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Rapid Release of Retinal from a Cone Visual Pigment following Photoactivation

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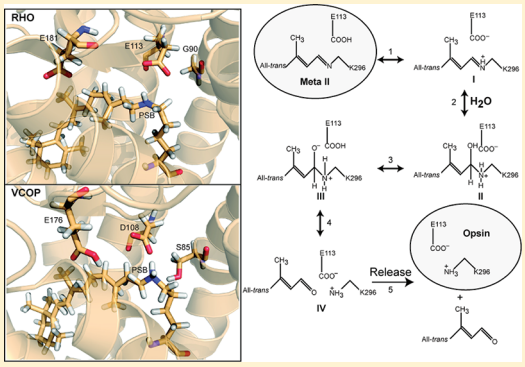
Supporting Information

ABSTRACT: As part of the visual cycle, the retinal chromophore in both rod and cone visual pigments undergoes reversible Schiff base hydrolysis and dissociation following photobleaching. We characterized light-activated release of retinal from a short-wavelength-sensitive cone pigment (VCOP) in 0.1% dodecyl maltoside using fluorescence spectroscopy. The half-time ($t_{1/2}$) of release of retinal from VCOP was 7.1 s, 250-fold faster than that of rhodopsin. VCOP exhibited pH-dependent release kinetics, with the $t_{1/2}$ decreasing from 23 to 4 s with the pH decreasing from 4.1 to 8, respectively. However, the Arrhenius activation energy (E_a) for VCOP derived from kinetic measurements between 4 and 20 °C was 17.4 kcal/mol, similar to the value of 18.5 kcal/mol for rhodopsin. There was a small kinetic isotope (D_2O) effect in VCOP, but this effect was smaller than that observed in rhodopsin. Mutation of the primary Schiff base counterion ($VCOP^{D108A}$) produced a pigment with an unprotonated chromophore (λ_{max} = 360 nm) and dramatically slowed ($t_{1/2} \sim 6.8$ min) light-dependent retinal release. Using homology modeling, a VCOP mutant with two substitutions (S85D and D108A) was designed to move the counterion one α-helical turn into the transmembrane region from the native position. This double mutant had a UV−visible absorption spectrum consistent with a protonated Schiff base (λ_{max} = 420 nm). Moreover, the $VCOP^{S85D/D108A}$ mutant had retinal release kinetics ($t_{1/2}$ = 7 s) and an E_a (18 kcal/mol) similar to those of the native pigment exhibiting no pH dependence. By contrast, the single mutant $VCOP^{S85D}$ had an ∼3-fold decreased retinal release rate compared to that of the native pigment. Photoactivated $VCOP^{D108A}$ had kinetics comparable to those of a rhodopsin counterion mutant, Rho^{E113Q}, both requiring hydroxylamine to fully release retinal. These results demonstrate that the primary counterion of cone visual pigments is necessary for efficient Schiff base hydrolysis. We discuss how the large differences in retinal release rates between rod and cone visual pigments arise, not from inherent differences in the rate of Schiff base hydrolysis but rather from differences in the properties of noncovalent binding of the retinal chromophore to the protein.



Vision in most vertebrates is functionally duplex.[1] Scotopic and photopic vision, mediated by rods and cones, respectively, have disparate sensitivity, spectral tuning, temporal properties, and recovery rates following exposure to light.[2−4] This division also occurs among the homologous phototransduction proteins that contribute to differences in cellular physiology, biochemistry, and phototransduction, although the molecular basis underlying these differences is still emerging. A primary focus has been the visual pigments, which consist of a seven-transmembrane α-helical bundle (opsin) and an 11-*cis*-retinal chromophore covalently attached via a Schiff base linkage.[5,6] The visual pigments determine the spectral sensitivity of a particular photoreceptor and share roughly 50% amino acid sequence identity.[7] The X-ray structures of rhodopsin[8] and related photobleaching conformations[9−12] have permitted a unique opportunity to understand the activation of G protein-coupled receptors (GPCRs) through the combination of computational approaches and a large repertoire of experimental approaches (recently reviewed[13]).

The primary isomerization event and subsequent photoreactions have been intensively studied.[14−17] Crystallography and UV−visible, ESR, and FTIR spectroscopy have led to a model of the photoactivation pathway in which the steric strain of all-*trans*-retinal drives the movement of extracellular loop EL2 and transmembrane helices H5 and H6 and the disruption of ionic interactions in the transmembrane bundle, termed the ionic lock.[13] This framework has been applied to other members of the GPCR class[18] and thus forms a unifying principle for the activation of these proteins by ligands. However, the molecular basis for functional differences between the visual pigments is not yet understood.

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There are four visual pigment properties that contribute to the distinctive photoresponses of rods and cones. First, the wavelength of light (λ_{max}) to which the pigment is maximally sensitive varies from the UV[19] to the far red.[20] Spectral tuning is regulated by the protonation state of the Schiff base,[21,22] noncovalent interactions distributed between multiple amino acid side chains with the polyene chain of retinal,[23] and structural water in the retinal binding pocket.[8,24] Second, the relative instability of the dark-adapted pigment influences the rate of thermal isomerization and thus activation of the phototransduction cascade in the dark, thus setting the absolute noise level.[25] Thus, cones express a more labile visual pigment and exhibit much more dark noise than rods.[26] In fact, rods transgenically expressing a cone opsin have more dark noise in either transgenic *Xenopus* or mice.[27] Following photoactivation, all-*trans*-retinal is eventually released from opsin after Schiff base hydrolysis,[28,29] a reduction of the pigment concentration, and visual sensitivity. The third property of visual pigments that contributes to rod−cone specialization is the lifetime of light-activated conformation R* (coinciding with *meta II*), which sets a constraint on the maximum number of transducins that can be activated. In fact, photoreceptors with transgenes having shorter *meta II* lifetimes produce smaller photoresponse amplitudes.[30,31] In rods, R* activity is terminated by downstream mechanisms before the Schiff base is hydrolyzed.[32] By contrast, the R*-*meta II* lifetime in cone pigments is much shorter, and this may contribute to the smaller photoresponse of the cones.[33] To reset the phototransduction cascade during response recovery and dark adaptation, regeneration of the visual pigment occurs via formation of a new covalent bond with 11-*cis*-retinal.[28] Fourth, rod and cone pigments have significantly different regeneration rates.[34] This contributes to variation between photoreceptor types in the overall adaptive changes to and recovery from steady light.[2,35,36] In summary, the biochemical differences between visual pigments are consistent with the hypothesis that visual pigments contribute to the differences in photoresponse sensitivity and kinetics between rods and cones, including their adaptation to background light.

The molecular determinants of the photochemical and biochemical properties of visual pigments have been investigated by site-specific mutagenesis of evolutionarily conserved amino acid residues. A primary focus has been the amino acids in the environment of the retinal−Schiff base linkage,[13] particularly in comparative studies of chicken rhodopsin and a green-sensitive cone pigment.[37] The Schiff base has been extensively studied in rhodopsin, where it has been shown to have multiple functions, including suppression of the constitutive activity of opsin, facilitation of regeneration, stabilization of Schiff base linkage, photoisomerization of spectral tuning as mentioned above, and a role in the determination of photointermediate lifetime.[21]

These studies identified a single amino acid (Rho^{E122}, using bovine rhodopsin numbering) that influences the rate of *meta II* decay and lifetime of light-activated conformation R*. In the *Xenopus* short-wavelength cone pigment (VCOP), mutation of the Schiff base counterion (Asp^{108}) causes a deprotonation of the Schiff base and stabilization of the R* conformation.[38] Counterion mutations show similar behaviors in UV-sensitive cone pigments, despite the fact that the Schiff base is normally deprotonated in the dark.[39] Additional amino acids, in particular a highly conserved proline (Pro^{189}) in extracellular loop 2, are important for the thermal stability of both dark-adapted and light-activated cone pigments.[40] These results emphasize the central importance of the chromophore−protein linkage for inactivating the light-activated conformation, and its significant contribution to the differences between rod and cone visual pigments. However, mechanistic insight is needed to explain the role of the cone pigment Schiff base counterion in retinal release.

In this study, we investigate the mechanism of release of retinal from light-activated VCOP in comparison to that of rhodopsin. We use the intrinsic fluorescence of opsin and its quenching by bound retinal to directly monitor interactions of retinal with opsin.[41] We demonstrate that retinal release in VCOP is ∼250-fold faster than in rhodopsin at neutral pH, but the Arrhenius activation energy of Schiff base hydrolysis is similar for both of them, implying a conserved reaction mechanism. We show that the counterion (Asp^{108}) is essential for rapid release following photoactivation and that movement of the counterion to a nearby position restores the rate to that of the native pigment. Furthermore, we show that the rate of release of the retinal chromophore from rod and cone visual pigments is the same in the absence of a counterion, indicating that the primary counterion is the catalytic residue required for Schiff base hydrolysis.

MATERIALS AND METHODS

Visual Pigment Expression and Purification. The epitope-tagged VCOP plasmids used for site-directed mutagenesis and protein expression have been described previously.[38] The mutants were expressed in COS1 cells by transient transfection, purified by immunoaffinity chromatography, and quantified by UV−visible spectroscopy. Except for Rho^{E113Q}, pigments were eluted in buffer Y1 [50 mM HEPES, 140 mM NaCl, and 3 mM $MgCl_2$ (pH 6.6)] with 0.1% *N*-dodecyl β-D-maltoside and 20% glycerol. Rho^{E113Q} was eluted in modified buffer Y1 at pH 8.0 with 0.1% *N*-dodecyl β-D-maltoside and 20% glycerol to deprotonate the Schiff base.

Fluorescence Spectroscopy. The procedure was modified from that of Farrens et al.[41] A FluoroMax-3 instrument (Jobin Yvon, Inc., Edison, NJ), fitted with a UV bandpass filter (U-360, Edmund Optics, Barrington, NJ) mounted in the sample chamber to eliminate >410 nm light to prevent the pigment from being bleached, was used to measure fluorescence changes. Protein samples (200 μL of 0.5 μM) were suspended in buffer A (0.1% *N*-dodecyl β-D-maltoside in 10 mM MES/Na_2HPO_4) at three different pH values (4.1, 6.0, and 8.0). Fluorescence was recorded from samples in a 0.2 mL quartz cuvette thermally controlled at 4, 6.7, 10, 12.5, 15, 18, and 20.4 °C with a circulating water bath (C6 Lauda). The excitation wavelength was 295 nm (slit width of 0.25 nm) and emission at 330 nm (slit width of 12 nm). There was noticeable pigment bleaching from the excitation beam provided in the FluoroMax-3 instrument, so a neutral density filter was introduced to minimize pigment loss during the experiment. This reduced the level of bleaching so that no correction of the fluorescence kinetic trace was needed (i.e., single-exponential fits to the initial phase of the responses were sufficient). Data from VCOP samples were integrated for 80 ms at 250 ms intervals, with the emission shutter constantly open. Data from rhodopsin samples were integrated for 2 s at 10 s intervals, with the emission shutter closed between collection periods. Samples were photoactivated using an external light source (model 66906 Research Arc Source, Newport Corp., Irvine, CA) containing a 200 W ozone-free Hg/Xe arc lamp (model 6292, Newport

Corp.). An IR cutoff filter (C53-711, Edmund Optics) was used; light was delivered to the cuvette via a quartz fiber optic light pipe (C38-955, Edmund Optics). Complete photoactivation of the samples was accomplished by illumination for either 500 ms (VCOP) or 25 s (rhodopsin). The half-time of the fluorescence change ($t_{1/2}$) was measured as previously described.[41,42] All fits in rhodopsin showed an r^2 value of >0.95, while in VCOP, the r and r^2 values were greater than 0.80 and 0.65, respectively.

Measurement of Fluorescence in D_2O. Water in purified samples was replaced with D_2O (Cambridge Isotope Laboratory, Inc.) at 4 °C by repetitive dilution and concentration using a Centricon YM-30 concentrator (Millipore). Fluorescence measurements were performed at 10 °C on protein diluted in buffer A at pH 4.1, 6.0, or 8.0 prepared with D_2O.

Homology Models. Homology models for $VCOP^{S85D/D108A}$ and $VCOP^{S85D}$ were generated according to the procedures previously described.[43−45]

RESULTS

Rate of Release of Retinal of VCOP. VCOP has a λ_{max} of 427 nm when incubated with 11-*cis*-retinal (Figure 1A) that

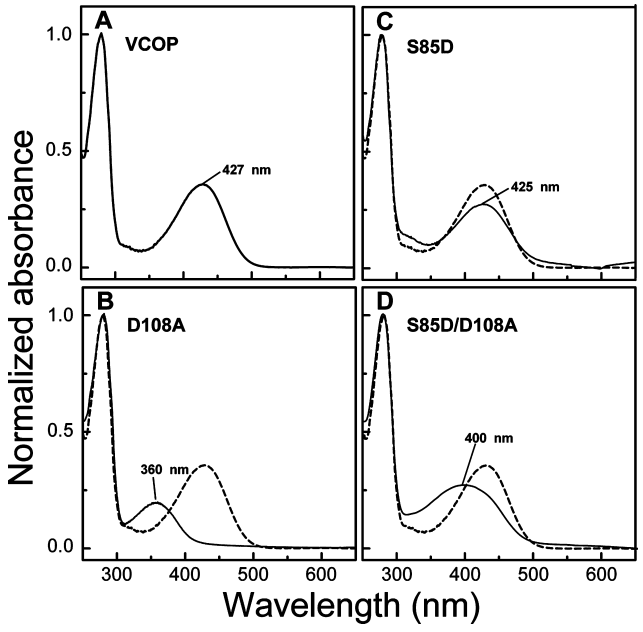

Figure 1. UV−vis spectroscopy of VCOP substitution mutants. Visual pigments were purified in dodecyl maltoside following addition of 11-*cis*-retinal. UV−vis spectra were obtained for wild-type VCOP (A), primary counterion mutant $VCOP^{D108A}$ (B), $VCOP^{S85D}$ (C), and the counterion replacement double mutant, $VCOP^{S85D/D108A}$ (D). The λ_{max} (±1 nm) is indicated for each pigment, and the VCOP spectrum (···) is included for comparison.

arises from a protonated Schiff base linkage.[23,46] Illumination of the pigment causes a conversion in seconds to an unprotonated Schiff base with a λ_{max} of 360 nm.[47] We measured the rate of retinal release following light activation at 10 °C and pH 6.0. The fluorescence increased rapidly following a bright flash and recovered slightly before reaching a steady state (Figure 2A). The fluorescence change was monophasic over the first 25 s (Figure 2A, inset). A single-exponential fit to the initial rising phase produced a half-time for the increase in fluorescence

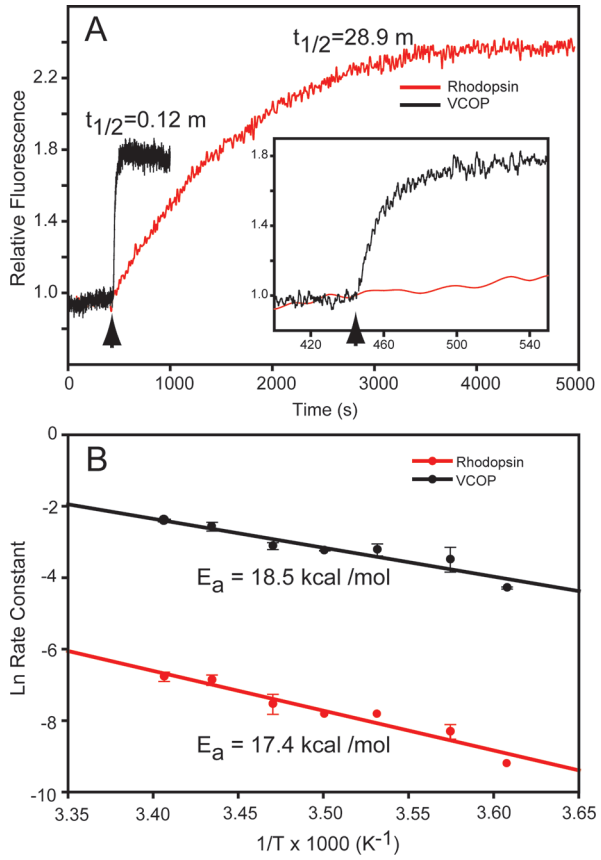

Figure 2. Changes in fluorescence intensity and Arrhenius activation energies following photobleaching. (A) Kinetics of the fluorescence increase at 330 nm of VCOP (black) and rhodopsin (red) after illumination with a 2 s flash of white light (arrow). The times to reach the half-maximal fluorescence intensity ($t_{1/2}$) were 28.9 and 0.12 min, respectively, at 10 °C in a solution containing 0.1% dodecyl maltoside (pH 6.0). The inset shows an expanded time scale of the fluorescence increase of VCOP. For recording fluorescence, the excitation was at 295 nm and the emission at 330 nm. Fluorescence was normalized to the initial value (set to 1). (B) Arrhenius plot of the natural log of the rates of fluorescence increase in illuminated rhodopsin and VCOP samples between 4 and 20 °C at pH 6.0. Activation energies (E_a), as indicated in the figure, were calculated from the negative reciprocal of the slope of the best fit linear regression line (—).

($t_{1/2}$) of 0.12 min. Changes in ionic strength from 0 to 300 mM NaCl had an only minor effect on the retinal release rates, ranging from 7.1 ± 2.0 to 14.5 ± 3.9 s, respectively (data not shown). In similar experiments, rhodopsin exhibited a much slower monophasic increase following a bright flash, with a $t_{1/2}$ of 28.9 min, in agreement with previous reports using this assay.[41] Thus, release of retinal from VCOP was 240-fold faster than that in rhodopsin under these conditions.

The rates of retinal release were determined at seven different temperatures ranging from 4 to 20 °C at pH 6.0. Arrhenius plots were utilized to estimate the activation energy of Schiff base hydrolysis (Figure 2B). The slopes from the plots obtained by linear regression produced activation energies of 18.5 kcal/mol for rhodopsin and 17.4 kcal/mol for VCOP. These results show that while the rates of retinal release are dramatically different, the activation energies for retinal release, which includes both Schiff base hydrolysis and all-*trans*-retinal dissociation, are very similar for both cone and rod visual pigments.

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Alteration of the Schiff Base Counterion. Computational models suggest that Ser^{85} and Asp^{108} form an electrostatic network around the Schiff base in VCOP.[48] Mutations of Ser^{85} and Asp^{108} not only caused spectral shifts of absorbance maxima but also destabilized photoactivation intermediates.[38,48] We investigated whether Ser^{85} could stabilize the protonated Schiff base in the absence of the primary counterion D^{108}. The single-mutant counterion $VCOP^{D108A}$ has an unprotonated Schiff base with a λ_{max} of 353 nm (Figure 1B; see also ref 38). Addition of another Asp in the Schiff base environment ($VCOP^{S85D}$) causes a minor blue shift in λ_{max} to 425 nm and a small accompanying broadening of the absorbance spectrum (Figure 1C). Position 85 is in a helix adjacent to Asp^{108} and approximately one turn of an α-helix toward the cytoplasmic face. This approach has been taken previously with bovine rhodopsin.[49,50] Thus, the mutant $VCOP^{S85D/D108A}$ has a potential counterion in a different location compared to the wild-type protein. This double-mutant pigment has a λ_{max} at 400 nm and a broad absorbance spectrum with significant intensity at >425 nm. The anomalous absorbance spectrum suggests inhomogeneous broadening and is not consistent with an unprotonated Schiff base.[51] Thus, removal of the counterion from its normal position and reintroduction nearby produce a pigment with a stabilized protonated Schiff base, although the chromophore environment is not entirely restored to that of a normal visual pigment.

A Schiff Base Counterion Is Required for Rapid Retinal Release. We investigated the role of the Schiff base counterion in retinal release by measuring the kinetics of light-induced fluorescence quenching for several counterion mutants at 10 °C and pH 6.0. We used 350 nm illumination to photobleach $VCOP^{D108A}$ because it has an absorbance maximum at 353 nm. For this mutant, no change in fluorescence was detected (Figure 3). Next, we tested the mutant with the counterion reintroduced toward the cytoplasmic face, $VCOP^{S85D/D108A}$. After irradiation with white light for 0.5 s, $VCOP^{S85D/D108A}$ exhibited a rate similar to that of VCOP, with a half-time of 6.9 s. Introduction of a second Asp into the Schiff base environment ($VCOP^{S85D}$) slowed the rate to a half-time of 22.1 s. Thus, changing the primary counterion to Ser^{85} did not alter the retinal release rate, while removal or increasing the level of electrostatic stabilization around the Schiff base slowed release. In the primary counterion mutant, we did not observe any fluorescence changes after more than 15 min (Figure 3), even though the light-activated $VCOP^{D108A}$ is able to stimulate GTP exchange on transducin.[38] We also introduced a Glu acid in place of the Asp at position 108 and observed retinal release kinetics similar to wild-type kinetics (Figure 1 of the Supporting Information).

pH Dependence and Isotope Effect of Schiff Base Hydrolysis. The chromophore environment is more accessible to solvent (e.g., H_2O or NH_2OH) in VCOP and other cone visual pigments.[36,46] This suggests that solvent may contribute catalytically in the hydrolysis of the Schiff base in cone visual pigments. Thus, we investigated the effects of pH on the rate and activation energy of retinal release at three different pH values (4.1, 6.0, and 8.0). As a control, we performed experiments with rhodopsin purified from bovine retina. The rate of retinal release in rhodopsin is not dependent upon pH, although the activation energy decreases with an increase in pH (Table 1 and ref 42). By contrast, at 10 °C, the rate of release of retinal from VCOP increased significantly as the pH increased (Figure 4 and Table 1). Overall, the activation energy decreased

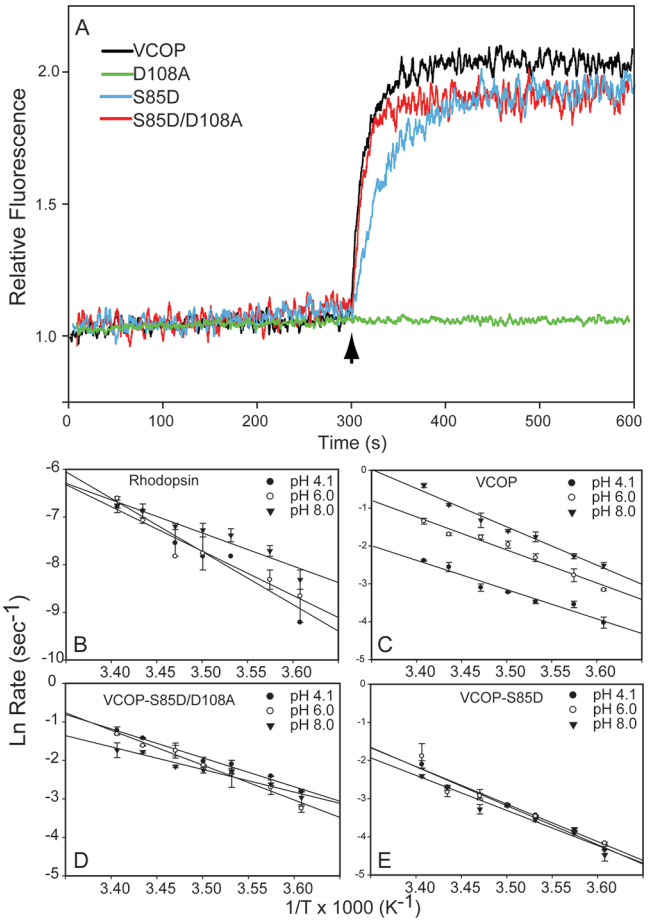

Figure 3. Fluorescence increases and activation energies of VCOP counterion mutants. (A) Fluorescence increases for VCOP (black), the primary counterion mutant $VCOP^{D108A}$ (green), $VCOP^{S85D}$ (blue), and the counterion replacement mutant $VCOP^{S85D/D108A}$ (red) were normalized individually to the initial fluorescence value (set to 1). Photobleaching was initiated after baseline fluorescence had been recorded by a 0.5 s flash of white light (arrow). The data were fit to a single exponential, and the half-time to reach the maximal level was calculated. The $t_{1/2}$ values were 7.1 s, 6.8 min, 22.1 s, and 6.9 s for VCOP, $VCOP^{D108A}$, $VCOP^{S85D}$, and $VCOP^{S85D/D108A}$, respectively. (B–E) Arrhenius plots of the rates of fluorescence increases in illuminated pigment samples between 4 and 20 °C at pH 4.1 (●), 6.0 (○), and 8.0 (▼). Activation energies are given in Table 1. Linear regression was used to generate the best fit lines (—).

Table 1. Retinal Release Rates and Activation Energies for Rho and VCOP (10 °C)

	pH	$t_{1/2}$ (s)	E_a (kcal/mol)
rhodopsin	4.1	1730	22.1
	6.0	1730	18.5
	8.0	1730	15.9
VCOP (D_2O)	4.1	22.9 (27.6)	15.4
	6.0	7.1 (8.6)	17.3
	8.0	4.0 (6.2)	19.9
$VCOP^{S85D/D108A}$	4.1	5.6	14.9
	6.0	6.9	18.0
	8.0	6.9	11.7
$VCOP^{S85D}$	4.1	21.4	20.3
	6.0	22.1	19.5
	8.0	22.8	18.3

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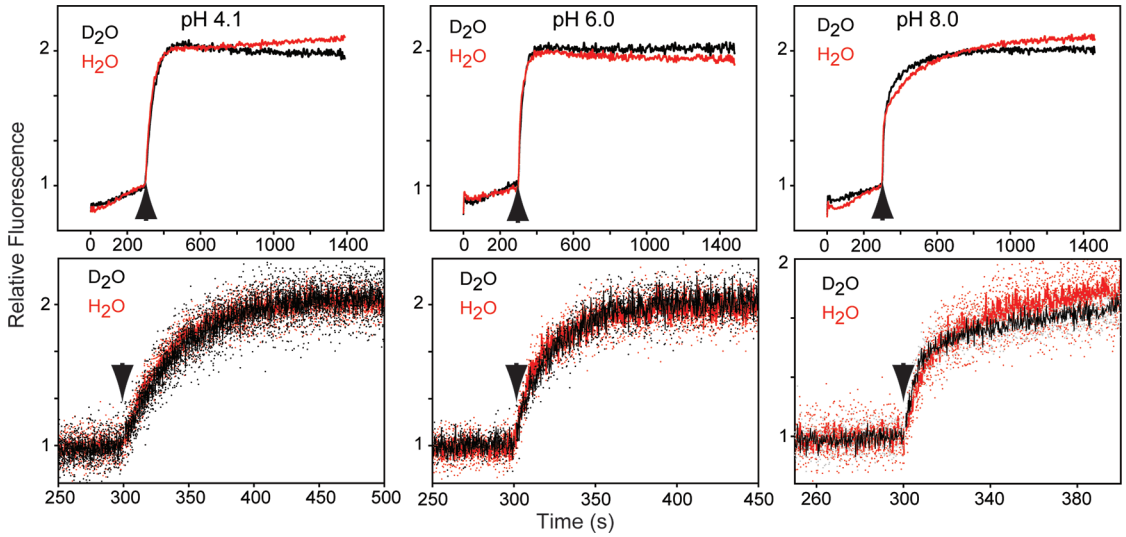

Figure 4. Kinetics of fluorescence increases of VCOP samples in H_2O (red) or D_2O (black) following photobleaching (arrow) at 10 °C and pH 4.1 (left), 6.0 (middle), or 8.0 (right). Bottom panels are an expanded time base of the top panels. Half-times are listed in Table 1.

~16% (Table 1). The rate of retinal release was measured in VCOP samples in which H_2O was replaced with D_2O (Figure 4) at 10 °C. Retinal release exhibited a similar trend of faster rates at elevated pD values. At pD 4.1 and 6.0, the half-times of retinal release were slightly increased 1.2-fold in D_2O (Table 1). At pD 8.0, the increase was slightly greater, 1.6-fold. Thus, in wild-type VCOP, the rate of retinal release shows a modest isotope effect, indicating proton transfer events may participate in Schiff base hydrolysis but are not the dominant catalytic moiety.

The rate of retinal release of the $VCOP^{S85D/D108A}$ mutant does not show a pH dependence in contrast to that of the wild-type protein (Table 1). However, the activation energy shows a complex behavior, with a peak at pH 6.0 and lower values at other pH values (Table 1). In the $VCOP^{S85D}$ mutant, neither the retinal release rate nor the activation energy was affected by pH (Table 1). However, compared to those of VCOP, there were a 3-fold increase in the rate and an elevated activation energy. In summary, these results show that mutations that alter interactions of hydrogen with the Schiff base significantly affect the pH dependence of retinal release.

Comparison of Counterion Mutants Rho^{E113Q} and $VCOP^{D108A}$. We compared the release of retinal on extended time frames from rod and cone visual pigments lacking a Schiff base counterion, Rho^{E113Q} and $VCOP^{D108A}$. Because the protonation state of the Schiff base in the Rho^{E113Q} mutant is determined by the pH,[52−54] we diluted the rhodopsin mutant in pH 8.0 buffer to deprotonate the Schiff base. For both counterion mutants, we used UV light, higher temperatures (20 °C), and extended photobleaching (25 s) to activate the unprotonated visual pigment. Remarkably, both Rho^{E113Q} and $VCOP^{D108A}$ exhibited very similar retinal release kinetics (Figure 5). We used three irradiation treatments, after which hydroxylamine was added to completely bleach the pigments. The average rate of retinal release for Rho^{E113Q} and $VCOP^{D108A}$ was 6.8 min. These observations strongly suggest that the primary counterion acts as the primary catalytic residue necessary for efficient Schiff base hydrolysis in both rod and cone visual pigments.

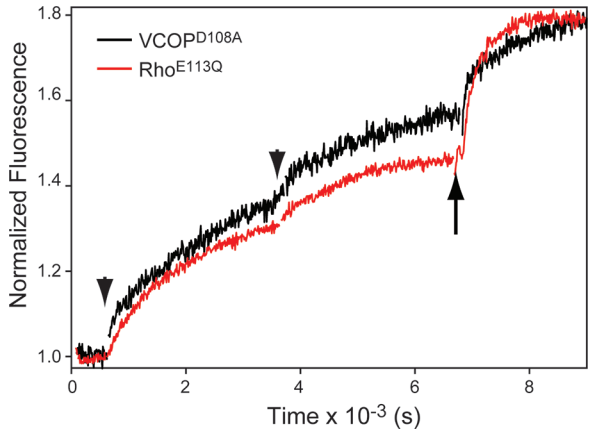

Figure 5. Comparison of the fluorescence increase of the bovine Rho^{E113Q} counterion mutant with $VCOP^{D108A}$ at 20 °C. The former is at pH 8.0 to deprotonate the Schiff base, and the latter is at pH 7.0. Two flashes were sequentially applied (arrowheads). The $t_{1/2}$ measured after after the first flash was 6.8 min for both samples. A final concentration of 10 mM hydroxylamine was added (arrow) to fully release all-*trans*-retinal. The fluorescence was normalized to the initial value (set to 1).

DISCUSSION

We have studied the fluorescence change during the decay of the *meta II* intermediate in VCOP and rhodopsin. We have found that the chromophore is more solvent accessible in the former, leading to a pH-dependent retinal release rate and activation energy of Schiff base hydrolysis. This study shows that the retinal release rate in VCOP is ~250-fold faster than in rhodopsin, which explains the faster decay of *meta II* in cone compared to rod pigments as reported by Govardovskii.[55] *Meta II* is the principal intermediate that couples to the G-protein transducin, starting the phototransduction cascade. R* is terminated in two ways: via phosphorylation and arrestin binding (see reviews in refs 32 and 56) and the release of all-*trans*-retinal to produce the apoprotein. Previous experiments have shown that cone *meta II* has a lower thermal stability than rhodopsin *meta II*.[34,37,47,57,58] The former has a short lifetime (~1 s), while the latter a much longer lifetime (>30 min). The

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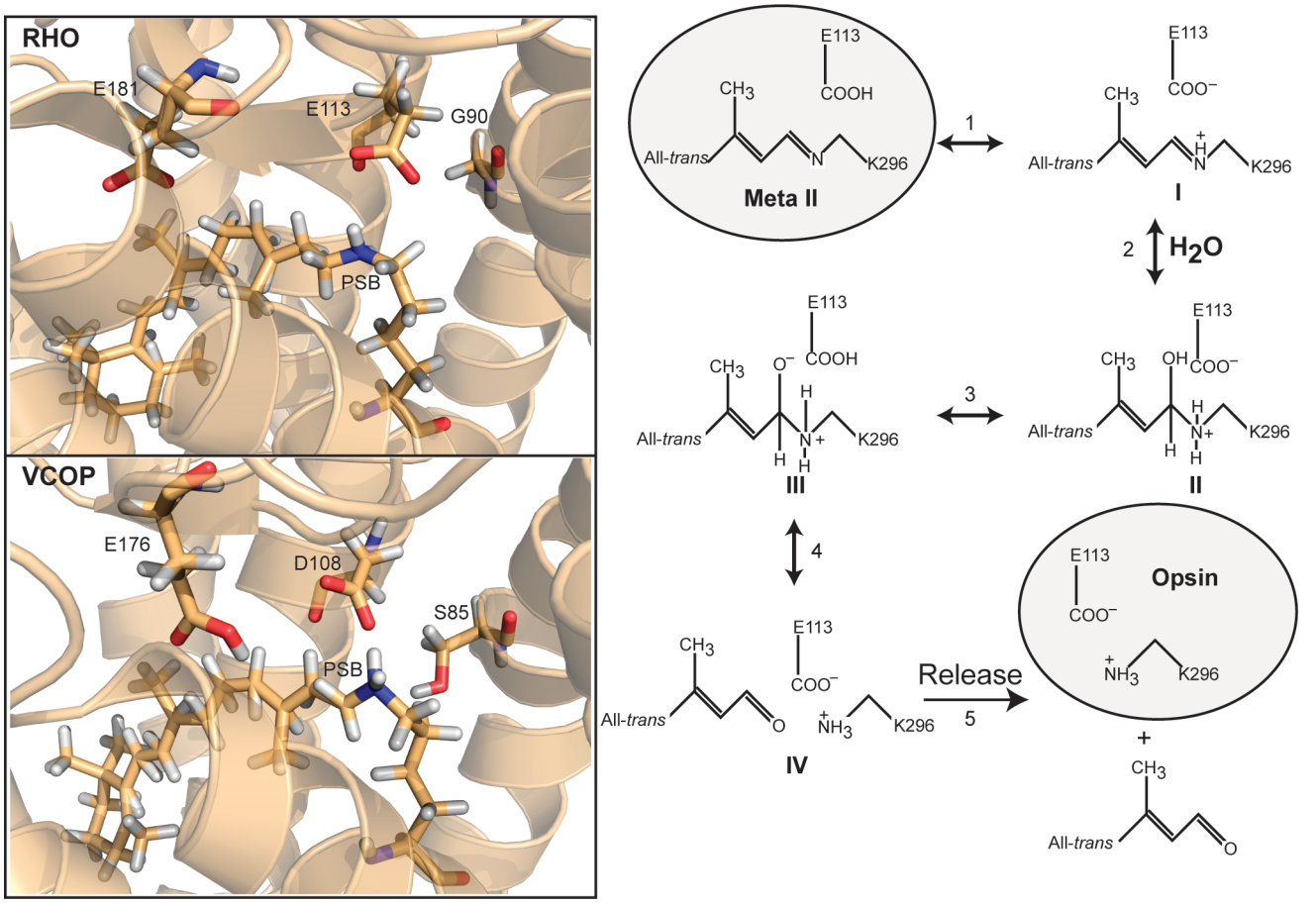

Figure 6. Three-dimensional models (left) of selected amino acids in the binding pockets in rhodopsin (RHO) and VCOP prepared using PyMOL.[68] Structural water has been omitted for the sake of clarity. The amino acid numbering is based upon individual protein sequences. The primary counterion is E113 for rhodopsin and D108 for VCOP. Proposed mechanism for Schiff base hydrolysis and retinal release (right). Two shaded conformations have been established experimentally, while the proposed intermediates are numbered **I**–**IV**. See Discussion for more details.

destabilized *meta II* in cone opsin may partially explain the physiological difference in late recovery between rods and cones. In humans, it takes >5 times longer for rods to recover following bright flashes than it does cones,[2,59] arising from the intrinsic difference between rhodopsin and cone opsin. Our results suggest that the hydrolysis of the Schiff base per se is similar in the two pigment classes, but we find support for the hypothesis that the molecular basis for these different properties resides primarily in the release of retinal from the light-activated visual pigments.

Mechanism of Schiff Base Hydrolysis. Following the mechanism for Schiff base hydrolysis discussed by Cooper et al.,[60] we propose a central catalytic role for the counterion in cone visual pigments (Figure 6). In this model, *meta II* has a deprotonated retinylidene Schiff base, protonated Glu^{113}/Asp^{108}, and undergoes a reversible and transient protonation, with the proton donor being Asp^{108}. Note that this state is distinct from *meta I*, which also has a similar configuration of Schiff base and counterion protonation states. There is evidence of such a state in rhodopsin, which may be either *meta* II_b,[61] *meta III*,[62] or a related post-*meta II* photoproduct. There is no direct evidence of such a species produced from photoactivation of cone visual pigments. However, given the similar activation energies for retinal release, the strong sequence homology among all visual pigments, and the known mechanisms for formation and hydrolysis of model Schiff base compounds,[60] it is reasonable to postulate that the formation of this intermediate (**I**) is the first step in hydrolysis. In **I**, the unprotonated Schiff base may be attacked by a water molecule (step 2) catalyzed by a nearby acidic residue (primary counterion) to form the protonated carbinolamine intermediate with a conformational change of the Schiff base from trigonal to tetrahedral, forming intermediate **II**. Abstraction of a proton by the acidic residue (step 3) produces the unstable intermediate **III** that decomposes (step 4), resulting in non-covalently bound all-*trans*-retinal, intermediate **IV**. Finally, the all-*trans* chromophore can dissociate from the protein (step 5), leaving the apoprotein with a deprotonated Glu^{113}/Asp^{108} ion-paired with Lys^{291}.

Experiments described in this paper support the proposed mechanism. The rate of retinal release in VCOP was increased by elevated pH, favoring the *meta II* conformation. Because the cone opsin has a more open chromophore environment,[46] acidic conditions may retard the proton transfer and formation of **I** in step 1 and/or the proton transfer in step 3, leading to slower retinal release. This also supports the idea that there is rapid exchange of solvent in the binding pocket, compared to that in rhodopsin. A kinetic isotope effect has been observed for rhodopsin and model Schiff base compounds.[63,64] However, we were unable to observe a similar effect for VCOP, finding a ratio of <1.2-fold compared to the ratio of 2.3–2.5-fold observed for rhodopsin. While we cannot rule out the existence

of nonexchangeable water molecules bound in the retinal binding pocket of VCOP, the sensitivity of the pigment to hydrolysis in the dark (by hydroxylamine and more slowly by water) suggests this is not likely. It is important to note that the rates of retinal release for VCOP are much faster than those of rhodopsin, and this may make it more difficult to observe an isotope effect. The Schiff base in VCOP may not readily re-form after hydrolysis, because it leaves the binding pocket so quickly. In rhodopsin, a substantial reverse reaction apparently occurs,[60] possibly because of sterically unfavorable conditions for release.[63] Moreover, hydrolysis of model Schiff compounds in detergent was studied under equilibrium conditions.[60] The similar activation energies, sequence homologies, and catalytic roles of the counterion all suggest that the catalytic mechanism of hydrolysis is quite similar in VCOP and rhodopsin. Thus, the lack of an observable kinetic isotope effect may reflect the absence of a significant reverse (Schiff base re-forming) step in cone pigments because of the rapid dissociation of retinal from the protein, compared to that of rhodopsin.

The primary counterion is necessary for rapid retinal release, as VCOP^{D108A} has very retarded kinetics. Substitution of an Asp in place of Ser^{85} restores near-normal retinal release in the counterion mutant. Given the proximity of these residues to the Schiff base (Figure 6) in the homology models, these results indicate that either the proton donor in step 1 or the proton acceptor in step 3 (or both) is the primary counterion. Although it is possible that two different amino acid side chains participate in each of these steps, the more likely scenario, given the behavior of VCOP$^{S85D/D108A}$, is that the primary counterion is the catalytically relevant residue as illustrated in Figure 6. Our homology model places Asp^{85} 2.6 Å deeper in the transmembrane bundle than Ala^{108}, placing the counterion in a more hydrophobic environment. This new environment would expel solvent more strongly and potentially stabilize the pK' of the counterion, thus rendering it less pH-sensitive. Additional support for the dual role of the counterion comes from the additional restraints imposed upon water molecules by both Asp^{108} and Asp^{85} (in VCOP^{S85D}). This mutant would require more free energy to alter the structure during Schiff base hydrolysis, leading to a slower rate of reaction in step 1. This is in fact observed in the retinal release measurements (Figure 3 and Table 1).

A remarkable prediction of this model is that the main catalytic power for Schiff base hydrolysis resides in the protonation of the counterion for both rod and cone visual pigments. When the primary counterion is neutralized, the reaction in step 1 is drastically slowed because of the absence of a proton donor. In such counterion mutants, the hydrolysis of the Schiff base is still mediated through a water. However, in a hydrophobic environment, the protonation of the Schiff base is not favorable, and thus, formation of the carbinolamine intermediate would be very slow, leading to a very slow hydrolysis rate. Because the formation of a Schiff base is expected to proceed by the reverse of the same reaction, re-formation would also be expected to be significantly inhibited in the counterion mutants. Thus, the re-formation of Meta II from non-covalently bound retinal in rhodopsin would not occur, and the proposed kinetic trap[63] would be removed. Thus, we predict that the rate-limiting step in retinal release for both rod and cone pigments in the absence of a primary counterion would become Schiff base hydrolysis. The slow rate of hydrolysis can be dramatically accelerated by hydroxylamine, which protonates the deprotonated Schiff base to initiate the nucleophilic attack in step 1. Deprotonated hydroxylamine absorbs the proton from the unstable carbinolamine intermediate and completes step 2 and step 3. This is supported in the retinal release experiments described here (Figure 5).

CONCLUSIONS

The primary counterion stabilizes the protonated Schiff base linkage in dark-adapted rod and cone visual pigments but is essential for normal Schiff base hydrolysis. Visual pigments without a counterion have extremely low rates of retinal release. Thus, even UV-sensitive visual pigments that have an unprotonated Schiff base[39,65] and thus do not require a counterion to neutralize the retinylidene linkage still require one for Schiff base hydrolysis and retinal release. Presumably, the reverse reaction, also known as pigment generation, will proceed through the reverse sequence of steps (Figure 6) and thus also require a primary counterion for efficient pigment formation. These experiments provide support for the primary role of the counterion at position 113 (rhodopsin numbering) in Schiff base hydrolysis and retinal release, and presumably in pigment formation in all vertebrate visual pigments. These results also are consistent with a number of previous reports about the primary role of the counterion in retinal−Schiff base chemistry.[63,66,67] Furthermore, our results provide evidence of the original suggestion that the counterion in bovine rhodopsin was necessary for efficient Schiff base hydrolysis.[52] The similar energetics of Schiff base hydrolysis and the catalytic mechanism for both rod and cone visual pigments strongly support the hypothesis that the intrinsic differences in retinal release between light-activated rod and cone pigments reside in the different dissociation rates for all-*trans*-retinal. This may be due to differences in interactions of the chromophore with retinal binding pocket side chains or in the accessibility of the non-covalently bound chromophore to pass through the seven-transhelix bundle. Finally, we note that these experiments were performed in dodecyl maltoside solutions, not in the native outer segment membrane. Future experiments should be directed toward examining how the native membrane could influence the release of all-*trans*-retinal or the binding of 11-*cis*-retinal to form the visual pigment.

ASSOCIATED CONTENT

Supporting Information

Kinetics of fluorescence increase following illumination of additional mutants VCOP^{D108E} and VCOP^{S85C} that alter the retinal Schiff base environment. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

VCOP, *Xenopus* short-wavelength-sensitive cone pigment; E_a, Arrhenius activation energy.

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