Engineering a "Steric Doorstop" in Rhodopsin: Converting an Inverse Agonist to an Agonist†

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ABSTRACT: The crystal structures of rhodopsin depict the inactive conformation of rhodopsin in the dark. The 11-cis retinoid chromophore, the inverse agonist holding rhodopsin inactive, is well-resolved. Thr118 in helix 3 is the closest amino acid residue next to the 9-methyl group of the chromophore. The 9-methyl group of retinal facilitates the transition from an inactive metarhodopsin I to the active metarhodopsin II intermediate. In this study, a site-specific mutation of Thr118 to the bulkier Trp was made with the idea to induce an active conformation of the protein. The data indicate that such a mutation does indeed result in an active protein that depends on the presence of the ligand, specifically the 9-methyl group. As a result of this mutation, 11-cis retinal has been converted to an agonist. The apoprotein form of this mutant is no more active than the wild-type apoprotein. However, unlike wild-type rhodopsin, the covalent linkage of the ligand can be attacked by hydroxylamine in the dark. The combination of the Thr118Trp mutation and the 9-methyl group of the chromophore behaves as a "steric doorstop" holding the protein in an open and active conformation.

Rhodopsin is arguably the best-characterized member of the G protein-coupled receptor (GPCR) superfamily. Its ligand in the dark is the 11-cis aldehyde form of vitamin A (11-cis retinal), which serves as an inverse agonist to the protein and is covalently bound via a protonated Schiff base linkage to the conserved Lys296 in the seventh transmembrane helix. Light converts the ligand to an agonist by isomerizing the 11-cis chromophore to the all-trans form. The protein conformational change that follows enables rhodopsin to activate its G protein, transducin.

An important steric interaction in forming the active metarhodopsin II (Meta II)[1] intermediate is the 9-methyl group of retinal. Ganter et al. (*1*) first demonstrated that regeneration of rhodopsin with a retinal analog lacking the 9-methyl group resulted in greatly inhibited formation of the active Meta II intermediate. Results along these lines formed the foundation of a steric-trigger mechanism for activation involving the 9-methyl group (*2*, *3*). Vogel et al. (*4*) and Meyer et al. (*5*) concluded that the effect of the missing 9-methyl group was a shift in the equilibrium between metarhodopsin I (Meta I) and Meta II to favor the inactive Meta I intermediate. These results suggested that movement of the 9-methyl group upon photoisomerization facilitates the transition from Meta I to Meta II.

The crystal structure of rhodopsin shows that in the dark (inactive) state, the chromophore is buried in the transmembrane region of the protein and the binding pocket is capped by extracellular domains comprised of the N-terminal tail and loop connecting helices 4 and 5 (*6*−*8*). In particular, there are several amino acid residues surrounding the 9-methyl group of retinal. The closest is Thr118 of helix 3 (Figure 1).

We have recently identified a cone pigment mutant where a larger residue at this position was one of three residues that formed a dark-active pigment (*9*). Janz and Farrens (*10*) showed that a mutation of Thr118 in rhodopsin with the smaller Ala had no significant effects on dark and light-activated properties of the pigment other than a slight blue shift in the dark spectrum. In addition, Patel et al. (*11*) have concluded from nuclear magnetic resonance data that the 9-methyl group of retinal moves away from Thr118 upon photoisomerization. With these data in mind, we specifically increased the steric bulk at position 118 in rhodopsin to induce a dark-active pigment that is dependent on the presence of the 9-methyl group of 11-cis retinal. The T118W mutant uses 11-cis retinal as an agonist. We interpret our results according to a model in which the mutation and 11-cis retinal acts as a "steric doorstop" forcing the protein into a more open and active conformation that is dependent on the larger group at 118 and the 9-methyl group of retinal.

MATERIALS AND METHODS

Expression and Purification of Wild-Type and Mutant Rhodopsins. Wild-type and mutant rhodopsins were generated from pMT4, an expression vector containing the

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[1] Abbreviations: Meta I, metarhodopsin I; Meta II, metarhodopsin II.

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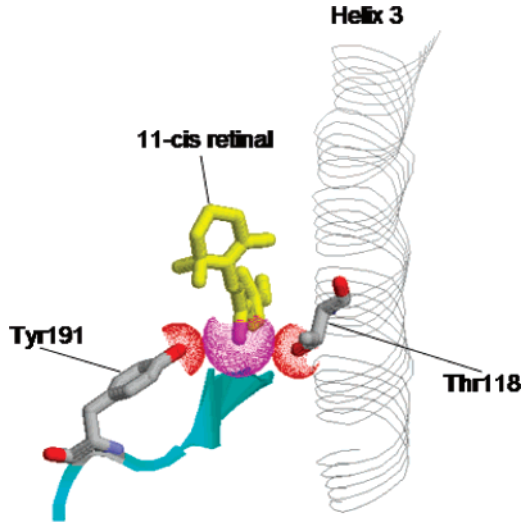

FIGURE 1: Location of Thr118 relative to 11-cis retinal from a crystal structure of rhodopsin (1gzm.pdb) (*8*). Part of helix 3 is shown with Thr118, 11-cis retinal, and part of the intradiscal loop between helices 4 and 5 (cyan) containing a β strand that roughly parallels the polyene chain of retinal and containing Tyr191 just beyond the β strand. The 11-cis retinal is colored yellow with the 9-methyl group colored purple. A sphere of dots representing the van der Waals radius of the 9-methyl group of retinal and the hydroxyl groups of Thr118 and Tyr191 is also shown to illustrate the proximity of the two amino acids flanking the 9-methyl group of retinal in the inactive dark state of rhodopsin.

synthetic bovine rhodopsin gene as described previously (*12*). Plasmids were transiently transfected into COS cells using the DEAE−dextran method as described before (*13*) except that the dimethyl sulfoxide treatment and subsequent washes were eliminated.

11-cis retinal was obtained from the National Eye Institute and Dr. Rosalie Crouch (Medical University of South Carolina, Charleston, SC), and the 9-demethyl 11-cis retinal was a gift from Dr. Crouch. Pigments were purified by 1D4-immunoaffinity chromatography essentially as described previously (*13*) except 11-cis retinal (or 9-demethyl 11-cis retinal) was incubated with the COS cell suspension for 1 h at room temperature and then overnight at 4 °C. The rationale for this modification was to increase pigment yield. We found that pigment formation of the T118W mutant at room temperature was much slower than wild-type rhodopsin (data not shown). We incubated our harvested COS cells at room temperature for 1 h at the end of the day and allowed the incubation to continue overnight at 4 °C. Reeves et al. (*14*) showed that higher pigment yields could be obtained from some rhodopsin mutants if cells were incubated with 11-cis retinal for a longer period of time or at higher concentrations.

COS cell membrane preparations containing wild-type and mutant opsins were performed essentially as described by Robinson (*15*) except that a Beckman SW 32 rotor was used throughout, and the final pellet was resuspended in 200 μL of the buffer and stored at −80 °C in 25 μL aliquots.

UV−Vis Spectrophotometry. Absorption spectra were recorded on a Varian Cary300 modified by the manufacturer for darkroom use.

Transducin Activation Assays. The ability of the opsins and pigments to activate bovine rod transducin was measured using a radioactive filter-binding assay essentially as described previously (*9*). The reaction contained 5 nM pigment,

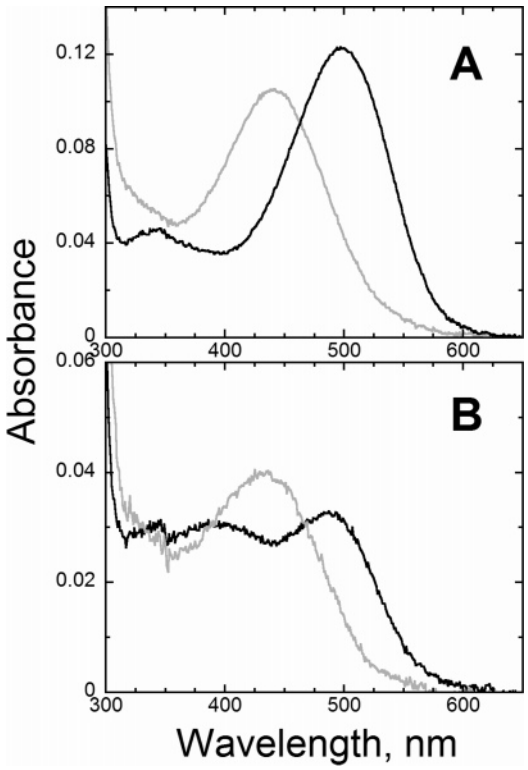

FIGURE 2: Absorption spectra of (A) wild-type and (B) T118W rhodopsin pigments (black traces) and their corresponding acid-denatured spectra with absorption maximum at 440 nm (gray traces).

2.5 μM transducin, and 3.0 μM GTPγS-35. The reaction pH was 7.5 (10 mM Tris) when assaying the purified pigments. The reaction pH was 6.5 (10 mM 2-[*N*-morpholino]-ethanesulfonic acid buffer instead of Tris) when assaying the membranes because opsin activity is higher and deactivation of the wild-type opsin upon addition of 11-cis retinal is more pronounced (*16*). Light activation was initiated with a 12 s pulse of light from a slide projector with a 300 W lamp. Each data point represent a 10 μL aliquot from the reaction mixture.

RESULTS AND DISCUSSION

Expression and Purification of T118W. The T118W mutant forms a pigment that can be purified (Figure 2). Unlike wild-type rhodopsin, the T118W pigment does not form a pigment with a single absorption maximum but rather appears to consist of a mixture of two or more components with discernible maxima at about 490 and 400 nm. The absorption bands did not change after adjusting the pH 0.5 units higher and lower (not shown) suggesting that we are not simply at the p*K* of the Schiff base. The chromophore is covalently bound to the opsin via a Schiff base linkage as both peaks collapse to a 440 nm band upon acid denaturation. The 440 nm maximum is characteristic of the spectrum of a protonated retinyl Schiff base free in solution (*17*).

Because our working hypothesis was that the bulkier Trp at position 118 would force the pigment into a more open conformation, we tested the integrity of the Schiff base linkage of the ligand to hydroxylamine assault. Unlike with wild-type rhodopsin where the absorption spectrum of the pigment in the dark is unaffected by the presence of hydroxylamine (*18*), the spectrum of the T118W does change with time in the presence of hydroxylamine (Figure 3A).

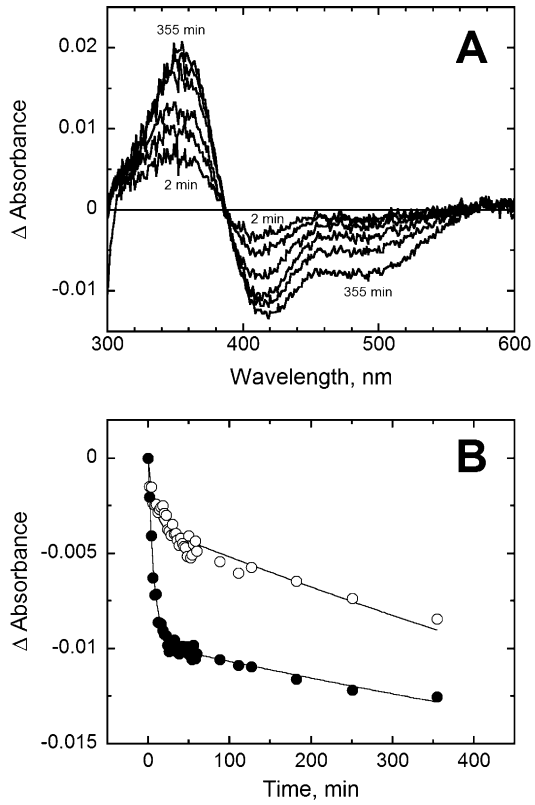

FIGURE 3: Mutant pigment instability in hydroxylamine. (A) Difference spectra of 1 μM T118W pigment in the presence of 50 mM hydroxylamine. A volume of 5 μL of a stock of 1 M hydroxylamine solution was added to 95 μL of purified T118W pigment, and spectra were recorded at room temperature. The spectrum of the pigment without hydroxylamine was then subtracted. Shown are representative difference spectra recorded at 2, 4, 8, 30, 88, and 355 min after addition of hydroxylamine. (B) Time course of pigment loss at 420 nm (filled circles) and 490 nm (open circles). Both traces could be fit with two exponentials with time constants of 7 and 1800 min. The amount of pigment loss for the 490 nm species at 355 min was determined to be 30% based on an assumed 100% pigment loss from a bleached spectrum taken at the end of the experiment.

The shorter wavelength species decayed considerably faster (exponential time constant of 7 min) than the long-wavelength species (time constant of about 1800 min) (Figure 3B), but it appears both species are sensitive to hydroxylamine. Although we cannot completely exclude the possibility that hydroxylamine attacks only the shorter wavelength species which drives the longer wavelength species to shorter one via mass action, the vastly different decay rates with hydroxylamine and lack of spectral shifts with changes in pH would argue against it. Janz and Farrens (*19*) have also described rhodopsins containing mutations around the chromophore with increased sensitivity to hydroxylamine. These pigments were more open and less stable than wild-type rhodopsin in the dark.

T118W Is Dark Active. The T118W pigment is dark active (i.e., the pigment is able to activate transducin in the dark) (Figure 4). The activity is dependent on the presence of 11-cis retinal. More specifically, it is dependent on the presence of the 9-methyl group of 11-cis retinal. When the mutant pigment is formed with 9-demethyl 11-cis retinal where the 9-methyl group of retinal is missing, the purified pigment is now inactive in the dark but still able to activate transducin in a light-dependent manner. (Figure 4). Again, this result

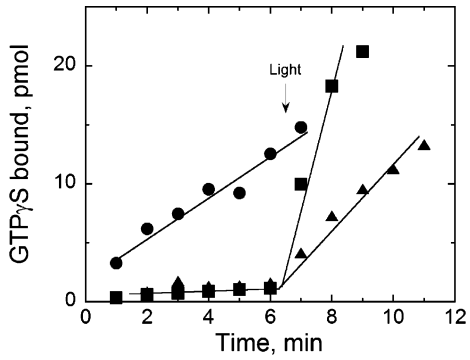

FIGURE 4: Activation of transducin by wild-type (squares), T118W (circles), and T118W regenerated with 9-demethyl retinal (triangles) rhodopsin pigments. At 6.5 min, samples were illuminated with a 12 s pulse of white light.

is consistent with the T118W mutation interacting with 11-cis retinal in a steric manner. Other less bulky residues at this position also resulted in a dark-active pigment (not shown), but the most robust activity was produced with Trp.

That the 9-demethyl retinal containing mutant pigment is able to be light-activated might seem counterintuitive because the 9-methyl group has been implicated as being critical to the formation of the active Meta II intermediate in wild-type rhodopsin (*1*, *20*). While initial spectroscopic and activity measurements showed that rhodopsin containing 9-demethyl retinal bleaches to form very little Meta II (*1*), subsequent studies demonstrated that Meta II does form with the Meta I to Meta II equilibrium shifted to favor the inactive Meta I species (*4*, *5*). The presence of transducin (*5*) or other compensatory mutations (*21*) can shift the equilibrium to form more Meta II. Recent studies have shown that rhodopsin containing 11-cis 9-demethyl retinal is readily able to activate transducin in a light-dependent manner especially in the presence of higher concentrations of transducin (*5*, *20*, *22*). Thus, the 9-methyl group of retinal may help in the efficient formation of the Meta II intermediate in wild-type rhodopsin, but it is not necessary for rhodopsin to activate transducin. The T118W rhodopsin mutant containing 9-demethyl retinal is inactive like wild-type rhodopsin containing 9-demethyl retinal, and thus both are able to activate transducin in a light-dependent manner. More importantly, light-dependent activation of the 9-demethyl retinal containing mutant indicates that the pigment in the dark is a functional and inactive protein, not merely a denatured and dead protein. This is in contrast to the same mutant pigment formed with 11-cis retinal which is very active in the dark.

In order to determine whether or not the apoprotein itself is constitutively active, we measured the ability of wild-type and T118W opsins from COS cell membrane preparations to activate transducin before and after addition of 11-cis retinal. The pH of these measurements is 6.5 where the differences between transducin activation by wild-type opsin and by pigment are more easily distinguishable than at pH values above 7 (*16*, *23*). Figure 5 shows that the apoproteins of both samples are essentially equally inactive. When 11-cis retinal is added to the samples, however, the two proteins behave quite differently. Wild-type rhodopsin becomes less active as is expected for an inverse agonist and in agreement with Cohen et al. (*16*). On the other hand, 11-cis retinal is an agonist to the T118W mutant protein. T118W is not

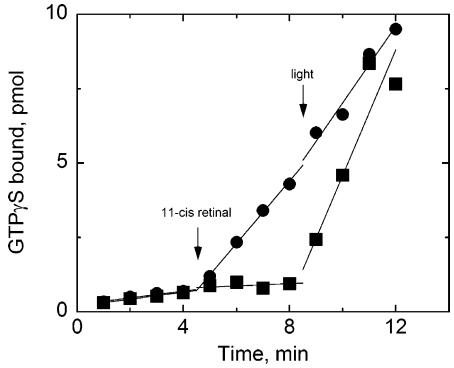

FIGURE 5: Activation of transducin by wild-type (squares) and T118W (circles) opsins at pH 6.5. Wild-type and T118W opsins in COS cell membranes were tested for their ability to activate transducin. At 4.5 min, 11-cis retinal is added to the reaction mixture. At 8.5 min, the samples are illuminated with >495 nm light for 12 s.

constitutively active, but it is dark active when bound with 11-cis retinal.

Constitutively and Dark-Active Rhodopsin Mutations. Previous reports showed that visual pigments required the presence of multiple mutations to be dark active, and often these dark-active mutants were less active than their apoproteins (*9*, *24*, *25*). Rhodopsins containing a mutation that affects an internal salt bridge between the highly conserved Glu113 and Lys296 (*16*, *23*) either by neutralizing one of the charges or adding a negative charge nearby (e.g., G90D) have been shown to be constitutively active; in other words, these mutants are able to activate transducin in a light- and ligand-independent manner (see the review by Rao and Oprian *26*). Binding of an 11-cis form of the ligand can deactivate these opsins. Similarly, some opsins with mutations not near the salt bridge, such as E134Q (*27*) and M257Y (*24*), are also constitutively active and deactivated upon binding 11-cis retinal. However, the cytoplasmic loops of these mutant holoproteins have adopted a structure more akin to the light-activated structure as determined by electron spin resonance studies on spin-labeled rhodopsin mutants (*25*, *28*). Double mutants comprised of one mutation that perturbs the salt bridge and one that alters the cytoplasmic surface conformation such as E113Q/M257Y and G90D/M257Y are both constitutively active and dark active relative to wild-type opsin and pigment activity (*24*, *25*). Interestingly, the dark-active mutant pigments described by Han et al. (*24*), E113Q/E134Q, E113Q/M257A, E113Q/M257N, E113Q/M257Y, E113Q/F261V, and E134Q/M257A, are all less active than their respective apoproteins indicating that 11-cis retinal still acts as an inverse agonist, and not an agonist.

On the other hand, in this study the apoprotein does not appear to be constitutively active, and pigment formation enables the protein to activate transducin in the dark. The 11-cis retinal ligand is clearly acting as an agonist now, whereas the 11-cis 9-demethyl retinal is an inverse agonist.

CONCLUSION

We have introduced steric bulk in the chromophore binding site of rhodopsin around the 9-methyl group of 11-cis retinal by replacing T118 with a Trp in the protein to form a dark-active pigment. The dark-active state is dependent on the presence of chromophore and, more specifically, the presence of the 9-methyl group. In addition to the dark activity, the covalent Schiff base linkage of the ligand is susceptible to attack by hydroxylamine. The bound ligand in wild-type rhodopsin does not react with hydroxylamine in the dark but does after exposure to light. Our interpretation is that steric interaction between the 9-methyl group of 11-cis retinal and Trp holds the protein in an open and active conformation and thus what was once an inverse agonist behaves as an agonist.

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REFERENCES

1. Ganter, U. M., Schmid, E. D., Perez-Sala, D., Rando, R. R., and Siebert, F. (1989) Removal of the 9-methyl group of retinal inhibits signal transduction in the visual process. A Fourier transform infrared and biochemical investigation, *Biochemistry 28*, 5954−5962.
2. Shieh, T., Han, M., Sakmar, T. P., and Smith, S. O. (1997) The steric trigger in rhodopsin activation, *J. Mol. Biol. 269*, 373−384.
3. Han, M., Groesbeek, M., Sakmar, T. P., and Smith, S. O. (1997) The C9 methyl group of retinal interacts with glycine-121 in rhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 94*, 13442−13447.
4. Vogel, R., Fan, G.-B., Sheves, M., and Siebert, F. (2000) The molecular origin of the inhibition of transducin activation in rhodopsin lacking the 9-methyl group of the retinal chromophore: A UV−vis and FTIR spectroscopic study, *Biochemistry 39*, 8895−8908.
5. Meyer, C. K., Böhme, M., Ockenfels, A., Gärnter, W., Hofmann, K. P., and Ernst, O. P. (2000) Signaling states of rhodopsin: Retinal provides a scaffold for activating proton transfer switches, *J. Biol. Chem. 275*, 19713−19718.
6. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal structure of rhodopsin: a G protein-coupled receptor, *Science 289*, 739−745.
7. Okada, T., Sugihara, M., Bondar, A.-N., Elstner, M., Entel, P., and Buss, V. (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 Å crystal structure, *J. Mol. Biol. 342*, 571−583.
8. Li, J., Edwards, P. C., Burghammer, M., Villa, C., and Schertler, G. F. X. (2004) Structure of bovine rhodopsin in a trigonal crystal, *J. Mol. Biol. 343*, 1409−1438.
9. Kono, M., Crouch, R. K., and Oprian, D. D. (2005) A dark and constitutively active mutant of the tiger salamander UV pigment, *Biochemistry 44*, 799−804.
10. Janz, J. M., and Farrens, D. L. (2001) Engineering a functional blue-wavelength-shifted rhodopsin mutant, *Biochemistry 40*, 7219−7227.
11. Patel, A. B., Crocker, E., Eilers, M., Hirshfeld, A., Sheves, M., and Smith, S. O. (2004) Coupling of retinal isomerization to the activation of rhodopsin, *Proc. Natl. Acad. Sci. U.S.A. 101*, 10048−10053.
12. Franke, R. R., Sakmar, T. P., Oprian, D. D., and Khorana, H. G. (1988) A single amino acid substitution in rhodopsin (lysine 248 → leucine) prevents activation of transducin, *J. Biol. Chem. 263*, 2119−2122.
13. Oprian, D. D. (1993) Expression of opsin genes in COS cells, *Methods Neurosci. 15*, 301−306.
14. Reeves, P. J., Hwa, J., and Khorana, H. G. (1999) Structure and function in rhodopsin: Kinetic studies of retinal binding to purified opsin mutants in defined phospholipid−detergent mixtures serve as probes of the retinal binding pocket, *Proc. Natl. Acad. Sci. U.S.A. 96*, 1927−1931.
15. Robinson, P. R. (2000) Assays for the detection of constitutively active opsins, *Methods Enzymol. 315*, 207−218.

16. Cohen, G. B., Oprian, D. D., and Robinson, P. R. (1992) Mechanism of activation and inactivation of opsin: role of Glu^{113} and Lys^{296}, *Biochemistry 31*, 12592−12601.
17. Morton, R. A., and Pitt, G. A. J. (1955) Studies on rhodopsin. 9. pH and the hydrolysis of indicator yellow, *Biochem. J. 59*, 128−134.
18. Wald, G., Brown, P. K., and Smith, P. H. (1955) Iodopsin, *J. Gen. Physiol. 38*, 623−681.
19. Janz, J. M., and Farrens, D. L. (2004) Role of retinal hydrogen bond network in rhodopsin Schiff base stability and hydrolysis, *J. Biol. Chem. 279*, 55886−55894.
20. Han, M., Groesbeek, M., Smith, S. O., and Sakmar, T. P. (1998) Role of the C_9 methyl group in rhodopsin activation: Characterization of mutant opsins with the artificial chromophore 11-*cis*-9-demethylretinal, *Biochemistry 37*, 538−545.
21. Fritze, O., Filipek, S., Kuksa, V., Palczewski, K., Hofmann, K. P., and Ernst, O. P. (2003) Role of the conserved $NPxxY(x)_{5,6}F$ motif in the rhodopsin ground state and during activation, *Proc. Natl. Acad. Sci. U.S.A. 100*, 2290−2295.
22. Das, J., Crouch, R. K., Ma, J.-x., Oprian, D. D., and Kono, M. (2004) Role of the 9-methyl group of retinal in cone visual pigments, *Biochemistry 43*, 5532−5538.
23. Robinson, P. R., Cohen, G. B., Zhukovsky, E. A., and Oprian, D. D. (1992) Constitutively active mutants of rhodopsin, *Neuron 9*, 719−725.
24. Han, M., Smith, S. O., and Sakmar, T. P. (1998) Constitutive activation of opsin by mutation of methionine 257 on transmembrane helix 6, *Biochemistry 37*, 8253−8261.
25. Kim, J.-M., Altenbach, C., Kono, M., Oprian, D. D., Hubbell, W. L., and Khorana, H. G. (2004) Structural origins of constitutive activation in rhodopsin: Role of the K296/E113 salt bridge, *Proc. Natl. Acad. Sci. U.S.A. 101*, 12508−12513.
26. Rao, V. R., and Oprian, D. D. (1996) Activating mutations of rhodopsin and other G protein-coupled receptors, *Annu. Rev. Biophys. Biomol. Struct. 25*, 287−314.
27. Cohen, G. B., Yang, T., Robinson, P. R., and Oprian, D. D. (1993) Constitutive activation of opsin: influence of charge at position 134 and size at position 296, *Biochemistry 32*, 6111−6115.
28. Kim, J.-M., Altenbach, C., Thurmond, R. L., Khorana, H. G., and Hubbell, W. L. (1997) Structure and function in rhodopsin: Rhodopsin mutants with a neutral amino acid at E134 have a partially activated conformation in the dark state, *Proc. Natl. Acad. Sci. U.S.A. 94*, 14273−14278.

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