Mechanisms of Spectral Tuning in Blue Cone Visual Pigments

VISIBLE AND RAMAN SPECTROSCOPY OF BLUE-SHIFTED RHODOPSIN MUTANTS*

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Spectral tuning by visual pigments involves the modulation of the physical properties of the chromophore (11-cis-retinal) by amino acid side chains that compose the chromophore-binding pocket. We identified 12 amino acid residues in the human blue cone pigment that might induce the required green-to-blue opsin shift. The simultaneous substitution of nine of these sites in rhodopsin (M86L, G90S, A117G, E122L, A124T, W265Y, A292S, A295S, and A299C) shifted the absorption maximum from 500 to 438 nm, accounting for 2,830 cm⁻¹, or 80%, of the opsin shift between rhodopsin and the blue cone pigment. Raman spectroscopy of mutant pigments shows that the dielectric character and architecture of the chromophore-binding pocket are specifically altered. An increase in the number of dipolar side chains near the protonated Schiff base of retinal increases the ground-excited state energy gap via long range dipole-dipole Coulomb interaction. In addition, the W265Y substitution causes a decrease in solvent polarizability near the chromophore ring structure. Finally, two substitutions on transmembrane helix 3 (A117G and E122L) act in combination with the other substitutions to alter the binding-pocket structure, resulting in stronger interaction of the protonated Schiff base group with the surrounding dipolar groups and the counterion. Taken together, these results identify the amino acid side chains and the underlying physical mechanisms responsible for a majority of the opsin shift in blue visual pigments.

At the fundamental level, the ability of the visual system to discriminate wavelengths of electromagnetic radiation is determined by the spectral response of the light-absorbing pigments that reside in the photoreceptor cells of the retina. Trichromatic color vision in humans is mediated by three types of cone photoreceptors. Each type of cone absorbs maximally at different wavelengths: blue, green, and red. The human blue cone pigment all the way to 560 nm (21), but three specific substitutions of polar amino acids at TM helices 4 and 6 (A180S, A184P, and A188S) completely substitute the blue-sensitive pigments with red-sensitive pigments. The human green cone pigment is sensitive from C-12 near the center of the polyene (17, 18). Most of the opsin shift in rhodopsin could be explained by weaker electrostatic interaction resulting from greater separation between the PSB group and the counterion compared with that of retinal PSB in solution (19).

The mechanism of spectral tuning between green- and red-sensitive pigments has also been addressed. The human green and red cone sequences share 96% sequence identity and differ at only 15 positions (20). The replacement of seven of these residues in the green cone sequence by those in the red cone sequence is necessary to shift the λ_max of the hybrid green pigment all the way to 560 nm (21), but three specific substitutions of polar amino acids at TM helices 4 and 6 (A180S, A184P, and A188S) completely substitute the blue-sensitive pigments with red-sensitive pigments. The human green cone pigment is sensitive from C-12 near the center of the polyene (17, 18). Most of the opsin shift in rhodopsin could be explained by weaker electrostatic interaction resulting from greater separation between the PSB group and the counterion compared with that of retinal PSB in solution (19).

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The abbreviations used are: TM, transmembrane; PSB, protonated Schiff base; meta, metarhodopsin.
Spectral Tuning in Blue-sensitive Visual Pigments

F277Y, and A285T) account for the majority of this ~1000-cm$^{-1}$ opsin shift (22, 23). The substitution of these three polar residues at analogous positions in bovine rhodopsin (A164S, F261Y, and A269T) also shifts its $\lambda_{\text{max}}$ to the red by ~700 cm$^{-1}$ (24). Recent Raman spectroscopy studies of the chromophore environments in the human green and red cone pigments have provided evidence that the primary origin of the opsin shift from 533 to 560 nm is the electrostatic interaction of the dipolar residues situated near the cyclohexyl ring with the change in dipole moment of the chromophore complex (25). The opsin shift from 500 to 533 nm (i.e. between rhodopsin and the green cone pigment) is thought to arise from the perturbation of PSB interactions, which is mediated by the binding of a chloride ion (25–27).

Although ample effort has been directed toward the issue of the green-red opsin shift, much less work has focused on the molecular mechanism of the larger opsin shift from 500 nm to the blue wavelengths. The gene for the human blue cone pigment has been cloned and sequenced (20), and recombinant blue cone pigment has been expressed transiently from a synthetic gene (3). However, no mutational studies have been reported on the blue cone pigment, presumably due to its low expression in mammalian cells. Measurements of its $\lambda_{\text{max}}$ range from ~420 to 435 nm (1–3, 21). Taking a median value of 425 nm, the opsin shift between rhodopsin and the human blue cone pigment amounts to approximately 3500 cm$^{-1}$. Several hypotheses have been proposed to explain the green-blue opsin shift, and candidate residues that may play a role in spectral tuning have been suggested. Comparative sequence analysis of blue- and green-sensitive pigments suggested that positively charged residues in the blue cone sequence, specifically arginine and histidine at positions equivalent to 107 and 108, respectively, in rhodopsin, may induce a blue shift by perturbing the PSB environment (28). Conservation of polar residues on TM helices 3 and 7 in the vicinity of the PSB also indicated that the blue shift might result from additional dipolar residues primarily on TM helices 3 and 7 in the vicinity of the PSB and its counterion (28). Conservation of polar residues respectively, in rhodopsin, may induce a blue shift by perturbing amino acids from the human blue cone sequence at selected positions.  

We report here a detailed mutagenesis study that addresses the mechanism of the green-to-blue opsin shift. We used bovine rhodopsin as a template to model the opsin shift by substituting amino acids from the human blue cone sequence at selected positions. These amino acids were identified from the alignment of bovine rhodopsin and the human, mouse, rat, and bovine blue cone sequences. A total of 12 positions in rhodopsin were evaluated. Substitutions at nine positions simultaneously (M86L, G90S, A117G, E122L, A124T, W265Y, A292S, A295S, and A299C) caused a $\lambda_{\text{max}}$ shift to ~438 nm and accounted for approximately 2830 cm$^{-1}$ (80%) of the opsin shift between the blue cone pigment and rhodopsin. Raman spectroscopy shows that the added dipolar groups by themselves modify the retinal electronic energies and induce a $\lambda_{\text{max}}$ shift by long range electrostatic effects on the change in dipole moment of the photoexcited chromophore. Furthermore, these substitutions in the presence of Gly$^{117}$, Leu$^{122}$, and Tyr$^{205}$ residues alter the architecture of the retinal-binding pocket and produce the observed ~60-nm blue shift.

Experimental Procedures

Preparation of Rhodopsin Mutants—Site-directed mutagenesis was performed as described previously (14, 33). Synthetic DNA duplexes containing the desired codon alterations were ligated to the synthetic rhodopsin gene in the PMT expression vector between the following pairs of restriction sites: BglII/HindIII for mutations in TM 2, NcoI/XhoI and Rsal/BspEI for mutations in TM 3, MluI/ApaI for mutations in TM 6, and ApaI/BspEI for mutations in TM 7. The altered genes were expressed in COS-1 cells following transient transfection using Lipo- 

fectAMINE or LipofectAMINE-Plus (Life Technologies, Inc.). COS cells expressing mutant apoprotein were harvested and incubated in the presence of 11-cis-retinal at 4 °C under dim red light. The pigment was solubilized in n-dodecyl-$\beta$-maltoside detergent (w/v %, 5% Tris-HCl, pH 6.8, 100 mM NaCl, 1 mM CaCl$_2$, 0.1 mM phenylmethylsulfonyl fluoride) and bound to the ID4 immunoaffinity resin (14). The resin was washed in buffer (5 mM sodium phosphate, pH 6, 5 mM NaCl, 0.02% (w/v) tridecyl maltoside, or 0.1% dodecyl maltoside) and eluted in wash buffer supplemented with the antigenic carboxyl-terminal peptide to the ID4 antibody (34). Aliquots of concentrated Tris-HCl and NaCl solutions were added to the eluted sample to bring their final concentrations to ~40 mM (pH 6.8) and 100 mM, respectively.

Spectroscopy—Absorption spectra (1-cm path length) were measured on a 19 Perkin-Elmer spectrophotometer as described previously (33). To bleach the pigment, the sample was illuminated for a minimum of 15 s with a 150-watt halogen light source. A photobleaching difference spectrum was obtained by subtracting the spectrum of the sample in the dark from the spectrum of the pre-bleached sample. Raman spectroscopy, ~3 µl of purified pigment (25–50 µM) in a glass capillary was excited with 795-nm light from a titanium:sapphire laser (Lexel 479) (25). The sample was cooled by blowing nitrogen gas passed through an isopropyl alcohol/CO$_2$(s) bath over the capillary. Scattered light at 90° from the excitation beam was detected by a CDD detector (Princeton Instruments, LN-1152) coupled to a double spectrograph (Spex 1401). Frequencies were calibrated using the Raman spectrum of cyclohexane and the emission spectrum of neon as standards. Reported frequencies are accurate to ±2 cm$^{-1}$, and the spectral resolution is 6 cm$^{-1}$.

Results

The amino acid sequences of rhodopsin and the human blue cone pigment are only approximately 46% identical (20, 28). Thus, compared with the case for the human green and red cone opsins, determining which amino acids may shift the $\lambda_{\text{max}}$ from the green to blue wavelengths is not straightforward. In order to minimize the number of sites to be studied by mutagenesis, three basic criteria were applied to select residues that were potentially functionally important. First, the amino acid side chains should be located near the retinal chromophore, which is predicted to lie approximately near the middle of the membrane bilayer at the level of Lys$^{296}$ (35, 36). Thus, residues that are positioned within one turn above and below Lys$^{296}$ on TM helices 2, 3, 6, and 7 in a secondary structural model (Fig. 1) were considered. Second, there should be an increase in the number of polar amino acids such as serine, threonine, or cysteine in the proximity of the Schiff base in the blue cone pigment. Data from previous Raman studies on visual pigments indicated that there was approximate positive correlation between the pigment $\lambda_{\text{max}}$ and increased electrostatic interaction of the retinal-PSB group with the surrounding protein environment (37). Specifically in the blue rod pigment (\(\lambda_{\text{max}}\) ~430 nm) from the toad Bufo marinus, it was shown that the Schiff base C=N stretching frequency of the 9-cis species of its chromophore was similar to the frequency measured for a model retinal-PSB molecule stabilized by a chloride counterion. This substituted ethanol (29). This indicated that the general characteristics of the protein environment around the PSB group may be modeled by the situation in methanol solution. These data further implied that the green-blue opsin shift may be achieved by (i) the presence of additional dipolar residues that solvate the PSB group, and (ii) stronger electrostatic interaction between the PSB group and its counterion and/or other polar groups. Therefore, this second criterion targeted nonpolar-to-dipolar changes of residues primarily on TM.
Mutations in TM Helices 2, 6, and 7—Seven sites on TM helices 2, 6, and 7 were selected after the initial screening: Met86, Val87, Gly90, Trp265, Ala292, Ala295, and Ala299. Amino acids at positions 90, 265, and 292 had been mutated in earlier studies (39–41), and these residues were found to be located sufficiently close to the chromophore to affect the $\lambda_{\text{max}}$ of the mutant pigment. Furthermore, the role of residues at positions 87, 90, 265, 295, and 299 in spectral tuning is suggested by the fact these amino acids are conserved in the human, mouse, rat, and bovine blue cone sequences (42). A glycine residue corresponding to position 83 in rhodopsin is also conserved in the blue cone sequences, but this site was not mutated because it had been shown in an earlier study that the D83G substitution does not alter the pigment $\lambda_{\text{max}}$ (43). The seven residues in rhodopsin were replaced by the respective residues from the human blue cone sequence (Fig. 1). The mutant opsins were expressed in COS cells, regenerated with 11-cis-retinal, and purified. The genotypes and the spectral properties of the mutant pigments are listed in Table I. A polar hydroxy group was inserted at positions 90, 292, and 295 by replacing the original residue by a serine. The $\lambda_{\text{max}}$ values of the G90S and A292S mutants were blue-shifted by $400 \text{ cm}^{-1}$, but the $\lambda_{\text{max}}$ of the A295S mutant was blue-shifted by only $200 \text{ cm}^{-1}$. The $\lambda_{\text{max}}$ values for the G90S and A292S mutants are consistent with earlier studies in which a glutamic acid at either site caused a blue shift of $20 \text{ nm}$ (39, 41). Val87 and Ala299 were replaced by cysteine whose sulfhydryl group has a smaller dipole moment compared with a hydroxy group. The V87C or A299C substitution resulted in a $80\text{-cm}^{-1}$ blue shift of the $\lambda_{\text{max}}$. The replacement of Met86 with a leucine is also estimated to induce a shift of $80 \text{ cm}^{-1}$. The $\lambda_{\text{max}}$ of the W265Y mutant was 485 nm, in agreement with earlier measurements (33, 40). This replacement induced the largest single opsin shift out of the initial set of substitutions examined.

The effects of multiple substitutions were examined by inserting two or more replacements within a single TM helix and constructing chimeras of these multiple-site mutants (Table I and II). Generally, the opsin shift due to multiple replacements within a TM helix was equal to the sum of the shifts of the single replacements. For example, the opsin shift of the A292S/A295S/A299C triple mutant was $660 (450 + 200 + 80) \text{ cm}^{-1}$. The $\lambda_{\text{max}}$ of chimera 2–7 containing the five mutations on TM helices 2 and 7 was 476 nm (Fig. 2), and its opsin shift of $1000 \text{ cm}^{-1}$ is close to the expected additive shift of 1190 cm$^{-1}$. Similarly, chimera 2–6–7 displayed a $\lambda_{\text{max}}$ of 460 nm, and the corresponding 1740-cm$^{-1}$ shift is essentially equal to the sum of the shifts observed in the single-site TM helix 2, 6, and 7.
Mutations in TM Helix 3—The alignment of the blue cone amino acid sequences shows conservation of two basic residues near the extracellular border of TM helix 3 (28, 42), and conservation of three residues (Gly, Leu, and Thr) in the TM portion. The corresponding residues in rhodopsin TM helix 3 are Thr<sup>117</sup> and Pro<sup>120</sup> near the extracellular border, and Ala<sup>122</sup>, Glu<sup>122</sup>, and Ala<sup>124</sup> in the TM portion. Since most of these residues are likely to be located near the chromophore, mutant rhodopsins with substitutions at these positions were constructed (Table I).

Individual replacement of Ala<sup>117</sup>, Glu<sup>122</sup>, and Ala<sup>124</sup> by Gly, Leu, and Thr, respectively, caused 3–5-nm blue shifts of the λ<sub>max</sub> corresponding to an opsin shift in the range of 120–200 cm<sup>−1</sup>. The λ<sub>max</sub> of the pigment with replacements at all three sites on TM helix 3 was at 489 nm, and the opsin shift in cm<sup>−1</sup> was nearly equal to the sum of the individual shifts.

Pro<sup>107</sup> and Thr<sup>108</sup> were replaced by Arg and His, respectively. The λ<sub>max</sub> of the double mutant at pH 6 is the same as that of native rhodopsin (Table I). It is blue-shifted by 4 and 2 nm at pH 4.5 and 8.5, respectively. The opsin shifts relative to the λ<sub>max</sub> values of native rhodopsin at the pH values tested were less than 100 cm<sup>−1</sup>. Furthermore, the mutant pigment containing all five substitutions on TM helix 3 (positions 107<sup>107</sup>, 108<sup>108</sup>, 117<sup>117</sup>, 122<sup>122</sup>, and 124<sup>124</sup>) showed the same λ<sub>max</sub> value as that of the TM helix 3 triple-site mutant (positions 117, 122, 124). Since Arg<sup>107</sup> is probably protonated, these data suggest, at least for the case of His<sup>108</sup>, that there is no change in its protonation state that influences the electrostatic environment of the chromophore.

Chimeras of the TM helix 3 triple-site mutant and the other TM helix mutants produced pigments with λ<sub>max</sub> values showing evidence of both negative and positive cooperative interactions between the substituted residues (Table II). The opsin shift of chimera 2–3–7 (λ<sub>max</sub> at 474 nm) is −540 cm<sup>−1</sup> less than the magnitude expected from the sum of the shifts of TM helix 2, 3, and 7 mutants. By contrast, the blue-rho pigment (chimera 2–3–6–7) displayed a blue-shifted λ<sub>max</sub> at −438 nm, and the resultant opsin shift was −570 cm<sup>−1</sup> greater than that expected from additive effects. The presence of the V87C substitution in TM helix 2 had no marked effect, and the λ<sub>max</sub> of chimera 2’–3–6–7 was still measured at −437 nm (Table II). The inclusion of the P107R/T108H mutation in TM helix 3 also had no effect, and this pigment (chimera 2–3’–6–7) had the same λ<sub>max</sub> as the blue-rho pigment. Subsequent biophysical studies were performed on the blue-rho pigment.

In order to determine the specific subset of substitutions responsible for this synergistic effect, the opsin shifts of various combinations of single-site replacements and TM helix mutants were examined (Table III). The combination of the W265Y mutation with the TM helix 3 mutations gave the expected additive shift, indicating that the positive cooperative effect requires one or more substitutions on TM helix 3 and all substitutions on TM helices 2, 6, and 7. The opsin shift data from the combination of single-site TM helix 3 mutants and chimera 2–6–7 show that the synergistic effect requires the E122L and A117G substitutions. In particular, a leucine at the 122 position alone combined with chimera 2–6–7 was expected to produce a pigment with a 455-nm λ<sub>max</sub> but instead its λ<sub>max</sub> was at −440 nm. The interaction between Leu<sup>122</sup> and residues on TM helices 2, 6, and 7 appears to account for most of the magnitude of the synergistic effect. The substitution of glycine at the 117 position elicits a comparatively smaller effect.

Regeneration of Blue-rho Opsin with Retinal Isomers—Wild-type opsin may be regenerated with 9-cis-retinal to produce an artificial pigment called isorhodopsin with a λ<sub>max</sub> of 485 nm (Fig. 3). In contrast, the opsin-binding pocket does not accommodate the all-trans isomer, and no pigment bound with an all-trans chromophore can be purified (data not shown). The ability of blue-rho opsin to regenerate with 9-cis- and all-trans-retinals was examined. Blue-rho opsin in COS cells was incubated with either 9-cis- or all-trans-retinal and subjected to the same purification procedure used to purify the pigment containing 11-cis-retinal. The 9-cis-retinal formed a covalent linkage with the blue-rho opsin to produce a 9-cis blue-rho pigment with a λ<sub>max</sub> at −425 nm. The −15-nm difference between the λ<sub>max</sub> values of the 9-cis and 11-cis blue-rho pigments is similar to that between isorhodopsin and rhodopsin. On the other
hand, no pigment was purified from the incubation of blue-rho opsin with all-trans-retinal. The absorption spectrum of the eluant contained a prominent 280-nm protein absorbance and a small band at 390 nm that is attributed to absorbance by residual unwashed free retinal. These data argue that no pigment with all-trans-retinal attached at lysine(s) including Lys296 may be recovered from our purification procedure. Moreover, since the purification is performed in aqueous buffer near neutral pH, a protonated Schiff base linkage is not expected to be stable unless it is protected from the bulk solvent as is the case with pigments.

Raman Spectroscopy—The vibrational Raman spectra of the retinal chromophore in the chimera 2-7 and blue-rho pigments were obtained using non-resonant excitation at 795 nm (Fig. 4). The fingerprint vibrational patterns of both spectra are consistent with the vibrational spectrum of an 11-cis-retinal PSB in a pigment. The C₆=C₅, C₁₂-C₁₃, C₁₄-C₁₅ stretching modes located at ~1216, 1237, and 1187 cm⁻¹, respectively, in the mutant pigments are basically at the same frequencies as found in the Raman spectrum of rhodopsin (44). Prominent C₁₁=C₁₂ A₂ hydrogen out-of-plane modes are detected at ~970 cm⁻¹ in both spectra. However, the 11H + 12H A₂ rocking modes in both pigments are downshifted by ~4 cm⁻¹ relative to the frequency in rhodopsin (1268 cm⁻¹). Also in the blue-rho spectrum, the C₁₄-C₁₅ stretch is apparently not detectable due to the lower signal-to-noise of the data, and the relative intensities of the fingerprint bands vary somewhat. The in-phase ethylenic stretching frequency of the chimera 2-7 pigment is shifted up 3–4 cm⁻¹ from the frequency in rhodopsin, and this mode is ~14 cm⁻¹ higher in the blue-rho pigment. The higher frequency is consistent with that expected from the empirical linear correlation between the absorption maximum and the ethylenic frequency of retinals and visual pigments (25, 45).

The C=N stretching frequencies of the chimera 2-7 pigment in H₂O and D₂O buffer are at 1657 and 1623 cm⁻¹, respectively, essentially unchanged from the frequencies of rhodopsin. By contrast, the C=N stretching mode of the blue-rho pigment is shifted up to 1658 and 1631 cm⁻¹ in H₂O and D₂O, respectively. This 27 cm⁻¹ D₂O shift is smaller than the ~33-
visual pigments. Residues at 12 positions in bovine rhodopsin were substituted with amino acids from the human blue cone sequences in order to evaluate their role in the opsin shift from 500 nm to bluer wavelengths. The amino acids at positions 86, 87, 90, 292, 295, and 299 on TM helices 2 and 7 are located in the vicinity of the PSB group and mainly differ in polarity. Additional conserved differences involving potential change in the size, charge, polarity, or polarizability of side chains constituting the binding pocket were identified at positions 107, 108, 117, 122, and 124 on TM helix 3 and at position 265 on TM helix 6. These sites were selected because the amino acid differences between the two pigments involved chemical changes that could alter the dielectric and electrostatic character of the chromophore-binding pocket.

We constructed a “blue-rho” mutant opsin that contained the following nine amino acid substitutions: M86L, G90S, A117G, E122L, A124T, W265Y, A292S, A295S, and A299C (Fig. 6). It bound 11-cis-retinal to yield a pigment with a λ<sub>max</sub> of 438 nm that could be purified under our standard purification procedure. Several lines of evidence support the view that this pigment is not a misfolded protein with a nonspecifically bound retinal. First, the widths of the Raman bands of this pigment are similar to those of rhodopsin and narrower than those observed in the spectrum of 11-cis-PSB molecule in solution. This feature is a characteristic of retinal chromophores in a defined homogeneous environment such as the inside of a protein. Second, the blue-rho opsin also regenerates with 9-cis-retinal to form a pigment with a λ<sub>max</sub> at 425 nm. On the other hand, no pigment nor a mixture containing unfolded opsin and retinal PSB was purified when the blue-rho opsin was incubated with all-trans-retinal. This regeneration behavior is identical to that of the isorhodopsin pigment.
of the wild-type opsin. Third, irradiation of the blue-rho pigment causes cis-trans isomerization of the chromophore and photolyzes the pigment based on the decrease of the UV absorbance between ~260 and 320 nm. However, the thermal decay of the protein is perturbed, and the nine mutations appear to inhibit the production of a meta-II-like species. This behavior is not atypical of some rhodopsin mutants containing single or double mutations that have been studied to date (33).

The nine substitutions in the blue-rho mutant shift the $\lambda_{\text{max}}$ of the pigment by ~2830 cm$^{-1}$ and account for ~80% of the total opsin shift between rhodopsin and the blue cone pigment. The $\lambda_{\text{max}}$ is unchanged by the addition of the P107R and T108H mutations (chimera 2–3’–6–7, Table II), indicating that these potential positively charged groups do not play a role, either directly or indirectly, in regulating the chromophore absorption. The hydroxy groups of Ser90, Ser292, and Ser295 individually blue shift the $\lambda_{\text{max}}$ by ~200–400 cm$^{-1}$. Smaller shifts in the range of 80–120 cm$^{-1}$ are induced by the sulfhydryl and hydroxy groups on Cys299, Cys292, and Thr124. A tyrosine residue in place of Trp265 alone induces a shift of ~620 cm$^{-1}$. The Met-to-Leu substitution at position 86 is estimated to cause a small 80-cm$^{-1}$ shift, whereas Gly117 and Leu122 individually elicit ~200-cm$^{-1}$ shifts. Interestingly, the blue shift induced by a leucine at position 122 is smaller than the shift caused by Ala or Glu substitution (40). The substitutions on TM helices 2, 6, and 7 collectively shift the $\lambda_{\text{max}}$ by ~1740 cm$^{-1}$, which is essentially equal to the sum of the individual shifts (1810 cm$^{-1}$). The inclusion of TM helix 3 substitutions (Gly117, Leu122, and Thr124) moves the $\lambda_{\text{max}}$ by ~2830 cm$^{-1}$, which is ~570 cm$^{-1}$ greater than the magnitude expected based on a simple sum. Additional mutational studies indicate that this synergistic or cooperative shift is a consequence of the presence of Gly117 and Leu122 on TM helix 3 and the substituted residues on TM helices 2, 6, and 7. Given the small shifts induced by Leu86, Cys117, Thr124, and Cys299, we infer that the majority of the opsin shift between rhodopsin and the blue-rho pigment, totaling 2600 to 2700 cm$^{-1}$, is caused by six substitutions as follows: G90S, A117G, E122L, W265Y, A292S, and A295S.

**Mechanism of Opsin Shift**—In order to understand the physical basis underlying $\lambda_{\text{max}}$ blue shift (i.e. the increase of $S_{\gamma}S_{\gamma}$ energy transition) induced by amino acid changes, we performed Raman spectroscopy on the mutant pigments. This technique provides chemical and structural information about the retinal chromophore and enables us to probe its interactions with the protein (46). The chromophores in both chimera 2–7 and blue-rho pigments display fingerprint modes with frequencies similar to those measured in rhodopsin. Thus, there appears to be no significant deviation of the ground-state electronic structure or geometry of the polyene backbone from that of the native 11-cis chromophore.

The comparison of the C=N stretching frequencies indicates interesting differences in the Schiff base environments among mutant pigments (Table IV). In the chimera 2–7 pigment, the C=N frequencies in H$_2$O and D$_2$O buffers are identical to those in rhodopsin. The frequency of the uncoupled C=N stretch (i.e. with deuterated nitrogen) is sensitive to the electrostatic interaction of the PSB group with the protein environment, whereas the magnitude of the difference between the C=N stretching frequencies in H$_2$O and D$_2$O is proportional to the strength of the hydrogen bonding of the Schiff base proton with its acceptor group (19). The data on the chimera 2–7 pigment reveal that the Schiff base environment in this mutant protein is the same as in rhodopsin. Therefore, Ser90, Ser292, Ser295, and Cys299 on TM helices 2 and 7, although located near the Schiff base, do not blue-shift the $\lambda_{\text{max}}$ by either directly or indirectly altering the hydrogen bonding of the Schiff base nitrogen or its electrostatic interaction with the protein. Instead, the increase in the $S_{\gamma}S_{\gamma}$ transition energy apparently originates from the through-space electrostatic interaction of the added dipole groups with the differential dipolar character of the chromophore. The additivity of the opsin shifts is consistent with this dipole-dipole mechanism since it indicates that the effects of the dipolar groups are weak perturbations that do not significantly change the retinal electronic structure. The dipole groups would initially be oriented around the PSB group to stabilize the ground-state chromophore charge distribution that is calculated to carry a net positive charge of approximately 0.23 (electron charge unit) between C-13 and nitrogen (47). Upon photoexcitation of retinal to the $S_1$ state, the net charge on these $\pi$ centers becomes negative. The surrounding
dipoles, unable to reorganize to stabilize the instantaneous charge redistribution, would raise the $S_1$ energy, and thereby increase the $S_0$-$S_1$ energy gap. The physical basis of this $\sim 1000$ cm$^{-1}$ opsin shift between the chimera 2–7 pigment and rhodopsin is the same as the mechanism operating between the green and red cone pigments. In the green-to-red opsin shift, a decrease in transition energy is accomplished by the interaction of the chromophore with the hydroxy dipoles on TM helices 4 and 6 near the cyclohexyl ring. The presence of these polar residues in the red cone sequence also does not alter the C≡N stretching frequencies (Table IV) (25).

Taken together, the Raman data on the mutant pigments show that dipolar groups can red shift or blue shift the retinal absorption depending on whether they are situated near the ring or the Schiff base, respectively, consistent with proposals by others (29, 31). Based on our results, it appears that a single Ser residue at the 292 position in the A292S rhodopsin mutant or the mouse green cone induces a blue shift by the long range dipole-dipole interaction and not by direct hydrogen bonding to the PSB group. In this case, the decrease in the induced dipole moment or polarizability accompanying a change from Trp to Tyr residue close to the ring would stabilize the $S_1$ state less and lead to an increase of the $S_0$-$S_1$ transition energy.

The synergistic blue shift of the $\lambda_{\text{max}}$ to $\sim 440$ nm in the blue-rho pigment is associated with the perturbation of the PSB environment. The increase of the C≡N stretch frequency in D$_2$O to $\sim 1631$ cm$^{-1}$ is consistent with a stronger electrostatic interaction of the PSB region of the chromophore with the protein, whereas the decrease in the magnitude of the D$_2$O shift is indicative of a weakening of the hydrogen bonding of the Schiff base proton. The substitutions on TM helix 3 in the presence of the substitutions on TM helices 2, 6, and 7 apparently induce a local structural reorganization that allows the protein groups to more strongly solvate the PSB moiety and thereby, reduce the electron delocalization of the chromophore. The insertions of Gly and Leu residues at the 117 and 122 positions, respectively, which are strictly conserved in the blue pigments, probably perturb the secondary structure of TM helix 3 and/or its position within the helical bundle and cause the repositioning or reorientation of the $S_0$-$S_1$ counterion and of the water (47, 48, 49) and the substituted polar amino acids nearby. The strengthened PSB-protein interaction and the $\lambda_{\text{max}}$ blue shift could be realized by the motion of the carboxylate oxygen(s) away from C-12 and closer to the nitrogen, and better solvation of the PSB moiety by dipolar groups of the serine residues and water in the binding pocket.

Conclusion—The substitution of nine amino acids from the blue cone sequence into the rhodopsin sequence yields a mutant pigment with a blue-shifted $\lambda_{\text{max}}$ at 438 nm and creates a protein environment around the PSB group that partially mimics the environment that exists in the blue-sensitive toad green rod pigment. By using the mutant blue-rho pigment as a model for the blue-sensitive pigments, we may draw some conclusions about the mechanisms that likely underlie the opsin shift between blue-sensitive pigments and rhodopsin. The results from this study exclude mechanisms that postulate effects from positive charges of arginine and histidine at the 107 and 108 positions, respectively. Except for these sites, no other basic residues that could interact directly with the chromophore are present in the putative TM domains of the blue cone pigment.

Our results indicate that the opsin shift to blue wavelengths originates from a combination of changes in the dielectric property and the architecture of the binding pocket, especially surrounding the PSB group. The dielectric change shifts the $\lambda_{\text{max}}$ to $\sim 460$ nm and involves two components. First, the W265Y substitution decreases polarizability close to the cyclohexyl ring. Second, there is an increase in the number of polar serine residues on TM helices 2 and 7 near the PSB group. The $\lambda_{\text{max}}$ shift to below $\sim 440$ nm requires two additional mutations, A117G and E122L, on TM helix 3. These substitutions act synergistically with the other residues on TM helices 2, 6, and 7 to induce rearrangement of $S_0$-$S_1$ energy gap. The physical basis of this $\lambda_{\text{max}}$ energy gap. The physical basis of this $\lambda_{\text{max}}$ shift is consistent with a stronger electrostatic interaction of the PSB region of the chromophore with the protein, which may partially mimic the environment that exists in the blue-sensitive toad green rod pigment. By using the mutant blue-rho pigment as a model for the blue-sensitive pigments, we may draw some conclusions about the mechanisms that likely underlie the opsin shift between blue-sensitive pigments and rhodopsin. The results from this study exclude mechanisms that postulate effects from positive charges of arginine and histidine at the 107 and 108 positions, respectively. Except for these sites, no other basic residues that could interact directly with the chromophore are present in the putative TM domains of the blue cone pigment.

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