95.5 | 4.5 | 0 |
| F134M | 500 | 40 | 97.2 | 2.2 | 0.7 |
| T204A[a] | 508 | 355 | 92.2 | 6.4 | 1.4 |
| BR/*p*pR′ | 509 | 393 | 71.9 | | 19.7 |
| 8.4 | | | | | |
| BR/*p*pR | 524 | 954 | 95.5 | 4.5 | 0 |

$\Delta\nu$ is the opsin shift value from wild-type *p*pR. Samples were measured in a dark-adapted state. The underlined opsin types are the three sites of the difference between BR/*p*pR′ and BR/*p*pR.

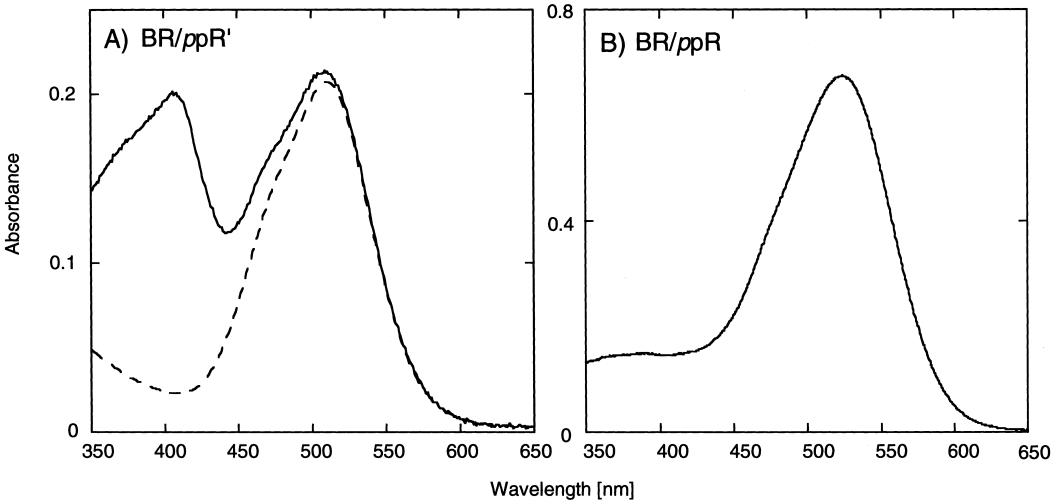


Fig. 3. Visible absorption spectra of mutant *p*pR with multiple substitutions. Panel A shows that of BR/*p*pR′ and B that of BR/*p*pR. The experimental conditions and the notations are the same as in Fig. 2. In panel A, the dotted line represents the calculated absorption curve of BR/*p*pR′ which eliminates the contribution from the contamination. For details, see text.

words, when polar amino acids exist near the β-ionone ring of the chromophore, its photo-exciting state becomes stable, which leads to the energy gap between the ground and excited states becoming small, and this gives rise to the red shift. The importance of Ser and Thr in color regulation was pointed out previously [17]. The N105D mutant did not show a shift of $\lambda_{max}$ although D is a polar residue; the reason may be that Asp-105 may be protonated, since Asp-115 in bR corresponding to Asp-105 in *p*pR is reported to be protonated.

### 3.2. Spectra of multiple mutants

As described in Section 1, seven amino acid residues around the retinal binding site are different between bR and *p*pR (see Fig. 1). We constructed a *p*pR mutant in which all these seven residues were substituted simultaneously by the corresponding residues of bR, and called it BR/*p*pR′. Three amino acid residues (see Fig. 1) were added when the difference between bR and *p*pR was extended to a 5 Å distance from the conjugated polyene chain or from any methyl group of the chain, and this mutant was termed BR/*p*pR. These mutants, therefore, have been thought to have the same amino acid residues as bR with respect to the retinal binding site. The mutant genotypes are denoted in the legend to Fig. 1.

As shown in Fig. 3, BR/*p*pR′ and BR/ppR showed their $\lambda_{max}$ at 509 and 524 nm, respectively. The amounts of expressed proteins of BR/*p*pR′ were not sufficient for a thorough purification (maybe due to its instability), so that its spectrum had a band around 400 nm as in some of Fig. 2. It seems that the spectrum of the longer wavelength region does not contain the contamination. Then, using the data of this region and well-known spectra of *p*pR, the whole spectrum of BR/*p*pR′ was calculated and it is shown by the dotted line. At this calculation, we assumed that the shape of the BR/*p*pR′ spectrum would follow a log normal equation as used in a previous paper [21]. From the observed and calculated spectra, $\lambda_{max}$ of BR/*p*pR′ was concluded to be 509 nm. This value is far from $\lambda_{max}$ of bR although the amino acid sequence of the so-called retinal binding pocket is the same as that in bR. The opsin shift of BR/*p*pR′ is 393 $cm^{-1}$ which is smaller than the sum of the shift of single mutants (479 $cm^{-1}$ = 120+80+0+120−40+159+40; see Table 1). As shown previously [21], Val-108 and Gly-130 produce a positive synergistic effect of these two residues on $\lambda_{max}$, and replacement of only these two sites results in a 394 $cm^{-1}$ shift [21]. Therefore, the shift of BR/*p*pR′ has come from these two residues.

Table 1 also shows the retinal isomer composition (see also Fig. 4). BR/*p*pR′ had a much larger content of 13-*cis* isomer than other mutants and there were some unidentified isomers of retinal. The existence of

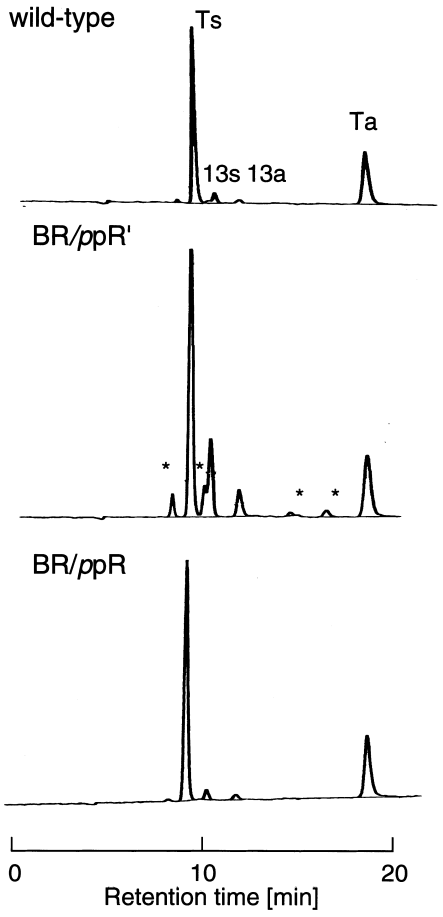


Fig. 4. HPLC pattern of chromophores extracted in the dark from wild-type *p*pR and multiple mutants. The detection beam was set at 360 nm. Ts, Ta, 13s and 13a stand for all-*trans* 15-*syn* retinal oxime, all-*trans* 15-*anti* retinal oxime, 13-*cis* 15-*syn* retinal oxime and 13-*cis* 15-*anti* retinal oxime, respectively. The peaks marked by an asterisk in the middle pattern are unidentified.

the 13-*cis* isomer might affect $\lambda_{max}$, but its effect may be small because of the small content of 13-*cis* isomer (only 20%). By illumination (5 min) and subsequent keeping in the dark (1 min), the isomer ratio did not change, meaning that there is no light–dark adaptation. On the other hand, a normal photocycle was observed (data not shown). Although the molecular interpretation of the existence of the 13-*cis* isomer is not known, this might imply the stronger interaction of chromophore retinal and surrounding amino acids than that of other mutants, because the all-*trans* form might be most stable if no interaction is exerted. If we accept this notion, it might indicate that the color tuning is regulated not by the amino acid residues surrounding the chromophore but by other factors.

The shift of BR/*p*pR is larger than that of BR/*p*pR′ with an opsin shift of 954 $cm^{-1}$, and larger than the sum of the shifts of single mutants (874 $cm^{-1}$). Interestingly, this mutant does not have a vibrational fine structure, indicating that the retinal molecule is not restricted, according to the interpretation of fine structure by Takahashi et al. [9]. We should, however, be aware of the following: it seems that the shoulder of the vibrational fine structure disappears when the main absorption band shifts to the longer wavelength. The retinal isomer composition was all-*trans* only, which is in sharp contrast to BR/*p*pR′. In BR/*p*pR, three additional positions (Met-109, Ala-131, Thr-204) are mutated to BR/*p*pR′; these residues might alter the structure of the retinal binding pocket although these sites are relatively far from the chromophore compared to the residues mutated in BR/*p*pR′. This might imply that the blue shift of $\lambda_{max}$ of *p*pR from bR is caused mainly by the structural difference around the chromophore rather than by the replacement of amino acid residues. The fact described above that the simple 'additivity rule' does not hold in BR/*p*pR may suggest a structural change around the chromophore, but its extent is still small because the shift is not large.

There are several aspects of the structural changes to be considered. FT-IR and resonance Raman spectroscopy showed that the frequency derived from the C–C stretch of the polyene chain in *p*pR does not differ from that of BR [27,35,36]. This fact indicates that the structure around the polyene chain is similar between these two pigments. Therefore, the difference may originate from the environment near the β-ionone ring or the Schiff base.

Less planar 6-s-*trans* conformation in *p*pR than in BR is suggested, which was deduced by less mode coupling in the C=C stretch [27]. The angle of the β-ionone ring and the polyene chain in *p*pR may be twisted because both the β-ionone ring and the polyene exist on the same plane in BR [34]. In conjugated molecules, if a single bond is twisted, the π bond character of this single bond becomes weaker and that of the neighboring double bond becomes stronger. As a result, π electrons in the conjugated ployene chain are localized, resulting in a blue shift of $\lambda_{max}$.

The difference in the environment near the Schiff base is inferred as follows. The shift value of C=N stretching vibrations of wild-type *p*pR by $H_2O$–$D_2O$ exchange is greater than that of BR [27,36]. This stronger hydrogen bond in the Schiff base of *p*pR than that of BR may be caused by the difference of the internal water molecule (probably W402 [34]) or/and the location of the counterion (Asp-85 for bR, Asp-75 for *p*pR). The presence of the internal water molecule and the counterion may stabilize the positive charge of the Schiff base and hence lower the energy level of the ground state of *p*pR. This stabilization leads to a greater energy gap between the ground and the excited state, resulting in a blue shift of $\lambda_{max}$.

### 3.3. Concluding remarks

We would like to point out two particular points in this paper. First, the addition or removal of amino acid residues having a hydroxyl group affects $\lambda_{max}$, which may lead to an alteration of the electrostatic environment around the chromophore due to their dipole moments. Second, even in BR/*p*pR the opsin shift is only approx. 45% of the shift value corresponding to the $\lambda_{max}$ shift from 500 to 570 nm. This result indicates that the structure of the retinal binding site between bR and *p*pR differs and that the structural factor is important in explaining the $\lambda_{max}$ difference between *p*pR and bR rather than amino acid substitutions. The possible origins are: (1) the angle between the β-ionone ring and the polyene chain of retinal in *p*pR differs from that in bR. (2) The environment of their Schiff bases differs. One possible origin is the difference of the distance between the Schiff base and the counterion. Hu et al. [15,37] found, for halide salts of all-*trans* protonated Schiff base of retinal with aniline, that the frequency of the maximum absorption is linearly related with the inverse square of the center-to-center distance between the Schiff base and halide. If his relationship would be applicable to the opsin shift from BR to *p*pR, the decrease in the distance between the Schiff base and the counterion by 0.5 Å is sufficient to account for. Resolution by X-ray crystallography may be needed to further analyze the color tuning between bR and *p*pR.

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