A *pharaonis* Phoborhodopsin Mutant with the Same Retinal Binding Site Residues As in Bacteriorhodopsin†

Kazumi Shimono,‡ Yuji Furutani,§,|| Hideki Kandori,*,§,|| and Naoki Kamo‡

Laboratory of Biophysical Chemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan, Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan, and Department of Applied Chemistry, Nagoya Institute of Technology, Showa-ku, Nagoya 466-8555, Japan

Received February 5, 2002; Revised Manuscript Received March 27, 2002

ABSTRACT: *pharaonis* phoborhodopsin (*p*pR, also called *pharaonis* sensory rhodopsin II, *p*sR-II) is a photoreceptor for negative phototaxis in *Natronobacterium pharaonis*. *p*pR has a blue-shifted absorption maximum (500 nm) relative those of other archaeal rhodopsins such as the proton-pump bacteriorhodopsin (BR; 570 nm). Among the 25 amino acids that are within 5 Å of the retinal chromophore, 10 are different in BR and *p*pR, and they are presumed to be crucial in determining the color of their chromophores. However, the spectral red shift in a multiple mutant of *p*pR, in which the retinal binding site was made similar to that of BR (BR/*p*pR), was smaller than 40% (λ_{max} = 524 nm) than expected. In the paper presented here, we report on low-temperature Fourier transform infrared (FTIR) spectroscopy of BR/*p*pR, and compare the infrared spectral changes before and after photoisomerization with those for *p*pR and BR. The C−C stretch and hydrogen out-of-plane (HOOP) vibrations of BR/*p*pR were similar to those of BR, suggesting that the surrounding protein moiety of BR/*p*pR becomes like BR. However, BR/*p*pR exhibited a unique IR band regarding the hydrogen bond of the protonated Schiff base. It has been known that *p*pR has a stronger hydrogen bond for the Schiff base than BR as judged from the frequency difference between their C═NH and C═ND stretches. We now find that replacement of the 10 amino acids of BR with *p*pR (BR/*p*pR) does not weaken the hydrogen bond of the Schiff base. Rather, the hydrogen bond in BR/*p*pR is stronger than that in the native *p*pR. We conclude that the principal factor of the smaller than expected opsin shift in BR/*p*pR is the strong association of the Schiff base with the surrounding counterion complex.

Phoborhodopsin (pR)[1] from *Halobacterium salinarum* and *pharaonis* phoborhodopsin (*p*pR) from *Natronobacterium pharaonis* are members of archaeal rhodopsins (*1*, *2*).[2] pR and *p*pR activate cognate transducer proteins for negative phototaxis of the bacteria. They possess a retinal chromophore that is embedded in a seven-transmembrane helical structure, like the well-studied proton-pump protein bacteriorhodopsin (BR) (*1*−*3*). In both *p*pR and BR, the retinal forms a Schiff base linkage, with Lys205 and Lys216, respectively, and the protonated Schiff base is stabilized by a negatively charged counterion, Asp75 and Asp85, respectively. Light absorption triggers cis−trans photoisomerization of the retinal chromophore upon formation of the K intermediate, which eventually leads to functional processes during both photocycles. Proton transfer reaction also takes place in both proteins, from the Schiff base to the counterion upon formation of the M intermediate.

Despite such similarity, *p*pR has some characteristics different from those of BR. One of the most prominent differences is the absorption maximum. The λ_{max} of *p*pR (500 nm) is considerably blue-shifted from that of BR (570 nm), which is suitable for the negative phototaxis against blue-green light (*2*). It is also different from those of halorhodopsin and sensory rhodopsin (570−590 nm), although these four proteins belong to one family. What is the molecular mechanism that sets the λ_{max} of *p*pR so far from others? Three factors have been considered to be the origin of the smaller spectral shift to a longer wavelength (called opsin shift) in *p*pR: (i) less planar retinal conformation, (ii) stronger interaction of the Schiff base with its counterion, and (iii) the presence of polar or polarizable groups. One might suggest that the coplanarity between the β-ionone ring and polyene chain could be lower for *p*pR and sufficient to explain its relatively small opsin shift. This explanation seems reasonable because the amino acids that are different from those of BR are mostly located in helices D and E that are in the proximity of the β-ionone ring. Figure 1 depicts 25

† This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, and by the Human Frontier Science Program.

* To whom correspondence should be addressed. Present address, Nagoya Institute of Technology; phone and fax, 81-52-735-5207; e-mail, kandori@ach.nitech.ac.jp.

‡ Hokkaido University.

§ Kyoto University.

|| Nagoya Institute of Technology.

[1] Abbreviations: pR, phoborhodopsin; *p*pR, *pharaonis* phoborhodopsin; BR, light-adapted bacteriorhodopsin; BR_K, K intermediate of BR; BR_L, L intermediate of BR; pR_K, K intermediate of *pharaonis* phoborhodopsin; pR_L, L intermediate of *pharaonis* phoborhodopsin; FTIR, Fourier transform infrared; HOOP, hydrogen out-of-plane.

[2] pR and *p*pR are also called sensory rhodopsin II (sR-II) and *pharaonis* sensory rhodopsin II (*p*sR-II), respectively.

10.1021/bi025636c CCC: $22.00
Published on Web 04/25/2002

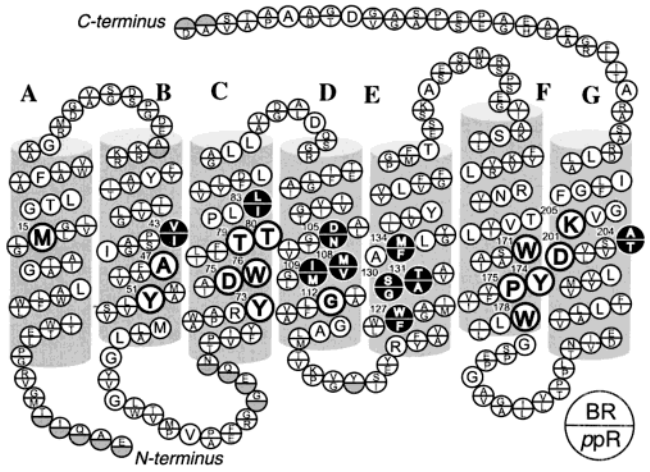

FIGURE 1: Comparison of amino acid sequences of bacteriorhodopsin (BR) and *pharaonis* phoborhodopsin (*p*pR). The transmembrane topography is based on the crystallographic three-dimensional model of BR (*34*). Single letters in a circle denote residues common to BR and *p*pR. The residues that are different in BR and *p*pR are denoted at the top and bottom of the circles, respectively. The bold filled circles compose the retinal binding site within 5 Å of the chromophore and numbered using the *p*pR numbering system. We constructed the multiple mutant (BR/*p*pR), whose genotype is I43V/I83L/N105D/V108M/M109I/F127W/G130S/A131T/F134M/T204A.

amino acids located within 5 Å of the retinal chromophore in the BR structure, where 10 amino acids are different between BR and *p*pR. Amino acids in the side of the β-ionone ring (helices D and E) tend to be altered between them, while those in the Schiff base side (helices C, F, and G) are highly conserved. Thus, the primary structures support the different colors being from the difference in ring and/or chain coplanarity. However, on the basis of their retinal analogue results, Takahashi et al. (*4*) argued that the difference between the absorption spectra of *H. salinarum* pR and BR was not due to a difference in coplanarization.

Recently, crystallographic structures of *p*pR have been reported from two groups (*5*, *6*). The atomic resolution structures show that the ring and polyene chain of *p*pR are as coplanaer as in BR, ruling out the ring and/or chain conformation as the cause of the difference in absorption maxima. The structural similarity is not only at the ring side but also at the Schiff base side; the water-containing pentagonal cluster structure was present in *p*pR as in BR (*5*, *6*). The two groups applied calculation studies based on the structure of *p*pR, and reported the principal factors that might regulate color independently (*7*, *8*). Although the two structures are almost identical, interestingly, their conclusions were different. Namely, one group concluded that the small opsin shift in *p*pR originates from the shorter distance between the Schiff base nitrogen and Asp201 (*7*), but the other group concluded that the small opsin shift in *p*pR originates from the different position of Arg72 (*8*). Thus, the color tuning mechanism remains undecided by the structural determinations.

One of the powerful methods for investigating the color tuning mechanism in rhodopsins is using mutants. Shimono et al. have been systematically studying the effects of amino acids characteristic of *p*pR. First, Val108 of *p*pR was replaced with Met, a corresponding amino acid in BR (Figure 1), and it was found that the V108M mutant has an absorption spectrum identical to that of wild-type *p*pR (*9*). Then, three characteristic amino acids of *p*pR (Val108, Gly130, and Thr204) were replaced with those of the corresponding ones of the others (Met, Ser, and Ala, respectively) (*10*). Nevertheless, the triple mutant (V108M/G130S/T204A) has a λ_{max} at 515 nm (*10*), and only 24% of the longer wavelength shift was achieved. Finally, a multiple mutant of *p*pR was constructed that has the same retinal binding site as BR (BR/*p*pR), where 10 amino acids of *p*pR were replaced with those of BR (I43V/I83L/N105D/V108M/M109I/F127W/G130S/A131T/F134M/T204A) (Figure 1) (*11*). The *p*pR mutant, however, exhibited a λ_{max} at 524 nm (*11*), in which only 37% of the longer wavelength shift was achieved. In addition, BR/*p*pR contains only an all-trans chromophore in the dark, as *p*pR, whereas bacteriorhodopsin has all-trans and 13-cis forms. These facts suggest that the shape of the retinal binding site in *p*pR differs from that in BR, even though their tertiary structures are very similar (*5*, *6*). Thus, the mutation study also raised questions about the mechanism of color determination. Similar overall structures between *p*pR and BR strongly suggested that the difference in their absorption originates from small structural alterations.

In the paper presented here, we attempt to compare the detailed structural differences of BR/*p*pR, *p*pR, and BR by means of low-temperature FTIR spectroscopy. FTIR spectroscopy is a powerful tool in studying molecular structure and structural changes of retinal proteins, which has been proven for *p*pR (*12*−*14*) and BR (*15*−*19*). Infrared spectral changes upon photoisomerization measured at 77 K were compared among *p*pR, BR/*p*pR, and BR. The chromophore vibrations of BR/*p*pR suggest that the surrounding protein moiety of BR/*p*pR becomes like BR. Nevertheless, we found remarkable structural alterations at the Schiff base region in BR/*p*pR. It is known that the hydrogen bond of the Schiff base is weaker in BR than in *p*pR (*13*, *20*). Introduction of the amino acids of BR into those surrounding the retinal chromophore of *p*pR, however, does not weaken the hydrogen bond of the Schiff base like in BR. Rather, the hydrogen bond in BR/*p*pR was stronger than that in native *p*pR. We conclude that strong interaction of the Schiff base with counterion is the principal reason for the smaller than expected opsin shift in BR/*p*pR.

MATERIALS AND METHODS

The BR/*p*pR protein with a histidine tag at the C-terminus was expressed in *Escherichia coli*, solubilized with 1.5% *n*-dodecyl β-D-maltoside, and purified with a Ni column as described previously (*11*, *13*). The purified BR/*p*pR sample was then reconstituted into L-α-phosphatidylcholine liposomes by dialysis, where the molar ratio of added PC was 50 times that of BR/*p*pR (*14*).

FTIR spectroscopy was applied as described previously (Bio-Rad, FTS-40; *13*, *14*). A 80 μL sample of BR/*p*pR in 2 mM phosphate buffer (pH 7.0) was dried on a BaF_2 window with a diameter of 18 mm. After hydration by either H_2O or D_2O, the sample was mounted in an Oxford DN-1704 cryostat, and cooled to 77 or 170 K. Illumination with 470 nm light at 77 K for 2 min converted the initial pigment to the K intermediate of BR/*p*pR, and subsequent illumination with $>$580 nm light returned the K intermediate to the initial state. The difference spectrum was calculated from the

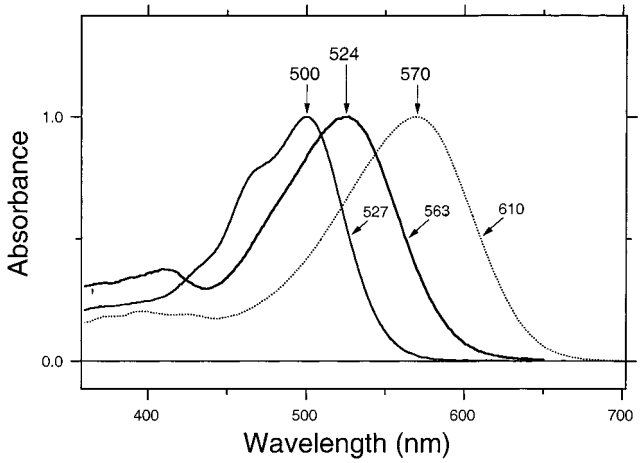

FIGURE 2: Visible absorption spectra of *p*pR, BR/*p*pR, and BR. Solid lines represent spectra of *p*pR (thin line) and BR/*p*pR (thick line) in 50 mM Tris (pH 7), 300 mM NaCl, and 150 mM imidazole containing 0.1% DM (*11*). The dotted line represents the spectrum of BR in water. Absorption maxima of *p*pR, BR/*p*pR, and BR are 500, 524, and 570 nm, respectively, while the wavelengths at which absorbances are half of the maxima at the longer wavelength side are 527, 563, and 610 nm, respectively.

spectra constructed with 128 interferograms before and after illumination. Twenty-four spectra obtained in this way were averaged for the spectrum of the K intermediate minus the initial state of BR/*p*pR. The identical measurement was also conducted at 170 K. Linear dichroism experiments revealed a random orientation of the molecules in the film. Therefore, the IR polarizer was not used in the measurements for *p*pR. The other spectra for *p*pR and BR were taken from Kandori et al. (*13*).

RESULTS

Figure 2 shows visible absorption spectra of *p*pR, BR/*p*pR, and BR measured at room temperature. As reported (*11*), the λ_{max} of BR/*p*pR is located at 524 nm, where only 37% of the spectral red shift (opsin shift) in wavenumber from *p*pR (500 nm) to BR (570 nm) was achieved by mutation of 10 amino acids surrounding the retinal chromophore. The absorption maximum of BR/*p*pR in detergent micelles (524 nm) was identical in PC liposomes and halobacterial lipids (*21*). It may not be appropriate to calculate the opsin shift from the maxima, because the spectral shapes are different among three proteins. There is a spectral shoulder in *p*pR, which seems to be lost in BR/*p*pR like BR (Figure 2). If we calculate the opsin shift of BR/*p*pR from the wavelengths at which absorbances are half of the maxima at the longer wavelength side, the difference between *p*pR and BR/*p*pR (1200 cm^{-1}) is 47% of that between *p*pR and BR (2560 cm^{-1}). Thus, even in this case, the opsin shift is less than half.

BR/*p*pR possesses only an all-trans chromophore in the dark state like *p*pR (*11*), whereas bacteriorhodopsin has both all-trans and 13-cis forms. Spectral half-widths of *p*pR, BR/*p*pR, and BR were 3400, 3700, and 3250 cm^{-1}, respectively. The broader spectral shape of BR/*p*pR implies the structural heterogeneity in the chromophore−protein interaction, which might contribute to the lack of the spectral shoulder. Below, we compared infrared spectral changes between the K intermediate and the initial state.

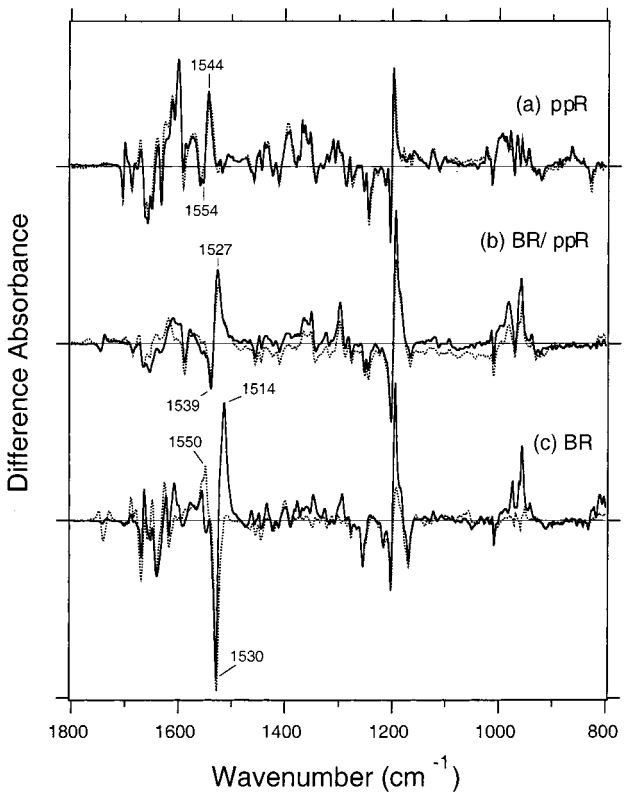

FIGURE 3: Difference infrared spectra of *p*pR (a), BR/*p*pR (b), and BR (c) at 77 K (solid line) and 170 K (dotted line) in the 1800−800 cm^{-1} region. The spectra correspond to the ppR_K minus *p*pR spectra (a), which are reproduced from ref *9*, and BR_K minus BR (c, solid line) and BR_L minus BR spectra (c, dotted line). One division of the *Y*-axis corresponds to 0.008 absorbance unit.

Infrared Spectral Changes of BR/ppR at Low Temperatures. The high thermal stability of the K intermediate is characteristic of *p*pR (*22*). In fact, Figure 3a shows identical spectra for *p*pR between 77 and 170 K, indicating that the protein structure of the K intermediate is preserved at these temperatures (*13*). In contrast, the K and L intermediates are formed for BR at 77 and 170 K (Figure 3c), respectively. Figure 3b shows difference infrared spectra of BR/*p*pR measured at 77 and 170 K. The lower-frequency shift of the C═C stretching vibration from 1539 to 1527 cm^{-1} (Figure 3b) corresponds to the formation of the K intermediate in BR/*p*pR, as for *p*pR [1554 (−)/1544 (+) cm^{-1} (Figure 3a)] and BR [1530 (−)/1514 (+) cm^{-1} (Figure 3c)]. These frequencies suggest that the opsin shift was small not only for BR/*p*pR but also for the K intermediate of BR/*p*pR. The chromophore bands such as the C═C stretch (1550−1500 cm^{-1}), C−C stretch (1250−1150 cm^{-1}), and HOOP vibration (1000−900 cm^{-1}) are identical between 77 and 170 K, indicating that the K intermediate is stable at these temperatures. The stability of the K intermediate at 170 K in BR/*p*pR is like that of *p*pR. It is noted, however, that there are considerable spectral differences in the 1800−1600 cm^{-1} region for BR/*p*pR (Figure 3b), suggesting that the K state possibly has different protein structures between 77 and 170 K. Introduction of the amino acids of BR presumably promotes partial relaxation of the protein environment, but the K-to-L thermal transition does not occur. Below we compare the difference spectra at 77 K in detail among *p*pR, BR/*p*pR, and BR.

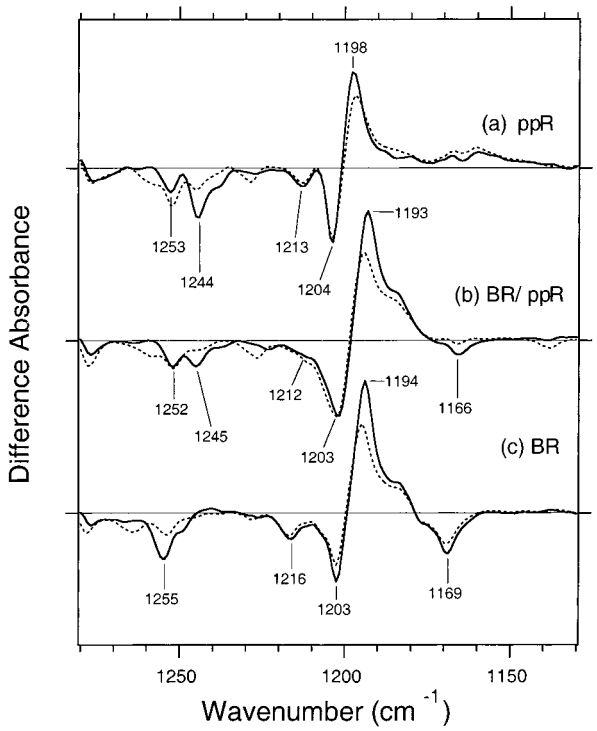

FIGURE 4: ppR$_K$ minus ppR spectra of the wild type (a) and BR/ppR (b) and BR$_K$ minus BR spectra of the wild type (c) in the 1280−1130 cm^{-1} region at 77 K, which correspond to C−C stretching vibrations of the retinal chromophore. The sample was hydrated with either H_2O (solid lines) or D_2O (dotted lines). One division of the *Y*-axis corresponds to 0.005 absorbance unit.

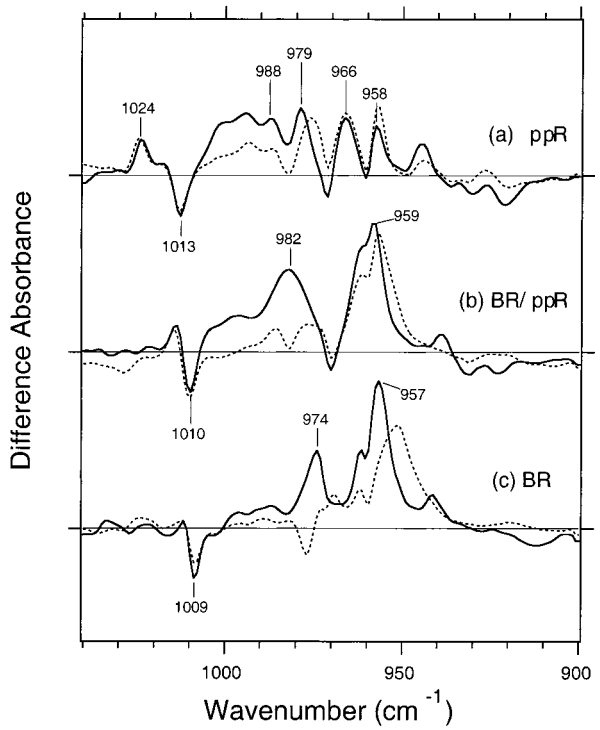

FIGURE 5: ppR$_K$ minus ppR spectra of the wild type (a) and BR/ppR (b) and BR$_K$ minus BR spectra of the wild type (c) in the 1040−900 cm^{-1} region at 77 K, which correspond to hydrogen out-of-plane (HOOP) vibrations of the retinal chromophore. The sample was hydrated with either H_2O (solid lines) or D_2O (dotted lines). One division of the *Y*-axis corresponds to 0.004 absorbance unit.

C−C Stretching and HOOP Vibrations of the Retinal Chromophore in BR/ppR. Both *p*pR and BR exhibit similar bands in the fingerprint region (1250−1100 cm^{-1}) that probe C−C stretching vibrations of the retinal chromophore (*13*). The result implies that the structure of the polyene chain is similar, which is consistent with the crystal structure of *p*pR (*5*, *6*). Figure 4 compares the fingerprint vibrations of *p*pR, BR/*p*pR, and BR. The negative bands in BR at 1255, 1216, 1203, and 1169 cm^{-1} can be attributed to the C−C stretching vibrations of the retinal chromophore at positions C12 and C13, C8 and C9, C14 and C15, and C10 and C11, respectively (Figure 4c) (*15*, *23*). The negative 1255 cm^{-1} band is composed of a mixture of D_2O-insensitive C12−C13 stretching and D_2O-sensitive N−H in-plane bending vibrations (*24*). The positive 1194 cm^{-1} band of BR originates from C14−C15 and C10−C11 stretches (*25*). A similar spectral feature was observed for BR/*p*pR (Figure 4b), suggesting common chromophore conformations among the three proteins.

One of the differences between *p*pR and BR in this frequency region is the lack of the 1169 cm^{-1} (−) band of BR in *p*pR (Figure 4a,c). The lack of this peak in *p*pR was interpreted to mean that the structure around the C10−C11 single bond is not changed in *p*pR upon photoisomerization (*13*). In fact, we observed a negative band at 1163 cm^{-1} in the ppR$_M$ minus *p*pR spectrum (Y. Furutani et al., unpublished observation). There is a clear negative band at 1166 cm^{-1} in BR/*p*pR like BR (Figure 4b). The frequency of the positive band in BR/*p*pR (1193 cm^{-1}) is closer to that in BR (1194 cm^{-1}) than that in *p*pR (1198 cm^{-1}). These observations demonstrate that the structure and structural changes in BR/*p*pR around the C10−C11 and C14−C15 stretches are more like those of BR than those of *p*pR.

The frequencies of the D_2O-sensitive N−H in-plane bending vibrations of the Schiff base are located at 1244 and 1255 cm^{-1} in *p*pR (Figure 4a) and BR (Figure 4c), respectively (*13*). The corresponding D_2O-sensitive band is at 1245 cm^{-1} in BR/*p*pR (Figure 4b). This observation indicates that the vibrational property of the Schiff base in BR/*p*pR is similar to that in *p*pR, unlike other chromophore vibrations.

A previous FTIR study showed remarkable spectral differences in the region of hydrogen out-of-plane (HOOP) vibrations of the retinal chromophore (1000−900 cm^{-1}) between BR and *p*pR, which provides information about the chromophore distortion upon photoisomerization (*13*). There are D_2O-sensitive intense bands at 974 and 957 cm^{-1} for BR (Figure 5c), which have been assigned as HOOP vibrations of C15−H and N−H stretches (*24*). The ppR$_K$ minus *p*pR spectrum exhibits more peaks (Figure 5a), which were interpreted in terms of more extended chromophore distortion in the K intermediate of *p*pR (*13*). Figure 5b clearly shows the presence of the BR-like positive peaks at 982 and 959 cm^{-1}, which probably correspond to those of BR (974 and 957 cm^{-1}, respectively). This fact suggests that the chromophore distortion in BR/*p*pR is more like that in BR, where structural changes after photoisomerization are localized at the Schiff base region.

The negative band of BR at 1009 cm^{-1} is the C−methyl in-plane bending vibration (*26*). Corresponding bands are observed at 1013 and 1010 cm^{-1} for *p*pR and BR/*p*pR, indicating that the environment of the methyl groups (C9

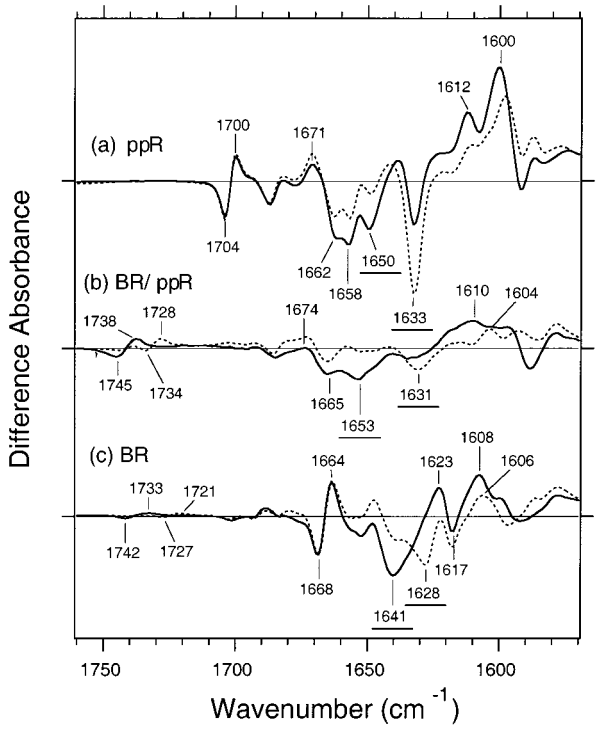

FIGURE 6: $p$ppR$_K$ minus ppR spectra of the wild type (a) and BR/ppR (b) and BR$_K$ minus BR spectra of the wild type (c) in the 1760−1570 cm^{-1} region at 77 K, most of which can be ascribed to protein. The sample was hydrated with either H_2O (solid lines) or D_2O (dotted lines). One division of the *Y*-axis corresponds to 0.007 absorbance unit.

and C13) in BR/ppR is similar to that in BR. In addition, the as yet unassigned positive band at 1024 cm^{-1} in the $p$ppR$_K$ minus ppR spectrum (Figure 5a) disappears for BR/ppR (Figure 5b).

Spectral comparison in the C−C stretching and HOOP vibrations reveals therefore that the chromophore structures of both the initial and K states in BR/ppR are more like those of BR than those of ppR. The results are consistent with the fact that amino acids surrounding the retinal chromophore of ppR are substituted for those of BR, but do not explain the origin of the small opsin shift in BR/ppR (Figure 2). Only one ppR-like band is observed, the N−H in-plane bending mode of the retinal Schiff base (Figure 4b), that suggests that the Schiff base environment in BR/ppR does not become completely BR-like.

C═N Stretching Vibration of the Retinal Schiff Base in BR/ppR. Figure 6 shows spectral changes in the 1760−1570 cm^{-1} region, where most signals originate from protein vibrations. One exception is the C═N stretching vibration of the retinal Schiff base that appears in the 1650−1600 cm^{-1} region. It is well-known that the difference in frequency between H_2O and D_2O in this mode provides information about the hydrogen bonding strength of the Schiff base. The C═N stretching vibrations of BR are located at 1641 cm^{-1} in H_2O and at 1628 cm^{-1} in D_2O (Figure 6c) (*27*), while those of ppR are located at 1650 cm^{-1} in H_2O and at 1633 cm^{-1} in D_2O (Figure 6a) (*13*). The difference in frequency between H_2O and D_2O is 13 cm^{-1} for BR and 17 cm^{-1} for ppR, indicating that the hydrogen bond of the Schiff base is stronger in ppR than in BR. A stronger hydrogen bond in ppR is consistent with its blue-shifted absorption spectrum, because stronger interaction at the Schiff base region leads to localization of the π-electrons of the retinal.

In BR/ppR, a negative band at 1653 cm^{-1} in H_2O exhibits a spectral downshift to 1631 cm^{-1} in D_2O, which most likely originates from the C═N stretch of the Schiff base (Figure 6b).[3] The difference of 22 cm^{-1} is greater than in either ppR (17 cm^{-1}) or BR (13 cm^{-1}). Thus, we concluded that BR/ppR has a stronger hydrogen bond than even ppR. Strong association of the protonated Schiff base with the counterion complex can explain the small red shift in visible absorption for BR/ppR (Figure 2).

Vibrational Bands of the Protein Moiety in BR/ppR. Figure 6 also shows vibrational bands of the protein. The large peaks in ppR at 1704 (−) and 1700 (+) cm^{-1} (Figure 6a) originate from the C═O stretching vibrations of the side chain of Asn105, because they shift to 1744 (−) and 1739 (+) cm^{-1}, respectively, for the N105D mutant (*28*). Since BR/ppR contains aspartate at position 105, the spectra lack the 1704 (−)and 1700 (+) cm^{-1} bands, but contain the D_2O-sensitive 1745 (−) and 1738 (+) cm^{-1} bands (Figure 6b).

In BR, the 1668 (−) and 1664 (+) cm^{-1} bands correspond to the amide I vibration of the α-helix (Figure 6c) (*29*), where a lower-frequency shift indicates a strengthened hydrogen bond upon photoisomerization. In contrast, ppR exhibits a higher-frequency shift for the amide I vibration of the α-helix [1662 (−) and 1671 (+) cm^{-1} (Figure 6a)], where the hydrogen bonding of the peptide backbone is weakened. In BR/ppR, the amide I bands of the α-helix are observed at 1665 (−) and 1674 (+) cm^{-1}, indicating that the structural changes are like ppR. Since the structure of the Schiff base region of BR/ppR is like ppR, the structural changes of αII helix are probably located at the Schiff base region. Another negative peak is seen for ppR at 1658 cm^{-1}, which is the typical frequency region of the amide I vibrations of the αI helix (*13*). In BR/ppR, there are no bands corresponding to this αI helix vibration (dotted line in Figure 6b), which is like BR. Thus, the structural changes of the α-helix in ppR are not in the Schiff base region.

The 1617 (−) and 1623 (+) cm^{-1} bands of BR originate from the amide I vibration of Val49 (Figure 6c) (*30*). There are no such spectral changes in ppR (Figure 6a), where the corresponding amino acid of ppR is Ile43 of helix B (Figure 1). In the case of BR/ppR, there are no such spectral changes (Figure 6b), although Ile43 is replaced with valine like in BR. Since these amino acids of helix B are located at the Schiff base region, the structure of ppR is possibly retained in BR/ppR.

DISCUSSION

Origin of the Small Opsin Shift in BR/ppR. The small size of the opsin shift in BR/ppR, after introducing 10 amino acids of BR into ppR, suggested that the shape of the retinal binding site in ppR differs from that in BR (*11*). However, the recent structural determination of ppR showed very similar protein architecture for ppR and BR (*5*, *6*), which

[3] The possibility of other vibrations such as amide I vibrations was not excluded in this study, because the isotope effect was not tested for the C═N stretch of the Schiff base. Nevertheless, amide I vibrations are generally insensitive to D_2O substitution. A large frequency shift of 22 cm^{-1} strongly suggests that the band originates from the C═N stretch of the Schiff base.

raised once again the question of the color tuning mechanism in archaeal rhodopsins. The present IR spectral comparison among *p*pR, BR/*p*pR, and BR reveals that the chromophore bands of BR/*p*pR tend to alter from *p*pR-like to BR-like, except for the Schiff base vibrations. The N−H bending frequency in BR/*p*pR is almost identical to that in *p*pR (Figure 4). The most significant observation was the C═N stretching vibrations of the protonated Schiff base. It is well-known that the spectral upshift of the C═N stretch in H_2O is caused by the coupling of the N−H bending vibration of the Schiff base, and the difference in frequency between H_2O and D_2O has been regarded as the marker of the hydrogen bonding strength of the Schiff base (*31*−*33*). The greater frequency difference in *p*pR (17 cm^{-1}) than in BR (13 cm^{-1}) suggests a stronger hydrogen bond of the Schiff base in *p*pR, which is consistent with the spectral blue shift in *p*pR. Interestingly, introduction of amino acids of BR into *p*pR does not alter the frequency difference from *p*pR-like to BR-like. Instead, the value (22 cm^{-1}) is even higher than that of *p*pR. Thus, we concluded the origin of the small opsin shift in BR/*p*pR is the strong hydrogen bond of the Schiff base.

The protein structure of BR/*p*pR is also *p*pR-like at the Schiff base region, as observed for the amide I vibrations of Ile43 in *p*pR (Val49 in BR) and αII helix (Figure 6). Thus, replacement of the amino acids within 5 Å of the retinal chromophore seems not to be sufficient to convert *p*pR into BR regarding the structure around the Schiff base, which was previously suggested by Luecke et al. (*5*). It is likely that the long-range interaction over 5 Å contributes the structure of the Schiff base, which might involve the position of Arg72 (*5*, *8*), or the position of Asp201 by moving helix G in the optimized structure of Hayashi et al. (*7*).[4] Therefore, further mutational study around the Schiff base region (along the helices) will lead to a better understanding of the color tuning mechanism, which is our future focus.

ACKNOWLEDGMENT

We thank M. Iwamoto and Y. Sudo for helping in sample preparation.

REFERENCES

1. Sasaki, J., and Spudich, J. L. (2000) *Biochim. Biophys. Acta 1460*, 230−239.
2. Kamo, N., Shimono, K., Iwamoto, M., and Sudo, Y. (2001) *Biochemistry (Moscow) 66*, 1277−1282.
3. Spudich, J. L., and Lanyi, J. K. (1996) *Curr. Opin. Cell Biol. 8*, 452−457.
4. Takahashi, T., Yan, B., Mazur, P., Derguini, F., Nakanishi, K., and Spudich, J. L. (1990) *Biochemistry 29*, 8467−8474.
5. Luecke, H., Schobert, B., Lanyi, J. K., Spudich, E. N., and Spudich, J. L. (2001) *Science 293*, 1499−1503.
6. Royant, A., Nollert, P., Edman, K., Neutze, R., Landau, E. M., Pebay-Peyroulla, E., and Navarro, J. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 10131−10136.
7. Hayashi, S., Tajkhorshid, E., Pebay-Peyroula, E., Royant, A., Landau, E. M., Navarro, J., and Schulten, K. (2001) *J. Phys. Chem. B 105*, 10124−10131.
8. Ren, L., Martin, C. H., Wise, K. J., Gillespie, N. B., Luecke, H., Lanyi, J. K., Spudich, J. L., and Birge, R. R. (2001) *Biochemistry 40*, 13906−13914.
9. Shimono, K., Iwamoto, M., Sumi, M., and Kamo, N. (1998) *J. Biochem. 124*, 404−409.
10. Shimono, K., Iwamoto, M., Sumi, M., and Kamo, N. (2000) *Photochem. Photobiol. 72*, 141−145.
11. Shimono, K., Ikeura, Y., Sudo, Y., Iwamoto, M., and Kamo, N. (2001) *Biochim. Biophys. Acta 1515*, 92−100.
12. Engelhard, M., Scharf, B., and Siebert, F. (1996) *FEBS Lett. 395*, 195−198.
13. Kandori, H., Shimono, K., Sudo, Y., Iwamoto, M., Shichida, Y., and Kamo, N. (2001) *Biochemistry 40*, 9238−9246.
14. Kandori, H., Furutani, Y., Shimono, K., Shichida, Y., and Kamo, N. (2001) *Biochemistry 40*, 15693−15698.
15. Maeda, A. (1995) *Isr. J. Chem. 35*, 387−400.
16. Kandori, H. (2000) *Biochim. Biophys. Acta 1460*, 177−191.
17. Rothschild, K. J. (1992) *J. Bioenerg. Biomembr. 24*, 147−167.
18. Siebert, F. (1995) *Methods Enzymol. 246*, 501−526.
19. Gerwert, K. (1999) *Biol. Chem. Hoppe-Seyler 380*, 931−935.
20. Gellini, C., Luttenberg, B., Sydor, J., Engelhard, M., and Hildebrandt, P. (2000) *FEBS Lett. 472*, 263−266.
21. Klare, J. P., Schmies, G., Chizhov, I., Shimono, K., Kamo, N., and Engelhard, M. (2002) *Biophys. J. 82*, 2156−2164.
22. Hirayama, J., Imamoto, Y., Shichida, Y., Kamo, N., Tomioka, H., and Yoshizawa, T. (1992) *Biochemistry 31*, 2093−2098.
23. Smith, S. O., Lugtenburg, J., and Mathies, R. A. (1985) *J. Membr. Biol. 85*, 95−109.
24. Maeda, A., Sasaki, J., Pfefferlé, J.-M., Shichida, Y., and Yoshizawa, T. (1991) *Photochem. Photobiol. 54*, 911−921.
25. Gerwert, K., and Siebert, F. (1986) *EMBO J. 5*, 805−811.
26. Yamazaki, Y., Sasaki, J., Hatanaka, M., Kandori, H., Maeda, A., Needleman, R., Shinada, T., Yoshihara, K., Brown, L. S., and Lanyi, J. K. (1995) *Biochemistry 34*, 577−582.
27. Siebert, F., and Mäntele, W. (1983) *Eur. J. Biochem. 130*, 565−573.
28. Kandori, H., Shimono, K., Shichida, Y., and Kamo, N. (2002) *Biochemistry 41*, 4554−4559.
29. Krimm, S., and Dwivedi, A. M. (1982) *Science 216*, 407−409.
30. Yamazaki, Y., Tuzi, S., Saitô, H., Kandori, H., Needleman, R., Lanyi, J. K., and Maeda, A. (1996) *Biochemistry 35*, 4063−4068.
31. Aton, B., Doukas, A. G., Callender, R. H., Dinur, U., and Honig, B. (1980) *Biophys. J. 29*, 79−94.
32. Rodman-Gilson, H. S., Honig, B., Croteau, A., Zarrilli, G., and Nakanishi, K. (1988) *Biophys. J. 53*, 261−269.
33. Baasov, T., Friedman, N., and Sheves, M. (1987) *Biochemistry 26*, 3210−3217.
34. Luecke, H., Schobert, B., Richter, H.-T., Cartailler, J. P., and Lanyi, J. K. (1999) *J. Mol. Biol. 291*, 899−911.
35. Balashov, S. P., Govingjee, R., and Ebrey, T. G. (1991) *Biophys. J. 60*, 475−490.

BI025636C

[4] It is known that BR is transferred into a P480 species at high pH, which absorbs at the same region as *p*pR (*35*). This suggests that the absorption maximum of BR may also undergo a large blue shift caused by long-range effects.