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V108M Mutant of *pharaonis* Phoborhodopsin: Substitution Caused No Absorption Change but Affected Its M-State[1]

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Crystallographic data reveal that Met-118 in bacteriorhodopsin (bR) contacts directly with the C_9 methyl group of retinal, and Khorana *et al.* [*J. Biol. Chem.* 268, 20305-20311 (1993)] suggest that this contact may regulate the absorption maximum (λ_{max}). We have replaced the amino acid (Val-108) corresponding to Met-118 of bR by methionine in *pharaonis* phoborhodopsin (*p*pR), whose λ_{max} is *ca.* 500 nm, while those of other bacterial rhodopsins such as bR, halorhodopsin, and sensory rhodopsin are red-shifted by 60-90 nm. By flash-photolysis measurement, we could not recognize a large spectral red-shift of the V108M mutant. On the other hand, the decay of ppR_M (M-intermediate) of the mutant was approximately three times as fast as that of wild-type, and an M-like intermediate (M′) whose λ_{max} is blue-shifted by 60 nm from that of M became appreciable. The replacement abolished the shoulder of the ppR_M spectrum. From these findings, we infer that the distance between the retinal and the 108-position in *p*pR is relatively long, and that in the M-state this distance is shortened.

Key words: absorption maximum, bacterial rhodopsins, *pharaonis* phoborhodopsin (*p*pR), *pharaonis* sensory rhodopsin II (*p*sRII), photocycle.

Bacterial rhodopsins expressed on halobacterial membranes are retinal proteins that are classified into 4 types: bacteriorhodopsin (bR) (*1, 2*), halorhodopsin (hR) (*3, 4*), sensory rhodopsin (sR or sensory rhodopsin I, sRI) (*5*), and phoborhodopsin (pR or sensory rhodopsin II, sRII) (*6-10*). bR works as a light-driven H^+ pump, and hR as a light-driven Cl^- pump. sR is a photoreceptor of positive and negative phototaxis of *Halobacterium salinarum*: its ground state, whose λ_{max} is 587 nm, is a receptor for positive taxis; and a long-lived photointermediate, S_{373}, is a receptor for negative taxis (*12*). *H. salinarum*, however, shows negative phototaxis below 520 nm which cannot be covered by S_{373} (*6, 11*). This negative phototaxis is attributed to pR, whose λ_{max} is 490 nm (*6*).

We purified a pR-like pigment from a haloalkaliphilic bacterium, *Natronobacterium pharaonis*, and studied its photochemical properties and retinal composition (*13-17*). This pigment was named *pharaonis* phoborhodopsin (*p*pR). pR and *p*pR are distinctly different from other bacterial rhodopsins: λ_{max} is about 500 nm, while those of others are 560-590 nm. λ_{max} is thought to be determined by the structure of the retinal binding site (retinal pocket), and there is high similarity in the secondary structure among these four types of retinal proteins. Why do pR and *p*pR differ from the others in λ_{max}?

Khorana and coworkers made a variety of bR mutants (*18, 19*). A Met-118 mutant showed a striking blue-shift of ~80 nm in its λ_{max}, which became similar to that of *p*pR (*19*). This residue was considered to be close to the β-ionone ring (*20*). [Analysis of recent structural data, however, reveals that the methyl group of the methionine interacts with the C_9 methyl of retinal but not with the β-ionone ring. See later.] A primary structure of *p*pR (*p*sRII) was determined by Seidel *et al.* (*21*). Alignment of putative amino acid sequences of 23 bacterial rhodopsins whose genes have been reported so far (see Fig. 1) reveals that pigment proteins with λ_{max} of ~570 nm have methionine at the position corresponding to Met-118 of bR, while the corresponding residue of *p*pR is a smaller amino acid, valine. This fact strongly suggests the size of this hydrophobic residue is a crucial factor in color regulation.

Recently we succeeded in expressing *p*pR functionally on *Escherichia coli* membrane (*22*). Using this expression system, we investigated whether this residue controls the color of *p*pR.

EXPERIENTIAL PROCEDURES

Bacterial Strains—*E. coli* JM109 was used as host for DNA manipulation, and BL21(DE3) was used for expressing the genes. Cells were grown in 2×YT medium supplemented with ampicillin (final concentration of 50 μg/ml).

Preparation of V108M Mutant—The gene for the single

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Abbreviations: bR, bacteriorhodopsin; CHES, 2-(cyclohexylamino)-ethanesulfonic acid; IPTG, isopropyl-1-thio-β-galactoside; *p*pR, *pharaonis* phoborhodopsin; λ_{max}, absorption maximum.

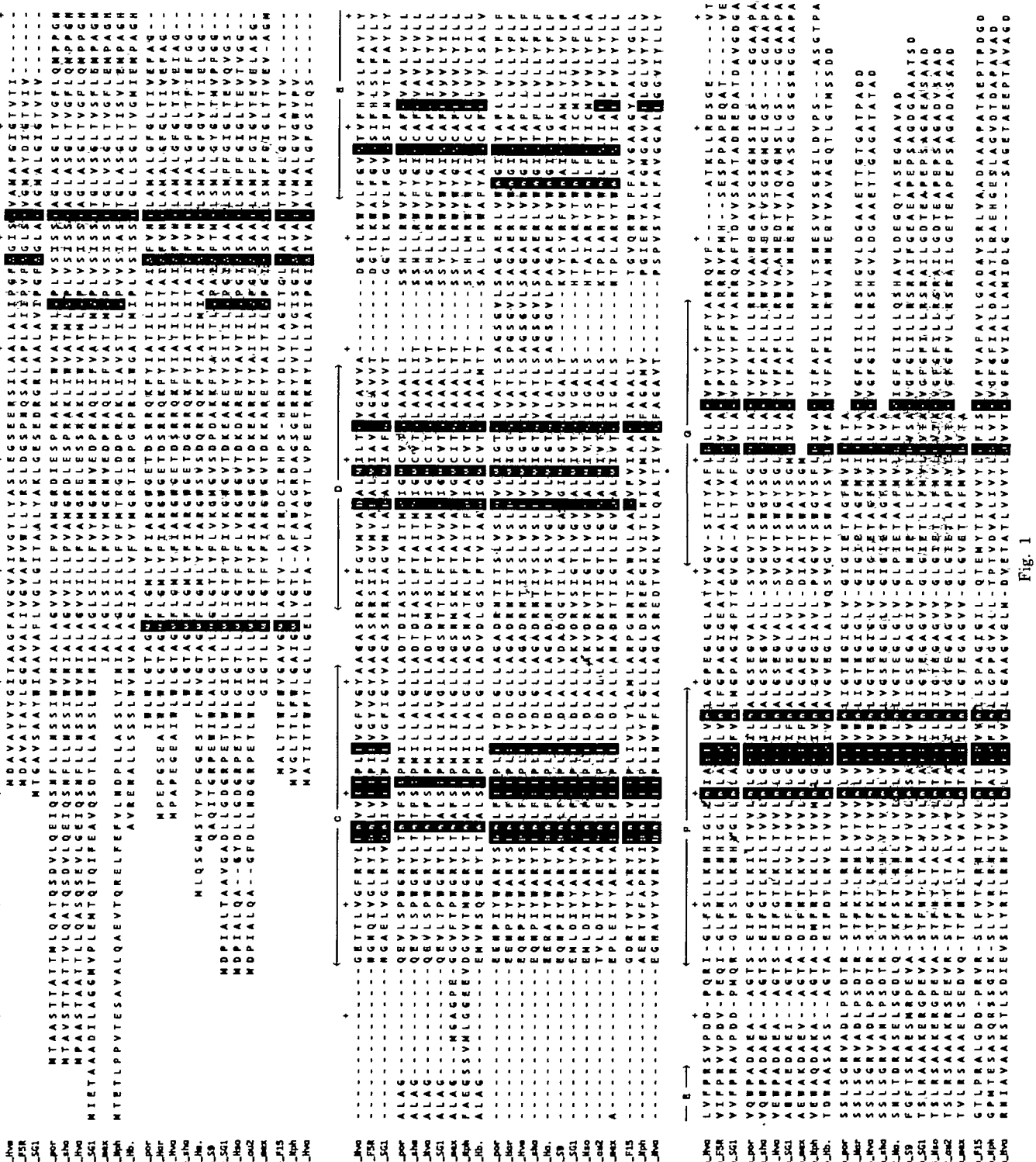

Fig. 1

Fig. 1. **Alignment of putative amino acid sequences of 23 bacterial rhodopsins reported so far.** Protein sequence comparisons were carried out by using the Pileup program of University of Wisconsin Genetics Computer Group (GCG) packages. The amino acid residues with maximum identical numbers in each position are masked by gray or black boxes. Black are those constituting the retinal binding pocket. The amino acid box marked by asterisk (*) in D helix is the attention site in this study. It is worth noting that methionine corresponding to the 118th amino acid of bR is conserved in all except pR, *p*pR and *v*pR. Some are partial sequences because full sequences have not been deposited. Names of bacterial rhodopsins and sources from top are: cso-Hva, *Haloarcula vallismortis* cruxsensory rhodopsin (GenBank™ D83748); sop-F5R, *Halobacterium salinarum* strain Flx5R sR (L05603); sop-SG1, *Hb.* sp. strain SG1 sR (X70290); hop-por, port hR (D43766); hop-sha, shark hR (D43765); ch3-Hva, *Ha. v.* cruxhalorhodopsin-3 (D31881); hop-SG1, SG1 hR (X70292); hop-mex, mex hR (D11136); hop-Nph, *Natronobacterium pharaonis* hR (J05199); hop-Hb., *Hb.* sp. hR (x04777); bop-por, port bR (D11057); co1-Har, *Ha. argentinensis* cruxrhodopsin-2 (S76743); bop-S9, S9 bR (J01727); bop-SG1, SG1 bR (X70291); ao3-Hso, *Halorubrum sodomense* archaerhodopsin-3 (D50848); ao2-au2, *Hb.* sp. strain aus-2 archaerhodopsin-2 (S56354); bop-mex, mex bR (D11065); pop-F15, Flx15 pR (U62676); pop-Nph, *N. p.* pR (J05199); pop-Hva, *Ha. v.* pR (Z35308).

amino acid replacement was prepared by PCR. For PCR, two pairs of sense and antisense oligonucleotide primers were designed based on the nucleotide sequence in the GenBank™ data base (accession no. Z35086); psp (5′-CATATGGTGGGACTTACGACC) and pap108M (5′-GCTAGCATCATCACGGTGTTG) and psp108 (5′-TCATGCTAGCCGGCTTCGCC) and pap (5′-AGAATAACGACGGACGTTCG). Underlined are the added restriction sites *Nde*I and *Nhe*I. The plasmid containing full-length *psopII* (opsin of *pharaonis* sensory rodopsin II, *pharaonis* phoborhodopsin) was used as template for PCR (*22*). Depending on the primer pair used, two PCR products were obtained, purified, and subcloned into the plasmid vector pGEM-T Easy (Promega). The *Nhe*I-*Sac*II fragment from the latter plasmid was ligated into the *Nhe*I and *Sac*II sites of the former plasmid. The *Nde*I-*Not*I fragment from this plasmid was ligated into the *Nde*I and *Not*I sites of pET21c (Novagen). DNA Sequencing was carried out using a kit (Applied Biosystems). The PCR products were analyzed using an automated sequencer (377 DNA sequencer, Applied Biosystems).

The wild-type and mutant *p*pR were expressed in *E. coli* BL21(DE3) by induction with 1 mM IPTG and 10 μM all-*trans* retinal. The preparation of crude membranes has been described previously (*22*).

Flash Spectroscopy—Crude membranes were resuspended in a medium composed of 4.0 M NaCl and 0.5% octylglucoside with a buffer (20 mM citrate for pH 5.0, 50 mM CHES for pH 9.0). After centrifugation (15,000 × *g*, 120 min at 4°C) to remove insoluble components, the supernatant was used for the measurements. The apparatus and procedure were essentially the same as in Ref. *15*. Short flashes were provided by a Xe-flash lamp (duration 200 μs) in combination with a 540 nm cut-off filter (Toshiba). For a single time scan, 500 data points were amplified 10 times, and stored in a microcomputer (PC-386M, Seiko EPSON) and 10 or 50 flash data were averaged for each wavelength.

RESULTS AND DISCUSSION

Figure 2 shows typical flash-induced difference spectra of V108M (right) and wild *p*pR (left) at pH 5.0 at 20°C. The negative band in these spectra originates from the depletion of the original *p*pR, and the absorption maximum of V108M mutant is estimated approximately to be 500 nm. Furthermore, this spectrum has a pronounced shoulder at 470 nm. These features are the same as those of the wild-type. Contrary to our expectation, therefore, the replacement of valine with methionine did not significantly change the absorption spectrum of the ground state of *p*pR.

The retinal proteins all have a cyclic reaction that is excited by light of specific wavelength, and this reaction is termed a photocycle. The *p*pR photocycle in the ms time range is $ppR \rightarrow ppR_M \rightarrow ppR_O \rightarrow ppR$ (*13, 15*). An M-intermediate, ppR_M is detected as the first product among photointermediates at room temperature in the ms time range. The absorption maxima of ppR_M and ppR_O are 390 and 560 nm (*15*), respectively. Careful examination of the *p*pRM-spectra (*i.e.*, comparison of curves 1 of Fig. 2) revealed the abolition or reduction of a shoulder in the mutant ppR_M spectrum.

The shift of maximum wavelengths and the time-dependent spectrum deformation of the negative bands as seen in Fig. 2 may have been due to the time-dependent absorption changes of ppR_M or ppR_O. It is reported that the decay rate of ppR_M (formation rate of ppR_O) becomes smaller under alkaline conditions, while the decay rate of ppR_O is almost independent of pH (*15*). This implies that under alkaline conditions, we can scarcely observe ppR_O. As seen in Fig. 3, which shows the difference spectra under alkaline conditions, this is the case for both wild-type and V108M. The time-dependent spectrum change in the negative bands is not observed, and an isosbestic point (*ca.* 430 nm) appears approximately on the base line. Since there is no absorption of wild-type ppR_M at *ca.* 500 nm (*15*), the wavelength at the peak of the negative band is considered to be λ_{max} of wild-type or V108M. Figure 3 shows clearly no changes in λ_{max} between wild-type and the mutant, which is contrary to our expectation described in "Introduction."

In addition, the shoulder of ppR_M of the mutant obviously disappeared, as was the case under acidic conditions (Fig. 2). It seems likely that the "isosbestic point" (since it is not an exact isosbestic point, we refer to it in this way) of the wild-type was red-shifted from that of V108M by *ca.* 20 nm. The exact reason is not known, but the lack of a shoulder may cause this shift, since there seems to be no absorption of ppR_O around 440 nm (*15*).

Figure 4 shows the flash-induced difference spectra at wavelengths below 350 nm, which corresponds to the spectrum edges of ppR_M. The ordinate values represent the flash-induced absorption changes relative to the change at 500 nm (λ_{max} of *p*pR or V108M) observed immediately after the flash. Interestingly, a small positive deflection (band) of the wild-type was seen whose maximum seemed to be at 335 nm. This near-UV positive band was also observed in a purified *p*pR sample from *N. pharaonis* (data not shown). This band may be caused by a new type of M-intermediate, which we call $ppR_{M'}$. The decay rate of $ppR_{M'}$ was 1.61 s^{-1}, while that of ppR_M is 5.97 s^{-1}: the decay of $ppR_{M'}$ is slower than that of ppR_M. What intermediate is

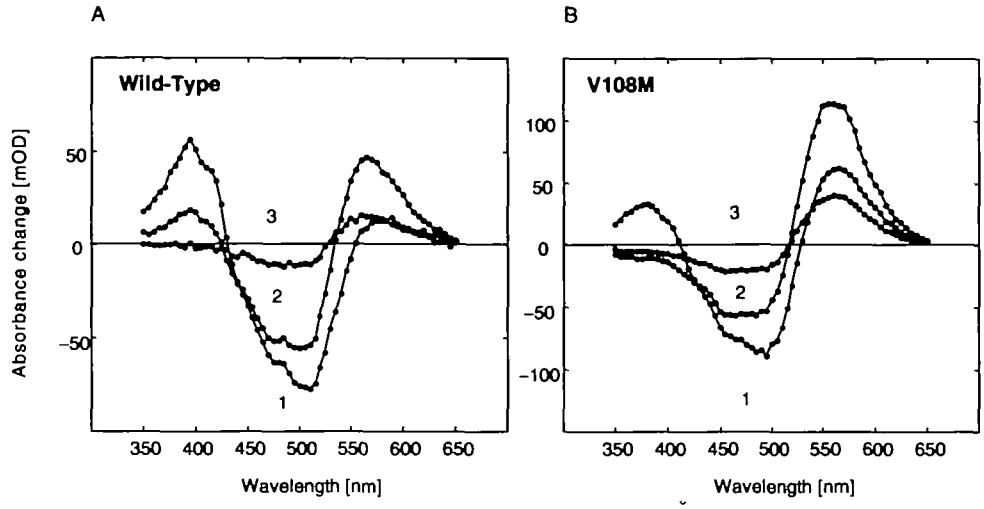

Fig. 2. **Flash-induced difference spectra of wild-type (A) and V108M mutant *p*pR (B) under acidic conditions.** Curve 1, spectrum at 30 ms after the flash; curve 2, 200 ms; curve 3, 1,000 ms. Flashes were supplied through an interference filter (540±10 nm) in combination with a cut-off filter (>523 nm). The solution contained 4.0 M NaCl and pH was adjusted to 5.0 with 20 mM citrate. Temperature was 20°C.

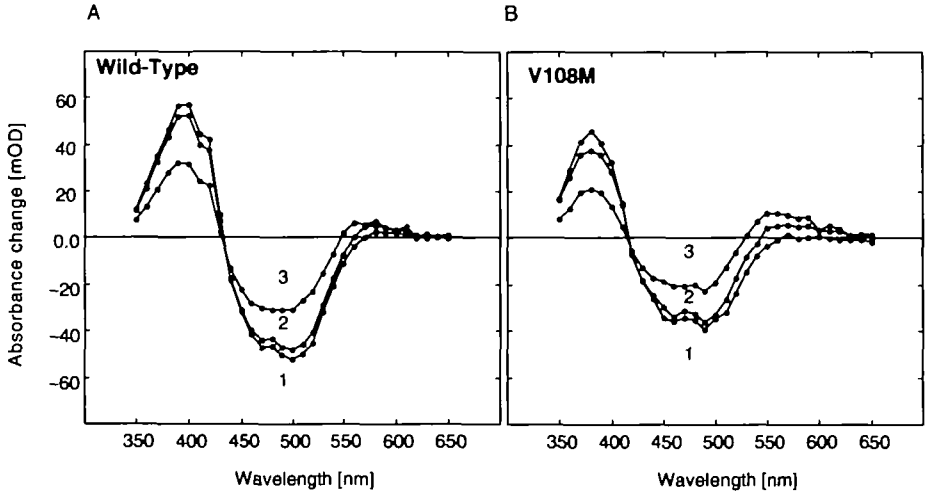

Fig. 3. **Flash-induced difference spectra of wild-type (A) and V108M mutant *p*pR (B) under alkaline conditions.** Curve 1, spectrum at 30 ms after the flash; curve 2, 200 ms; curve 3, 1,000 ms. The solution contained 4.0 M NaCl, and pH was adjusted to 9.0 with 50 mM CHES. Temperature was 20°C.

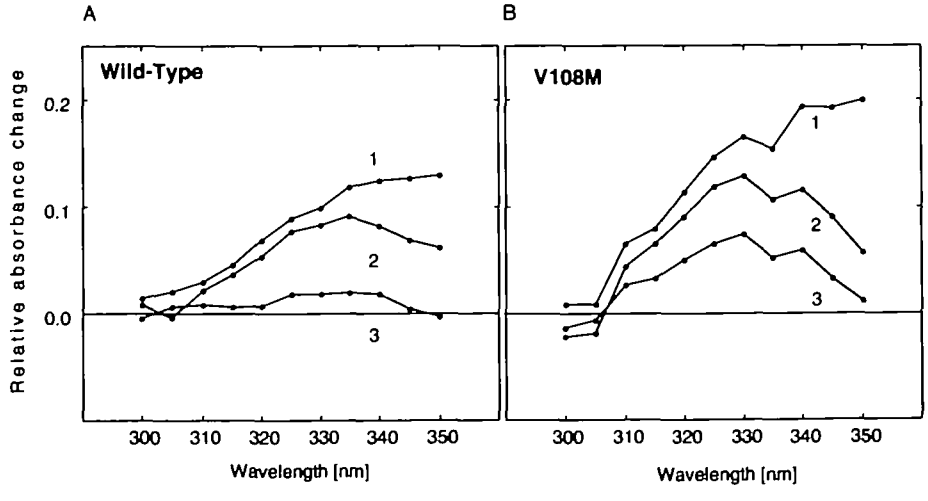

Fig. 4. **Flash-induced difference spectra below 350 nm of wild-type (A) and V108M mutant *p*pR, showing an increase in M′-intermediate of the mutant.** Curve 1, spectrum at 60 ms after the flash; curve 2, 200 ms; curve 3, 1,000 ms. The values of absorbance change are those relative to the depletion of the original *p*pR by the flash.

generated after the decay of ppR$_{M'}$ is not known at present.

Comparison of the near-UV bands between the wild-type and V108M revealed intensification of the mutant band. The absorbance change at 320 nm was clearly bi-phasic for V108M mutant (data not shown).

The decay rates of ppR$_M$ (the formation rate of ppR$_O$) were 5.97 s^{-1} at pH 5.0 and 0.58 s^{-1} at pH 9.0 for the wild-type; 21.6 s^{-1} at pH 5.0 and 0.58 s^{-1} at pH 9.0 for V108M. The decay rates of ppR$_O$ were 2.12 s^{-1} at pH 5.0 and 3.76 s^{-1} at pH 9.0 for the wild-type; 1.31 s^{-1} at pH 5.0

and 1.29 s^{-1} for V108M.

Our observations can be summarized as follows: (i) replacement of valine 108 with methionine did not alter the *p*pR spectrum; (ii) the shoulder of ppR_M disappeared; (iii) $ppR_{M'}$ became appreciable; (iv) the decay rate of ppR_M increased about threefold at pH 5, while no appreciable change was found at pH 9; (v) the decay rate of ppR_O became slower, but the ppR_O-spectrum showed no apparent change. Therefore, it is considered that this replacement changes the properties of the photointermediates, especially the M-intermediates (disappearance of the shoulder, increase in the decay rate of ppR_M, and increase in $ppR_{M'}$ content) without affecting the spectrum of *p*pR, implying that in the M state, the replacement of valine with a more bulky methionine increases the interaction between 108 position (located in helix D) and retinal. Note that λ_{max} of the M-intermediate is considered to be mainly determined by de-protonation of the Schiff-base. Since properties of earlier photointermediates than ppR_M cannot be investigated by our present apparatus, we do not know whether this increase in the interaction is peculiar to ppR_M or occurs before ppR_M.

According to Khorana and coworkers, Met-118, which was considered to locate very close to the β-ionone ring, was suggested to be important for color regulation in bR (*18, 19*). Alignment of putative amino acid sequences of 23 bacterial rhodopsins (Fig. 1) reveals that rhodopsins with λ_{max} of 560-590 nm have methionine at the position corresponding to Met-118 of bR. These facts might lead to the hypothesis that the distance between β-ionone ring and the nearest hydrophobic amino acid residue determines the color of the pigments. Recently, the three-dimensional structure of bR has been published (*20, 23, 24*). Analysis of it in the Protein Data Bank (entry code 2BRD) with RasMac Molecular Graphics program (Macintosh version 2.6; originally developed by Roger Sayle, enhancement by the Multichem. Facility, UCB) indicated that the distance between the methyl group of Met-118 and the C_9 methyl group of the retinal is 3.29 Å, and that of the C_5 methyl group of the retinal (β-ionon ring) is 4.12 Å. This means the direct interaction between the methyl group of Met-118 and the C_9 methyl group of the retinal: the van der Waals radii overlap each other. Therefore, Khorana's hypothesis should be amended as follows: the direct interaction or contact of the methyl group of Met-118 with the C_9 methyl group of the retinal (not with β-ionon ring) may shift λ_{max} to longer wavelength. If this hypothesis is adopted, the present data showing no change in λ_{max} of *p*pR might imply the distance between the methyl group of Met-108 in V108M *p*pR mutant and the C_9 methyl group of the retinal may be longer than that in bR. Since wild-type *p*pR has valine instead of bulky methionine, and since Met-118 of bR and Val-108 of *p*pR are present in helix D, the distance between helix D and the C_9 methyl group of the all-*trans* retinal may be longer than those of rhodopsins with λ_{max} of 560-590 nm.

Our finding that the replacement with the bulky amino acid affects the properties of the M-intermediates (ppR_M and $ppR_{M'}$) suggests the distance between helix D and 13-*cis* retinal becomes shorter in the M-state than in the ground-state of *p*pR. If it is indeed the case that the distance between helix D and retinal is longer in *p*pR than bR, this close distance at the M-state might be equivalent to the larger light-induced conformational change of *p*pR than bR, which may favor the signal transduction to *p*Htr-II.

We should keep in mind that the hypothesis described above may not be the only mechanism accounting for the color regulation. In addition, molecular insight into the decay time constants awaits further investigation. Further studies on other mutants may be necessary.

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