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Importance of Alanine at Position 178 in Proteorhodopsin for Absorption of Prevalent Ambient Light in the Marine Environment†

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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. A recent mutation study revealed that the observed color change was highly specific to position 178 [Yoshitsugu, M., Yamada, J., and Kandori, H. (2009) *Biochemistry 48*, 4324−4330]. Thus, in the study presented here, we replaced Ala178 with 19 different amino acids and measured the absorption spectra and the pK_a of the Schiff base counterion, Asp97. Most of the mutants exhibited a spectral red shift and increased pK_a of Asp97. None of charged amino acids at position 178 influences color tuning of PR specifically, being similar to the Arg replacement studied earlier. Only Cys and Thr replacements exhibited color and a pK_a similar to that of the wild type. Ser, Val, and Gly mutants behave like the wild type only with respect to the λ_{max} of the species with deprotonated Asp97. We conclude that the E−F loop region contains a unique structure in PR, disruption of which causes large-scale rearrangement of α-helices. Ala178 in PR contributes to the blue-shifted absorption (525 nm at neutral pH) and lowering of the counterion pK_a, which is important for proton-pump function in the marine environment, even though its position is far removed from the chromophore binding domain.

Visual and archaeal-type rhodopsins contain 11-*cis*- and 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*). The interaction between the chromophore and protein 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 reason for the spectral red shift is that 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 positions, respectively, contribute to the color tuning in BR significantly. It should be noted that the reported color changes caused by mutations in BR were mostly limited to the amino acids located near the retinal chromophore (*8*). In general, mutations of the outlying amino acids do not cause color change, unless a mutation changes the chromophore−protein interaction in 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*).

PR is a light-driven proton pump found in marine γ-proteobacteria (*14*). Because of the widespread distribution of proteobacteria in 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 (λ_{max} ∼ 490 nm) and green-absorbing (λ_{max} ∼ 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 presumably occurs because of the direct contact with the retinal chromophore. This is in contrast to our recent finding for 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

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[1]Abbreviations: BR, bacteriorhodopsin; PDB, Protein Data Bank; PR, proteorhodopsin.

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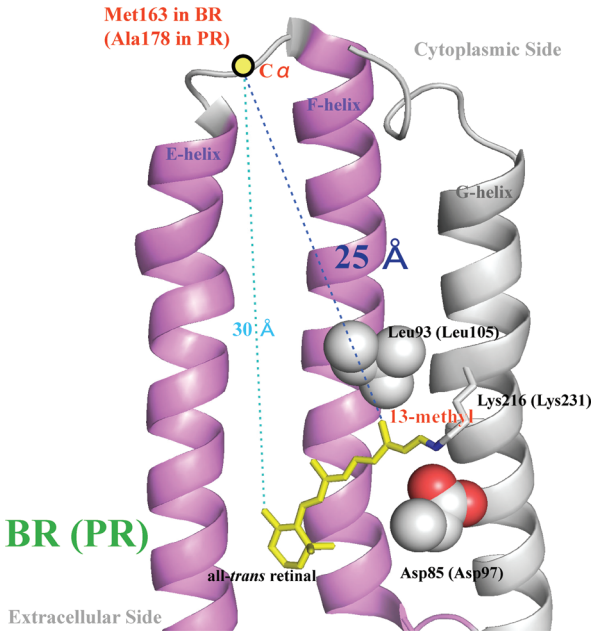

FIGURE 1: X-ray crystallographic structure of bacteriorhodopsin (BR) [PDB entry 1IW6 (*34*)] viewed from the side of B and C helices. Top and bottom regions correspond to the cytoplasmic (CP) and extracellular (EC) sides, respectively. The retinal chromophore bound to Lys216 is colored yellow. Asp85 and Leu93 are 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 modified from Figure 1 of ref *11*.

observation, because Ala178 should be distant from the retinal chromophore. One concern was that the mutation destabilizes the protein structure so that the weakened chromophore−protein interaction results in the observed spectral change. However, the previous thermal stability experiment at 75 °C clearly showed that the protein stability is not reduced by the A178R mutation (*11*). Figure 1 shows that the nearest retinal atom to the Cα atom of Met163 in BR, the 13-methyl carbon of the chromophore, is 25 Å away. pH titration revealed that the 20 nm spectral red shift is caused by (1) the red shifts of absorption spectra of both protonated and deprotonated forms by 7−10 nm and (2) the increase in the pK_a of the counterion by 1.0 pH unit (*11*).

We recently examined the mechanism of this unusual color change by using additional mutations and some analytical methods (*17*). Introduction of Arg into the corresponding position 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. The systematic introduction of Arg into the 176−180 loop segment showed that the observed color change is position-specific, being fully effective at position 178 and half-effective at position 179. These results suggest that introduction of a positive charge at position 178 disrupts the structure of the E−F loop. If the positive charge were a key element of the color change, we would expect only the A178K mutant to exhibit the red shift similar to that of A178R. We thus replaced Ala178 with 19 different amino acids and measured the absorption spectra and the counterion pK_a values. Most of the mutants exhibited a spectral red shift and an increased

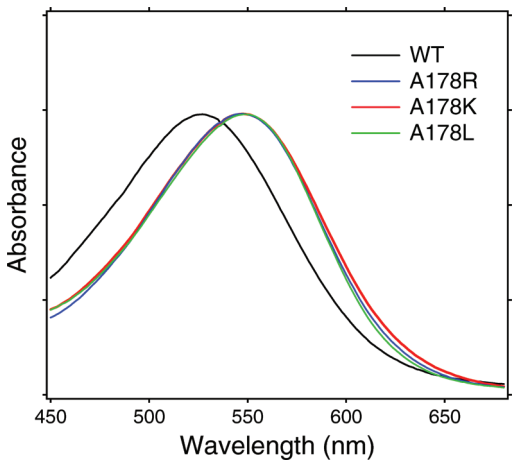

FIGURE 2: Absorption spectra of wild-type (black), A178R (blue), A178K (red), and A178L (green) PR at pH 7.0. The spectra were measured for the detergent-solubilized sample at 20 °C. The λ_{max} is located at 527 nm (wild type), 548 nm (A178R), 547 nm (A178K), or 548 nm (A178L).

counterion pK_a, except for A178C and A178T. Ser, Val, and Gly mutants behaved like the wild type only with respect to the λ_{max} of the species with deprotonated Asp97. All other amino acids exhibit red-shifted λ_{max} values for both deprotonated and protonated forms of Asp97 by 10−15 nm and pK_a values of Asp97 increased by 0.6−1.2. We conclude that the chromophore−protein interaction in PR is dependent on the amino acid at position 178.

MATERIALS AND METHODS

Preparation of the Proteorhodopsin Samples. The expression plasmids were constructed as described previously (*11*, *18*). 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) were replaced with serine as a starting template (*19*, *20*). We regarded this protein as the wild type and introduced additional mutations. For preparation of the expression plasmids of the mutants, a QuikChange site-directed mutagenesis kit (Stratagene) was used according to the standard protocol (*18*). The PR proteins possessing a six-histidine tag at the C-terminus were expressed in *Escherichia coli* strain BL21 (DE3) (Stratagene), solubilized with 0.1% *n*-dodecyl β-D-maltoside (DM), and purified via Ni^{2+} column chromatography as described previously (*11*, *21*).

UV−Visible Spectroscopy. Absorption spectra were recorded for solubilized PR (0.1% DM, 150 mM NaCl, and 10 mM citric acid monohydrate, MES, MOPS, HEPES, CHES and CAPS) at 20 °C by using a Shimadzu UV-2400PC UV−visible spectrophotometer (*11*). The initial pH was 4−5, from which the pH was increased to 10 by addition of NaOH, where the absorption spectrum was measured every 0.25 pH unit. Then, the pH was decreased to 4 by addition of HCl, where the absorption spectrum was measured every 0.25 pH unit. We confirmed that absorption spectra at the same pH produced by additions of either NaOH or HCl were identical, indicating that pH titration was reversible between pH 4 and 10 for all proteins. The spectra were not reversible at pH >10 for many proteins, which suggests denaturation. The λ_{max} was plotted as a function of pH, and the Henderson−Hasselbalch equation was used for determination of the pK_a values.

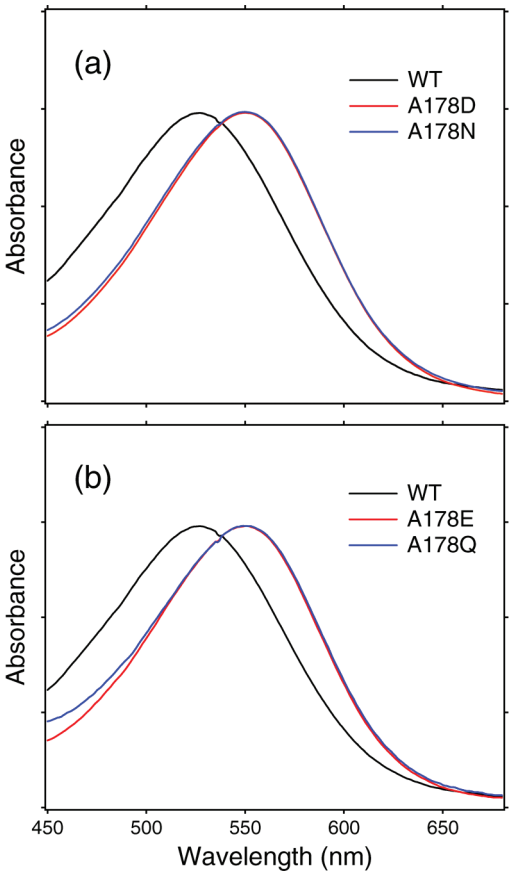

FIGURE 3: (a) Absorption spectra of wild-type (black), A178D (red), and A178N (blue) PR at pH 7.0. (b) Absorption spectra of wild-type (black), A178E (red), and A178Q (blue) PR at pH 7.0. The λ_{max} is located at 527 nm (wild type), 550 nm (A178D), 549 nm (A178N), 551 nm (A178E), or 549 nm (A178Q).

RESULTS

Spectral Comparison of the A178 Mutants at pH 7.0. We first examined absorption spectra of the wild-type and 19 A178 mutant proteins at pH 7.0. If the positive charge were a key element in the color change of A178R, one would expect the A178K mutant to exhibit a red shift similar to that of A178R. Figure 2 shows that the spectrum of A178K (red curve) is almost identical to that of A178R (blue curve), with λ_{max} values of 547 and 548 nm, respectively. Nevertheless, the positive charge at position 178 is not necessarily the determinant of the color, because the λ_{max} of A178L (green curve in Figure 2) is also located at 548 nm. As seen in Figure 2, the spectra of A178R, A178K, and A178L are superimposable with each other, being red-shifted by ~20 nm from that of the wild type.

We next examined the effect of a negative charge at position 178. Figure 3a shows that the λ_{max} values of A178D (red curve) and A178N (blue curve) are located at 550 and 549 nm, respectively. Similarly, the λ_{max} values of A178E (red curve in Figure 3b) and A178Q (blue curve in Figure 3b) are located at 551 and 549 nm, respectively. Thus, all these mutations yielded significant red shifts, indicating that a negative charge at position 178 does not affect the color change of PR specifically.

Figures 2 and 3 show that the observed red shift of >20 nm does not originate from introduction of charged groups at position 178. Instead, it is likely that Ala at position 178 is structurally unique. This suggests that the side chain volume at position 178 may have to be small, which we examined next. Figure 4 shows

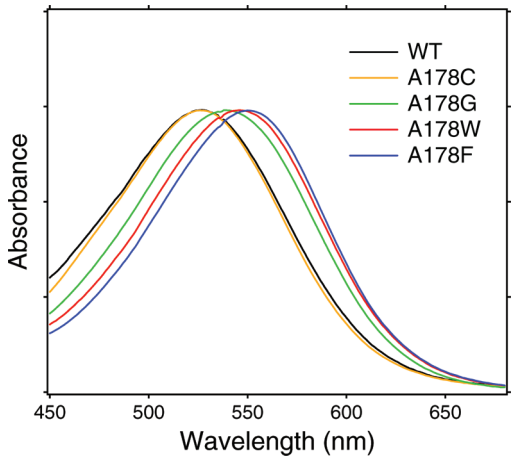

FIGURE 4: Absorption spectra of wild-type (black), A178C (yellow), A178G (green), A178W (red), and A178F (blue) PR at pH 7.0. The λ_{max} is located at 527 nm (wild type), 526 nm (A178C), 539 nm (A178G), 546 nm (A178W), or 550 nm (A178F).

the λ_{max} values of A178W (red curve) and A178F (blue curve) at 546 and 550 nm, respectively, which also classifies them as the red-shifting mutations. On the other hand, A178C (yellow curve in Figure 4) exhibited an absorption spectrum identical to that of the wild type. This strongly suggests the importance of the side chain volume at position 178. It should be noted that the λ_{max} of A178G (green curve in Figure 4), the mutation to the smallest amino acid, is located at 539 nm, exhibiting a considerable red shift. In summary, many mutants exhibit a spectral red shift of ~20 nm like A178R, demonstrating that the side chain volume at this position is an important element of the color change.

pH Titration of the A178 Mutants. It is known that the pK_a of the Schiff base counterion in PR, Asp97, is located at neutral pH (*22*, *23*), indicating that both protonated and deprotonated forms exist at pH 7.0. In fact, a previous pH titration study revealed the pK_a values of wild-type and A178R mutant PR to be 7.2 and 8.2, respectively, indicating that the red shift in A178R of ~20 nm originates from (i) the red shifts of 7 and 10 nm for the protonated and deprotonated forms, respectively, and (ii) the counterion pK_a that is increased by 1.0 pH unit. It is thus important to examine the λ_{max} values of both protonated and deprotonated forms, and the pK_a value by pH titration, which we performed on all A178 mutants. The initial pH was in the range of 4−5, and we first added NaOH stepwise and measured absorption spectra. After the sample pH reached 10.0, HCl was added to lower the pH to 4.0. We confirmed that the absorption spectra at the same pH were identical between the experiments with increasing and decreasing pH.

Figure 5 shows absorption spectra of the wild type and 19 A178 mutants at pH 4 (dotted curves) and pH 10 (solid curves). Since the absorption spectra of all these proteins at various pH values exhibit a single isosbestic point (data not shown), only two forms, with protonated and deprotonated Asp97, are in equilibrium. Figure 6 shows pH titration curves of the wild type and 19 A178 mutants. Most of the data can be fitted by a curve derived from the Henderson−Hasselbalch equation with $n = 1$. Nevertheless, two kinds of deviations should be noted for some mutants. The λ_{max} is red-shifted at low pH because of protonation of Asp97, while the λ_{max} is then blue-shifted at pH 4−5 in some mutants, such as A178H, A178Y, and A178R (Figure 6). This suggests the presence of another titratable group at pH

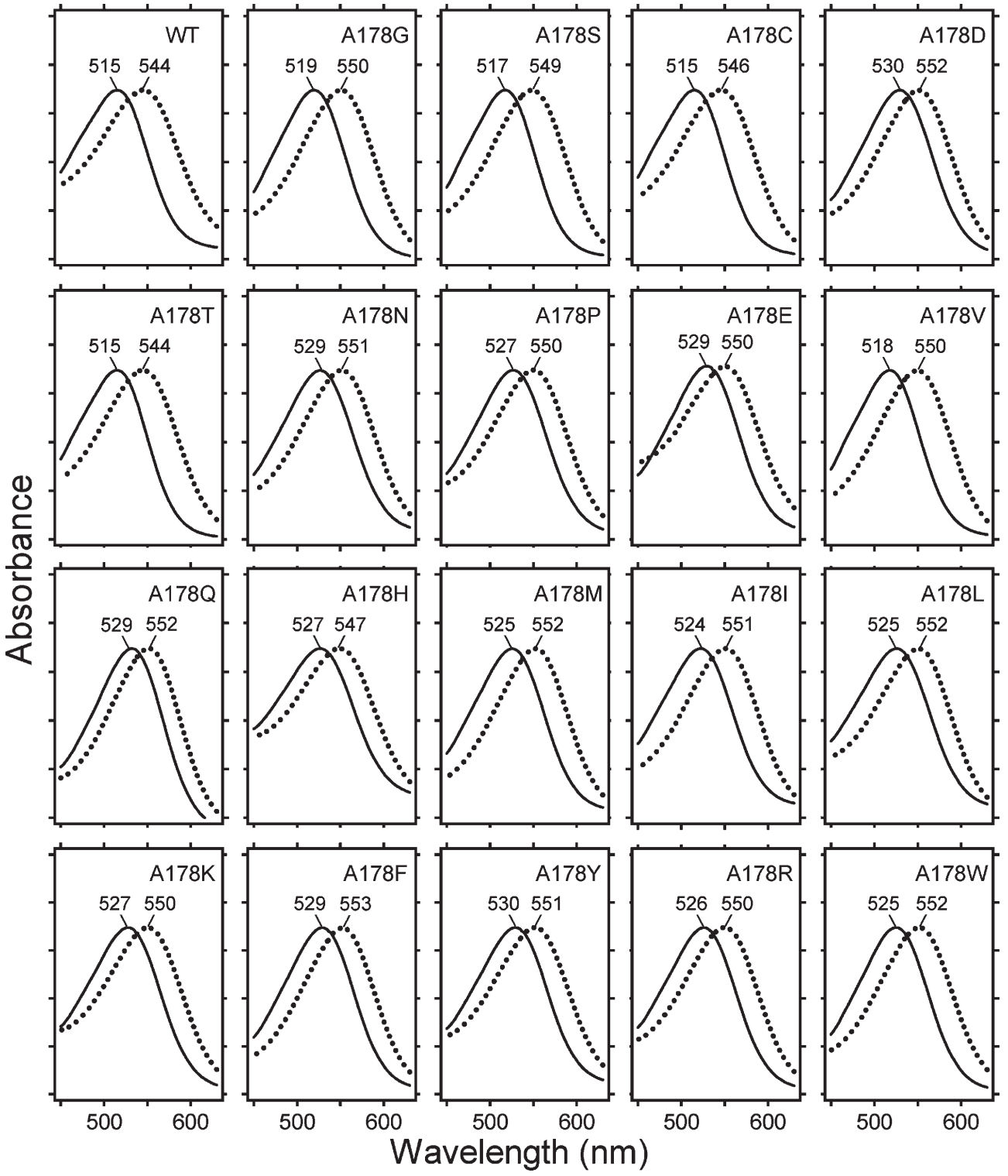

FIGURE 5: Absorption spectra of the wild type and 19 A178 mutants at pH 4 (···) and pH 10 (—).

< 5 in these mutants. This might originate from chloride binding to the species with protonated Asp97, because similar spectral shifts were reported for BR (*24*) and SRII (*25*, *26*). It also should be noted that the λ_{max} is not constant at pH 9−10 in several mutants, such as A178N, A178E, A178M, A178L, and A178K (Figure 6). One possible reason may be the elevated counterion pK_a values in these mutants. Unfortunately, the pH titration was not reversible at pH > 10, presumably because of protein decomposition, which is particularly noticeable for the mutants. This fact produces inaccuracy in the pK_a determination for these mutants, but Figure 6 provides reasonably quantitative estimates of the pK_a values (summarized in Table 1).

Correlation of λ_{max} Values, Counterion pK_a Values, and Side Chain Volumes of Amino Acids. From the data shown in Figure 6, the λ_{max} values of species with both protonated and deprotonated Asp97 and the counterion pK_a values were obtained for 20 amino acids at position 178 (Table 1). As we reported previously, the mutation of Ala178 to Arg affected not only the color but also the pK_a of the Schiff base counterion (Asp97) (*11*, *17*). Figure 7 shows the correlation between the λ_{max} of the

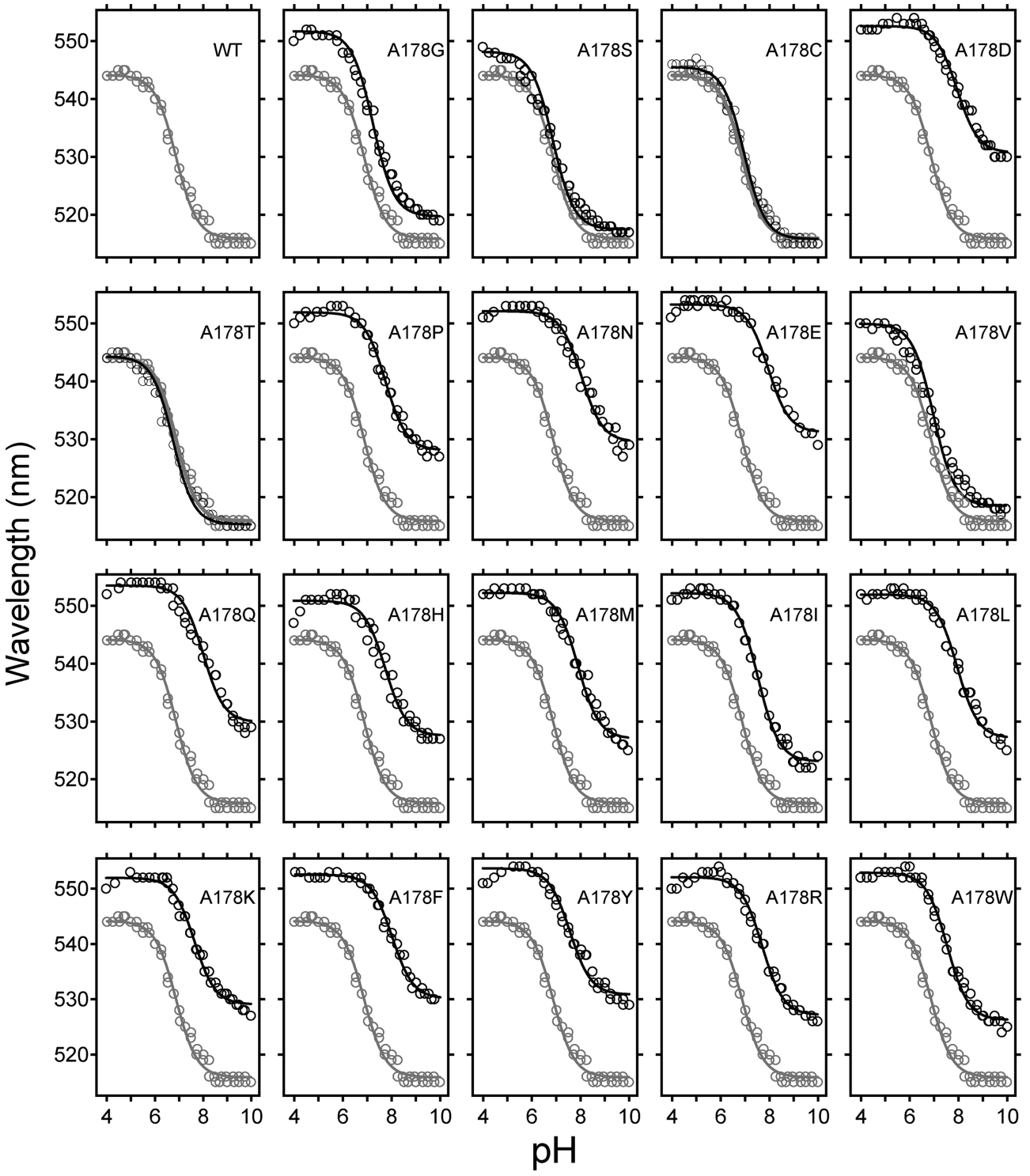

FIGURE 6: pH titration of the wild type (gray circles) and 19 A178 mutants (black circles). Lines represent the fitting according to the Henderson−Hasselbalch equation.

species with deprotonated (a) and protonated (b) Asp97 and the pK_a of Asp97, which are obtained from the values in the fitting curve of the Henderson−Hasselbalch equation in Figure 6 (see the values of curve fitting in Table 1). A linear relationship is obvious in Figure 7a, whereas it is less clear in Figure 7b. However, it is likely that the spectral red shifts are correlated with the pK_a increase of Asp97. This suggests the important role of the hydrogen bonding network in the Schiff base region to control the color and pK_a of the Schiff base counterion.

Table 1 also allows quantitative analysis of the dependencies on the side chain volume of amino acids (*27*). Figure 8a shows the correlation between the side chain volume of the amino acid at position 178 and the λ_{max} of the species with deprotonated Asp97. Though the correlation is not strict, generally, the λ_{max} is red-shifted when the volume is larger. In addition, the data points can be classified into two categories (dashed circles in Figure 8a), where five amino acids, Gly, Ser, Cys, Thr, and Val, are in the same group as the wild type (Ala). This suggests the presence of two kinds of retinal binding pockets, controlled by the side chain volume of the amino acid at the center of the E−F loop (position 178).

Figure 8b shows the correlation between the volume of the amino acid at position 178 and the λ_{max} of the species with

Table 1: Properties of the Wild Type and 19 Mutant Proteins at Position 178

	volume[a] (cm^3/mol)	λ_{max} measured (nm)			λ_{max} obtained from curve fitting (nm)		
		pH 4	pH 7	pH 10	pH 4	pH 10	pK_a
wild type	52	544	527	515	544.0	515.9	6.87
A178G	34.8	550	539	519	551.7	519.8	7.24
A178S	51.9	549	529	517	548.1	517.5	6.87
A178C	65	546	526	515	545.5	515.8	6.96
A178D	65.4	552	550	530	552.6	531.0	7.94
A178T	68.4	544	526	515	544.2	515.3	6.75
A178N	69.6	551	549	529	552.1	529.9	8.04
A178P	72.6	550	548	527	551.9	528.3	7.75
A178E	77.5	550	551	529	553.3	531.5	7.94
A178V	82.4	550	533	518	549.9	518.5	6.97
A178Q	85.5	552	549	529	553.5	530.2	8.00
A178H	90.4	547	547	527	550.9	527.7	7.72
A178M	96.9	552	549	525	552.2	527.3	7.89
A178I	99.1	551	544	524	552.2	523.2	7.53
A178L	99.1	552	548	525	551.9	527.4	7.94
A178K	100.1	550	547	527	552.0	529.2	7.64
A178F	112.8	553	550	529	552.5	530.5	8.02
A178Y	114.7	551	547	530	553.7	531.0	7.56
A178R	118.9	550	548	526	552.1	527.3	7.70
A178W	135.5	552	546	525	552.9	526.4	7.54

[a] From ref *27*.

protonated Asp97. Similar to that of the deprotonated form (Figure 8a), the λ_{max} is red-shifted when the volume is larger. In contrast, the two clusters are less clearly defined for the protonated form, where only Cys and Thr are in the same group as the wild type (Ala). Unlike that for the deprotonated form (Figure 8a), Val is in the major group (Figure 8b), while Ser and Gly are not included in either of the two groups.

Figure 8c shows that there is the same correlation between the side chain volume of the amino acid at position 178 and the pK_a of Asp97 like those for λ_{max}. The pK_a of Asp97 is increased when the volume is larger. The data points are classified into two categories (dashed circles in Figure 8c), where four amino acids, Ser, Cys, Thr, and Val, are in the same group as the wild type (Ala). Gly is isolated from both of the two groups. The positive correlation between the volume of amino acid at position 178 and the pK_a of Asp97 is also consistent with two kinds of retinal binding pockets, controlled by the side chain volume of the amino acid at the center of the E−F loop (position 178).

DISCUSSION

Recently, we found that replacement of Ala178 with Arg in the E−F loop of PR shifts the λ_{max} of the absorption spectrum to the red by 20 nm (*11*). 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. A subsequent mutation study revealed that the observed color change was highly specific to position 178 (*17*), and in this study, we thus replaced Ala178 with 19 different amino acids. First, we expected the importance of the positive charge at position 178 for the color tuning, because A178R possesses an additional positive charge at the cytoplasmic surface compared with the wild type. The results in Figures 2 and 3 clearly exclude the charge effect on the color tuning of A178R PR. On the other hand, the size of the side chain is important, as Figures 7−9 show. The Cys and Thr mutants are similar to the wild type (Ala). The Ser, Val, and Gly mutants behave like the wild type only with respect to the λ_{max} of the

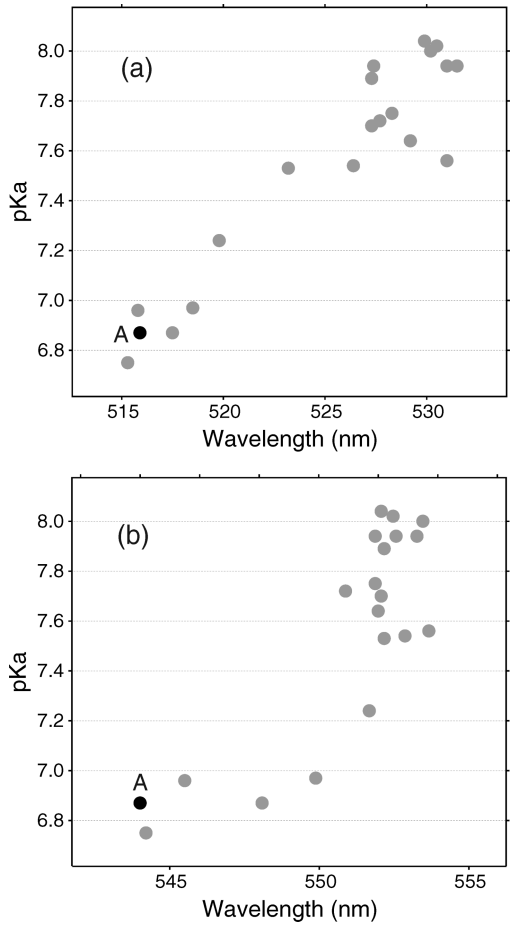

FIGURE 7: Correlation between the λ_{max} values of the species with deprotonated (a) and protonated (b) Asp97 and the pK_a value of Asp97. The λ_{max} values of the deprotonated (a) and protonated (b) forms are obtained from the values at pH 10 and 4, respectively, in the fitting curve of the Henderson−Hasselbalch equation in Figure 6 (see the values of curve fitting in Table 1). The pK_a of Asp97 is similarly obtained from the Henderson−Hasselbalch equation in Figure 6 (see the pK_a values in Table 1).

species with deprotonated Asp97. All other amino acid replacements exhibit red-shifted λ_{max} values for the forms with both deprotonated and protonated Asp97 by 10−15 nm and increased pK_a values of Asp97 by 0.6−1.2 pH units. These observations indicate that the chromophore−protein interaction in PR is dependent on the amino acid type at position 178. They further suggest the presence of the two conformational states, controlled by the side chain volume of the amino acid at position 178.

By use of three-dimensional solid-state NMR, Shi et al. revealed that the E−F loop region in PR 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) (*28*). Ala178 is located at the connecting region of the two helices, the cytoplasmic short helix and helix F, and the β-turn is stabilized by a hydrogen bond of the peptide backbone. This indicates that the region around Ala178 forms an ordered structure, and replacement of Ala178 with other amino acids may disrupt the structure. Since BR also possesses such an extra helix in the E−F loop region (*29*), the correlation with color change is not clear at present. In fact, introduction of Arg into the corresponding position in BR (M163R mutant) does not change the absorption spectra at all (*17*), whereas we did not test other mutations such as Ala. A comprehensive mutation study for other rhodopsins will

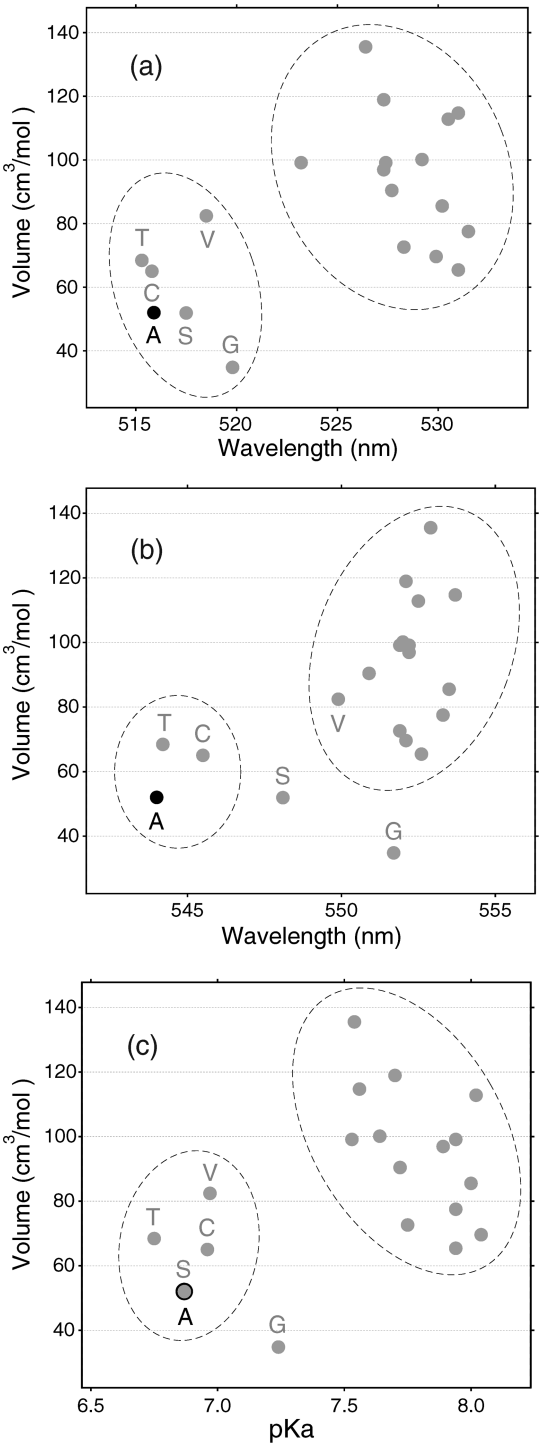

FIGURE 8: Correlation between the λ_{max} values of the species with deprotonated (a) and protonated (b) Asp97 and the side chain volume of the amino acid at position 178. The λ_{max} values of the deprotonated (a) and protonated (b) forms are obtained from the values at pH 10 and 4, respectively, in the fitting curve of the Henderson−Hasselbalch equation in Figure 6 (see the values of curve fitting in Table 1). (c) Correlation between the pK_a of Asp97 and the side chain volume of the amino acid at position 178. The pK_a of Asp97 is obtained from the Henderson−Hasselbalch equation in Figure 6 (see the pK_a values in Table 1).

lead to an improved understanding of the long-range mutation effect.

Then, one may question whether Ala is conserved in PR. If Ala178 is important for color tuning of PR, Ala should be highly conserved among various PRs. Figure 9 indeed shows that Ala is

(a)

	176	177	178	179	180	ref.
Green PR	N	T	A	S	P	14
BAC EBAC40E8	N	T	A	S	P	15
RED27	N	T	A	S	P	16
medA15r11b9	N	T	A	S	P	29
medA15r10b5	T	T	A	S	P	29
PR_108_08_14	A	S	A	N	A	30
PR_707_07_18	A	S	G	N	A	30
PR_707_07_09	S	L	A	N	P	30
PR_108_08_24	G	L	S	N	D	30
PR_108_08_18	G	L	A	N	P	30
PR_707_07_04	N	E	A	P	E	30
PR_707_07_12	E	Q	A	P	E	30
PR_707_07_08	E	K	A	P	A	30
PR_108_08_13	Q	E	A	P	A	30
PR_707_07_24	E	K	A	P	P	30
PR_108_08_16	Q	S	A	P	P	30
PR_108_08_22	N	S	A	P	P	30
PR_108_08_17	K	S	G	N	K	30
PR_707_07_02	E	K	A	S	P	30

(b)

	176	177	178	179	180	ref.
Blue PR	S	T	A	S	P	15
MEDPR51	G	S	G	N	A	31
MEDPR55G04	S	S	G	N	A	31
MEDPR42E12	N	S	G	N	A	31
BAC13K9BAC	S	C	G	S	V	31
AtlaNA39r14_9	S	S	G	S	A	*
REDPR17H08	E	S	A	P	E	31
MEDPR45	K	S	G	N	K	31
PR_108_08_04	S	Q	G	T	E	30
MEDPR46D6	S	D	G	T	P	31
PalE1	S	T	A	S	P	15

FIGURE 9: Amino acid sequences at positions 176−180 in green PR (a) and blue PR (b), which are obtained from refs *14*, *15*, and *30*−*32*. An asterisk represents an unpublished report by Sabehi et al., which appears in GenBank (entry 71743405).

mostly conserved in green PR, where Ala is replaced with Gly or Ser in some exceptional cases (*14*, *15*, *30*−*32*). This study shows that Gly and Ser behave much like the wild type, particularly for the form with deprotonated Asp97 (Figure 8a), which possesses proton pumping activity (*33*). A previous study reported the importance of position 179, where the observed color change was fully effective at position 178 and half-effective at position 179, but not effective at positions 176, 177, and 180 (*17*). Figure 9 shows that the amino acids at position 179 are Ser, Asn, and Pro. Thus, these amino acids contribute to the specific color tuning of green PR by forming a unique secondary structure in the E−F loop region. It should be noted that the red-shifted A178R mutant has similar proton pumping activity at pH 8.2 (*17*), implying a significant effect on color and the pK_a of the Schiff base counterion, but not on the function. Figure 9 also shows that three and eight of the blue PRs possess Ala and Gly at position 178, respectively. They have Ser, Asn, Pro, or Thr at position 179. These facts suggest that blue PR has the similar color tuning mechanism, whereas the mechanism would be somehow modified because there are more Gly residues at position 178.

In conclusion, this comprehensive mutation study revealed that Ala178 is important for absorption of the prevalent ambient light by green PR. The mechanism of color tuning is fully preserved only in the case of the replacement with Cys or Thr. As marine environments are dominated by 480−520 nm light, its absorption is important for the proton pump function. In parallel, PR has the pK_a of the Schiff base counterion at neutral pH, where only the alkaline form can pump protons in the outward direction (*33*). Thus, absorption of the prevalent ambient

light and the low pK_a of the counterion are crucial for the function of PR. This study demonstrates that Ala178 contributes to the proper wavelength absorption and lowering of the pK_a, even though its position is distant from the chromophore binding domain.

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