Specificity of the chromophore-binding site in human cone opsin

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ABSTRACT: The variable composition of the chromophore-binding pocket in visual receptors is essential for vision. The visual phototransduction starts with the cis–trans isomerization of the retinal chromophore upon absorption of photons. Despite sharing the common 11-cis-retinal chromophore, rod and cone photoreceptors possess distinct photochemical properties. Thus, a detailed molecular characterization of the chromophore-binding pocket of these receptors is critical to understanding the differences in the photochemistry of vision between rods and cones. Unlike for rhodopsin (Rh), the crystal structures of cone opsins remain to be determined. To obtain insights into the specific chromophore–protein interactions that govern spectral tuning in human
visual pigments, here we harnessed the unique binding properties of 11-cis-6mr-retinal with human blue, green, and red cone opsins. To unravel the specificity of the chromophore-binding pocket of cone opsins, we applied 11-cis-6mr-retinal analog-binding analyses to human blue, green, and red cone opsins. Our results revealed that among the three cone opsins, only blue cone opsin can accommodate the 11-cis-6mr-retinal in its chromophore-binding pocket, resulting in the formation of a synthetic blue pigment (B6mr) that absorbs visible light. A combination of primary sequence alignment, molecular modeling, and mutagenesis experiments revealed the specific amino acid residue 6.48 (Tyr262 in blue cone opsins and Trp281 in green and red cone opsins) as a selectivity filter in human cone opsins. Altogether, the results of our study uncover the molecular basis underlying the binding selectivity of 11-cis-6mr-retinal to the cone opsins.

Both families of visual G protein-coupled receptors (GPCRs), implicated in twilight vision rhodopsin (Rh) and responsible for color vision cone opsins, consist of the universal chromophore, 11-cis-retinal, bound to the opsin protein moiety via a protonated Schiff base linkage (1). Besides several structural and functional similarities between Rh and cone opsins, including (i) heptahelical transmembrane architecture, (ii) an ultrafast photo-induced cis-trans isomerization of the chromophore, and (iii) signal transduction through the interaction with heterotrimeric G protein, cone opsins exhibit a relatively faster activation, inactivation and regeneration, than Rh (2,3). These differences are attributed to the specific interactions between 11-cis-retinal and the chromophore-binding pocket of each pigment. The distinct chromophore-protein interactions also determine the light absorption properties of these receptors (4).

On the molecular level, Rh has been extensively studied by X-ray crystallography (5-9), NMR (10-12), FTIR (13-15), and resonance Raman spectroscopic techniques (16,17). These structural studies determined that the chromophore-binding pocket of Rh is largely comprised of hydrophobic residues that enable accommodation of the retinal chromophore molecule and dictate its photochemistry.

Previously, various retinal analogs have been used to probe the specificity of Rh’s chromophore-binding pocket and its functional implications (18-20). Recent comprehensive structural and biophysical analyses of the rod pigment regenerated with the 11-cis-6mr-retinal chromophore analog (Rh6mr) showed unique photocyclic behavior of Rh6mr with an atypical cis-dicis retinal photoisomerization, which was entirely mediated by the chemical properties of 11-cis-6mr-retinal featuring a locked C11=C12 cis-trans isomerization (21). In addition, determination of the crystal structure of Rh6mr and molecular mechanics studies led to the conclusion that 11-cis isomer of 6mr-retinal is the most favorable configuration within the chromophore-binding pocket of Rh.

In contrast to Rh, fewer studies have been conducted to unravel the molecular details of the chromophore-binding pocket of cone opsins, which is in part due to their instability in vitro. Previous studies to delineate the spectral tuning and the G protein activation mechanism of cone opsins, focused on regenerating red cone opsin with retinal analogs with varying polyene chain lengths and district geometric isomers (22). More recently, the 11-cis-6mr-retinal was utilized to elucidate the basis of the chromophore-binding specificity of green cone opsin (23). Interestingly, green cone opsin was unable to bind 11-cis-6mr-retinal chromophore, suggesting a tighter/rigid chromophore-binding pocket relative to Rh. In this study, we investigated the ability of human blue, green, and red cone opsins to bind the 11-cis-6mr-retinal. We found that among three cone opsin, 11-cis-6mr-retinal formed a Schiff base only with blue cone opsin, resulting in the formation of pigment (B6mr) exhibiting an absorption base at 440 nm and we discerned that the specific amino acid environment of the blue cone opsin chromophore-binding pocket contributed to this property.

RESULTS

Regeneration of rod and cone opsins with 11-cis-6mr-retinal

Human blue, green and red cone opsins were expressed in insect cells, and their
regenerative properties with 11-cis-6mr-retinal were tested in the isolated membranes. Rod opsin was prepared in ROS membranes isolated from bovine retinas as described in the Material and Methods. Opsin membranes were incubated either with 11-cis-retinal or 11-cis-6mr-retinal followed by pigments purification by 1D4 immunoaffinity chromatography. As expected, UV-visible spectroscopy of the samples regenerated with 11-cis-retinal revealed absorption maxima ($\lambda_{\text{max}}$) of 415 nm, 530 nm and 560 nm for blue, green and red cone opsin, respectively. These spectral properties were similar to that obtained previously (24,25). Interestingly, upon incubation with 11-cis-6mr-retinal only the blue cone opsin showed formation of light absorbing pigment with ~25 nm red-shifted $\lambda_{\text{max}}$ at 440 nm, as compared to blue cone opsin regenerated with 11-cis-retinal. In contrast, both green and red cone opsins were unable to bind 11-cis-6mr-retinal (Fig. 1a). As expected, Rh could be regenerated with 11-cis-6mr-retinal resulting in a pigment (Rh6mr) with $\lambda_{\text{max}}$ at 505 nm, featuring 8 nm red-shift as compared to Rh.

To decipher the basis of chromophore selectivity between the visual receptors, we performed multiple sequence alignment focusing on the amino acid residues in and around the chromophore-binding pocket. The major difference was identified in the primary sequences of cone opsins at amino acid residue 6.48, which plays a critical role in the chromophore entry and spectral tuning. While green and red cone opsins comprise a Trp at the position 6.48 (Trp281), blue cone opsin features a Tyr at the corresponding position (Tyr262) (Fig. 1b) (26). The different interactions between the residue 6.48 and $\beta$-ionone ring of the chromophore in cone opsins cause a large spectral shift of the $\lambda_{\text{max}}$ in blue as compared to green and red cone opsins (27). Thus, these differences could underlie the binding specificity of 11-cis-6mr-retinal to cone opsins. Moreover, the 6.48 residue is highly conserved among class A GPCRs and plays a critical role in the ligand binding and receptor activation (28,29).

**In silico binding analysis of 11-cis-6mr-retinal to cone opsins**

The difference in the binding ability of 11-cis-6mr-retinal to blue and green cone opsins could be attributed to differences in the amino acid composition within their chromophore-binding pocket. To obtain further insights into the potential steric hindrance caused by the 6.48 residue, ROSETTA suite was used to calculate the pairwise interaction energies of 11-cis-retinal with Tyr262 residue of blue cone opsin and Trp281 residue of green cone opsin. Similarly, the pairwise interaction energies of the swapped mutants (Tyr262Trp in blue cone opsin and Trp281Tyr in green cone opsin) were also determined. These models were used as templates for calculating the binding free energies and pairwise binding energies between 11-cis-6mr-retinal and residue 6.48 in both WT and the swapped mutants of blue and green cone opsin. Significant differences in the interaction energy profiles of 11-cis-6mr-retinal with all protein models were observed. Binding of 11-cis-6mr-retinal was energetically more favorable in WT blue cone opsin than in its swapped Tyr262Trp mutant. Interestingly, the Trp281Tyr mutant of green cone opsin displayed a lower binding energy for 11-cis-6mr-retinal as compared to its WT counterpart (Table 1). The molecular modeling studies showed that the orientation of substituted residues did not change, and in both swapped mutants their side chains extended towards the retinal chromophore (Fig. 2a and b). However, such amino acid substitutions would likely cause global rearrangements in the chromophore-binding pocket allowing accommodation of 11-cis-6mr-retinal in the larger space of the Trp281Tyr green cone opsin as compared to its WT counterpart. Similarly, these spatial changes would also result in the impairment of 11-cis-6mr-retinal fit in the Tyr262Trp blue cone opsin mutant due to the exchange of the smaller Tyr into the bulkier Trp residue (Fig. 2). Overall, our molecular modeling analyses suggest a role for residue 6.48 in the chromophore-binding specificity of cone opsins.

**In vitro binding of 11-cis-6mr-retinal to the swapped mutants of blue and green cone opsin**

To verify our computational predictions, we prepared the Tyr262Trp and Trp281Tyr mutants for blue and green cone opsin, respectively.
in the pcDNA3.1(+) vector and expressed them in HEK-293 cells. Notably, the expression level of WT green cone opsin was higher as compared to WT blue cone opsin. However, the amino acid substitutions did not significantly affect the expression profiles of the respective cone opsins (Fig. 3a). Opsin pigments were regenerated either with 11-cis-retinal or 11-cis-6mr-retinal, and then purified by 1D4 immunoaffinity chromatography. The UV-visible absorption spectra revealed a significant reduction (~60%) in the ability of Tyr262Trp blue cone opsin mutant to bind 11-cis-6mr-retinal, thereby validating our in silico predictions. In contrast, the regeneration of green Trp281Tyr cone opsin with 11-cis-6mr-retinal was significantly enhanced as compared to WT green cone opsin (Fig. 3b). However, this mutation also altered the spectral properties of green cone opsin upon binding of natural chromophore 11-cis-retinal. Binding of 11-cis-retinal to Trp281Tyr green cone opsin resulted in the formation of pigment with the $\lambda_{\text{max}}$ at 511 nm, ~19 nm blue-shifted as compared to WT green cone opsin (Fig. 3b and Fig 1a).

**Specificity of the chromophore-binding pockets in rod and cone opsins**

The amino acid residue 6.48 regulates the binding of retinal into the chromophore-binding pocket of opsin pigments. Binding of 11-cis-6mr-retinal is permitted in Tyr$^{6.48}$-containing blue cone opsin, but not in Trp$^{6.48}$-containing green and red cone opsins. Interestingly, Rh also comprises a Trp in position 6.48, and binds 11-cis-6mr-retinal efficiently (5,30). This suggests that perhaps Trp265$^{5.48}$ is not the only amino acid involved in the chromophore-binding selectivity. Multiple sequence alignment of cone opsins and Rh identified Val132$^{3.47}$ in close vicinity of the retinal-binding pocket of green and red cone opsins. In contrast, blue cone opsin and Rh feature smaller amino acid residues at the corresponding positions. While Rh contains an alanine (Ala117$^{3.32}$) (Fig. 1) (26,31), blue cone opsin comprises a glycine residue (Gly114$^{3.29}$). Conformation of 11-cis-6mr-retinal relative to Trp265$^{5.48}$ and Ala117$^{3.32}$ is depicted in the crystal structure of Rh6mr (Fig. 4). While small Ala residue does not perturb the binding of 11-cis-6mr-retinal to rod opsin, an extra methyl group of Val132$^{3.47}$ in green and red cone opsins along with a bulkier Trp$^{6.48}$ residue could sterically hinder the cyclo-hexyl ring of 11-cis-6mr-retinal, and thereby inhibit its entry into the chromophore-binding pocket of these cone opsins.

**Biophysical properties of B6mr**

While Rh6mr features an 8 nm red-shift in the $\lambda_{\text{max}}$ as compared to Rh, B6mr displays a 25 nm red-shift in its $\lambda_{\text{max}}$ (Fig. 5a and b, black spectrum) (21). The photobleaching experiments were performed through a 400-440 nm band pass filter for B6mr and through a 480-520 nm band pass filter for Rh6mr. As described previously, Rh6mr requires a prolonged illumination of at least 1 min to reach its photo-stationary Meta-II-like state absorbing at 497 nm (Fig. 5b, red spectrum), whereas 5 s illumination of Rh in detergent solution at neutral pH is enough to convert 11-cis-retinal to all-trans-retinal and transition to Meta II state. Rh6mr features a 24-fold lower photosensitivity (quantum yield of 0.027) relative to Rh (quantum yield of 0.65) (32). Interestingly, B6mr required even longer illumination period of at least 30 min to achieve its photo-stationary Meta-II-like state with a $\lambda_{\text{max}}$ of 390 nm. The addition of an acid to the sample (pH = 2) resulted in a red-shift due to the Schiff base protonation, suggesting that the Meta-II-like photoproduct of B6mr possess an intact, deprotonated Schiff base (Fig. 5a, red spectrum, Fig. 6a, blue spectrum). A shorter illumination of 5 min showed the $\lambda_{\text{max}}$ at 410 nm, corresponding to a mixture of activated and inactive states of B6mr (Fig. 5a, yellow spectrum). Thus, these results indicate that B6mr has even lower photosensitivity than Rh6mr. Indeed, the quantum yield of isomerization of B6mr was calculated as 0.0047, which is about 6-fold lower than that of Rh6mr (0.027) and 138-fold lower than for Rh (0.65) (Fig. 5e and Fig. 7). As shown previously, the Meta-II-like state of Rh6mr does not decay into opsin and free 11-cis-6mr-retinal upon light illumination, but instead converts back to its inactive state after 2 h (Fig. 5b, dotted black spectrum and Fig. 7) (21). Interestingly, the Meta-II-like state of B6mr did not revert back to its inactive state within a time range of 20-24 h (Fig. 5a, dotted red spectrum). Comparative retinoid isomeric composition analyses of the inactive and photoactivated Meta-II-like states of Rh6mr and B6mr revealed similar light-stimulated changes in the isomeric composition of 11-cis-6mr-retinal in both pigments.
In the absence of opsin moiety, 11-cis-6mr-retinal exists as a mixture of 4 isomers: 9, 11,13-tricis-, 11,13-dicis-, 11-cis- and 9,11-dicis-6mr-retinal. These isomers elute as peak 1, 2, 3 and 4, respectively from a normal phase HPLC chromatography column (21,33). The inactive state of both Rh6mr and B6mr showed a predominant peak that corresponds to 11-cis-6mr-retinal (peak 3). HPLC analyses of light exposed Rh6mr and B6mr displayed a significant decrease in the intensity of peak 3 and a simultaneous increase of peak 2, which corresponds to the 11,13-dicis-6mr-retinal in both samples, suggesting that 11-cis converts to 11,13-dicis isomer upon light illumination of B6mr (Fig. 5c and d), similarly as in Rh6mr.

Previous studies showed that the inactive state Rh is inaccessible to bulk water, however, binding of 11-cis-6mr-retinal significantly increased solvent accessibility in the inactive state of Rh6mr (21). Thus, we evaluated the accessibility of bulk water to B6mr in comparison to WT blue cone opsin. Both WT blue cone opsin and B6mr were sensitive to hydroxylamine (NH₂OH) in the dark with the half-life of the chromophore release ($T_{1/2}$) of 6.67 ± 0.54 min and 8.99 ± 0.71 min, respectively. A faster retinal hydrolysis in the presence of NH₂OH, suggests increased accessibility of bulk water into the chromophore-binding pocket (Fig. 5f). This indicates that binding of 11-cis-6mr-retinal facilitated an additional opening of the cytoplasmic side in B6mr. Next, we assessed the thermal stability of B6mr relative to Rh6mr (Fig. 6b). The results showed that the half-life of the chromophore release in B6mr ($T_{1/2}$ =12±3 min) was significantly shorter than in Rh6mr ($T_{1/2}$ =80±3 min), suggesting a less stable Schiff base formation with Lys743 of B6mr as compared to Rh6mr.

**DISCUSSION**

In addition to the native chromophore 11-cis-retinal, rod opsin can bind retinal geometric isomers and various synthetic retinal analogs (34-36). In this study, we analyzed the binding properties of 11-cis-6mr-retinal to human cone opsin receptors and compared their biochemical and biophysical properties to Rh. As previously reported, rod opsin can efficiently accommodate 11-cis-6mr-retinal in the retinal-binding pocket (4,22,30,34). However, 11-cis-6mr-retinal does not fit very well into the chromophore-binding pocket of green cone opsin (23). Here, we extended this investigation and explored the capability of blue and red cone opsins to bind 11-cis-6mr-retinal. Upon incubation of isolated opsin membranes with 11-cis-6mr-retinal, we found that only blue cone opsin could bind this retinal analog and form a functional pigment absorbing in a blue wavelength range. Similar to Rh6mr, B6mr features a significant change in its spectral properties, displaying a 25 nm red-shifted $\lambda_{max}$. This change could be attributed to the differences in the interaction profile of 11-cis-6mr-retinal and 11-cis-retinal with the retinal-binding pocket of blue cone opsin. Additionally, distortion in the C₁₂–C₁₅=C₁₄–C₁₅ dihedral angle of 11-cis-6mr-retinal might contribute to the observed spectral shift. The crystal structure of bovine Rh shows a distortion of the retinal polyene chain at positions C₁₁ and C₁₂, which likely plays a key role in achieving an ultrafast retinal isomerization rate (5). Thus, incorporation of a 6mr-ring between C₁₀ and C₁₃ to the retinal would release the structural constrains and induce depolarization of π-electron on the polyene chain, resulting in the spectral red-shift.

Furthermore, the conformation of the C₆–C₇ single bond is also one of the key factors for spectral tuning. For the bovine Rh, C₆–C₇ bond is 6-s-cis conformer, resulting in the nonplanar structure from polyene chain to β-ionone ring (37). This nonplanar conformation causes spectral blue shift due to localization of π-electron along the polyene chain. Therefore, the chromophore-binding pocket of B6mr might promote the polyene chain and β-ionone ring planar structure to lead spectral red shift.

Conversion of Rh to the active Meta-II state in response to light associates with a 118 nm blue shift in the $\lambda_{max}$ (498 nm → 380 nm), occurring as a consequence of the Schiff base deprotonation (4,30,38). In Rh6mr, such transition to the active Meta-II-like state is accompanied by a much smaller 8 nm blue shift in the $\lambda_{max}$ (505 nm → 497 nm) (21), likely due to the protonated Schiff base, as in Meta-I state of Rh, also featuring a small 8 nm blue-shift (4,30). However, photostationary Meta-II-like state of B6mr is associated with much larger 50 nm blue-shift in the
of the chromophore is essential for the spectral and functional properties of the visual receptors.

Another facet of regulating the spectral tuning in visual pigments involves a network of protein-bound water molecules. It is well known that several water molecules are situated in the vicinity of the retinal chromophore in Rh. These water molecules form an intricate hydrogen bonding network that undergoes significant changes during light-induced activation (41-44). Additionally, some water molecules also facilitate the Schiff base hydrolysis resulting in the chromophore release (31,45). In cone opsins, an internal water molecule network in the chromophore-binding pocket regulates the spectral properties of cone opsins and has significant effects on the rates of G protein activation (39,46,47). Remarkably, a specific water cluster in the chromophore-binding pocket of blue cone opsin was shown to be influenced by the Tyr262Trp mutation (39). Therefore, this water-mediated hydrogen bonding network embedded in the chromophore-binding pocket might play a role in providing the structural flexibility required for the binding of 11-cis-6mr-retinal to blue cone opsin.

This study also revealed key structural differences in the chromophore-binding pocket of human cone opsins based on their unique ability to accommodate 11-cis-6mr-retinal. The amino acid residue 6.48 is the key selectivity filter that works in conjunction with surrounding residues to determine binding of 11-cis-6mr-retinal in the chromophore-binding pocket. The presence of smaller Tyr in the retinal-binding pocket instead of bulkier Trp was crucial for the ability to accommodate 11-cis-6mr-retinal to blue cone opsin and Trp281Tyr green cone opsin mutant. Interestingly, this single amino acid substitution in green cone opsin permitted the binding of 11-cis-6mr-retinal. In our previous study (23) we have discovered the role of green cone opsin N-terminus for the retinal-binding selectivity. Both green and red cone opsins possess a longer N-terminus as compared to blue cone opsin and Rh. Interestingly, this feature associates with their inability to bind 11-cis-6mr-retinal. Removal of the first 16 N-terminal amino acid residues of green cone opsin enables the binding of 11-cis-6mr-retinal, suggesting that such extended N-terminus contributes to protein rigidity.
The molecular modeling analysis revealed that the N-terminus of green cone opsin forms a short α-helix that interacts with transmembrane helices TM5 and TM6, most likely stabilizing these helices. Interestingly, the interface formed by TM5 and TM6 is the most favorable entrance into the retinal-binding pocket. Removal of the N-terminus resulted in increased protein flexibility, allowing 11-cis-6mr-retinal to gain access into the chromophore-binding pocket. Thus, it is likely that among other specific signatures of the chromophore-binding pocket, blue cone opsin and rod opsin are inherently more flexible due to their shorter N-terminus as compared to green cone opsin. Trp281 is directly adjacent to the polyene chain of 11-cis-retinal chromophore and its substitution to smaller and more flexible Tyr residue, together with the rearrangement of the associated structural water molecules might introduce enough conformational flexibility to enable the entry and binding of 11-cis-6mr-retinal even in the presence of the N-terminus intact. Overall, both the N-terminus and the retinal binding pocket composition define the selectivity of retinal binding.

Together, these findings provide viable information for designing a new class of retinal-based drug treatment for color blindness. Further investigations will aid in pinpointing all determinants of the chromophore-binding specificity in cone opsins required to advance these developments.

In summary, similar to green cone opsin (23), incubation of red cone opsin with 11-cis-6mr-retinal did not result in the formation of a light absorbing pigment. Interestingly, 11-cis-6mr-retinal formed a Schiff base with blue cone opsin, resulting in the formation of pigment (B6mr) exhibiting an absorption maximum at 440 nm. These results indicate that the chromophore-binding pocket of blue cone opsin is more flexible as compared to green or red cone opsins and could accommodate the bulkier cyclo-hexyl ring of 11-cis-6mr-retinal. The primary sequence alignment of human Rh and cone opsins in combination with the molecular modeling analysis revealed a highly conserved residue homologous to Trp265 (residue 6.48) (28,48) as the key selectivity filter for the chromophore-binding in cone opsins. Furthermore, the molecular modeling analyses indicated a more stable interaction between the 11-cis-6mr-retinal and the swapped Trp281Tyr green cone opsin mutant with a lower binding energy relative to wild-type (WT) green cone opsin. However, mutation of the Tyr262 residue to Trp in blue cone opsin completely ablated its binding with 11-cis-6mr-retinal. In agreement with these, in silico binding energy calculations, the substitution of Trp281 residue to Tyr in green cone opsin enabled binding of 11-cis-6mr-retinal. Moreover, unlike Rh6mr, B6mr did not exhibit the photocyclic behavior upon illumination. Altogether, these findings imply that the chromophore-binding pocket of blue cone opsin is optimized to facilitate the Schiff base hydrolysis for faster chromophore release as compared to Rh.

EXPERIMENTAL PROCEDURES

Chemicals – n-Dodecyl-β-D-maltoside (DDM) was purchased from Affymetrix Inc. (Maumee, OH). 11-cis-Retinal was a generous gift from Dr. Rosaline Crouch (Medical University of South Carolina, Charleston, SC). 11-cis-6-membered-ring-Retinal (11-cis-6mr-retinal) was synthesized as described previously (21,23) and provided by Novartis (Cambridge, MA).

Constructs – Human blue, green and red cone opsin cDNA cloned into a pUC57 vector was synthesized by Genentech (San Francisco, CA). The last 12 amino acids in each cone opsin were replaced by the rod opsin C-terminal amino acid sequence (TETSQVAPA, called 1D4 tag) to enable protein purification. These constructs were subcloned into a pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) as per the manufacturer’s protocol. The resulting constructs were used for protein purification and UV-visible spectroscopy experiments and the sequence of each construct was confirmed by DNA sequencing.

Mutagenesis – The blue cone opsin Thr262Trp and the green cone opsin Trp281Thr mutants were full length constructs with intact N-terminus. Mutants of blue and green cone opsin were generated with the Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) according to the manufacturer’s procedures.

Expression of cone opsins in insect cells and membrane isolation – Expression constructs of
human blue, green and red cone opsins were prepared in pFastBac HT vectors (Invitrogen) with their N-terminal His tags removed. Constructs were transformed into DH10 Bac to obtain a Bacmid for transfection with X-tremeGene 9 (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, three to four days after transfection with P1 virus, the cell supernatant was collected. P2 and P3 viruses were collected similarly and stored. For the larger scale expression, Sf9 cells at density 3.5x10^6 cells per ml of culture were infected with P3 stage virus at a 1 to 100 volume ratio, and cultured for 2 days with shaking at 135 rpm in a 28 °C incubator. Cells were collected 48 h post-infection, and the cell pellet was either kept at -80 °C or used immediately for membrane isolation.

To isolate membranes, first cells were homogenized on ice with a Dounce homogenizer in a hypotonic buffer: 25 mM HEPES, pH 7.5, 10 mM MgCl₂, 20 mM KCl containing EDTA-free complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The homogenate was then centrifuged at 100,000 g for 30 min. The supernatant was discarded and the membrane pellet was homogenized in the same buffer and centrifuged at 100,000 g for 30 min again. Next, membranes were washed three to four times with 25 mM HEPES, pH 7.5, 1.0 M NaCl, 10 mM MgCl₂, 20 mM KCl containing EDTA-free complete protease inhibitor cocktail. Finally, the washed membranes were resuspended in 50% (v/v) glycerol, flash frozen with liquid nitrogen, and stored at -80 °C or used immediately. Pigments were reconstituted with either 11-cis-retinal or 11-cis-6mr-retinal. Retinal was added to the membranes from a DMSO stock solution to a final concentration of 10 µM, and then incubated in the dark for 1 h at 4 °C. Regenerated pigments were purified by 1D4 immunoaffinity chromatography.

**Preparation of opsin membranes and pigment regeneration** – Bovine rod outer segment (ROS) membranes were isolated from 100 frozen retinas under dim red light as previously described (51). To prepare opsin-containing membranes, ROS were suspended in 10 mM sodium phosphate, pH 7.0, and 50 mM hydroxylamine with Rh concentration of 2 mg/ml, placed on ice, and illuminated with a 150 Watt bulb for 30 min at a distance of 15 cm. Then membranes were centrifuged at 16,000 g for 10 min. Supernatant The supernatant was discarded and the pellet was washed four times with 10 mM sodium phosphate, pH 7.0, followed by 4 washes with 2% BSA and then with 4 washes with 10 mM sodium phosphate, pH 7.0. Final membrane wash was performed in 20 mM bis-tris propane (BTP), 100 mM NaCl, pH 7.5 twice. Rh and opsins concentrations were measured with a UV-visible spectrophotometer (Cary 50, Varian, Palo Alto, CA) and quantified using the absorption coefficients ε_{500nm} = 40,600 M⁻¹cm⁻¹ and ε_{280nm} = 81,200 M⁻¹cm⁻¹, respectively (52). Regeneration of Rh or Rh6mr with 11-cis-retinal or 11-cis-6mr-retinal, respectively was accomplished by incubating opsins membranes with the respective retinal (10 µM) overnight at 4 °C. Regenerated pigments were purified by 1D4 immunoaffinity chromatography.

**Pigment purification by 1D4 immunoaffinity chromatography** – HEK-293T cells transiently transfected with the WT blue, green or red cone opsin constructs, and the blue Thr262Trp or green Thr262Trp mutant constructs were harvested from forty 10-cm plates and centrifuged at 800 g. Cell pellets were suspended in 50 mM HEPES, 150 mM NaCl, 20 mM n-dodecyl-β-D-maltopyranoside (DDM), pH 7.0, containing protease inhibitor cocktail, and incubated for 1 h at 4 °C on a rotating platform. Alternatively, insect cell membranes with
reconstituted cone opsins or regenerated bovine ROS membranes were solubilized with the above buffer. The lysate then was centrifuged at 100,000 g for 1 h at 4 °C and cone or rod pigments were purified from the supernatant by immunoaffinity chromatography with an anti-Rh C-terminal 1D4 antibody immobilized on cyanogen bromide-activated agarose. Four-hundred µl of 6 mg 1D4/ml agarose beads were added to the supernatant and incubated for 1 h at 4 °C on the rotating platform. The resin was then transferred to a column and washed with 10 ml of buffer composed of 50 mM HEPES, 150 mM NaCl, and 2 mM DDM, pH 7.0. Pigments were eluted with buffer composed (150 mM HEPES, 150 mM NaCl, and 2 mM DDM, pH 7.0) supplemented with 0.6 mg/ml of the TETSQVAPA peptide.

**UV-visible spectroscopy of opsin pigments** – UV-visible spectra of freshly purified cone or rod pigment samples were recorded in the dark with a Cary 50 UV-visible spectrophotometer (Varian, Palo Alto, CA). Spectra of the samples were recorded after being exposed to white light delivered from a Fiber-Light illuminator (150 W lamp) (Dolan-Jenner, Boxborough, MA) at a distance of 10 cm for the indicated time. The band pass 400-440 nm filter was used for illumination of blue cone opsin and 480-520 nm filter was used for green cone opsin and Rh. To obtain a difference spectra of purified cone pigments, the spectrum of the sample recorded in the dark was subtracted from the spectrum of the light illuminated sample. To check the protonation state of the protein Schiff base 1 µl of concentrated H2SO4 was added to the sample loaded to the cuvette to decrease the sample pH to 2 and the spectrum was recorded immediately.

**HPLC analyses** – Regenerated with either 11-cis-6mr-retinal samples of blue cone opsin (B6mr) or bovine Rh (Rh6mr) after their purification by 1D4 immunoaffinity chromatography were denatured for 30 min at room temperature with 50% CH3OH in 100 mM NH2OH. The resulting retinal oximes were extracted with 600 µl of hexane and their isomeric content was determined by normal phase HPLC with a Luna 10 µm PRE Silica (3) 100 Å, 250 x 4.6 mm column (Beckman, San Ramon, CA). Retinoids were eluted isocratically with 10% ethyl acetate in hexane at a flow rate of 1.4 ml/min. Their signals were detected by absorption at 360 nm (53,54).

**Photosensitivity measurements** – Photosensitivity measurements of B6mr and Rh6mr were performed as previously described (32,55-58). Briefly, B6mr samples maintained at 20 °C were irradiated with light from a 150 W Fiber light source delivered through a 400-440 nm band pass interference filter. The cuvette (width, 4 mm; path length, 1 cm) was placed in a cell holder maintained at 20 °C and the absorbance change was measured with a UV-visible spectrophotometer. The light intensity was controlled by using a neutral density (ND) filter (Thorlabs Inc. USA) with a maximum intensity was set to either bleach <90% of Rh or deliver 450 μW to B6mr due of its low quantum yield as compared to Rh. B6mr was illuminated for 120 min. UV-visible spectra were recorded at 1.5, 3.5, 5.5, 7.5, 12.5, 20, 37.5, 60, 75, 90, and 120 min. The amount of residual pigment after each irradiation was corrected with the dark spectrum of the pigment. The incident photon flux (s⁻¹ cm²) was calculated from the power (W) of the incident light measured by a power meter (Thorlabs Inc., Newton, NJ). The intensity of the incident light was continuously monitored to correct for any power fluctuations. The percentage of residual pigment was plotted against the incident photon count and fitted with an exponential function. The slope of the fitting line corresponded to the relative photosensitivity of the pigment at the irradiating wavelength (32,55,56). Rh photosensitivity was used as a control for all measurements, and the photosensitivity of B6mr was determined as a value relative to that of Rh.

**Thermal stability** – Purified Rh6mr or B6mr diluted with 50 mM HEPES, 150 mM NaCl, and 2 mM DDM, pH 7.0 to a final volume of 0.4 ml were incubated at 55 °C in the dark. The absorbance spectra were collected every 2 min for 40 min. The absorbance maximum at the initial time point was anticipated to be 100% and used to calculate the percentage of the remaining pigments at specific time points and then plotted as a function of time. These plots were used to calculate the half-lives (T1/2) of chromophore release upon thermal denaturation.
**Modeling** – The model of the green and blue cone opsins were generated with the ROSETTA software suite (59) as described previously (23). The crystal structure of Rh (PDB ID 1U19) (60) and the bovine Rh sequence (UniProtKB – P02699) were used as the template for the human green or blue cone opsin protein sequences (UniProtKB - P04001 and P03999, respectively). The most energetically favorable models were selected.

Retinal was parameterized for use within ROSETTA (61). Because the retinal ligand is bound to Lys312 in green cone opsin and Lys293 in blue cone opsin by a protonated Schiff base, the ligand-residue complex was treated as a non-canonical amino acid, allowing the structure to be flawlessly used within the ROSETTA framework. The coordinates for the retinal-lysine complex were extracted from the 2.2 Å resolution crystal structure of Rh (PDB ID 1U19), and a ROSETTA non-canonical amino acid parameter file was created. The fixed-backbone design (FBBD) protocol in ROSETTA was used to mutate Lys312 in green cone opsin or Lys293 in blue cone opsin to the retinal-lysine complex, which was introduced with the conformation present in the crystal structure. All other coordinates in the cone opsins structures were kept constant. To remove any structural inconsistencies or clashes introduced into the cone opsin models, the structure was relaxed with the ROSETTA membrane fast relax protocol (62), and the most energetically favorable models were selected. These modeling procedures were repeated to produce a model with 11-cis-6mr-retinal chromophore in the binding site.
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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

B.J., K.K., S.G. and K.P. conceived and designed the experiments. B.J., K.K., S.G., J.T.O., N.S.A., W.S. and M.M.S. conducted the experiments. B.J., K.K. S.G., J.T.O. and K.P. wrote the manuscript. B.J. and K.P. coordinated and oversaw the research project. All authors discussed the results and commented on the manuscript.

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**Abbreviations**

B6mr, 11-cis-6mr-retinal-bound blue cone opsin; BTP, bis-tris propane; DDM, n-dodecyl-β-D-maltopyranoside; GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; Meta II, metarhodopsin II; ROS, rod outer segments; Rh, rhodopsin; Rh6mr, 11-cis-6mr-retinal-bound rhodopsin; SDS, sodium dodecyl sulfate; TM, transmembrane; UV-visible, ultraviolet–visible, WT, wild-type; 11-cis-6mr-retinal, 11-cis-6-membered-ring-retinal
**Table 1.** Binding energy of 11-cis-6ring-retinal to blue and green cone opsin

|                     | Binding energy of 11-cis-6mr-retinal (kcal/mole) |
|---------------------|---------------------------------------------------|
|                     | WT       | Mutant       |
| Blue cone opsin     | -9.4 (-2.7) | -8.0 (-2.1) |
| Green cone opsin    | -8.4 (-2.0) | -9.8 (-2.6) |

The binding free energies and the energies of interaction between 11-cis-6mr-retinal and the 6.48 residue (the Tyr262 or Trp281 residues) were calculated in WT or swapped mutants of blue and green cone opsin.
Figure 1. Binding of 11-cis-retinal chromophore and its locked 11-cis-6mr-retinal analog to human blue, green and red cone opsins, and bovine rod opsin. (a) UV-visible absorption spectra of reconstituted blue cone opsin, Rh, green, and red cone opsins with the native chromophore 11-cis-retinal (blue, black, green, and red black dashed line, respectively) and UV-visible absorption spectra of blue cone opsin, Rh, green, and red cone opsins bound with 11-cis-6mr-retinal analog (blue, black, green, and red solid line, respectively). (b) Partial amino acid sequence alignment of human blue, green and red cone opsins and bovine Rh (black). Conserved residues in the chromophore-binding pocket are shown. The amino acid numbers are based on bovine Rh sequence.
Figure 2. Comparison of the retinal-binding pockets in WT and mutated cone opsins. (a) WT blue cone opsin (dark blue) and the Tyr262Trp mutant (light blue) (top panel) are shown. WT green cone opsin (dark green) and the Trp281Tyr mutant (light green) (bottom panel) are shown. Tyr residues (yellow sticks) and Trp residues (orange sticks) are shown. 11-cis-6mr-Retinal was modeled into the binding pocket of WT and mutated cone opsins and is shown with light gray sticks in WT blue and green cone opsins and with dark gray sticks in mutated blue and green cone opsins. (b) Magnified view of 11-cis-6mr-retinal conformations in the binding pocket of WT and mutated blue and green cone opsins (top and bottom panel, respectively) relative to the residues of interest Tyr and Trp are shown. The same 11-cis-6mr-retinal orientations as in the merged panels of (a) and after 60-degree rotation along the Y-axis are shown.
Figure 3. Binding of 11-cis-retinal chromophore and its locked 11-cis-6mr-retinal analog to green cone opsin and the Trp281Tyr mutant. (a) SDS-polyacrylamide gel of regenerated and purified pigments. Pigment regeneration was performed in HEK-293T cell pellets, which then were purified by 1D4 immunoaffinity chromatography. Proteins were separated on the SDS-polyacrylamide gel (2 µg was loaded in each lane) and the gel was stained with Coomassie blue staining. Proteins were deglycosylated with PNGase F before loading onto the gel. (b) UV-visible absorption spectra of reconstituted Trp281Tyr green cone opsin regenerated with 11-cis-retinal (11-cis) (dark green), and spectra of Trp281Tyr green cone opsin regenerated with 11-cis-6mr-retinal (6mr) (light green), and also UV-visible absorption spectra of reconstituted WT green cone opsin regenerated with 11-cis-retinal (WT (11-cis)) (dark green, dashed) and with 11-cis-6mr-retinal (WT (6mr)) (dark green dotted line) are shown.
Figure 4. The crystal structure of Rh6mr (PDB: 5TE5; (21)) is shown on the left. Magnified region of Rh6mr displaying 11-cis-6mr-retinal in the chromophore-binding pocket is shown on the right. 11-cis-6mr-Retinal is shown with black sticks. Trp265^6.48 and Ala 117^{3.32} are shown with orange and cyan sticks and balls, respectively.
Figure 5. Spectral properties of blue cone opsin reconstituted with 11-cis-6mr-retinal compared to rod opsin reconstituted with 11-cis-6mr-retinal. (a) UV-visible absorption spectra of blue cone opsin regenerated with 11-cis-6mr-retinal (B6mr) in dark conditions (black spectrum) and after illumination for 5 min (orange spectrum) and 30 min (red spectrum); sample illuminated for 30 min was then kept for 1200 min in the dark (red dotted spectrum). (b) UV-visible absorption spectra of rod opsin regenerated with 11-cis-6mr-retinal (Rh6mr) in dark conditions (black spectrum) and after illumination for 1 min (red spectrum). Sample illuminated for 30 min were then kept for 1200 min in the dark (black dotted spectrum). (c) HPLC elution profile of retinoid oximes extracted from dark state B6mr (black line) or from B6mr illuminated for 5 min (red line). (d) HPLC elution profile of retinoid oximes extracted from dark state Rh6mr (black line) or from Rh6mr illuminated for 1 min. (e) Photosensitivity of B6mr. Samples were illuminated with light from a 150-W fiber light source delivered through a 400-440 nm band pass interference filter at 20 °C. The percentage of residual pigment was plotted against the incident photon count and
fitted with an exponential function. The slope of the fitting line corresponds to the relative photosensitivity of the pigment at the irradiating wavelength. (f) Accessibility of the bulk solvent to the protonated Schiff base in blue cone opsin regenerated with 11-cis-retinal (Blue opsin, light blue) or 11-cis-6mr-retinal (B6mr, dark blue). The UV-visible absorption spectra were recorded every 2 min at 20°C. The percentage of residual pigment was plotted as a function of time and fitted with an exponential function.

**Figure 6.** Thermal stability and acidification of blue cone opsin reconstituted with 11-cis-6mr-retinal. (a) UV-visible absorption spectra of dark state B6mr (black line), light activated Meta II-like B6mr (red line) and Meta II-like B6mr after acid denaturation (yellow line). The pH of the sample was adjusted to 2 with H$_2$SO$_4$ to protonate the Schiff base. (b) The thermal stability of purified Rh6mr or B6mr was determined. Samples were incubated at 37°C in the dark and their absorbance spectra were recorded every 2 min for 40 min. The change in the absorbance maximum was calculated as a percentage of residual pigment assuming the absorbance at the initial point as 100% and plotted as a function of time. These plots were used to calculate the half-lives ($T_{1/2}$) of chromophore release. Each experiment was performed in triplicate. **Inset,** difference in spectrum of acidified sample indicates that the Schiff base in Meta II-like B6mr is in the equilibrium of protonated and deprotonated states.
Figure 7. Photochemical modeling of B6mr in comparison to Rh6mr. B6mr and R6mr feature the red-shifted $\lambda_{\text{max}}$ relative to WT blue cone opsin and Rh (at 440 nm and 505 nm, respectively). Upon prolonged light illumination B6mr is converted to the active Meta-II-like state and 11-cis-6mr-retinal (6mr) is released from the binding pocket, while Rh6mr displays reversible photochemical behavior resulting in back conversion of its Meta-II-like state to the ground R6mr state. Calculated quantum yield ($\phi$) of B6mr and R6mr were 0.0047 and 0.027, respectively.
