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Color-Changing Mutation in the E−F Loop of Proteorhodopsin[†]

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ABSTRACT: It is usually assumed that only amino acids located near the retinal chromophore are responsible for color tuning of rhodopsins. However, we recently found that replacement of Ala178 with Arg in the E−F loop of proteorhodopsin (PR), an archaeal-type rhodopsin in marine bacteria, shifts the λ_{max} from 525 to 545 nm at neutral pH [Yoshitsugu, M., Shibata, M., Ikeda, D., Furutani, Y., and Kandori, H. (2008) *Angew. Chem., Int. Ed. 47*, 3923−3926]. Since the location of Ala178 is distant from the retinal chromophore (∼25 Å), the molecular mechanism of the unusual mutation effect on color tuning is intriguing. Here we studied this mechanism by using additional mutations and some analytical methods. Introduction of Arg into the corresponding amino acid in bacteriorhodopsin (BR, M163R mutant) does not change the absorption spectra, indicating that the effect is specific to PR. Introduction of Arg into the A−B or C−D loop yields little (3 nm) or no color change, respectively. T177R and P180R mutants exhibited absorption spectra identical to that of the wild type, while N176R and S179R mutants exhibit λ_{max} values of 528 and 535 nm, respectively. Therefore, the observed color change is position-specific, being fully effective at position 178 and half-effective at position 179. Salt affects the absorption spectra of wild-type and A178R PR similarly. FTIR spectroscopy at 77 K indicated similar chromophore structures for wild-type and A178R PR, and A178R PR pumps protons normally. We infer that the E−F loop has a unique structure in PR and the mutation of Ala178 disrupts the structure that includes the transmembrane region, leading to the observed changes in color and pK_a.

Visual or archaeal-type rhodopsins contain either 11-*cis*- or all-*trans*-retinal inside the core of seven transmembrane helices, respectively (*1*). The retinal chromophore is bound to a lysine residue of the seventh helix via a protonated Schiff base linkage. The color tuning mechanism is one of the important topics in the rhodopsin field, because the color of a common molecule, either 11-*cis*- or all-*trans*-retinal Schiff base, is determined by the surrounding amino acids of the protein (*2*−*7*). While we do not fully understand the mechanism, it is likely that color tuning is determined by various interactions between the retinal chromophore and protein, such as the electrostatic effect of charged groups, interactions of the dipole with polar amino acids, aromatic amino acids, hydrogen bonding interactions, and steric contact effects (*1*−*7*).

These interactions may be experimentally tested by site-directed mutagenesis. For instance, many mutations were introduced into bacteriorhodopsin (BR),[1] an archaeal-type rhodopsin functioning as a light-driven proton pump (*8*). For example, in the case of the D85N mutant of BR, the color changes from purple (λ_{max} ∼ 560 nm) to blue (λ_{max} ∼ 600 nm) (*9*). The spectral red shift occurs because the negatively charged counterion [Asp85 (Figure 1)] is neutralized. In contrast, the color changes from purple (∼560 nm) to reddish (∼530 nm) for the L93A and L93T mutants, where specific chromophore−protein interaction is modified (Figure 1) (*10*). Thus, electrostatic and steric effects at Asp85 and Leu93, respectively, contribute to the color tuning in BR significantly. It should be noted that the reported color changes by mutations in BR were mostly limited to the amino acids located near the retinal chromophore (*8*). In general, mutations of distant amino acids do not cause color change, unless a mutation destabilizes the retinal binding site. This is true not only for BR but also for other archaeal-type and visual rhodopsins (*2*, *4*). Therefore, it has been generally accepted that amino acids located near the retinal chromophore are mainly responsible for color tuning. However, we recently presented an unusual mutation effect on color tuning of proteorhodopsin (PR), an archaeal-type rhodopsin, where color was changed by a single amino acid replacement in the cytoplasmic E−F loop far from the retinal molecule (*11*). Likewise, the possibilities of remote chromophore color changes in PR were suggested by the analysis of natural sequence variability (*12*) and random mutagenesis (*13*).

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[1]Abbreviations: BR, bacteriorhodopsin; PR, proteorhodopsin; PR_K, K intermediate of PR.

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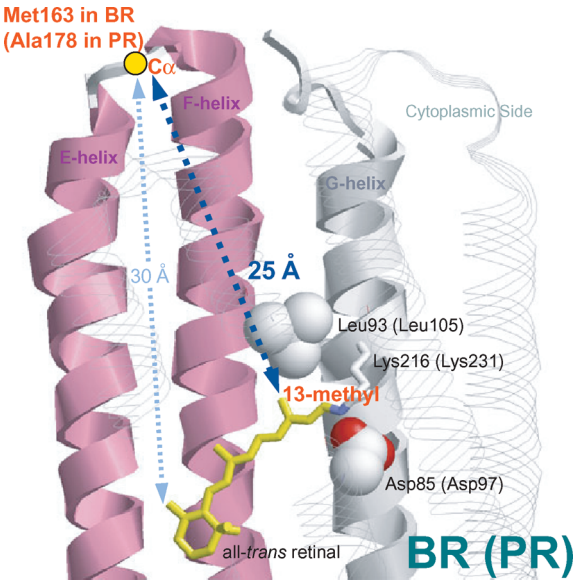

FIGURE 1: X-ray crystallographic structure of bacteriorhodopsin (BR) [Protein Data Bank entry 1IW6 (*31*)] viewed from the B−C helix side. Upper and lower regions correspond to the cytoplasmic (CP) and extracellular (EC) sides, respectively. The retinal chromophore bound to Lys216 is colored yellow. Asp85 and Leu93 are also shown as space-filling models. The Cα atom of Met163 is shown as a yellow circle located at the center of the E−F loop. The nearest atom in the retinal chromophore is the 13-methyl carbon, and the distance between them is 25 Å. The corresponding amino acid in green proteorhodopsin (PR) is Ala178, and the A178R mutant exhibits an absorption spectrum red-shifted by 20 nm at pH 7.0 (*11*). This figure is reproduced from Figure 1 of ref *11*. Copyright 2008 Wiley-VCH.

PR is a light-driven proton pump found in marine γ-proteobacteria (*14*). Because of the widespread distribution of proteobacteria in the worldwide oceanic waters, PR may contribute significantly to the global solar energy input in the biosphere. Extensive genome analysis revealed the presence of thousands of PRs, which can be classified into blue-absorbing ($\lambda_{max} \sim 490$ nm) and green-absorbing ($\lambda_{max} \sim 525$ nm) PR (*15*). Previous studies showed that one of the determinants of color tuning of PR is at position 105, where blue and green PR possess Gln and Leu, respectively (*16*). The corresponding amino acid in BR is Leu93, and it is in direct contact with the retinal chromophore (Figure 1). Although the structure of PR has not been determined, the L/Q switch for color tuning is presumably caused by the direct contact with the retinal chromophore. This is in contrast to our recent finding of the color change for the A178R mutant, where the λ_{max} was shifted by 20 nm at pH 7.0 (*11*). This was an entirely unexpected observation, because Ala178 should be distant from the retinal chromophore. Figure 1 shows that the atom closest to the Cα atom of Met163 in BR is the 13-methyl carbon of the retinal chromophore, 25 Å away. pH titration revealed that the 20 nm spectral red shift is caused by (1) the red shifts of both protonated and deprotonated forms by 7−10 nm and (2) the increase in the pK_a of the counterion by 1.0 (*11*).

In this paper, we further examined properties of mutations at the cytoplasmic loops of PR. First, we examined the homologous mutation of BR, where Met163 was replaced with Arg. The absence of spectral change in the M163R mutant of BR clearly showed that the observed color change is specific to PR. Then, various mutations were tested for several amino acids in the cytoplasmic loops of PR (Figure 2). We mutated Ser55, Ala115, and Ala178 to Arg; the latter two were reported previously (*11*). In addition, four amino acids near Ala178 were also mutated to Arg (N176R, T177R, S179R, and P180R). These mutations revealed that the color change was specific to each position. We expected that salt conditions may affect the color of A178R PR. Therefore, we measured absorption spectra with and without 2 M NaCl, but the observed salt effect was similar between wild-type and A178R PR. In addition, we compared the properties of wild-type and A178R mutant PR by use of low-temperature FTIR spectroscopy and an assay of proton pumping activity. The molecular mechanism of unusual color change of PR by distant mutation will be discussed on the basis of this observation.

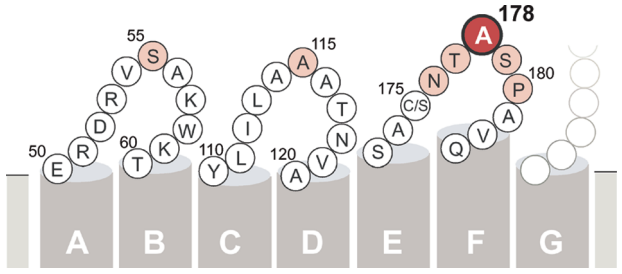

FIGURE 2: Amino acids of the cytoplasmic loop region in proteorhodopsin. Ala178 and Ala115 were replaced with Arg in the previous study (*11*), while other amino acids are replaced with Arg one by one in this study. Corresponding amino acids of the E−F loop (S173−Q183) in bacteriorhodopsin are S158-K-A-E-S-M163-R-P-E-V-A168.

MATERIALS AND METHODS

Preparation of the Proteorhodopsin Samples. The expression plasmids were constructed as described previously (*11*, *17*). To avoid oxidation of one or more of the cysteine residues, we constructed a triple mutant in which all three cysteines (Cys107, Cys156, and Cys175) are replaced with serine as a starting template (*18*). We regarded this protein as the wild type and introduced additional mutations. For preparation of the expression plasmids of the mutants, a Quickchange site-directed mutagenesis kit (Stratagene) was used according to the standard protocol (*17*). The PR proteins possessing a six-histidine tag at the C-terminus were expressed in *Escherichia coli*, solubilized with 0.1% *n*-dodecyl β-D-maltoside (DM), and purified by Ni column chromatography as described previously (*11*, *19*).

Preparation of the M163R Mutant Bacteriorhodopsin Sample. Plasmid construction, site-directed mutagenesis, and transformation for *Halobacterium salinarum* for M163R BR were performed by the methods described previously (*20*).

UV−Visible Spectroscopy. Absorption spectra were measured for solubilized PR (0.1% DM and 100 mM NaCl) at 20 °C by use of a Shimadzu UV-2400PC UV−visible spectrometer (*11*).

FTIR Spectroscopy. Low-temperature FTIR spectroscopy was performed as described previously (*17*, *21*−*23*). For the FTIR measurements, the wild-type and A178R mutant proteins were reconstituted into

L-α-phosphatidylcholine (PC) liposomes (PR:PC molar ratio of 1:50), from which DM was removed via gently stirring with detergent-adsorbing Biobeads [100 mg of Biobeads/mg of protein (Bio-Rad)]. The liposomes were washed three times with a buffer at pH 10.0. Then, 100 μL of the PR sample was dried on a BaF_2 window with a diameter of 18 mm. After hydration by placement of ~1 μL of H_2O beside the dry film and after the system had been sealed with a silicon rubber O-ring and another BaF_2 window, the sample was placed in a cell, which was mounted in an Oxford DN-1704 cryostat in the Bio-Rad FTS-40 spectrometer. Illumination with 460 nm light at 77 K for 2 min converted PR to PR_K, and subsequent illumination with >640 nm light forced PR_K to revert to PR as described previously (*23*). The difference spectrum was calculated from the spectra constructed from 128 interferograms before and after the illumination, and 16 spectra obtained in this way were averaged to yield the PR_K minus PR spectra.

Light-Induced pH Changes. The proton pumping activity of each protein was measured by monitoring pH changes in spheroplast suspension by a glass electrode. Spheroplasts containing the wild-type or A178R mutant protein in unbuffered solution (150 mM NaCl and 150 mM $MgSO_4$) were illuminated at >500 nm through a glass filter (AGC Techno Glass Y-52), and the pH changes were monitored (Horiba pH meter F-55). The light source was a 1 kW tungsten−halogen projector lamp (Rikagaku Master HILUX-HR). The samples were then illuminated after addition of CCCP to a final concentration of 10 μM. Proton pumping activities were calibrated by adding 10 μL of 0.01 N HCl to the suspension.

RESULTS

Absorption Spectrum of the BR Mutant at the Position Corresponding to Position 178 in PR. Figure 3 compares absorption spectra of wild-type and M163R mutant bacteriorhodopsin (BR). The identical spectra clearly demonstrate that introduction of Arg into the E−F loop does not affect the absorption of BR, which is consistent with the previous reports (*8*). In fact, many mutation studies have been performed on BR, but none of them reported such a distant color change. Stern and Khorana reported no color change for R164Q BR (*24*). The position of Arg164 is next to Met163, the amino acid in BR corresponding to Ala178 in PR (Figure 1). Introduction of cysteines into loops in BR at various positions and their spin labeling have been extensively studied, and there were no reports of color changes (*25*, *26*). Thus, the observed mutation effect of Ala178 to Arg is specific for PR.

Absorption Spectra of the PR Mutants in the Cytoplasmic Loops. Figure 4 shows absorption spectra of the PR mutants of the A−B (S55R), C−D (A115R), and E−F (A178R) loops, where the results for A115R and A178R are reproduced from the previous paper (*11*). The spectrum of A115R (black dotted curve) is identical to that of the wild type (gray curve), having a λ_{max} at 525 nm. On the other hand, we observed a clear spectral red shift for A178R (red curve), where the λ_{max} is shifted by 20 nm at pH 7.0. Such an unexpected observation was interpreted

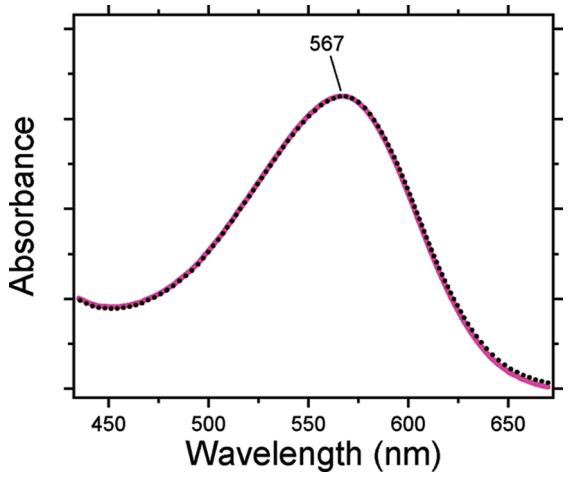

FIGURE 3: Absorption spectra of wild-type (black dotted curve) and M163R mutant (red curve) bacteriorhodopsin (BR).

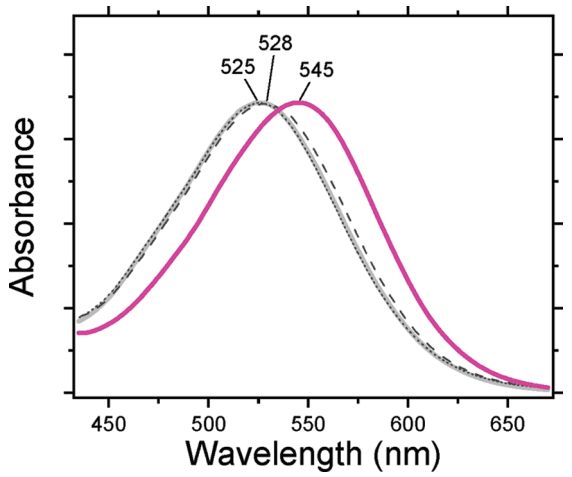

FIGURE 4: Absorption spectra of wild-type (gray curve), S55R (black dotted curve), A115R (black dashed curve), and A178R (red curve) PR at pH 7.0. The λ_{max} is located at 525 nm (wild type and A115R), 528 nm (S55R), and 545 nm (A178R).

in terms of specific long-range interaction between the retinal chromophore and the E−F loop, at a distance of 25 Å (Figure 1). To further examine the specificity of the E−F loop, we tested a mutation in the A−B loop. The black dashed curve in Figure 4 corresponds to the absorption spectrum of S55R, whose λ_{max} is red-shifted by 3 nm at pH 7.0. Since the A−B loop contains many charged amino acids [two acidic and four basic amino acids of 11 (Figure 2)], introduction of Arg into position 55 may cause slight protein structural alteration. Nevertheless, the red shift in S55R is much smaller than that in A178R, indicating that the observed mutation effect in the cytoplasmic region is specific to the E−F loop.

We next examined the positional mutation effect in the E−F loop, where Arg was introduced at positions 176−180 one by one. Figure 5 shows absorption spectra of N176R (black solid curve), T177R (black dotted curve), A178R (red curve), S179R (thin pink curve), and P180R (black dashed line). Two mutants, T177R and P180R, exhibit absorption spectra identical to that of the wild type (gray curve), while N176R exhibits a slightly red-shifted absorption spectrum (by ~3 nm). Only S179R shows a considerable spectral red shift, having a λ_{max}

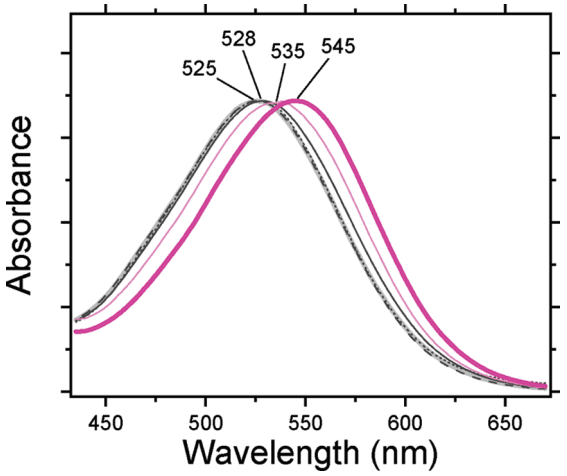

FIGURE 5: Absorption spectra of wild-type (gray curve), N176R (black solid curve), T177R (black dotted curve), A178R (red curve), S179R (pink curve), and P180R (black dashed curve) PR at pH 7.0. The λ_{max} is located at 525 nm (wild type, A177R, and P180R), 528 nm (N176R), 535 nm (S179R), and 545 nm (A178R).

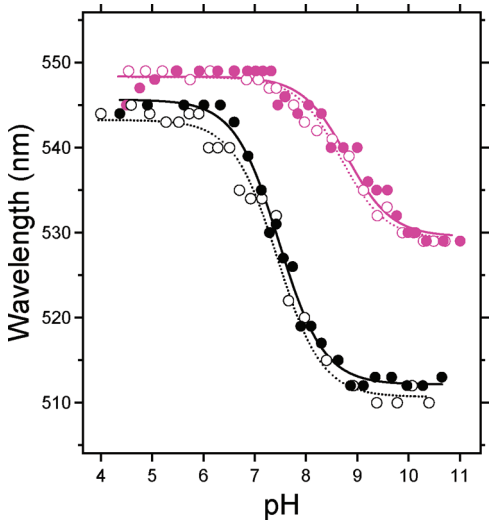

FIGURE 6: Effect of salt on the absorption spectra of A178R PR. pH titration of wild-type (black) and A178R (red) PR containing 0 (○) and 2 M NaCl (●).

at 535 nm. Thus, a color change via introduction of Arg into the E−F loop is not universal, but considerably site-specific. Position 178 is the most influential, while position 179 is half-influential. This may suggest that the E−F loop forms a unique conformation, in which position 178 (and possibly 179) plays an important role.

Properties of A178R PR. (i) Effect of Salt on the Absorption Spectra. We previously performed pH titrations of the wild-type and A178R mutant proteins (*11*) and concluded that the observed 20 nm red shift at neutral pH is caused by (i) the increase in the pK_a of the counterion (Asp97) by 1.0 and (ii) the spectral red shifts in the PR forms with protonated and deprotonated Asp97 of 7 and 10 nm, respectively. Since the chromophore structure of wild-type and A178R mutant PR was predominantly all-*trans* (>90%) at both pH 4 and 10, the possibility of different isomeric content was excluded as the origin of color change (*11*). We also excluded the possibility of destabilized protein structure for A178R by studying the thermal stability of A178R at high temperatures in ref *11*. A similar decomposition time course of wild-type and A178R PR at 75 °C (pH 7.0) strongly suggests that the protein stability is not reduced by the A178R mutation.

Additionally, it may be necessary to take into the account salt conditions. Previously, we measured absorption spectra of A178R in the presence of 100 mM NaCl. Since a positively charged Arg is introduced into the cytoplasmic loop, the 20 nm red shift may be affected by the salt concentration. Therefore, we next tested the effect of salt on the color change. Figure 6 shows the titration curves for wild-type (black curves) and A178R (red curves) PR. In both cases, the λ_{max} values are shifted to the longer wavelengths in the presence of 2 M NaCl compared to those in its absence (0 M). This indicates that salt affects the color and pK_a of PR, but the effect is not specific to A178R PR. It is likely that a color change via introduction of Arg into position 178 is not simply explained by surface charges.

(ii) Local Structure around the Retinal Chromophore. The color change of A178R PR suggests chromophore−protein interactions different from those of the wild type. Since such a change in interaction may be easily monitored by light-induced difference FTIR spectroscopy at cryogenic temperatures (77 K), we compared light-induced difference FTIR spectra between the original and K intermediate states. Figure 7 shows the PR_K minus PR difference infrared spectra of wild-type (dotted curve) and A178R (solid curve) PR in the 1800−900 cm^{-1} region. The spectra were measured at pH 10, ensuring that Asp97 is deprotonated for both wild-type and A178R PR. The intense bands at 1542(−)/1523(+) cm^{-1} of the wild type (dotted curve) can be assigned to the ethylenic C=C stretching vibrations of the retinal chromophore. The lower-frequency shift of the band indicates formation of the red-shifted intermediate, PR_K, upon absorption of light by PR at 77 K. Vibrational bands in the 1280−1050 cm^{-1} region can be assigned to the C−C stretching vibrations of the retinal chromophore, which monitor the retinal configuration (*23*). The positive band at 1192 cm^{-1} shows that photoisomerization takes place from the all-*trans* to 13-*cis* form, because the 1192 cm^{-1} band originates from the mixed C14−C15 and C10−C11 stretching vibrations of the 13-*cis*-retinal chromophore. The positive peak at 1377 cm^{-1} was recently suggested to originate from the COO^- stretching vibration of Asp227 (*23*).

The PR_K minus PR difference infrared spectrum of A178R (solid curve) is almost identical to that of the wild type (Figure 7). The only exception was observed for the ethylenic C=C stretching vibrations of the retinal chromophore at 1550−1500 cm^{-1}. The peaks at 1542(−)/1523(+) cm^{-1} of the wild type (dotted curve) are slightly shifted to the lower frequencies, which is consistent with the spectral red shift in UV−visible region. On the other hand, there are no other spectral changes, suggesting little structural modification between wild-type and A178R PR. It should be noted, however, that the observed lower-frequency shifts of the ethylenic C=C stretching vibrations (1−2 cm^{-1}) in Figure 7 were smaller than the expected values (~4 cm^{-1}) from the spectral red shift at room temperature (10 nm for the unprotonated Asp97). This may imply that the spectral red shift is reduced

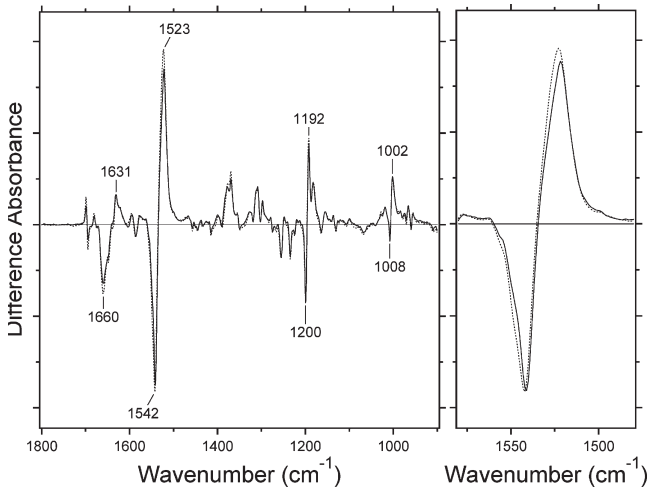

FIGURE 7: PR_K minus PR difference infrared spectra of wild-type (dotted curve) and A178R (solid curve) PR measured at 77 K. The samples were prepared at pH 10, and dry films were rehydrated. One division of the *y*-axis corresponds to 0.0005 unit.

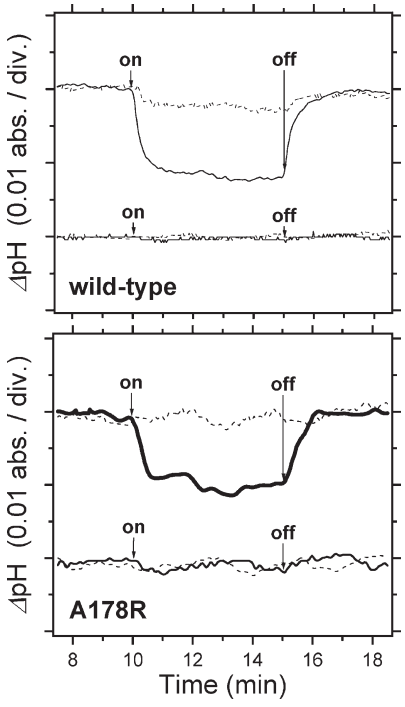

FIGURE 8: Light-driven proton pumping activity in spheroplast vesicles containing wild-type (top panel) and A178R (bottom panel) PR. "On" and "off" indicate the onset and offset of illumination (with yellow light, > 500 nm), respectively, and a negative signal corresponds to an increase in pH (outward proton pump). The top traces correspond to the measurements at pH 7.2 (top) and 8.2 (bottom), the counterion pK_a values of wild-type and A178R PR, respectively. The bottom traces correspond to those at pH 5.0. Dotted lines represent identical measurements in the presence of 10 μM CCCP.

at 77 K. Therefore, we compared visible absorption spectra of wild-type and A178R PR at 77 K, and the apparent spectral red shift in A178R PR was consistent with the FTIR observation. Because of increased scattering in the visible region, however, the λ_{max} values were not quantitatively analyzed.

(iii) Proton Transport. Light-driven proton transport was assessed for spheroplast membrane vesicles of wild-type and A178R PR by monitoring the pH changes with a glass electrode. The top panel of Figure 8 shows light-induced pH changes of wild-type PR at pH 7.2 (top) and 5.0 (bottom). In the case of the wild type at pH 7.2 (the counterion pK_a value), illumination caused a net acidification of the medium (solid curve), indicating that protons are pumped out of the vesicles. Addition of 10 μM CCCP almost abolished the observed light-induced pH changes (dotted curve). In contrast, no proton pumping activity was observed at pH 5.0, which reproduced the previous report (*27*, *28*). Similarly, illumination of the membrane vesicles containing A178R at pH 8.2 (the counterion pK_a value) caused net acidification of the medium (thick solid curve in the bottom panel), indicating that protons are pumped out of the vesicles. Addition of 10 μM CCCP almost abolished the observed light-induced pH changes (dotted curve in the bottom panel). Since the *y*-axis is normalized between wild-type and A178R PR by visible absorption spectra, similar pH changes suggest that A178R has proton pumping activity similar to that of the wild type.

DISCUSSION

The previous study of green PR demonstrated that the λ_{max} of A178R is shifted by 20 nm at pH 7.0 (*11*). Such a spectral red shift is caused by (1) the red shifts of both protonated and deprotonated forms of 7−10 nm and (2) the increase in the counterion pK_a of 1.0. As already described, various interactions between the retinal chromophore and protein contribute to the color tuning of rhodopsins, but the experimentally observed effects have been mostly local (*1*−*7*). Thus, the unusual color change by a single amino acid replacement distant from the retinal chromophore (~25 Å) generates various questions about the molecular mechanism.

It has to be noted that mutations often destabilize protein structure, so that the weakened chromophore−protein interaction results in color change. To exclude this possibility, we previously tested the protein stability by keeping the sample at 75 °C for 5 min (pH 7.0), where it is gradually thermally decomposes. Similar decomposition rates between the wild-type and A178R proteins strongly suggest that the protein stability is not reduced by the A178R mutation (*11*). The study presented here further reports unmodified protein structure by showing the almost identical PR_K minus PR difference FTIR spectra for wild-type and A178R PR (Figure 7). This observation implies that the A178R mutation does not change the local chromophore−protein interaction significantly, being consistent with the unchanged thermal stability. A178R also exhibits normal proton pumping activity (Figure 8).

While the data in Figure 7 provide useful structural information for A178R PR, they are also something puzzling. Molecular vibrations are highly sensitive to the environment and thus are a good probe of structural changes near the retinal chromophore (*29*). In other words, we expected some interaction changes between the chromophore and protein induced by the A178R mutation. Again, it is impossible that the positive charge of the mutated Arg178 directly influences the absorption spectra of PR, because the distance is ~25 Å. Instead, the A178R mutation in the E−F loop of PR probably causes

structural changes in the chromophore domain, which leads to the observed changes in color and counterion pK_a value. If it is, we expected that highly sensitive FTIR signals would be able to extract structural alterations in retinal and/or protein. However, Figure 7 showed that the only clear difference was seen for the C=C stretching vibrations, which are directly correlated with the color change. The origin of the absence of other differences is unclear at present, and frequency shifts in the ethylenic C=C stretching vibrations (1−2 cm^{-1}) smaller than the expected values (~4 cm^{-1}) from the spectral red shift at room temperature (10 nm for the unprotonated Asp97) suggest that the structural alteration at room temperature may be reduced at 77 K. It also might be reasonable to suggest that electronic states of the retinal polyene are more sensitive than the structure monitored by FTIR spectroscopy. To improve our understanding, structural and kinetic analyses of late intermediates are important. In fact, it is likely that many color tuning mutations in various rhodopsins also influence their photocycles. It is thus intriguing whether A178R PR has a photocycle similar to that of the wild type, which is our future focus.

Since the A178R mutant possesses an additional positive charge at the cytoplasmic surface compared with the wild type, we also examined the effect of salt on the absorption of wild-type and A178R PR (Figure 6). The results clearly exclude the specific effect of salt on color tuning of A178R PR, because the observed salt effects were similar between wild-type and A178R PR. It seems that chloride ion does not bind to Arg178 in the mutant. This may further suggest the origin of color change coming from structural modification transmitted through the transmembrane region.

Many mutation studies have been performed on BR, the best studied archaeal-type rhodopsin, but none of them reported such a distant color change. Stern and Khorana reported no color change for R164Q BR, though the λ_{max} values of both dark- and light-adapted states were slightly different from that of wild-type BR (*24*). The position of Arg164 is next to Met163, the amino acid in BR corresponding to Ala178 in PR (Figure 1). Various cysteine mutations in the loops of BR and their spin labeling have been extensively studied, while there are no reports of color change (*25*, *26*). Identical absorption spectra of wild-type and M163R BR, the position corresponding to Ala178 in PR, in Figure 3 directly show that the unusual effect of mutation on color is specific for PR.

The nature of the suggested long-range interaction channel in PR is interesting. However, because of the lack of X-ray crystallographic structure of PR, the possibility of a full explanation of the observed color change is limited. The structure of BR shows the presence of hydrophobic bulky groups in the cytoplasmic domain, such as Leu93, Asp96, Leu97, and Leu100 of the C helix and Val167, Phe171, Leu174, Thr178, and Trp182 of the F helix, where the corresponding amino acids in PR are Leu105, Glu108, Phe109, and Ile112 of the C helix and Val182, Tyr186, Met189, Ile193, and Trp197 of the F helix, respectively (Figure 9). The presence of similar bulky groups in PR suggests the absence of a direct hydrogen bonding network between the retinal chromophore and Arg178 in the E−F loop. Instead, it is possible

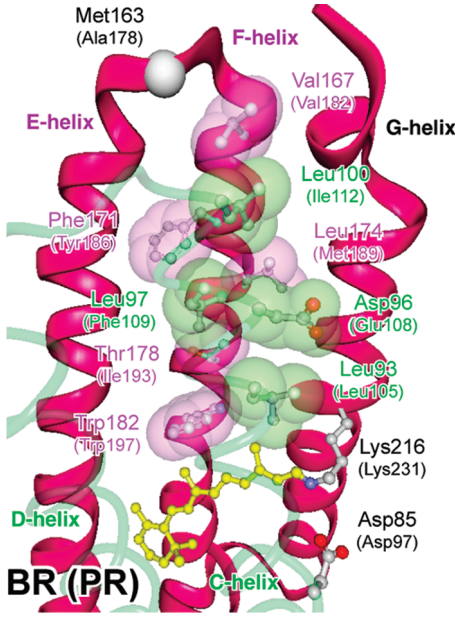

FIGURE 9: Amino acid residues located between the retinal molecule and the position of Met163 in BR (Ala178 in PR) [Protein Data Bank entry 1IW6 (*30*)]. This figure is reproduced from Figure S2 of ref *11*. Copyright 2008 Wiley-VCH.

that the A178R mutation causes rearrangement of these helices, leading to the observed changes in color and counterion pK_a. It should be noted that Kim et al. reported remote chromophore color changes with mutations such as L67R, C107R, and F234S, which caused large red shifts (20−25 nm) (*13*). These amino acids are embedded in the transmembrane region, being closer to the retinal chromophore than Ala178 in the E−F loop. Nevertheless, they are all located at the cytoplasmic side, and such observation is not typical for BR (*13*). Thus, it seems easy to induce a spectral red shift by mutation in the cytoplasmic domain of PR, and the color change of A178R could be explained by the same mechanism.

A color change via the introduction of Arg into the cytoplasmic loop was specific to the E−F loop, as it was not detected for either the A−B or the C−D loop (Figure 4). In addition, the mutation was specific to the position. In fact, among five Arg mutants of Asn176, Thr177, Ala178, Ser179, and Pro180, only two mutations had significant spectral effects. A178R was fully effective in terms of color change (λ_{max} at 545 nm), while S179R was half-effective (λ_{max} at 535 nm) (Figure 5). It should be noted that five amino acids between positions 176 and 180 are all neutral (Figure 2), so that the position 178 (and 179) is specific as the determinant of color change. We infer that the E−F loop constitutes a unique structure in PR, but not in BR, in which position 178 (and 179) plays an important role. In this regard, a three-dimensional solid-state NMR study of PR was reported after the present work had been prepared (*30*). In the paper, interestingly, Shi et al. revealed that the E−F loop region contains a short extramembrane helical segment from Glu170 to Asn176, connected to the extended helix F (starting from Pro180) by a short β turn (Thr177, Ala178, and Ser179). Such secondary structure in wild-type PR is probably correlated with the unique color change caused by the A178R mutation. In the study presented

here, Arg was introduced into the cytoplasmic loops, where Arg has both bulky and positively charged functional groups. Because there is no salt effect, the bulkiness may cause the breaking of the β turn, leading to the rearrangement of the helices. Since position 178 is highly specific, we plan to introduce all 19 mutations into this position. The absorption spectra will provide further information about the mechanism of the unusual mutation effect.

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