Regulation of Phototransduction in Short-Wavelength Cone Visual Pigments via the Retinylidene Schiff Base Counterion†

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ABSTRACT: Short-wavelength visual pigments (SWS1) have λ_{max} values that range from the ultraviolet to the blue. Like all visual pigments, this class has an 11-*cis*-retinal chromophore attached through a Schiff base linkage to a lysine residue of opsin apoprotein. We have characterized a series of site-specific mutants at a conserved acidic residue in transmembrane helix 3 in the *Xenopus* short-wavelength sensitive cone opsin (VCOP, $\lambda_{max} \sim 427$ nm). We report the identification of D108 as the counterion to the protonated retinylidene Schiff base. This residue regulates the pK_a of the Schiff base and, neutralizing this charge, converts the violet sensitive pigment into one that absorbs maximally in the ultraviolet region. Changes to this position cause the pigment to exhibit two chromophore absorbance bands, a major band with a λ_{max} of ∼352−372 nm and a minor, broad shoulder centered around 480 nm. The behavior of these two absorbance bands suggests that these represent unprotonated and protonated Schiff base forms of the pigment. The D108A mutant does not activate bovine rod transducin in the dark but has a significantly prolonged lifetime of the active MetaII state. The data suggest that in short-wavelength sensitive cone visual pigments, the counterion is necessary for the characteristic rapid production and decay of the active MetaII state.

Daytime vision occurs in cone photoreceptors. Even though rods and rhodopsin dominate our retina and have been the predominant subject of study, cones dominate our vision. Cones operate over a wide range of illumination intensities and spectral sensitivities from the ultraviolet to the red. Their response behavior is akin to those controlled by hormones or neurotransmitters, rather than to the rod single-photon detector. Moreover, cone visual pigments recycle rapidly in bright light.

The hallmark of visual pigment structure, one that distinguishes the opsins from all other G-protein-coupled receptors, is the covalent bond between the light sensitive ligand, 11-*cis*-retinal, and the opsin apoprotein, through a Schiff base linkage with the ϵ-amino group of a membrane-embedded lysine residue (*1*). This linkage enables the extremely rapid and efficient light-driven isomerization of the bound chromophore to *all-trans*-retinal and the concomitant storage of a substantial portion of the light energy in an excited protein conformation (*2*). The covalent occupancy of the ligand binding pocket stabilizes the apoprotein in a conformation that is unable to interact with other proteins in the phototransduction cascade, and thus is crucial for the regulation of photoreceptor noise and sensitivity (*3*). The covalent linkage is important in spectral tuning of visual pigments and, protonation of the Schiff base is necessary for visual pigments to have wavelength maxima above ∼440 nm. The regulation and role of Schiff base protonation in cone pigments has not been investigated.

The vertebrate visual pigments contain a conserved lysine residue in the seventh transmembrane helix (K296 in bovine rhodopsin and K291 in *Xenopus* violet and mouse UV opsins) to which the chromophore is attached. In bovine rhodopsin, the Schiff base is protonated and stabilized by a salt bridge mediated through a negatively charged counterion (E113) in transmembrane helix 3 (*4*). The E113 residue is unprotonated in the dark state and becomes protonated upon light-dependent formation of the metarhodopsin II (MII) state (*5*−*7*). Removal of the counterion results in a dramatic change in the pK_a of the Schiff base and light-independent activation of transducin and slowed formation of the later photointermediates (*8*−*10*). We have previously shown that the short-wavelength cone opsin (VCOP)[1] of *Xenopus* has a protonated Schiff base, while the mouse UV opsin has properties indicative of an unprotonated Schiff base (*11*, *12*). Here we show that the protonated Schiff base in short-wavelength visual pigment absorbing light in the violet (λ_{max}

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[1] Abbreviations: VCOP, violet cone opsin; SWS, short-wavelength sensitive.

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$\sim$ 427 nm) is stabilized by an acidic residue in the third transmembrane helix and that the interaction of this counterion with the Schiff base is crucial for the rapid photobleaching required for photopic vision.

MATERIALS AND METHODS

Site-Directed Mutagenesis and Visual Pigment Expression. Site-specific mutagenesis was carried out using the QuikChange mutagenesis kit (Stratagene). Mutants were prepared in a pBlueScript derivative, and the entire coding region from each mutant was sequenced to verify the mutation. The mutant cDNAs were cloned into the expression vector pMT-VCOP (*13*, *14*) for the transient expression in COS1 cells. The transfection and purification protocol was essentially as described previously (*11*, *13*, *14*) with the following modifications. The DNA used in the transfection was 15−23 μg/15 cm plate containing $\sim 1.2 \times 10^7$ cells. The COS1 cells from 15 plates were harvested 55 h after DNA addition, except for the D108E, G112D, and G112A mutants which were harvested at approximately 68 h. The washed cells were resuspended in 15 mL of buffer Y1 [50 mM HEPES, 140 mM NaCl, and 3 mM $MgCl_2$ (pH 6.6)], and 11-*cis*-retinal was added (final concentration of 5 μM). The cells were incubated overnight at 4 °C on a nutator. Following solubilization and incubation with 1D4 Sepharose (*15*) in a slurry for 2.5 h, the protein-bound resin was washed, loaded in a Poly-Prep column (0.8 cm × 4 cm, Bio-Rad), and eluted with 5 mL of the competing peptide (40 μM) in buffer Y1 with 0.1% DM and 20% glycerol. For some experiments, the pigment was exchanged into 10 mM NaP_i (pH 6.6) and 0.1% DM by washing it two times by ultrafiltration (Centricon YM-30, Amicon).

Absorption Spectroscopy and Illumination. UV−visible absorption spectra of pigment samples were recorded on a Beckman DU-640 single-beam spectrophotometer as described previously (*14*). Spectra were normalized as described in the figure legends and analyzed using the FitSpectra program which allows the absorbance due to light scattering using a $1/\lambda^4$ correction to be subtracted (R. R. Birge, unpublished, available upon request). For pH titrations of D108H, the pigment was washed extensively with 10 mM sodium phosphate buffer (pH 6.6) containing 0.1% DM and titrated with calibrated quantities of dilute phosphoric acid and NaOH. At each pH, four spectra were averaged. These spectra were then corrected for dilutions, and the light scattering component was removed using a $1/\lambda^4$ correction and normalized at 280 nm using FitSpectra. To fit the titration data, the absorbance values at 385 and 485 nm at different pHs were normalized to the maximum value and fit to the Henderson−Haselbach equation using SigmaPlot (Jandel). Bleaching of the D108H pigment was performed by illuminating the pigment with a projector lamp (300 W, EXR-5, Wiko, Inc.) from a distance of 30 cm with either a 550 nm filter (03FIV079, Melles Griot) or directly (white light) for the desired duration. The total radiant energies of the white light and the 550 nm filtered light, measured by an optometer (Graseby Optronics), were 8 mW and 220 μW, respectively. The temperature was maintained at 22 °C. For the bleaching of visual pigments, a 380 nm light (18 μW filtered through a band-pass filter, Oriel Corp.) was used for 5−10 min at 10 °C before the samples were acid denatured. Four sets of spectra were obtained and averaged to generate the final spectrum. The spectra were imported into SigmaPlot for preparation of the figures. The spectroscopic properties of the protein-bound chromophore were calculated using MNDO-PSDCI molecular orbital theory, including full single- and double-configuration interaction within the chromophore π-electron manifold (*12*). This method allows the entire protein binding site to be included in the SCF calculation while constraining the configuration interaction to the chromophore.

Transducin Activation Assays. Purification of bovine transducin from the frozen retinae and the [^{35}S]GTPγS exchange assays have been performed essentially as described previously (*16*). In short, the assays were carried out at 22 °C in reaction buffer containing 0.01% DM, 10 mM Tris acetate (pH 7.0), 100 mM NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 5 μM [^{35}S]GTPγS (900−19000 cpm/pmol). Visual pigment (21 nM, calculated using an extinction coefficient of 40 000 M^{-1} cm^{-1} at λ_{max}) was added to the reaction mixture in a transparent microcentrifuge tube. Finally, 1 μM bovine transducin was added to the mix and equilibrated to 22 °C for 1 min. The nucleotide exchange reaction was initiated by exposing the sample to white light from the projector described above. Control experiments were carried out to determine the level of binding of [^{35}S]GTPγS to transducin in the absence of pigment and also with bovine rhodopsin. The illumination time varied as described in the figure legend.

To determine the decay of the active state of the pigments, visual pigments were illuminated for 1 min in the absence of transducin and the aliquots were added to transducin for various time intervals, and incubated for an additional 10 min in dark. The kinetics of activation were determined by illuminating a reaction mixture for 1 min and then removing aliquots at various times.

RESULTS AND DISCUSSION

To determine the role of the conserved acidic residue in transmembrane 3, we prepared a series of site-specific mutations that changed the putative counterion at position 108, the position equivalent to the counterion position in bovine rhodopsin (*4*). The mutants were expressed in COS1 cells, and the pigment was generated by incubation with 11-*cis*-retinal and purified in dodecyl maltoside (DM). Replacement of the aspartic acid at position 108 with an alanine resulted in a shift of the dark λ_{max} from 427 to 353 nm with a reduction in the overall regeneration yield of 25−40% (Figure 1a). Acid treatment of visual pigments at pH 1.8 denatures the protein, removes noncovalent interactions between the retinal and opsin, but preserves the Schiff base linkage, which is protonated under these conditions (*11*, *13*, *14*, *17*). When the D108A mutant was acid-denatured, the resulting absorbance spectrum red-shifted with a λ_{max} of 440 nm (Figure 1a, inset). The absorbance spectra of both wild-type VCOP and the D108A mutant were not sensitive to pH between 4 and 9 (data not shown), indicating that the pK_as for both pigments were outside this range. These results are consistent with the mutant pigment containing a deprotonated Schiff base in the dark and indicate that D108 serves to stabilize the protonated Schiff base in the dark. We thus identify this residue as the counterion.

Replacement of the counterion with a similarly sized neutral amino acid (D108Q) caused a complete loss in the

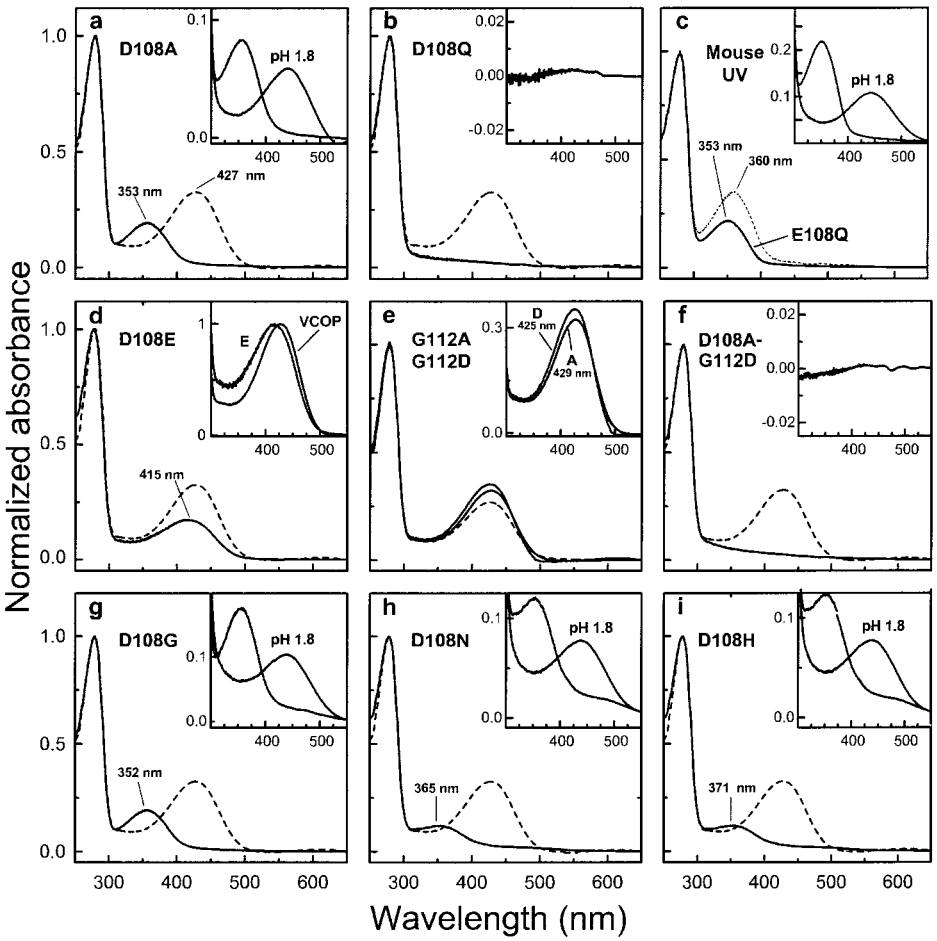

FIGURE 1: UV−visible absorption spectra of counterion mutants in the short-wavelength visual pigments. Protein was purified from transfected COS1 cells in detergent solution (pH 6.6) following incubation with 11-*cis*-retinal. The dark spectra of individual mutants (normalized at 280 nm) are superimposed upon that of the wild-type protein. *Xenopus* violet cone opsin mutants, their spectra shown as solid lines (panels a, b, and d−i), form visual pigments to various extents and with various λ_{max} values (indicated for each mutant) compared to wild-type pigment (dashed line). Panel c shows the wild-type mouse UV cone opsin (dotted line) and counterion mutant E108Q (solid line). Insets in panels a, c, and g−i show the unnormalized pigment peak (pH 6.6) and the resulting spectrum following acid denaturation at pH 1.8. Insets in panels b and f show dark-minus-light difference spectra of D108Q and the D108A/G112D double mutant which failed to bind retinal. The inset in panel d shows the D108E mutant normalized to the pigment peak, illustrating the blue-shifted λ_{max} compared to that of the wild type. The inset in panel e shows the unnormalized spectra of the G112A (A) and G112D (D) mutants.

ability of the protein to stably bind retinal (Figure 1b), in contrast to results obtained with the equivalent mutant rod opsin (*18*, *19*). This observation suggests that the geometry surrounding the Schiff base is more constrained in the violet cone opsin than in the rod opsin. The mouse short-wavelength visual pigment, MUV, absorbs in the ultraviolet region of the spectrum (Figure 1c) and is stable as an unprotonated Schiff base (*11*). Replacement of the counterion in MUV with an isosteric amino acid residue (E108Q) does not cause a major change in the dark absorbance properties, although there is a modest reduction in the level of the regenerated pigment (Figure 1c). The absence of a change in the absorbance properties in the MUV when the putative counterion position is neutralized supports the notion that the Schiff base is unprotonated in this pigment. It also suggests that, although both VCOP and MUV are related members of the same opsin subfamily [SWS1 (*20*)], the environment of the Schiff base is significantly different within this group, most likely reflecting the requirements for differently stabilized and protonated Schiff bases in the violet and ultraviolet opsins.

We manipulated the electrostatic environment of the Schiff base via mutagenesis. The *Xenopus* violet cone opsin contains an aspartic acid at the counterion position, while all other opsins have a glutamic acid (*4*, *20*). Mutation of the VCOP counterion, D108E, produced a 12 nm blue shift in λ_{max} to 415 nm and reduced the regeneration yield by almost 50% (Figure 1d). The blue shift would be expected from the movement of the counterion closer to the Schiff base (*11*), further supporting the identification of this position as the counterion. In bovine rhodopsin, the counterion position in the third transmembrane helix was flexible, as movement of the acidic residue one turn into the membrane (i.e., in the double mutant E113Q/A117D) caused essentially no change in the spectral properties (*21*). In the case of VCOP, we first replaced the residue one turn into the helix from the counterion (G112) with either alanine or glutamic acid. We found no significant shifts in the λ_{max}, although there was a slight increase in the yield of the regenerated pigment (Figure 1e). We also examined the spectra of the G112D mutant at pH 4.0 and 7.7, and found identical λ_{max} values (data not shown). Therefore, the presence of another negatively charged residue in the vicinity of the counterion was not sufficient to destabilize the interaction of the counterion with the retinylidene Schiff base. When we removed the counterion in combination with addition of an acidic residue one

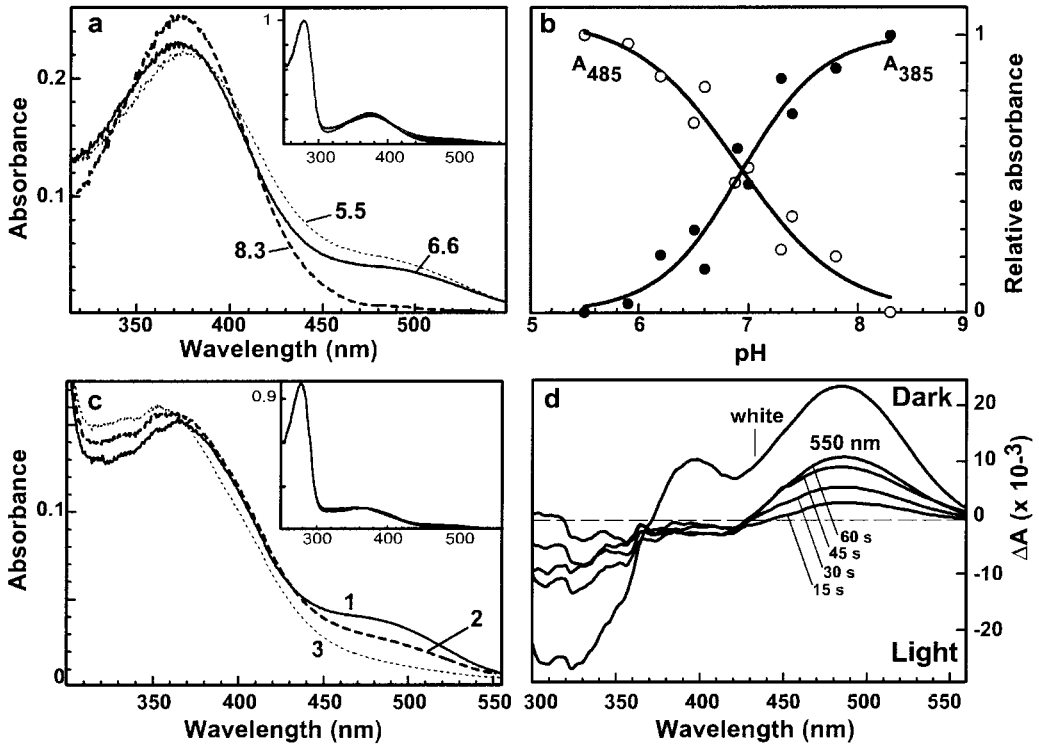

FIGURE 2: pH titration and photobleaching profile of the VCOP D108H mutant. UV−visible absorption spectra (normalized at 280 nm) of the purified pigment in 10 mM sodium phosphate buffer (pH 6.6) containing 0.1% DM titrated to the indicated pH (a). The inset shows the entire spectra. Panel b shows relative changes as a function of pH in absorbance at 385 nm (●) and 485 nm (○), wavelengths that produce the maximum difference. Solid lines are fits to a single ionizable group with a pK_a of 7. The mutant pigment was illuminated at pH 6.6 first with light filtered through a 550 nm band-pass followed by white light. Panel c shows the dark spectrum (curve 1), the spectrum after exposure of the pigment to 550 nm light for 1 min (curve 2), and the spectrum after exposure to white light for 1 min (curve 3). The inset shows the entire spectra. Panel d shows the dark-minus-light difference spectra after bleaching with 550 nm filtered light and white light as indicated in the figure. The 550 nm light caused a reduction in the absorbance with a λ_{max} of ∼485 nm, and the white light further reduced the absorbance at both 485 and 385 nm.

α-helical turn away (D108A/G112D), the protein failed to reconstitute with 11-*cis*-retinal (Figure 2f). Thus, the counterion location is constrained, and significant relocation while maintaining stability is not possible in VCOP.

We further investigated the counterion position by making additional neutral substitutions. The three mutants in this class (D108G, D108N, and D108H) all exhibited a significant reduction in the level of stable binding of 11-*cis*-retinal and unusual absorbance spectra (Figure 1g−i). There were two chromophore peaks in each mutant, a major peak with a λ_{max} of ∼352−372 nm and a minor, broad shoulder centered around 480 nm. Both chromophore peaks were derived from covalently attached retinal, since acid denaturation could shift all of the absorbance to 440 nm (Figure 1g−i, insets). D108H regenerated poorly, but exhibited the largest 480 nm component of the mutants we tested. The two components were observed even in pigments prepared in phosphate buffer, free of chloride ions (data not shown). When a solution of the D108H mutant at pH 6.6 was increased to 8.3, there was a complete loss of the 480 nm component and a concomitant increase in absorbance at 376 nm (Figure 2a). The spectral shift was reversible, and additional absorbance at 480 nm was recovered if the pH was reduced to 5.5, below which the protein denatured (data not shown). The interconversion between the two absorbing species was not complete; at all pHs, the 360 nm species was predominant (Figure 2a). We plotted the portion of the absorbance that was titratable and fit the changes in absorbance to a simple two-state model with a pK_a of 7 (Figure 2b). Illumination of the D108H mutant with orange light (λ_{max} = 550 nm) led to a selective loss of the long-wavelength component (Figure 2c,d). Due to the initial small amount of pigment and changes in the UV portion of the spectra, including an increase in light scattering, an accurate measure of the photoproducts from the orange light illumination was not possible. Illumination with white light caused further bleaching of the pigment, and the appearance of a peak at 380 nm representing the formation of MetaII (see below). These data are in support of the model in which the two absorbance maxima represent the unprotonated and protonated Schiff base forms of the pigment in this mutant. MNDO-PSDCI calculations on a model of the VCOP binding site containing the D108H mutation indicate that the protonated Schiff base species has a λ_{max} of ∼480 (data not shown), in good agreement with the experimental observations. It is not clear why the two forms cannot be completely interconverted, and future experiments are required to resolve this issue. One possibility is that during biosynthesis, the mutant folds into two different conformations, only one of which has a titratable Schiff base.

One of the fundamental differences between rod and cone opsins is the lifetime of the light-activated species, MetaII, and the rate of hydrolysis of the Schiff base (*4*). In detergent solutions, the MetaII form of rhodopsin is stable for many minutes (*22*) while cone pigments undergo rapid hydrolysis (*23*). When purified VCOP was exposed to white light, the λ_{max} changed from 427 to 389 nm (Figure 3a, curve 2). Reducing the pH of the illuminated sample to 1.8 caused a slight reduction in the extinction coefficient at 389 nm

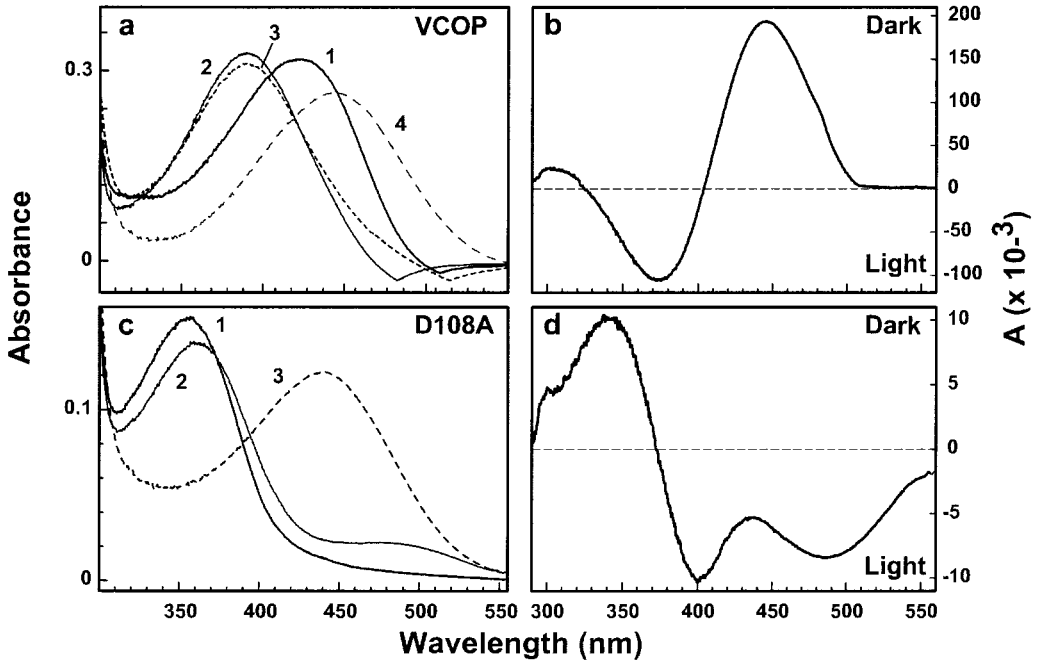

FIGURE 3: Illumination of VCOP and D108A with 380 nm filtered light. The purified pigment at pH 6.6 in 0.1% DM was illuminated for 5−10 min at 10 °C. Panel a shows the UV−visible absorption spectra of VCOP in the dark (curve 1), after illumination for 5 min (curve 2), and then following acid denaturation at the end of illumination (curve 3). For reference, acid denaturation of an unbleached sample is shown (curve 4). Panel b shows the dark-minus-light difference spectra of VCOP (panel a, curve 1 minus curve 2). Panel c shows the absorption spectra of D108A in the dark (curve 1), after illumination for 10 min (curve 2), and then following acid denaturation at the end of illumination (curve 3). Panel d shows the dark-minus-light difference spectra of D108A (panel c, curve 1 minus curve 2).

(Figure 3a, curve 3), but did not result in a shift to 440 nm as seen for the sample that was not exposed to light (Figure 3a, curve 4). These results show that the retinal is not stably bound in a Schiff base linkage following the exposure of the wild-type protein to light, consistent with the rapid decay of MetaII. In contrast, the counterion mutant D108A exhibits completely altered behavior. Illumination of the sample caused a reduction in 353 nm absorbance and a red shift to 365 nm (Figure 3c, curve 2). In addition, a shoulder on the main absorbance peak appeared at ∼490 nm. Upon acid denaturation, all of the chromophore absorbance was converted to a spectrum with a λ_{max} at 440 nm, showing that the retinal remained attached to the protein in a Schiff base following light exposure (Figure 3c, curve 3). The light-exposed sample had two main absorbing species, which were significantly red-shifted compared to the dark spectra (Figure 3d) and have absorbance properties expected for the unprotonated (365 nm) and protonated (485 nm) forms of the *all-trans*-retinal−opsin complex. These two species most likely represent the MetaI and MetaII forms of the mutant, and are dramatically stabilized compared to the wild-type pigment. In the wild-type pigment, the MetaI and MetaII forms absorb with λ_{max} values of 420 and 388 nm, respectively (*12*). The counterion thus plays an important role in the absorbance properties of these two photointermediates. The significant red shift of the MetaI intermediate is similar to the shift we have observed in the MetaI intermediates of mouse UV opsin trapped at low temperatures (manuscript in preparation). Thus, the Schiff base counterion is required for the rapid hydrolysis observed in cone opsins.

We examined the signal transduction properties of the counterion mutant using a biochemical assay of transducin activation, the guanyl nucleotide (GTPγS) exchange reaction. Wild-type visual pigments containing an 11-*cis*-retinal chromophore are dependent upon light to form the MetaII conformation that interacts with transducin (*4*). Although rhodopsin has a long-lived active state, the cone opsins are active for a much shorter time (*13*). However, the cone opsins are able to undergo rapid regeneration during continuous illumination, and thus can extend their activity by recycling (*13*). We investigated the effect of light on the activation of transducin by the counterion mutant, D108A. Illumination was essential for both the wild type and D108A to stimulate nucleotide exchange (Figure 4a), in contrast to the rhodopsin counterion mutant which was active in the dark (*24*). In continuous light, both the wild type and D108A stimulated nucleotide exchange to similar extents, with each molecule of pigment catalyzing a total of approximately 30 turnovers during the reaction (Figure 4a). The kinetics of activation were studied by briefly illuminating the visual pigments, and then following the time course of activation in the dark. Under these conditions, no recycling occurs and the lifetime of the MetaII species contributes significantly to the overall integral of activation. The initial rates of activation were similar: for D108A, we found 6.1 mol of GTPγS exchanged (mol of pigment)$^{-1}$ min^{-1} compared to 3.9 for both the wild type and D108E (Figure 4b). However, the most striking difference was seen in the duration of the reaction. Whereas the reactions catalyzed by the wild-type and D108E pigments were essentially complete in 1 min, the reaction of the D108A mutant continued at the same rate until the substrates were depleted (Figure 4b). The absence of decay in the active state in D108A was also seen in an experiment in which the visual pigment was first exposed to light followed by an incubation period before addition of transducin. The wild-type pigment decayed very rapidly, with a half-time of 1−2 min, similar to our previous report (*13*). D108A, on the other hand, did not show any decay over the 6 min period prior to

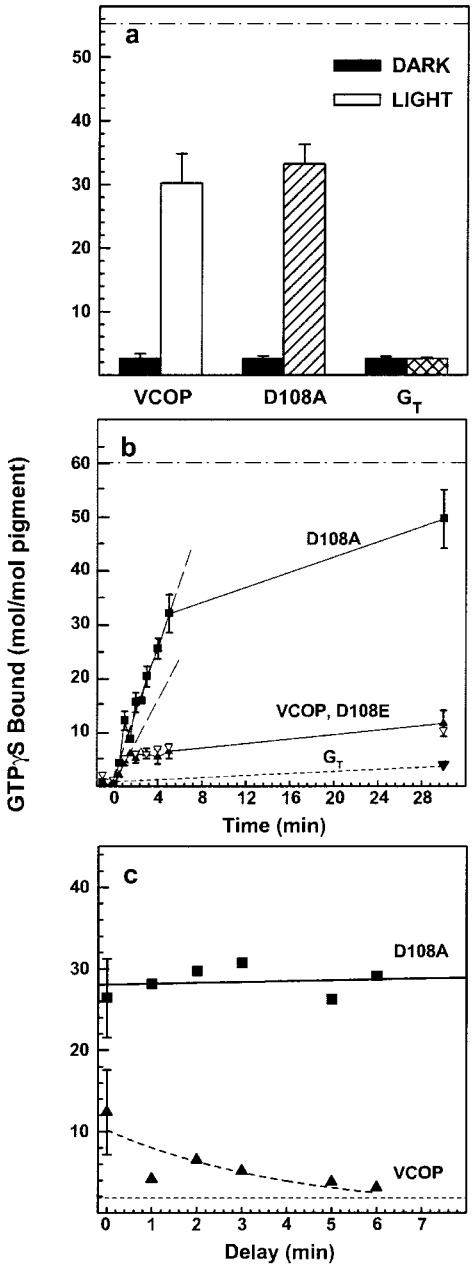

FIGURE 4: Transducin activation by counterion mutants in a short-wavelength visual pigment. Guanyl nucleotide exchange on the bovine rod transducin (1 μM) was assessed using 5 μM [^{35}S]GTPγS and 21 nM visual pigment in 0.01% DM. Panel a shows GTPγS bound (moles per mole of pigment) following a 10 min exposure to continuous white light illumination. The dark bars show the activity of the pigments in dark. G_T indicates the exchange activity in the absence of added visual pigment. The upper dashed line indicates the GTPγS exchange catalyzed by bovine rhodopsin under identical assay conditions. Panel b shows the kinetics of activation of VCOP (▲), D108A (■), and D108E (▽). The reaction mixture was illuminated for 1 min, and the samples were removed at the indicated time and immediately filtered. The residual activity was determined by incubating the remaining sample for up to 30 min in the dark. The dashed lines represent the linear regression fit to the initial rate for each curve. The GTPγS exchange catalyzed by bovine rhodopsin (−·−) and that without added visual pigment [G_T (▼)] are indicated. Panel c shows the decay of the active state of VCOP and D108A after illumination. The pigments were exposed to illumination for 1 min in the presence (0 min delay) or absence of transducin. Aliquots were then added to transducin with a delay of 1−6 min and incubated for an additional 10 min in the dark. The dotted line indicates the nucleotide exchange in the absence of visual pigment.

transducin addition (Figure 4c). The half-time of D108A was estimated to be approximately 165 min compared to 40 min for bovine rhodopsin under similar experimental conditions (data not shown). These results are in agreement with those from spectroscopy (Figure 3) and show that the D108A mutant has a dramatically slowed decay of the light-activated conformation, which remains competent to activate transducin.

The results reported here identify the Schiff base counterion as D108 in VCOP. This residue regulates the pK_a of the Schiff base, and neutralization of the acidic residue causes a change from a protonated form (427 nm) to an unprotonated form (353 nm). This phenotype is similar to that observed with comparable mutants in rhodopsin. However, a number of mutations that were tolerated in rhodopsin prevented stable retinal binding in the case of violet cone opsin. Several of the cone opsin mutants did exhibit a titratable retinal Schiff base, but did not bind retinal or titrate stoichiometrically, unlike the rhodopsin mutants. Our data support a model in which the environment of the Schiff base is more constrained or experiences significant interactions with other residues. In rhodopsin, the deprotonation of the Schiff base is a crucial event in the formation of the later photointermediates that interact with transducin. The absence of an acidic residue at this position leads to slowed production of the MetaII form of rhodopsin. Our results support the idea that the counterion, while playing an important role in spectral tuning, has a major function in regulating the photobleaching pathway in cone visual pigments. We have shown that in short-wavelength cone visual pigments, the counterion is necessary for the rapid production and decay of the active state required for the appropriate system response characteristics of photopic vision.

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