BIOCHEMISTRY

 Volume 47, Number 41 October 14, 2008

Accelerated Publications

E113 Is Required for the Efficient Photoisomerization of the Unprotonated Chromophore in a UV-Absorbing Visual Pigment[†]

Kei Tsutsui, Hiroo Imai,[‡] and Yoshinori Shichida*

Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

Received July 23, 2008; Revised Manuscript Received September 8, 2008

ABSTRACT: Protonation of the retinal Schiff base chromophore is responsible for the absorption of visible light and is stabilized by the counterion residue E113 in vertebrate visual pigments. However, this residue is also conserved in vertebrate UV-absorbing visual pigments (UV pigments) which have an unprotonated Schiff base chromophore. To elucidate the role played by this residue in the photoisomerization of the unprotonated chromophore in UV pigments, we measured the quantum yield of the E113Q mutant of the mouse UV cone pigment (mouse UV). The quantum yield of the mutant was much lower than that of the wild type, indicating that E113 is required for the efficient photoisomerization of the unprotonated chromophore in mouse UV. Introduction of the E113Q mutation into the chicken violet cone pigment (chicken violet), which has a protonated chromophore, caused deprotonation of the chromophore and a reduction in the quantum yield. On the other hand, the S90C mutation in chicken violet, which deprotonated the chromophore with E113 remaining intact, did not significantly affect the quantum yield. These results suggest that E113 facilitates photoisomerization in both UV-absorbing and visible light-absorbing visual pigments and provide a possible explanation for the complete conservation of E113 among vertebrate UV pigments.

It is important for vision that the phototransduction cascade in photoreceptor cells efficiently converts a photon signal to an electrical signal. The photon is initially captured by visual pigments which are embedded in the membrane of the photoreceptors. A visual pigment consists of a protein moiety (opsin) and a chromophore (11-*cis*-retinal), which covalently binds to K296[1] in transmembrane helix 7 of the opsin via a Schiff base linkage. The chromophore becomes excited upon photon absorption and undergoes a quite efficient cis−trans photoisomerization reaction (*1*), followed by a conformational change in the opsin and an activation of the G protein transducin (*2*).

Most visual pigments, including rhodopsins, absorb maximally in the visible region of light. The visible light sensitivity of the visual pigments is achieved by protonation of the Schiff base of the retinal chromophore. The protonated Schiff base is stabilized by a "counterion" residue, E113, in bovine rhodopsin (*3*−*5*) and presumably in all other vertebrate visible light-absorbing pigments (*6*−*8*). In contrast,

[†] This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (19045018) and for Scientific Research (S) (20227002) and a Grant for the Global Center of Excellence Program "Formation of a Strategic Base for Biodiversity and Evolutionary Research: From Genome to Ecosystem" from the Japanese Ministry of Education, Culture, Sports, Science, and Technology to Y.S. K.T. was supported by a Japanese Society for the Promotion of Science Research Fellowship for Young Scientists.

* To whom correspondence should be addressed. Telephone: +81-75-753-4213. Fax: +81-75-753-4210. E-mail: shichida@rh.biophys.kyoto-u.ac.jp.

[‡] Present address: Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan.

[1] The numbers of all amino acid residues in this paper are based on the bovine rhodopsin numbering system.

10.1021/bi801377v CCC: $40.75
Published on Web 09/20/2008

Table 1: Experimental Conditions and Photoreactive Properties of Visual Pigments

pigment	pH	λ_{max}[a] (nm)	method[b]	C_{NH_2OH}[c] (mM)	λ_{irrad}[d] (nm)	ε_{irrad}[e] ($M^{-1}cm^{-1}$)	S[f]	φ[g]
bRh[h] WT[i]	6.5	500	UV−vis/HPLC	48	500	40600[j]	1.00	0.65[k]
bRh E113Q[o]	5.5	500	UV−vis	4.8	500	35600 (1000)[l]	0.87 (0.02)	0.65 (0.02)
	8.2	385	HPLC	4.8	390	38100 (1000)	0.61 (0.03)	0.42 (0.02)
mUV[m] WT[o]	6.5	358	HPLC	9.5	359	43600 (1200)	0.84 (0.06)	0.51 (0.04)
mUV F86Y	6.5	420	UV−vis	2.4	417	39400 (800)	0.67 (0.01)	0.45 (0.01)
mUV E113Q	6.5	353	HPLC	2.4	359	44800 (1100)	0.51 (0.09)	0.30 (0.05)
cV[n] WT	6.5	415	UV−vis	9.5	417	39900 (900)	0.83 (0.06)	0.55 (0.04)
cV S90C	6.5	370	HPLC	2.4	359	36900 (900)	0.65 (0.10)	0.47 (0.07)
cV E113Q	6.5	350	HPLC	2.4	359	45700 (800)	0.44 (0.01)	0.25 (0.01)

[a] Wavelength at the absorption maximum. [b] Method for estimating the photosensitivity. [c] Final concentration of hydroxylamine used in the photosensitivity measurement. [d] Irradiation wavelength. [e] Molar extinction coefficient at λ_{irrad}. [f] Photosensitivity at λ_{irrad} (relative to wild-type bovine rhodopsin). [g] Quantum yield at λ_{irrad}. [h] Bovine rhodopsin. [i] Wild type. [j] From ref *34*. [k] From ref *19*. [l] The values in parentheses are standard deviations calculated from two to four independent experiments. [m] Mouse UV. [n] Chicken violet. [o] Values from ref *11*.

UV-absorbing visual pigments (UV[2] pigments) have an unprotonated Schiff base chromophore (*9, 10*). Nevertheless, all of the known vertebrate UV pigments also have a glutamic acid residue at the same sites, and its role is not well understood.

In a previous study, we measured the quantum yield of photoisomerization in the mouse UV cone pigment (mouse UV) and found that the quantum yield was higher than that of the alkaline form of the E113Q mutant of bovine rhodopsin, which also had an unprotonated chromophore (*11*). This result suggested that the protein assists photoisomerization of the unprotonated chromophore in mouse UV.

The unexpected high quantum yield and the seemingly paradoxical conservation of E113 in mouse UV prompted us to test the possibility that E113 plays a role in the efficient photoisomerization of the unprotonated chromophore. In fact, introduction of the E113Q mutation into mouse UV resulted in a reduction of the quantum yield. At the same time, to investigate in more detail the influence of Schiff base protonation on the photoisomerization efficiency, we used the chicken violet-sensitive cone pigment (chicken violet) (*12*), which belongs to the same subgroup of vertebrate visual pigments as mouse UV (group S or SWS1) but is very likely to have a protonated chromophore. We introduced mutations that alter the protonation state of the Schiff base while keeping E113 intact in mouse UV and chicken violet (*9, 13*) and found that the quantum yields were not significantly affected. On the basis of these results, possible mechanisms of photoisomerization facilitated by E113 and implications of the conservation of E113 in UV pigments are discussed.

MATERIALS AND METHODS

Sample Preparation. The cDNAs of mouse UV (*14*) and chicken violet (*12*) were tagged by the Rho1D4 epitope sequence (*15*) and introduced into expression vectors pcDLSRα296 (*16*) and pUSRα (*17*), respectively. The cDNAs of site-directed mutants were constructed using the QuickChange mutagenesis kit (Stratagene). The wild-type and mutant opsins were expressed in the HEK 293T cell line and regenerated with 11-*cis*-retinal as previously reported (*18*). The reconstituted pigments were extracted with buffer A [1% (w/v) DM, 25 mM HEPES, 70 mM NaCl, and 1.5 mM $MgCl_2$ (pH 6.5)] and purified by adsorption on an antibody-conjugated column and elution with buffer B [0.3 mg/mL 1D4 peptide, 0.02% DM, 50 mM HEPES, 140 mM NaCl, and 3 mM $MgCl_2$ (pH 6.5)], unless noted otherwise.

Photosensitivities of the Visible Light-Absorbing Visual Pigments. The photosensitivities of wild-type chicken violet and the F86Y mutant of mouse UV were determined by UV−vis spectrophotometry using a Hitachi U-4100 spectrophotometer as previously described (*11*). Briefly, the sample was successively irradiated at 3 °C in the presence of hydroxylamine (the final concentrations of hydroxylamine are summarized in Table 1). For irradiation, light from a 1 kW tungsten lamp (Master HILUX-HR, Rikagaku) which had been passed through a band-path filter (KL42, Toshiba) which was centered at 417 nm (half-bandwidth of 18 nm) was used. Finally, the sample was completely bleached with >420 nm light (VY44 filter, Toshiba) to define the baseline. The absorbance change was monitored at 430 nm of the minus-baseline difference spectra. The amounts of residual pigment at each step of irradiation were determined from the absorbances after correction for hydroxylamine bleaching (see the Supporting Information of ref *11*), plotted on a semilogarithmic scale against the incident photon number and fitted to a single-exponential function. The slope of the fitting line relative to that of the wild-type bovine rhodopsin when it was irradiated at 500 nm (which was measured at the same time in each experiment) was defined as the photosensitivity of the pigment.

Photosensitivities of the UV-Absorbing Visual Pigments. The photosensitivities of the S90C mutant of chicken violet and the E113Q mutants of chicken violet and mouse UV were measured by chromophore extraction and HPLC analysis, as previously reported (*11*). Briefly, a single sample was divided into four aliquots and irradiated at 3 °C for incremental periods with light which had been passed through an interference filter (359 nm; half-bandwidth, 11 nm; Optical Coatings Japan) in the presence of 2.4 mM hydroxylamine. Then chromophore extraction and HPLC analysis were performed to determine the amounts of residual pigment in each sample. Again, the correction for hydroxylamine bleaching (see the Supporting Information of ref *11*) was made. The photosensitivity was defined as described above.

Molar Extinction Coefficients. The molar extinction coefficients were determined by the acid denaturation method, as previously described (*11*). Correction for the presence of two different protonation states, which was made in the previous paper (*11*), was not performed, because of the

[2] Abbreviations: UV, ultraviolet; UV pigment, ultraviolet-absorbing visual pigment; DM, dodecyl β-D-maltoside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; λ_{max}, wavelength at the absorption maximum; FTIR, Fourier transform infrared spectroscopy; QM/MM, quantum mechanical/molecular mechanical.

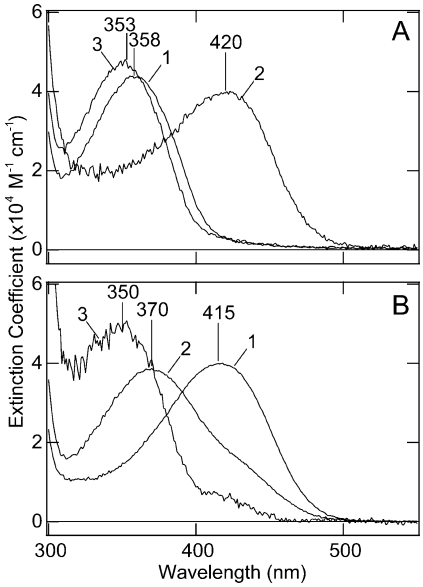

FIGURE 1: Absorption spectra of the visual pigments at pH 6.5 and 3 °C. Molar extinction coefficients were determined by the acid denaturation method. (A) Wild-type (curve 1, from ref *11*), F86Y mutant (curve 2), and E113Q mutant (curve 3) mouse UV. (B) Wild-type (curve 1), S90C mutant (curve 2), and E113Q mutant (curve 3) chicken violet. The λ_{max} values (nanometers) are indicated for each spectrum.

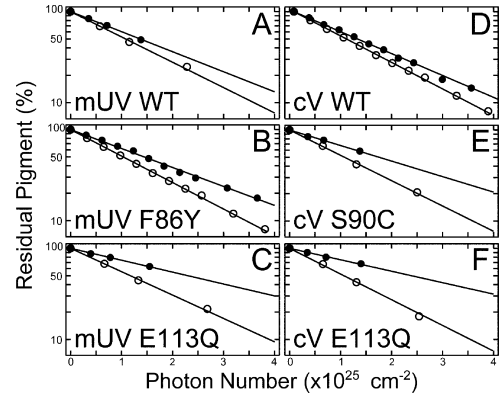

FIGURE 2: Photosensitivity measurements of the visual pigments. Irradiation wavelengths were 500, 417, and 359 nm for rhodopsin, violet pigments, and UV pigments, respectively. The amounts of residual pigments are plotted vs incident photon number on a semilogarithmic scale and fitted to an exponential function: (A) wild-type mouse UV (from ref *11*), (B) F86Y mutant of mouse UV, (C) E113Q mutant of mouse UV, (D) wild-type chicken violet, (E) S90C mutant of chicken violet, and (F) E113Q mutant of chicken violet. In each panel, white circles and black circles represent data for the wild-type bovine rhodopsin and the pigment being tested, respectively. A representative set of data from two to four experiments is shown for each panel. Photosensitivities were determined from the slopes of the fitting lines relative to that of wild-type rhodopsin. Photosensitivity values are summarized in Table 1.

difficulty in changing the protonation state with pH. However, each pigment appeared to contain almost exclusively the protonated or unprotonated state (>90%), and the effect of the correction on quantum yield should be relatively small (<10%). The values are summarized in Table 1.

Quantum Yields. The quantum yields (φ) of visual pigments were calculated using the following relationship (*11*):

$$S \propto \varepsilon\varphi$$

where S and ε are the photosensitivity and molar extinction coefficient, respectively. The values of quantum yields were determined relative to that of wild-type bovine rhodopsin [0.65 (*19*)].

RESULTS

For the sake of clarity, the molar extinction coefficients and the photosensitivity values are not described in the text (all values are summarized in Table 1).

Quantum Yields of the E113Q Mutants. The λ_{max} of the E113Q mutant of the mouse UV cone pigment (mouse UV) was located at 353 nm, which was blue-shifted from that of the wild type by 5 nm, as previously reported (*7, 9, 20*) (curve 3 in Figure 1A). The photosensitivity, which was defined as the slope of the fitting line (Figure 2C), was reduced compared with that of the wild type (Figure 2A). The quantum yield of the E113Q mutant was 0.30 ± 0.05, which was significantly ($p < 0.01$) lower than that of the wild type (0.51 ± 0.04) (*11*). This result demonstrated that E113 is required for the efficient photoisomerization of the unprotonated chromophore in mouse UV.

We also measured the quantum yields of the wild type and the E113Q mutant of the chicken violet cone pigment (chicken violet), which belongs to the same subgroup (S or SWS1) of the vertebrate visual pigments as mouse UV (*12*). Chicken violet is very likely to have a protonated chromophore, because its λ_{max} is located in the visible region, and protonation of the chromophore has been demonstrated by FTIR spectroscopy in the *Xenopus* violet cone pigment, which is phylogenetically closely related to chicken violet (*21*). The wild type had its λ_{max} at 415 nm (curve 1 in Figure 1B), as previously reported (*13, 22, 23*). In contrast, the E113Q mutant had its λ_{max} at 350 nm (curve 3 in Figure 1B), which was consistent with an unprotonated Schiff base. This confirmed that the chromophore was protonated in the wild-type chicken violet, with E113 being the counterion (*7*). The mutant contained almost exclusively the unprotonated form at neutral pH as opposed to the E113Q mutant of bovine rhodopsin which contained approximately the same amount of both forms (*11*). It has been reported that this is also the case for the D113A, -G, -N, and -H mutants of *Xenopus* violet (*7*), the F86Y/E113Q double mutant of mouse UV (*9*), and the mouse UV mutant with seven mutations (which enable violet sensitivity) and E113Q (*20*). The successful purification of the E113Q mutant of chicken violet is in contrast to the failures of the E113Q (D113Q) mutants of human, bovine, and *Xenopus* violet pigments to bind their chromophores (*9, 20, 24*). This discrepancy may be due to differences in the conditions or may be associated with differences in binding pocket conformation among these pigments. The photosensitivity of wild-type chicken violet (Figure 2D) was similar to that of mouse UV (Figure 2A). The quantum yield of wild-type chicken violet was calculated to be 0.55 ± 0.04, which was similar to that of mouse UV (0.51 ± 0.04). On the other hand, the E113Q mutant exhibited reduced photosensitivity (Figure 2F). The quantum yield was 0.25 ± 0.01, which was significantly ($p < 0.01$) lower than that of the wild type, indicating that E113 is also required for the efficient photoisomerization in chicken violet.

Quantum Yields of Mutant Pigments with an Altered Protonation State. We then generated two mutant pigments in which the protonation state of the Schiff base was altered:

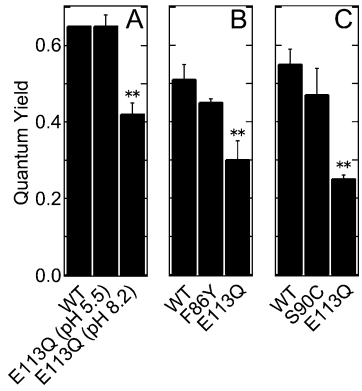

FIGURE 3: Quantum yields of visual pigments. (A) Bovine rhodopsin (from ref *11*). (B) Mouse UV (the value for the wild type is from ref *11*). (C) Chicken violet. Error bars denote standard deviations ($n = 2-4$). Asterisks denote significant difference compared with the value of the wild type ($p < 0.01$). Quantum yield values are also summarized in Table 1.

the F86Y mutant of mouse UV (*9*), which had a protonated chromophore, and the S90C mutant of chicken violet (*13*), which was suggested by a QM/MM calculation to have an unprotonated chromophore (*25*). The F86Y mutant of mouse UV (curve 2 in Figure 1A) and the S90C mutant of chicken violet (curve 2 in Figure 1B) had their λ_{max} values at 420 and 370 nm, respectively, as previously reported (*9, 13*). The S90C mutant of chicken violet had a shoulder around 450 nm, which could not be eliminated by changing the pH (5.3−7.5) of the solution (data not shown), as previously reported (*13*).

We then measured the quantum yields of these mutant pigments. The photosensitivities were slightly reduced (Figure 2B,E) compared with those of the respective wild-type pigments (Figure 2A,D). The quantum yields were calculated to be 0.45 ± 0.01 for the F86Y mutant of mouse UV and 0.47 ± 0.07 for the S90C mutant of chicken violet. These values were slightly lower than those of the respective wild-type pigments (0.51 ± 0.04 for mouse UV and 0.55 ± 0.04 for chicken violet), but the reductions were not significant ($p > 0.01$). These observations suggest that the protonation state of the Schiff base does not markedly affect the efficiency of photoisomerization in mouse UV and chicken violet. All the results are summarized in Figure 3 and Table 1.

DISCUSSION

Effect of the E113Q Mutation on the Photoisomerization Efficiency of Mouse UV, Chicken Violet, and Bovine Rhodopsin. The reduced quantum yield of the E113Q mutant (Figure 3B) clearly shows that E113 is required for the efficient photoisomerization of mouse UV. On the other hand, the results obtained with the F86Y mutant (Figure 3B) suggest that the protonation state of the Schiff base does not significantly affect the photoisomerization efficiency in mouse UV.

The E113Q mutation also reduced the quantum yield in chicken violet (Figure 3C). In chicken violet, however, the effect of the E113Q mutation is rather complicated, because under our conditions the introduction of the E113Q mutation results simultaneously in the deprotonation of the Schiff base. That is, both the deprotonation of the Schiff base and the loss of E113 could be responsible for the reduced quantum yield of the mutant. However, the latter interpretation is favored by the current finding that the S90C mutation, which is very likely to deprotonate the Schiff base (*25*), did not significantly affect the quantum yield while the E113Q mutation greatly reduced the quantum yield in chicken violet (Figure 3C). These results suggest that the reduced quantum yield of the E113Q mutant is due to the loss of E113 itself rather than the deprotonation of the Schiff base.

On the other hand, the previously reported findings that the E113Q mutant of bovine rhodopsin exhibited a quantum yield almost identical to that of the wild type at pH 5.5 and the lower quantum yield at pH 8.2 (Figure 3A) (*11*) seem to be consistent with the hypothesis that the protonation state of the Schiff base affects the quantum yield. In fact, in our previous paper, we interpreted this result as being due to the difference in the protonation state of the Schiff base. However, this observation can also be interpreted otherwise (see below).

Although the precise mechanism is unknown, our conclusion is that E113 is required for the efficient photoisomerization in UV-absorbing visual pigments and probably in visible light-absorbing pigments. Birge, Knox, and colleagues have previously reported that the E113Q mutant of mouse UV exhibited a reduced amount of photoproduct compared with the amount of the wild type when irradiated at cryotemperatures (*26*), and the quantum yield of the D113A mutant of *Xenopus* violet was lower than that of wild-type mouse UV (*27*). These observations are consistent with our results.

Possible Mechanisms of the Facilitation of Photoisomerization by E113. On the basis of the experimental evidence that E113 is essential for efficient photoisomerization, one could consider several mechanisms by which photoisomerization would be facilitated.

One of the mechanisms could be the presence of a negative point charge near the chromophore. In fact, several experimental and theoretical studies have pointed out the impact of a negative point charge on the photoisomerization dynamics in retinal proteins (*28*−*30*). E113 is negatively charged in bovine rhodopsin and presumably in chicken violet. E113 in mouse UV is considered to be protonated in the dark state (*10*), but direct evidence of this has not been obtained. The results obtained with the E113Q mutant of bovine rhodopsin (see above) can also be interpreted in terms of this negative charge hypothesis. It has been shown that a chloride ion from the bulk solution acts as the counterion in the acidic form of the E113Q mutant of bovine rhodopsin (*31*), and this may substitute for the negative charge of E113 (*32*), leading to the wild-type-like efficient photoisomerization.

Alternatively, E113 may affect photoisomerization through a steric or polar interaction rather than an electrostatic one. It is also possible that E113 contributes to the quantum yield by maintaining an appropriate conformation and/or hydrogen-bonding network in the binding pocket which enables the efficient photoisomerization.

To distinguish among these possibilities, experiments using mutants which contain other amino acids at position 113 are now in progress.

Role of E113 in UV Pigments. The counterion residue E113 is essential for the absorption of visible light in visual pigments. If its function as a counterion were critical, E113

would not seem to be required for an unprotonated chromophore, but this residue is actually conserved among all known vertebrate UV pigments. It has been proposed that E113 in UV pigments plays multiple roles, as a counterion in a photointermediate (*26*), in the regulation of the meta II lifetime (*20, 26*), and in the suppression of the constitutive activity of opsin (*33*). This study suggests another role for E113 in UV pigments, facilitation of photoisomerization of the unprotonated chromophore. This may be important particularly for increasing the absolute sensitivity of UV-sensitive photoreceptors. In other words, if one assumes a hypothetical photoreceptor cell containing visual pigments whose quantum yield is reduced to the same extent as we observed with the E113Q mutant (almost halved), it would be expected to have half the absolute sensitivity of an intact photoreceptor. This would be likely to seriously impair the visual ability of the animal.

ACKNOWLEDGMENT

We thank Dr. S. Koike for providing us with the HEK 293T cell line, Prof. F. Tokunaga for providing us with a pUSRα expression vector, and Prof. R. S. Molday for the generous gift of a Rho1D4-producing hybridoma. We are also grateful to Drs. Y. Imamoto and T. Yamashita for valuable discussions and Dr. E. Nakajima for her critical reading of our manuscript and invaluable comments.

REFERENCES

1. Kandori, H., Shichida, Y., and Yoshizawa, T. (2001) Photoisomerization in rhodopsin. *Biochemistry (Moscow, Russ. Fed.) 66*, 1197–1209.
2. Shichida, Y., and Morizumi, T. (2007) Mechanism of G-protein activation by rhodopsin. *Photochem. Photobiol. 83*, 70–75.
3. Zhukovsky, E. A., and Oprian, D. D. (1989) Effect of carboxylic acid side chains on the absorption maximum of visual pigments. *Science 246*, 928–930.
4. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309–8313.
5. Nathans, J. (1990) Determinants of visual pigment absorbance: Identification of the retinylidene Schiff's base counterion in bovine rhodopsin. *Biochemistry 29*, 9746–9752.
6. Ebrey, T. G. (2000) pKa of the protonated Schiff base of visual pigments. *Methods Enzymol. 315*, 196–207.
7. Babu, K. R., Dukkipati, A., Birge, R. R., and Knox, B. E. (2001) Regulation of phototransduction in short-wavelength cone visual pigments via the retinylidene Schiff base counterion. *Biochemistry 40*, 13760–13766.
8. Nickle, B., Wilkie, S. E., Cowing, J. A., Hunt, D. M., and Robinson, P. R. (2006) Vertebrate opsins belonging to different classes vary in constitutively active properties resulting from salt-bridge mutations. *Biochemistry 45*, 7307–7313.
9. Fasick, J. I., Applebury, M. L., and Oprian, D. D. (2002) Spectral tuning in the mammalian short-wavelength sensitive cone pigments. *Biochemistry 41*, 6860–6865.
10. Dukkipati, A., Kusnetzow, A., Babu, K. R., Ramos, L., Singh, D., Knox, B. E., and Birge, R. R. (2002) Phototransduction by vertebrate ultraviolet visual pigments: Protonation of the retinylidene Schiff base following photobleaching. *Biochemistry 41*, 9842–9851.
11. Tsutsui, K., Imai, H., and Shichida, Y. (2007) Photoisomerization efficiency in UV-absorbing visual pigments: Protein-directed isomerization of an unprotonated retinal Schiff base. *Biochemistry 46*, 6437–6445.
12. Okano, T., Kojima, D., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1992) Primary structures of chicken cone visual pigments: Vertebrate rhodopsins have evolved out of cone visual pigments. *Proc. Natl. Acad. Sci. U.S.A. 89*, 5932–5936.
13. Yokoyama, S., Radlwimmer, F. B., and Blow, N. S. (2000) Ultraviolet pigments in birds evolved from violet pigments by a single amino acid change. *Proc. Natl. Acad. Sci. U.S.A. 97*, 7366–7371.
14. Onishi, A., Hasegawa, J., Imai, H., Chisaka, O., Ueda, Y., Honda, Y., Tachibana, M., and Shichida, Y. (2005) Generation of knock-in mice carrying third cones with spectral sensitivity different from S and L cones. *Zool. Sci. 22*, 1145–1156.
15. Oprian, D. D., Asenjo, A. B., Lee, N., and Pelletier, S. L. (1991) Design, chemical synthesis, and expression of genes for the three human color vision pigments. *Biochemistry 30*, 11367–11372.
16. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) SR α promoter: An efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol. 8*, 466–472.
17. Kayada, S., Hisatomi, O., and Tokunaga, F. (1995) Cloning and expression of frog rhodopsin cDNA. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 110*, 599–604.
18. Imai, H., Terakita, A., and Shichida, Y. (2000) Analysis of amino acid residues in rhodopsin and cone visual pigments that determine their molecular properties. *Methods Enzymol. 315*, 293–312.
19. Kim, J. E., Tauber, M. J., and Mathies, R. A. (2001) Wavelength dependent cis-trans isomerization in vision. *Biochemistry 40*, 13774–13778.
20. Shi, Y., Radlwimmer, F. B., and Yokoyama, S. (2001) Molecular genetics and the evolution of ultraviolet vision in vertebrates. *Proc. Natl. Acad. Sci. U.S.A. 98*, 11731–11736.
21. Kusnetzow, A., Dukkipati, A., Babu, K. R., Singh, D., Vought, B. W., Knox, B. E., and Birge, R. R. (2001) The photobleaching sequence of a short-wavelength visual pigment. *Biochemistry 40*, 7832–7844.
22. Fager, L. Y., and Fager, R. S. (1981) Chicken blue and chicken violet, short wavelength sensitive visual pigments. *Vision Res. 21*, 581–586.
23. Okano, T., Fukada, Y., Artamonov, I. D., and Yoshizawa, T. (1989) Purification of cone visual pigments from chicken retina. *Biochemistry 28*, 8848–8856.
24. Fasick, J. I., Lee, N., and Oprian, D. D. (1999) Spectral tuning in the human blue cone pigment. *Biochemistry 38*, 11593–11596.
25. Yokoyama, S., Starmer, W. T., Takahashi, Y., and Tada, T. (2006) Tertiary structure and spectral tuning of UV and violet pigments in vertebrates. *Gene 365*, 95–103.
26. Kusnetzow, A. K., Dukkipati, A., Babu, K. R., Ramos, L., Knox, B. E., and Birge, R. R. (2004) Vertebrate ultraviolet visual pigments: Protonation of the retinylidene Schiff base and a counterion switch during photoactivation. *Proc. Natl. Acad. Sci. U.S.A. 101*, 941–946.
27. Ramos, L. S., Chen, M. H., Knox, B. E., and Birge, R. R. (2007) Regulation of photoactivation in vertebrate short wavelength visual pigments: Protonation of the retinylidene Schiff base and a counterion switch. *Biochemistry 46*, 5330–5340.
28. Song, L., El-Sayed, M. A., and Lanyi, J. K. (1993) Protein Catalysis of the Retinal Subpicosecond Photoisomerization in the Primary Process of Bacteriorhodopsin Photosynthesis. *Science 261*, 891–894.
29. Lenz, M. O., Woerner, A. C., Glaubitz, C., and Wachtveitl, J. (2007) Photoisomerization in proteorhodopsin mutant D97N. *Photochem. Photobiol. 83*, 226–231.
30. Cembran, A., Bernardi, F., Olivucci, M., and Garavelli, M. (2004) Counterion controlled photoisomerization of retinal chromophore models: A computational investigation. *J. Am. Chem. Soc. 126*, 16018–16037.
31. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1991) The role of the retinylidene Schiff base counterion in rhodopsin in determining wavelength absorbance and Schiff base pKa. *Proc. Natl. Acad. Sci. U.S.A. 88*, 3079–3083.
32. Logunov, S. L., el-Sayed, M. A., and Lanyi, J. K. (1996) Catalysis of the retinal subpicosecond photoisomerization process in acid purple bacteriorhodopsin and some bacteriorhodopsin mutants by chloride ions. *Biophys. J. 71*, 1545–1553.
33. Kono, M. (2006) Constitutive activity of a UV cone opsin. *FEBS Lett. 580*, 229–232.
34. Wald, G., and Brown, P. K. (1953) The molar extinction of rhodopsin. *J. Gen. Physiol. 37*, 189–200.

BI801377V