A Dark and Constitutively Active Mutant of the Tiger Salamander UV Pigment†

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ABSTRACT: A triple mutant (F86L/T93P/S118T; bovine rhodopsin numbering) of the tiger salamander UV cone pigment appears to be trapped in an open conformation that is metarhodopsin-II-like. The pigment is able to activate transducin in the dark, and the ligand-free apoprotein is also able to activate transducin constitutively. The pigment permits protons and chloride ions from solution access to the active site as it displays a pH- and NaCl-dependent absorption spectrum not observed with the wild-type pigment. However, the wild-type properties of light-dependent activity and a pH-independent absorption spectrum are recovered upon reconstitution of the triple mutant with 11-*cis*-9-demethyl retinal. These results suggest that binding the native chromophore cannot deactivate the protein because of steric interactions between the protein, possibly residue 118, and the 9-methyl group of the chromophore. Furthermore, the absorption spectrum of the 9-demethyl retinal regenerated pigment exhibits a band broader and with lower extinction at the absorption maximum than either the human blue or salamander UV wild-type pigments generated with the same retinal analogue. The broad spectrum appears to be comprised of two or more species and can be well-fit by a sum of scaled spectra of the two wild-type pigments. Binding the chromophore appears to trap the pigment in two or more conformations. The triple mutant reported here represents the first example of a dark-active cone pigment and constitutively active cone opsin.

Visual pigment proteins belong to the superfamily of G protein-coupled receptors. Several mutations in rhodopsin, the visual pigment responsible for dim light vision, have been found to be associated with retinitis pigmentosa and night blindness and shown to be constitutively active (for a review, see ref *1*). Some of these mutations have provided insight into rhodopsin activation and deactivation, most notably the identification of an internal salt bridge that maintains rhodopsin in an inactive state (*2*, *3*). Much less is known about cone pigment proteins. Shichida and co-workers have identified amino acid residues that can account for some of the differences of chromophore binding and hydrolysis rates between cone pigments and rhodopsin (*4*−*7*). Birge, Knox, and co-workers have begun to characterize light activation of a class of short wavelength sensitive cone pigments (SWS1)[1] (*8*−*12*). Thus far, to our knowledge, a constitutively active mutant of a cone pigment protein has not been reported.

The SWS1 class is comprised of cone pigments absorbing maximally in the blue to near UV regions of the spectrum (*13*, *14*). Many of these pigments absorb in the near UV at about 360 nm (*15*−*18*), as well as pigments that absorb in the visible region as high as 438 nm for the bovine blue (*19*), 427 nm for the *Xenopus* violet (*9*), and 414 nm for the human blue (*20*) cone pigments. Although the SWS1 subgroup spans a fairly broad spectral range, the proteins themselves are highly homologous (60−90% amino acid sequence identity), and all of the pigments use the same 11-*cis* retinal chromophore. As a consequence, the SWS1 group has been the recent focus of spectral tuning studies from a number of laboratories (*19*, *21*−*25*).

All data to date on SWS1 pigments generally have led to two basic conclusions regarding their absorption maxima. First, the spectral sensitivity of pigments that absorb in the UV is likely due to retinal bound as an unprotonated Schiff base (*8*, *10*, *18*, *19*, *22*, *26*). Second, spectral tuning among members of this family of pigments is not straightforward, particularly when the human blue cone pigment is included. With SWS1 pigments, complete spectral conversion from one pigment to another via point mutations is either incomplete or requires many amino acid substitutions (*19*, *21*−*24*, *27*). In birds, the residue corresponding to position 90 in bovine rhodopsin produced a fairly large spectral shift depending on whether the residue was a Cys or Ser (*23*, *24*), but this residue had little effect on nonavian SWS1 pigment absorption (*19*). Position 86, however, had a large effect on the absorption maxima of two mammalian SWS1 pigments, although it was not sufficient to fully account for the spectral

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[1] Abbreviations: SWS1, short-wavelength sensitive class 1 cone pigment; PBS, 10 mM sodium phosphate buffer at pH 7.0 containing 150 mM NaCl; DM, *n*-dodecyl-*β*-D-maltoside; MES, 2-[*N*-morpholino]-ethanesulfonic acid; MBS, 10 mM MES buffer at pH 6.0 containing 150 mM NaCl.

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differences between the bovine blue and mouse UV pigments, and substitution to the human blue pigment residue had no effect on absorption maximum (*19*). Shi et al. (*22*) incorporated several residues to make a mouse UV pigment spectrum mimic the spectrum of the human blue pigment and vice versa. The need for several substitutions was most striking because single mutations had little effect on the absorption maximum. Furthermore, the spectral shape of several mutants with absorption maxima between the mouse UV and human blue pigments' absorption maxima was often broader than the classical shape of visual pigment spectra (*22*).

We describe here the first example of a cone pigment mutant that can activate transducin in the dark and whose opsin is constitutively active. This mutant has the potential to be a useful model to study the active state of cone pigments and might give clues to the confusing nature of spectral tuning of SWS1 pigments.

EXPERIMENTAL PROCEDURES

Expression and Mutagenesis. The cDNA for the salamander UV pigment (*18*) and a synthetic gene for the human blue pigment (*28*) had been modified by addition of the coding sequence for the carboxy-terminal eight amino acids from bovine rhodopsin (ETSQVAPA) to the 3′ end of the genes. This sequence permits purification of the expressed proteins by means of immunoaffinity chromatography using the anti-rhodopsin 1D4 antibody (National Cell Culture Center, Minneapolis, MN) (*29*) as described earlier (*28*). Mutations were introduced by standard procedures using cassettes or PCR. The genes were subcloned as *Eco*RI−*Not*I cassettes into the mammalian pMT3 expression vector (*30*). COS-1 cells were transiently transfected using the DEAE−dextran method as previously described (*31*). On the 3rd day after transfection, cells were harvested and stored at −80 °C until needed.

Purification of the Pigments. Pigments were generated essentially as has been described for rhodopsin (*31*) with minor modifications as follows. Thawed cell pellets from 20 10-cm tissue culture plates were resuspended in 10 mL phosphate-buffered saline [10 mM sodium phosphate and 150 mM NaCl at pH 7.0 (PBS)] containing 10 μM 11-*cis* retinal or 11-*cis*-9-demethyl retinal and incubated in the dark at 4 °C for at least 2 h. Cells were lysed by addition of an equal volume of 2% dodecyl maltoside (DM) (w/v) in 10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer and 150 mM NaCl at pH 6.0 (MBS), and nuclei were removed by centrifugation at 1380*g* for 5 min. The clarified supernate was incubated with 1D4-conjugated Sepharose 4B for 2 h at 4 °C to allow binding of the solubilized pigments. The matrix was then washed extensively with 0.1% (w/v) DM in MBS. The pigments (200 μL) were eluted with 88 μM of a synthetic peptide corresponding to the carboxy-terminal 18 amino acids of bovine rhodopsin dissolved in the wash buffer.

11-cis-9-Demethyl Retinal. 11-*cis*-9-Demethyl retinal was synthesized according to Corson et al. (*32*) and the purity confirmed by high-performance liquid chromatography analysis and absorption spectroscopy before use.

Absorption Spectra. UV−visible absorption spectra were recorded using an Hitachi U-3210 spectrophotometer, modified for dark room use by the manufacturer. Path length for all measurements was 1 cm, and sample volume was typically 100 μL. A circulating water bath attached to the cuvette holder maintained the sample temperature at 4 °C. The pH of the samples was adjusted by addition of 5 μL aliquots of a 1 M stock solution of sodium citrate or HEPES buffer at the desired pH. The reported pH was determined by measuring the pH of solutions of identical composition but with a larger volume and containing no protein. Acid denaturation of the pigments and trapping of the chromophore as a protonated Schiff base was accomplished by addition of 2 μL of concentrated HCl to the sample.

The extinction coefficients for the salamander UV pigment (*18*) and the human blue pigment (*20*) have been published. Extinction coefficients for the mutants were determined as previously described (*20*). We assumed that the extinction coefficient of the acid-trapped, 11-*cis*-9-demethyl retinal-reconstituted pigments was identical to that of the 11-*cis*-retinal-bound forms. The absorption maxima were determined by fitting a fourth order polynomial about the absorption maximum using the curve fitting features of KaleidaGraph (Synergy Software) as described previously (*20*).

Assay for Activation of Transducin. Transducin was purified from bovine retina according to the procedure of Wessling-Resnick and Johnson (*33*) and then subjected to ion-exchange chromatography on DE-52 (*34*). As a final step, the protein was dialyzed against 10 mM Tris buffer (pH 7.5) containing 50% (v/v) glycerol and 2 mM $MgCl_2$ and then stored at −20 °C. The pigments were assayed for their ability to catalytically activate transducin by following the binding of [^{35}S]GTPγS as has been described previously (*35*). The reaction mixture contained 10 mM Tris/1 mM MES buffer (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 115 mM NaCl, 2.5 μM transducin, 0.01% DM, and 5 nM pigment (as determined from the extinction coefficient). For assaying the apoprotein and pigment controls, membrane fractions were isolated from COS cells (*3*, *36*). Equal amounts of membranes were incubated with either ethanol for the opsin or 100 μM retinal (11-*cis* or 9-demethyl dissolved in ethanol) in the dark for at least 1 h. This was diluted 1:10 for the assay reaction mix. Transducin activation assay conditions were essentially identical to those used for the purified pigments except for the absence of the MES buffer and detergent. Concentration of mutant protein, roughly estimated by western blots, was about 1−5 nM in the assays. However, because the composition of all of the reaction mixtures was the same except for the addition of 11-*cis* retinal or 9-demethyl retinal, the relative activity of all of the samples could be compared.

RESULTS

Salamander UV Pigment Triple Mutant. The salamander UV pigment triple mutant F86L/T93P/S118T[2] was generated as part of a series of experiments designed to identify key amino acid residues that distinguished the absorption maximum of the UV pigment from that of the human blue pigment. Absorption spectra for the salamander and human

[2] For consistency and clarity, all amino acid residues are identified according to their corresponding numbers in bovine rhodopsin.

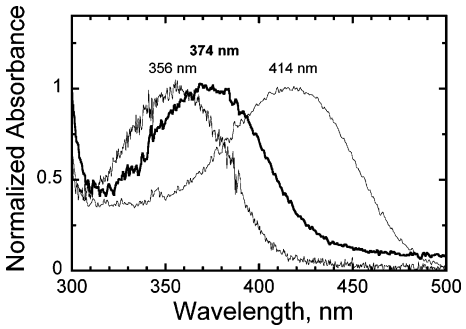

FIGURE 1: Absorption spectra of the wild-type salamander UV (λ_{max} = 356 nm) and human blue (λ_{max} = 414 nm) pigments and the salamander UV triple mutant (F86L/T93P/S118T) (thick black trace; λ_{max} = 374 nm). All pigments were reconstituted with 11-*cis* retinal. The position of the absorption maximum is noted above each spectrum. Spectra were normalized to an absorbance of 1 at λ_{max}. Each pigment was converted to a 440 nm absorbing species upon acid denaturation (not shown). The pH of all samples was 6.0.

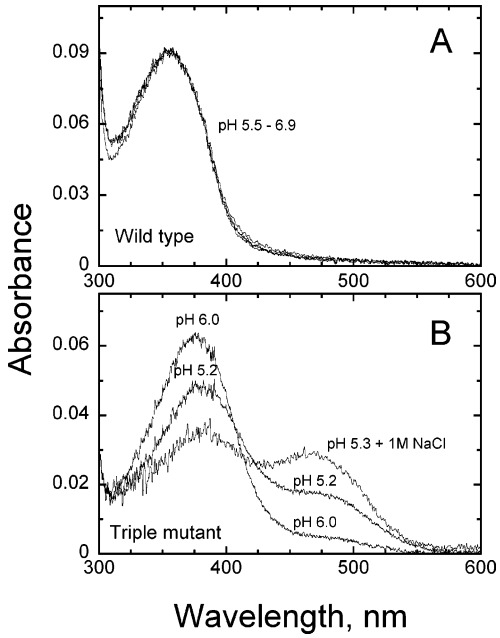

FIGURE 2: Effect of pH and NaCl concentration on the spectrum of the salamander UV triple mutant. Each spectrum was adjusted for dilution upon buffer addition and labeled according to pH. Pigment samples contain 150 mM NaCl except where noted otherwise. (A) Wild-type UV pigment. (B) UV triple mutant.

SWS1 wild-type pigments and the salamander triple mutant are presented in Figure 1. While the mutations clearly result in a red shift of the absorption maximum (from 356 nm for the salamander UV wild-type pigment to 374 nm for the triple mutant), the absorption maximum of the triple mutant falls well short of the maximum for the human blue pigment (414 nm).

However, the absorption spectrum of the mutant is sensitive to pH and NaCl concentration (Figure 2B). Unlike the wild-type UV pigment, the Schiff base of the triple mutant can be titrated under mildly acidic pH conditions. At pH 6, the spectrum is dominated by the 374 nm species, but a residual absorbance at longer wavelengths is clearly also present (Figures 1 and 2). As the pH is lowered to pH 5.2 (below this pH the pigment was unstable), there is a loss of the 374 nm absorbing species and an increase in absorbance at 475 nm. The conversion is accompanied by an apparent isosbestic point at 403 nm (intermediate spectra not shown) and is promoted by an increase in the NaCl concentration from 150 mM to 1 M (Figure 2B). This behavior is not observed in the wild-type pigments; absorption spectra for the wild-type salamander UV (Figure 2A) and human blue (not shown) pigments are independent of pH from 5.2 to 7.0 (up to 9.0 for UV pigment) and NaCl concentration, as is also the case for other wild-type SWS1 pigments (*8*, *22*). This is not seen with any other chimeric or mutant pigments involving SWS1 pigments (*8*, *22*, *25*) except the D108H mutation in the *Xenopus* violet cone pigment (*8*). A full conversion to the 475 nm pigment could not be achieved because the Schiff base hydrolyzed destroying the pigment at lower pH values. This was most clearly noticeable when the pH was dropped below 5.2 at 150 mM NaCl where there was initially a significant amount of the 475 nm pigment. This pigment subsequently converted to the 380 nm free retinal absorption spectrum with each successive spectrum taken during a 15 min period (not shown).

This long wavelength absorbance is clearly distinct from the long wavelength shoulders to the main absorption band of some of the chimeric constructs between the mouse UV and human blue cone pigments noted by Shi et al. (*22*). The amount of the long-wavelength absorbing species in those pigments does not vary with changes in pH, and its absorption maximum is well below 440 nm. In chimeric pigments involving the human blue cone pigment from Shi et al. (*22*) and us (not shown), the long-wavelength component seems to closely resemble the absorption of the human blue pigment itself.

The pH- and NaCl-dependent spectral properties of the triple mutant are consistent with protonation of the Schiff base at lower pH values and are highly reminiscent of the E113Q mutant of bovine rhodopsin in which the Glu113 counterion to the protonated Schiff base is changed to Gln (*37*−*40*). As was the case for the rhodopsin mutant, we interpret the spectral properties of the salamander UV triple mutant as follows. At pH 6.0, the Schiff base of the chromophore is largely unprotonated, and the absorption spectrum displays an absorption maximum in the near UV at 374 nm. As the pH is decreased, the Schiff base becomes protonated and the absorption maximum shifts to 475 nm. Protonation of the Schiff base requires a counterion to the positively charged nitrogen, and this counterion is supplied by a chloride ion recruited from solution (accounting for the effect of NaCl on the pK_a). Alternatively, the high salt concentration could have lowered the surface pH because of screening of the net positive charge on the protein surface and hence form more of the 475 nm pigment. Nevertheless, the data are consistent with a partially titratable Schiff base where the pigment has 11-*cis* retinal primarily bound as an unprotonated Schiff base because the main absorption band is at 374 nm in the pH range studied. At pH 7.0, there is no 475 nm pigment indicating that the Schiff base is completely unprotonated (not shown).

Activity of the Salamander UV Triple Mutant Pigment and Opsin. As has been reported previously (*18*), the wild-type salamander UV pigment activates bovine transducin in a light-dependent reaction that terminates quickly, presumably as a consequence of the rapid decay of the activated metarhodopsin-II-like intermediate via hydrolysis of the

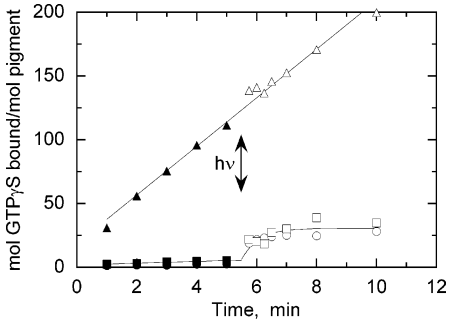

FIGURE 3: Activation of bovine transducin by wild-type salamander UV pigment (●, ○) and UV triple mutant (▲, △) reconstituted with 11-*cis* retinal and salamander UV triple mutant reconstituted with 11-*cis*-9-demethyl retinal (■, □). The first 5 time points were taken from reactions carried out in the dark (●, ▲, ■), while the last 7 time points were taken after the reactions were exposed to light (○, △, □) at $t = 5.5$ min. Pigments were detergent-solubilized and immunopurified.

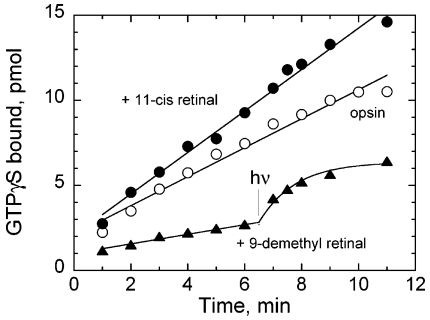

FIGURE 4: Activation of bovine transducin by salamander UV triple mutant opsin (○) reconstituted with 11-*cis* retinal (●) and UV triple mutant reconstituted with 11-*cis*-9-demethyl retinal (▲). The first 6 time points were taken from reactions carried out in the dark, while the last 7 time points were taken after reactions were exposed to light at $t = 6.5$ min. Membrane preparations from the COS cells transfected with the triple mutant were used to maintain the integrity of the apoprotein.

Schiff base, protein denaturation, and/or relaxation to an inactive conformation. Therefore, high levels of activated transducin do not accumulate (Figure 3). In contrast, the UV pigment triple mutant activates transducin in the dark (Figure 3), and because the active state of the pigment is not transient, activated transducin accumulates to much higher levels than observed for the wild-type reaction. The lack of any changes to the rate of transducin activation by the triple mutant pigment after illumination suggests that the chromophore remains bound to the opsin and that 11-*cis* and all-*trans* retinals are both capable of maintaining an active pigment. The three single mutants (F86L, T93P, and S118T) only activated transducin in a light-dependent manner like the wild-type pigment (not shown).

One concern was that the pigment activity in the dark might be an artifact from a constitutively active opsin. Indeed, when we assayed the ability of membrane preparations of the mutant apoprotein to activate transducin, we discovered that it was constitutively active (Figure 4). However, the pigments described in the previous paragraph were detergent-solubilized and immunopurified under conditions where opsins are generally unstable and denature (*36*). To measure the ability of the apoprotein to activate transducin, we maintained the protein in a membrane environment. This also allowed us to assay the activity from the same amount of triple mutant protein with and without

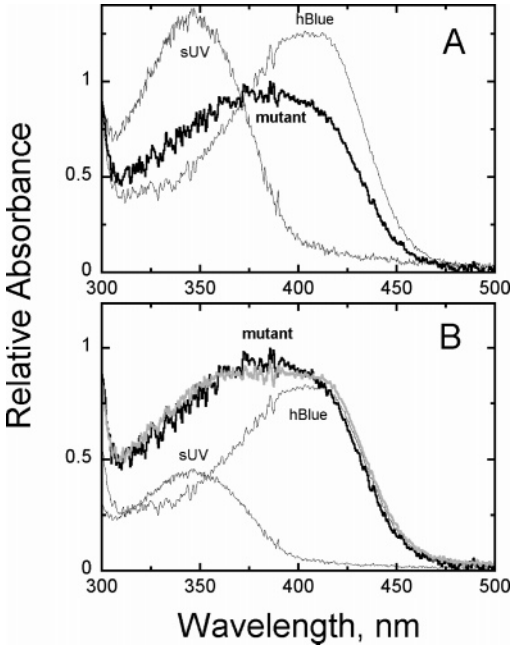

FIGURE 5: Absorption spectra of wild-type salamander UV, human blue, and salamander UV triple mutant reconstituted with 11-*cis*-9-demethyl retinal. (A) Each absorption spectrum was normalized through reference to the corresponding acid-trapped spectrum (not shown). (B) Simulation of the 11-*cis*-9-demethyl triple mutant spectrum by a linear combination of spectra from wild-type salamander UV (33%) and wild-type human blue (67%) pigments reconstituted with 11-*cis*-9-demethyl retinal. The 11-*cis*-9-demethyl triple mutant spectrum (thick black trace) is shown superimposed onto the simulated spectrum (gray trace).

exogenously added retinal because each reaction tube contained membranes that were equally divided from the same preparations. Adding 11-*cis* retinal did not quench the constitutive activity as would be expected if the activity were solely due to the apoprotein. Because pigments do form with 11-*cis* retinal (Figure 1), we conclude that the opsin can activate transducin constitutively and the pigment can activate transducin in the dark.

Phenotypic Rescue with 11-cis-9-Demethyl Retinal. Reconstitution of the triple mutant with the 11-*cis*-9-demethyl retinal inactivated the pigment in the dark and rescued the wild-type light-dependent activation of transducin, as shown in Figures 3 and 4. The 11-*cis*-9-demethyl triple mutant pigment now activates transducin in a light-dependent manner that terminates quickly with a time course essentially identical to that of the wild-type pigment reconstituted with 11-*cis* retinal. Why does the bleached pigment become inactive so quickly? In Figure 3, the protein has been solubilized and purified in detergent, and loss of activity is probably due to denaturation of the bleached pigment. In Figure 4, the membrane environment protects the bleached pigment from denaturation, but there is an excess of 11-*cis*-9-demethyl retinal available to reform the inactive pigment.

Reconstitution of the triple mutant with the 11-*cis*-9-demethyl retinal yielded a pigment with an absorption maximum of about 385 nm (Figure 5) that also rescued many of the wild-type spectral properties. The long-wavelength species with a maximum at 475 nm observed for the mutant reconstituted with 11-*cis* retinal is no longer present, and the absorption spectrum, like that of the wild-type pigment, is independent of both pH and chloride ion concentration

(not shown). Despite the lack of pH sensitivity, the absorption band is broad with a lower extinction at its absorption maximum than either of the wild-type pigments (Figure 5A).

DISCUSSION

Working Model. We interpret the pH- and salt-dependent spectral properties of the triple mutant to indicate that the mutant has a more open conformation than does the wild-type pigment such that protons and chloride ions from solution have access to the chromophore-binding pocket. We also note a recent study by Kefalov et al. (*41*) showing that opsin transiently activates transducin during binding of 11-*cis* retinal to the protein [this effect is not observed in red cones (*41*, *42*)]. Because the retinal chromophore in the X-ray crystal structure of rhodopsin (*43*) is completely hidden in a spacefilling model of the pigment, it is clear that the protein must adopt a more open conformation to allow retinal to enter the active site. Presumably, it is this open conformation that is metarhodopsin-II-like and gives rise to the transient activity observed during retinal binding in the experiments of Kefalov et al. (*41*). Furthermore, numerous studies have highlighted the importance of the 9-methyl group in activation (*32*, *44*−*47*), and the X-ray crystal structure of rhodopsin (*43*) shows that the 9-methyl group of the retinal chromophore is in van der Waals contact with the side chain of Thr118, one of the amino acid positions substituted in the UV pigment triple mutant.

Our working hypothesis is that the 9-methyl group of retinal acts as a steric "doorstop" to the mutant protein by holding the protein in an active conformation. We envision a process similar to the transient activation and subsequent deactivation upon binding 11-*cis* retinal in wild-type visual pigments (*41*). However, in the triple mutant, binding 11-*cis* retinal forms a pigment where the protein cannot be closed because of the steric clash between the 9-methyl group and protein and consequently prevents the protein from leaving an active conformation. It is unlikely that 11-*cis* retinal is binding nonspecifically in a completely different binding pocket because the absorption maximum of the protonated Schiff base form is greater than the absorption maximum for retinal bound as a protonated Schiff base in solution, 440 nm. The 475 nm absorption of the triple mutant indicates that the chromophore is bound and buried inside a protein environment.

Broad Absorption Spectrum of the 9-Demethyl Triple Mutant Pigment. When the salamander UV pigment triple mutant is reconstituted with 11-*cis*-9-demethyl retinal, it displays a much broader absorption spectrum with a maximum centered near 385 nm and a markedly lower extinction at its absorption maximum compared to the wild-type salamander UV and human blue pigments generated with 9-demethyl retinal (Figure 5A). The broad spectrum resembles some of the mouse UV/human blue pigment (*22*) and salamander UV/human blue pigment chimera (not shown) spectra. In fact, the absorption spectrum appears to be a linear combination of roughly 33% 11-*cis*-9-demethyl wild-type UV and 67% 11-*cis*-9-demethyl wild-type human blue pigments (Figure 5B), and the ratio of the two forms does not change with pH. This result is interesting because the mutations were originally selected as part of a spectral-tuning study. Although, the single-point mutations had very little effect on the absorption maximum, it appears that, with just three residues, there is complete conversion of a UV pigment to the human blue pigment, at least with two-thirds of the population.

This suggests that binding the chromophore trapped the pigment in at least two conformations. Spectral tuning of SWS1 pigments may not necessarily be a simple sum of small incremental changes from each amino acid replacement as it is in converting between the human red and green cone pigments (*48*). The cooperative effects of multiple substitutions lead to 11-*cis* retinal binding in a preferred conformation. Perhaps this "all or nothing" aspect reflects conditions that favor binding the chromophore as either a protonated or unprotonated Schiff base.

CONCLUSION

We report the first example of a dark and constitutively active mutant of a cone pigment. The mutant pigment displayed properties consistent with a stable metarhodopsin-II-like "open" conformation of the protein. The pH- and salt-independent absorption spectrum and the light-dependent activity of the wild-type UV pigment were rescued in the triple mutant by reconstitution with 11-*cis*-9-demethyl retinal. We conclude that the high pH form of the mutant with an absorption maximum at 374 nm contains a chromophore with an unprotonated Schiff base, and the ability to protonate it by increasing the hydrogen ion concentration is a consequence of the fact that the protein is trapped in a conformation with the active site accessible from the external solution. The protein was closed by binding a retinal analogue lacking the 9-methyl group. This closing process, however, trapped the pigment in two conformations that displayed spectral bands similar to a mixture of pure UV and human blue pigments instead of a single pigment with an intermediate absorption maximum.

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