Chromophores

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Color Change of Proteorhodopsin by a Single Amino Acid Replacement at a Distant Cytoplasmic Loop**

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Visual and archaeal-type rhodopsins contain either 11-*cis*- or all-*trans*-retinal, respectively, inside the seven transmembrane helices.[1] The retinal chromophore is bound to a lysine residue of the seventh helix through 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 the 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 the protein, such as electrostatic effects of charged groups, dipolar amino acids, and 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), an archaeal-type rhodopsin functioning as a light-driven proton pump.[8] In the case of BR, the color changes from purple ($\lambda_{max} \approx 560$ nm) to blue ($\lambda_{max} \approx 600$ nm) for the D85N mutant.[9] The reason for the spectral red shift is that the negatively charged counterion (Asp85; Figure 1) is neutralized. By contrast, the color changes from purple ($\lambda_{max} \approx 560$ nm) to reddish ($\lambda_{max} \approx 530$ nm) for the L93A and L93T mutants, wherein a specific chromophore–protein interaction is modified (Figure 1).[10] Thus, electrostatic and steric effects at Asp85 and Leu93, respectively, contribute significantly to the color tuning in BR. It should be noted that the reported color changes by mutation of BR were limited to the amino acids near the retinal chromophore.[8] In general, mutation of distant amino acids does not cause color change unless the mutation destabilizes the retinal-binding site. This is not only the case for BR but also for other archaeal-type and visual rhodopsins.[2,4] Therefore, it has been generally accepted that only the amino acids near the retinal chromophore are responsible for color tuning. This paper, in contrast, presents an unusual mutation effect on the color tuning of proteorhodopsin (PR), an archaeal-type rhodopsin, where the color was changed by a single amino acid replacement at the cytoplasmic E–F loop, which is distant from the retinal molecule.

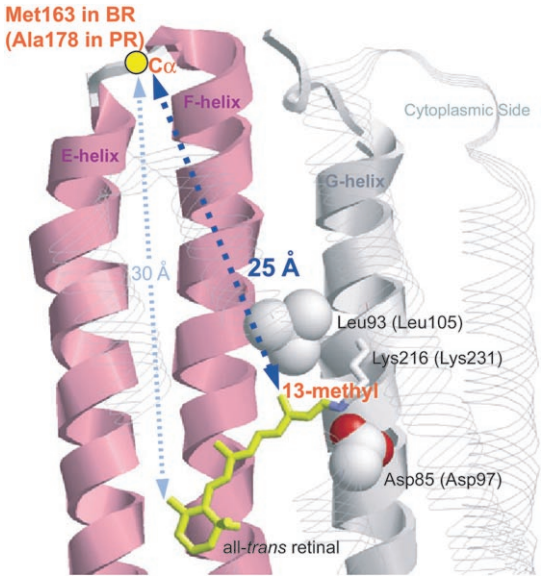

Figure 1. X-ray crystallographic structure of bacteriorhodopsin (BR) (Protein Data Bank entry: 1IW6[29]), which is viewed from the B–C helix side. The upper and lower regions correspond to the cytoplasmic (CP) and extracellular (EC) sides, respectively. The retinal chromophore, which is bound to Lys216, is colored yellow. Asp85 and Leu93 are shown by space-filling models. The Cα atom of Met163, which is located at the center of the E–F loop, is shown by a yellow circle. The nearest atom to this in the retinal chromophore is the 13-methyl carbon atom; the distance between them is 25 Å. The corresponding amino acid in green-absorbing proteorhodopsin (PR) is alanine, and we replaced Ala178 with arginine (A178R mutant) in the present study.

PR is a light-driven proton pump found in marine γ-proteobacteria.[11] PR may contribute significantly to the global solar-energy input into the biosphere because of the widespread distribution of proteobacteria in the worldwide oceanic waters. Extensive genome analysis revealed the presence of thousands of PRs, which can be classified into blue-absorbing ($\lambda_{max} \approx 490$ nm) and green-absorbing ($\lambda_{max} \approx 525$ nm) PRs.[12] Previous studies have shown that one of the determinants of color tuning in PR is at position 105, where blue- and green-absorbing PRs possess Gln and Leu, respectively.[13] The corresponding amino acid in BR is Leu93, which is in direct contact with the retinal chromophore (Figure 1). Although the structure of PR has not been determined, the Leu/Gln switch for color tuning is presumably attributable to direct contact with the retinal chromophore.

In this work, we prepared two mutants based on a triple cysteine mutant (TCM) of green-absorbing PR. It is known

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that the wild-type PR is unstable in vitro because of oxidation of one or more of the cysteine residues, which can be avoided by mutating all three cysteines (Cys107, Cys156, and Cys175) into serines.[14] In this study, we regarded this protein (TCM: C107S/C156S/C175S) as the wild-type PR. We confirmed the identical λ_{max} values between them, as described previously.[15] We then replaced two alanines at the center of the C–D (Ala115) and E–F (Ala178) loops with arginines. The corresponding amino acids in BR are Ala103 and Met163 (Figure 1), respectively. We originally aimed to investigate the influence of a positive charge in the loops on the proton-pumping dynamics, whereas we found an unexpected color change. Figure 2 shows that the absorption spectrum of

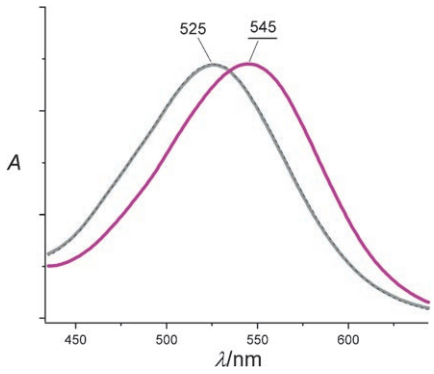

Figure 2. Absorption spectra of the wild-type (dotted line), A115R mutant (gray solid line), and A178R mutant (pink solid line) PRs at pH 7.0. The λ_{max} value is red shifted by 20 nm for A178R.

A115R is identical to that of the wild type, with the λ_{max} value at 525 nm. This is reasonable, because Ala115 must be located very distant from the retinal chromophore. Nevertheless, we observed a clear spectral red shift for A178R, with the λ_{max} value being shifted by 20 nm at pH 7.0 (Figure 2). This observation was unexpected because Ala178 should be also distant from the retinal chromophore. Figure 1 shows that the atom of the retinal chromophore nearest to the Cα atom of Met163 in BR is the 13-methyl carbon atom, which is at a distance of 25 Å.

It is, however, noted that the counterion of the Schiff base in PR is Asp97, the pK_a value of which is about 7.[16,17] This indicates that both protonated and deprotonated forms exist at a neutral pH value. Therefore, we next measured a pH titration with the wild-type and A178R mutant proteins. Figure 3a compares the λ_{max} values of the wild-type and A178R mutant PRs at various pH values; from these results, the pK_a values were determined to be 7.2 and 8.2, respectively. The pK_a value of the wild type is consistent with a previous report.[18] Figure 3b and c shows that the λ_{max} value of the A178R mutant is red shifted from that of the wild type by 7 and 10 nm for the protonated and deprotonated forms, respectively. Together with the increase of 1.0 in the pK_a value (Figure 3a), these spectral shifts yield the observed 20 nm red shift at the neutral pH value (Figure 2).

The question then arises of why the A178R mutation caused a color change. According to HPLC analysis, the chromophore structure of both the wild-type and A178R

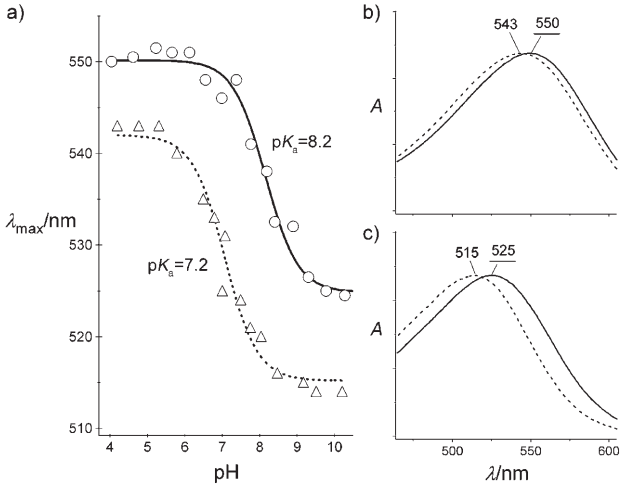

Figure 3. a) pH Titration of the wild-type (△ and •••••) and A178R mutant (○ and ——) PRs. b) Absorption spectra of the wild-type (•••••) and A178R mutant (——) PRs at pH 4.0 (with Asp97 protonated). c) Absorption spectra of the wild-type (•••••) and A178R mutant (——) PRs at pH 10.0 (with Asp97 deprotonated).

mutant PRs was predominantly all *trans* (>90%) at both pH 4 and 10 (data not shown); this excludes the possibility of different isomeric content as the origin of the color change. One concern is that the mutation destabilizes the protein structure so that the weakened chromophore–protein interaction results in the observed spectral change. Such a mutation effect often happens, and we have to be careful with making conclusions from the mutation study. We then tested the protein stability by keeping the sample at 75 °C for 5 min (pH 7.0), during which time some portions thermally decompose. Figure S1 in the Supporting Information compares the thermal stability, and a similar time dependence in this experiment for the wild-type and A178R proteins strongly suggests that the protein stability is not reduced by the A178R mutation.

The present study with green-absorbing PR demonstrated that the λ_{max} value of A178R is shifted by 20 nm at pH 7.0 (Figure 2). Such a spectral red shift is caused by 1) the red shifts of both the protonated and deprotonated forms by 7–10 nm and 2) the increase in the pK_a value by 1.0. The color originates from the energy gap of the π–π* transition between electronically excited and ground states. The positive charge originally located at the Schiff base is more delocalized in the excited state, and more or less charge delocalization results in a smaller or larger energy gap, respectively. As already described, various interactions between the retinal chromophore and the protein contribute to the color tuning of rhodopsins,[1–7] but the experimentally observed effects have been mostly local.[8] This is the first time that such a long-range effect has been clearly observed for rhodopsins.

The molecular mechanism of the distant color tuning is then in question. It must be noted that the single amino acid replacement (Ala178 to Arg) was introduced into TCM, not the native PR, where Ser175 from the cysteine mutations is close to the mutated position. However, the identical λ_{max} values between TCM and the native PR[15] suggest no

influence of the TCM mutation on the color tuning. The present color change presumably originates from a specific interaction between the retinal chromophore and Arg178 in the E–F loop, because there is no spectral change for A115R PR (Figure 2). The presence of a specific-interaction channel between the E–F loop and the retinal is also suggested by the increased pK_a value. The pK_a value is obtained for the protonation state of Asp97, which works as the Schiff base counterion.[16,17] Therefore, it is likely that the A178R mutation somehow alters the hydrogen-bonding network in the retinal Schiff base region and this leads to a pK_a increase of 1.0.

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, although the λ_{max} values of both the dark- and light-adapted states were slightly different from those of the wild-type BR.[19] The position of Arg164 is next to Met163, the amino acid in BR that corresponds to Ala178 in PR (Figure 1). Various mutations of cysteine into loops in BR and their spin labelings have been extensively studied, although there are no reports of color change.[20,21] Thus, the present observation might be specific for PR, not for BR. The M163R mutation of BR will directly answer the question of such specificity, which is one of our future aims.

The nature of the suggested long-range-interaction channel in PR is interesting. An explanation of the observed color change could, however, be limited on the structural basis because there is no X-ray crystallographic structure of PR. The structure of BR shows the presence of hydrophobic bulky groups, such as Leu93, Asp96, Leu97, and Leu100 of the C helix and Val167, Phe171, Leu174, Thr178, and Trp182 of the F helix, in the cytoplasmic domain, while 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 (see Figure S2 in the Supporting Information). The presence of similar bulky groups in PR suggests no direct hydrogen-bonding network between the retinal chromophore and Arg178 in the E–F loop. Instead, it is possible that the A178R mutation causes a rearrangement of these helices, which leads to the observed changes of color and pK_a value. Interestingly, it is well established that an opening motion of the E–F loop takes place during the proton-pump cycle of BR and this helps proton uptake from the cytoplasmic aqueous side.[22] Such a motion is also common for sensor rhodopsins, such as bovine rhodopsin[23] and microbial sensory rhodopsin.[24] This motion probably also takes place in PR for proton uptake. There may be a correlation between the present finding and the loop motion during the functional processes. A comprehensive mutation study in the future will reveal the color-tuning mechanism and the correlation with the function of the protein.

Experimental Section

The expression plasmids were constructed as described previously.[25] To avoid oxidation of one or more of the cysteine residues, a triple mutant was constructed in which all three cysteines (Cys107, Cys156, and Cys175) were replaced with serines as a starting template.[15] This protein was regarded as the wild type. An additional mutation was introduced at positions 115 and 178. For preparation of the expression plasmids of the mutants, a Quickchange site-directed mutagenesis kit (Stratagene) was used according to the stantdard protocol.[25] The PR proteins that carry 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.[26] Absorption spectra were measured for the solubilized PRs (0.1% DM, 100 mM NaCl) at 20°C by use of a Shimadzu UV-2400PC UV/Vis spectrometer. HPLC analysis was performed as described previously.[27] Protein stability was measured as described previously.[28]

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