Spectral Tuning in the Mammalian Short-Wavelength Sensitive Cone Pigments†

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ABSTRACT: The wild-type mouse ultraviolet (UV) and bovine blue cone visual pigments have absorption maxima of 358 and 438 nm, respectively, while sharing 87% amino acid identity. To determine the molecular basis underlying the 80 nm spectral shift between these pigments, we selected several amino acids in helices II and III for site-directed mutagenesis. These amino acids included: (1) those that differ between mouse UV and bovine blue; (2) the conserved counterion, Glu113; and (3) Ser90, which is involved in wavelength modulation in avian short-wavelength sensitive cone pigments. These studies resulted in the identification of a single amino acid substitution at position 86 responsible for the majority of the spectral shift between the mouse UV and bovine blue cone pigments. This is the first time that this amino acid by itself has been shown to play a major role in the spectral tuning of the SWS1 cone pigments. A single amino acid substitution appears to be the dominant factor by which the majority of mammalian short-wavelength sensitive cone pigments have shifted their absorption maxima from the UV to the visible regions of the spectrum. Studies investigating the role of the conserved counterion Glu113 suggest that the bovine and mouse SWS1 pigments result from a protonated and unprotonated Schiff base chromophore, respectively.

Mammalian color vision is mediated predominantly by two cone photoreceptor cell classes containing either a short-wavelength (SWS1)[1] or a long-wavelength (M/LWS) sensitive visual pigment that give rise to dichromatic color vision. Exceptions to this are Old World primates, including humans, and some New World primates that possess trichromatic color vision. The vertebrate SWS1 class includes pigments with absorption maxima (λ_{max}) ranging from 360 nm, as found in mouse and zebra finch (*1*−*3*), to 460 nm, as found in several artiodactyls (*4*), resulting in an extremely large range of λ_{max} values within a single visual pigment class. The relative protein similarities shared by members of the SWS1 class, along with the approximately 100 nm spectral region to which these pigments are tuned, make this class an ideal model system for spectral tuning studies.

The mechanisms of spectral tuning within the SWS1 cone pigments have been a topic of increasing interest in recent years. While all visual pigments are composed of a retinal chromophore covalently bound to a protein containing seven transmembrane α-helical segments, the SWS1 pigments share relatively little protein sequence similarity with rhodopsin (Rh1), less than 50%, when compared with members of their own class. Although a previous attempt was made to use blue-shifted rhodopsin mutants as a model to study SWS1 pigments (*5*), more direct examinations of the spectral tuning properties within the SWS1 pigments have been done in both birds (*6*, *7*) and mammals (*8*, *9*). These studies have shown that wavelength modulation between UV and visible regions of the spectrum by the avian and mammalian SWS1 pigments is accomplished in two different ways. The avian SWS1 pigments rely on a single amino acid substitution, Cys90→Ser, to shift the absorption maximum from the UV to the visible regions of the spectrum (*6*, *7*). However, the wavelength modulation between the mouse UV and the human blue cone pigments is accounted for by the introduction of amino acid substitutions at eight specific sites (*9*). Interestingly, at least five amino acid substitutions must be present in order to observe the majority of the shift, with single amino acid substitutions at each individual site failing to shift the absorption spectrum away from wild type (*8*).

Here we describe the amino acid substitutions responsible for the majority of the wavelength modulation between the mouse (λ_{max} = 358 nm) and bovine (λ_{max} = 438 nm) SWS1 cone pigments. Through a series of mutagenesis experiments, we have identified a single amino acid substitution that accounts for almost the entire 80 nm shift between the mouse and bovine SWS1 pigments, and that this amino acid substitution appears to be involved in protonating the chromophore Schiff base in the bovine blue pigment. We also show that the amino acid substitutions involved in the wavelength modulation between the mouse UV and bovine blue pigments are different from those previously identified to be involved in the wavelength modulation between the avian UV and blue pigments (*6*, *7*) as well as between the mammalian UV and blue pigments (*8*, *9*).

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[1] Abbreviations: SWS1, short-wavelength sensitive class 1 cone pigment; M/LWS, middle/long-wavelength sensitive cone pigment; RH1, rhodopsin class 1 rod pigment; ERG, electroretinogram; DM, *n*-dodecyl β-D-maltoside; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of the Mouse UV and Bovine Blue Cone Opsin Genes. The bovine blue coding sequence was PCR-amplified from bovine retinal cDNA using oligonucleotide primers designed from previously published sequences (*10*) and then subcloned into the mammalian expression vector pMT3 used for transient expression in COS cells (*11*, *12*). The mouse UV coding sequence was isolated from a Balb/c mouse retinal cDNA λZapI library (Stratagene, LaJolla, CA) probed with human blue hs36 (*13*) as described (*14*) and deposited as AF190670 in GenBank. The cDNA was then subcloned into pMT3. All procedures for DNA manipulation, mutation of the opsin genes, DNA sequence analysis, and transient expression of the opsin genes in COS cells were performed as previously described (*11*, *15*, *16*), except that nonchimeric mutants were generated using the QuickChange site-directed mutagenesis kit from Stratagene.

Reconstitution and Purification of Visual Pigments. Transfected COS-1 cells were harvested 72 h after initial exposure to DEAE-dextran and DNA. Procedures for reconstitution of the pigments with 11-*cis*-retinal, solubilization of the COS cell membranes with 1% DM, and purification of the proteins by immunoaffinity chromatography on the 1D4-Sepharose 4B matrix have been described previously (*17*). The pigments were purified in 10 mM MES buffer, pH 6.0, containing 150 mM NaCl and 0.1% DM. Several pigment samples that reconstituted poorly were concentrated in order improve the quality of their absorption spectra. The human blue wild-type and L86Y mutant were reconstituted and purified as described above except that they were purified in 0.01% DM and then concentrated approximately 10-fold using Microcon-30 concentrators from Amicon, Inc. (Beverly, MA). We estimate that the final concentration of DM was approximately 0.1%. To determine the effect of pH on the mouse UV F86Y/E113Q spectrum, individual pigment samples were washed and eluted from the 1D4-Sepharose 4B matrix at either pH 5, 6, or 7 in buffers containing 10 mM citric acid, MES, and HEPES, respectively, and either 150 mM or 1 M NaCl. Samples were then concentrated as described above.

Absorption Spectroscopy. UV−visible absorption spectra were recorded using an Hitachi model U-3210 spectrophotometer that was specifically modified by the manufacturer for use in a dark room. Data were acquired with the aid of a Gateway 2000 4DX2-50V microcomputer using Spectra Calc software from Galatic Industries Corp. (Salem, NH). All spectra were recorded on samples with a 1.0 cm path length in a thermostated cell holder with the temperature maintained at 4 °C. Absorption maxima were determined from the first derivative of a fourth-order polynomial fit to a 40 nm region surrounding each maximum. Procedures for the determination of the extinction coefficient of the mouse UV and bovine blue cone pigments have been described previously (*18*).

Difference Spectra. The absolute absorption maximum of some mutants (e.g., the bovine blue S90C and human blue L86Y) was difficult to determine due to the relatively greater contribution of light scattering to the absorbance on the short-wavelength end of the spectrum. To remove contributions made by light scattering, difference spectra were generated as follows: After an absorption spectrum was recorded, the sample was exposed to light in the presence of 50 mM hydroxylamine (pH 6.0), and the spectrum of the resulting retinal oxime was recorded (λ_{max} = 362 nm). A difference spectrum was obtained by subtracting the absolute spectrum of the retinal oxime from that of the dark sample.

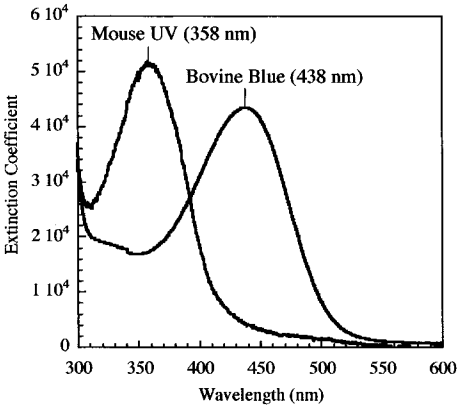

FIGURE 1: Absolute absorption spectra of the mouse UV and bovine blue cone pigments. The position of the absorption maximum is noted above each spectrum.

Assay for Activation of Transducin. Light-dependent activation of bovine rod cell transducin was assayed by following the binding of [^{35}S]GTPγS as described (*16*, *19*). The concentrations of purified pigments used in transducin assays were based on absorption spectra and adjusted to 15 nM with buffer consisting of 10 mM MES (pH 6.0), 150 mM NaCl, and 0.01% DM.

RESULTS

Absorption Spectra of SWS1 Wild-Type Pigments. The absolute absorption spectra of the mouse UV and bovine blue cone pigments purified by immunoaffinity chromatography from COS cells transiently transfected with cDNA for the SWS1 opsin genes exhibit maxima at 358 and 438 nm, respectively, as shown in Figure 1. The absorption maximum for the mouse UV pigment is in good agreement with those published previously [358 nm from ERG recordings (*1*); 359 nm for the purified pigment (*3*)]. However, our determination of the absorption maximum for the bovine blue cone pigment is at a somewhat longer wavelength than that reported for the purified pigment (431 nm) (*8*), and at a somewhat shorter wavelength than that determined from ERG recordings (451 nm) (*4*). The extinction coefficient of the mouse UV pigment at 358 nm is 51 800 M^{-1} cm^{-1}, while that of the bovine blue pigment at 438 nm is 43 400 M^{-1} cm^{-1}.

Bovine Blue S90C Mutant Pigment. Previous studies of the avian SWS1 pigments have shown that the Ser90→Cys substitution in the wild-type pigeon (λ_{max} = 393 nm) and chicken (λ_{max} = 415 nm) violet pigments is responsible for 34 and 46 nm blue-shifts, respectively, while the reciprocal substitution (Cys90→Ser) in the wild-type zebrafinch UV pigment (λ_{max} = 359 nm) is responsible for a 38 nm red-shift (*7*). These single Ser/Cys substitutions at position 90 are responsible for the majority of the wavelength modulation between the avian violet and UV pigments (*6*, *7*). Although mouse UV and bovine blue pigments both have Ser at position 90 (Table 1 and Figure 2), we tested the bovine blue S90C mutant for an effect of the mutation on the absorption spectrum. Difference spectra for the bovine blue

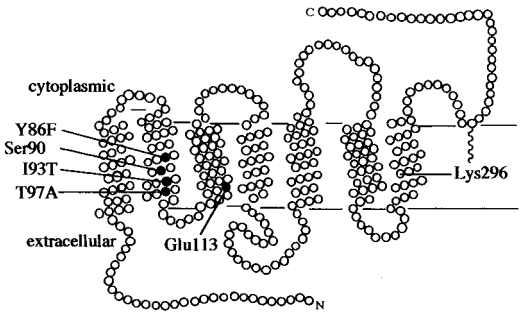

FIGURE 2: Schematic diagram of the SWS1 cone opsin. Filled circles represent individual amino acids selected for mutagenesis. Identification in each case is as follows: The amino acid in the bovine blue opsin is followed by the position number (rhodopsin numbering), followed by the amino acid at that position in the mouse UV opsin. Ser90 and Glu113 are conserved in both the bovine and mouse SWS1 cone opsins, and their positions are indicated. Lys296, the site of chromophore attachment, is also indicated.

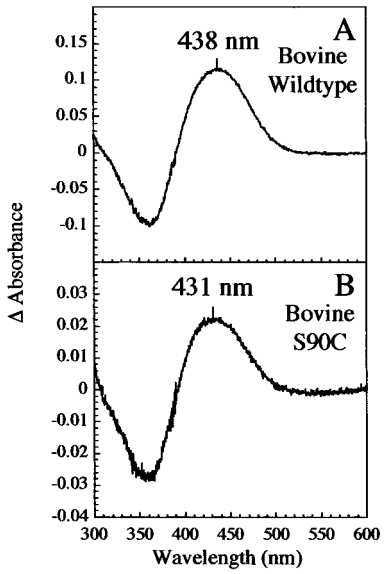

FIGURE 3: Difference spectra of the bovine blue wild-type pigment (A) and S90C mutant (B). The position of the difference maximum is noted above each spectrum.

Table 1: Variation at Nine Amino Acid Sites in Three Mammalian SWS1 Cone Pigments

species	λ_{max} (nm)	46	49	52	81	86	90	93	114	118
mouse	358	Phe	Phe	Thr	Leu	Phe	Ser	Thr	Ala	Ser
human	414	Thr	Leu	Phe	Phe	Leu	Ser	Pro	Gly	Thr
bovine	438	Phe	Phe	Thr	Leu	Tyr	Ser	Ile	Ala	Cys

wild-type and S90C mutant were generated as described under Experimental Procedures and are shown in Figure 3. The difference maximum of the bovine blue S90C mutant (λ_{max} = 431 nm) is blue-shifted 7 nm from that of wild type (λ_{max} = 438 nm). When compared to the approximately 40 nm blue-shift associated with the avian S90C mutants (*7*), it is clear that this mutation has a greatly reduced effect in the bovine blue pigment.

Wavelength Modulation between the Mouse and Bovine SWS1 Pigments. Chimeric proteins of the mouse UV and bovine blue cone opsins were constructed to determine the protein regions responsible for the 80 nm shift in λ_{max} between the two pigments. A chimeric pigment consisting

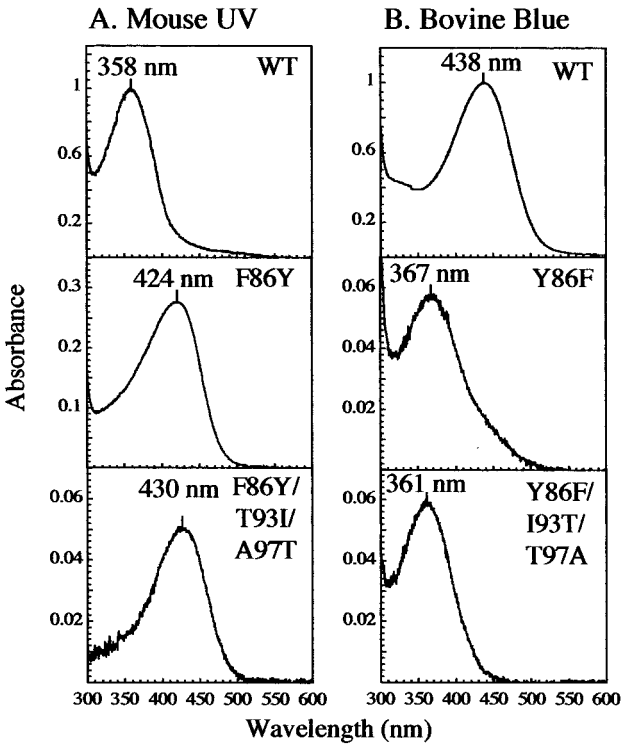

FIGURE 4: Absorption spectra of the wild-type mouse and bovine SWS1 pigments and mutants. (A) Spectra for mouse UV F86Y and F86Y/T93I/A97T mutants; (B) spectra for bovine blue Y86F and Y86F/I93T/T97A mutants. The position of the absorption maximum is noted above each spectrum.

of bovine blue amino acids 1−150 and mouse UV amino acids 151−348 displays a λ_{max} of 430 nm, while the reciprocal chimeric pigment displays a λ_{max} of 358 nm (data not shown). From these experiments, we concluded that helices I−III were responsible for most of the difference. To determine specific amino acids involved in the spectral differences between these two pigments, amino acid residues in helix II were selected for site-directed mutagenesis. Selection was based on relative proximity to the Schiff base region of the chromophore as inferred from the X-ray crystal structure of rhodopsin (*20*) as well as nonconservative amino acid differences between these two pigments. Only three amino acid substitutions, at positions 86, 93, and 97 (Figure 2), met these criteria. The substitutions were first made in the mouse UV opsin (F86Y, T93I, and A97T). While little change was observed for substitutions at positions 93 and 97, the single amino acid substitution Phe86→Tyr resulted in a dramatic red-shift of 66 nm (λ_{max} = 424 nm) accounting for 83% of the total wavelength shift (Figure 4A, middle). Further red-shifting was observed in the F86Y/A97T double mutant (λ_{max} = 426 nm), but not in the F86Y/T93I mutant (λ_{max} = 424 nm, data not shown). However, when the three mutations were incorporated together, the resulting absorption maximum of 430 nm accounted for 90% of the total shift from wild type (Figure 4A, bottom). The Tyr86→Phe substitution at position 86 in the bovine blue pigment resulted in a dramatic blue-shift of 71 nm (λ_{max} = 367 nm) accounting for 89% of the total shift from wild type (Figure 4B, middle). It should be noted that the bovine blue Y86F mutant absorption spectrum is unusually broad, resulting from additional absorption along the long-wavelength arm. The bovine blue Y86F/I93T and Y86F/T97A double mutant

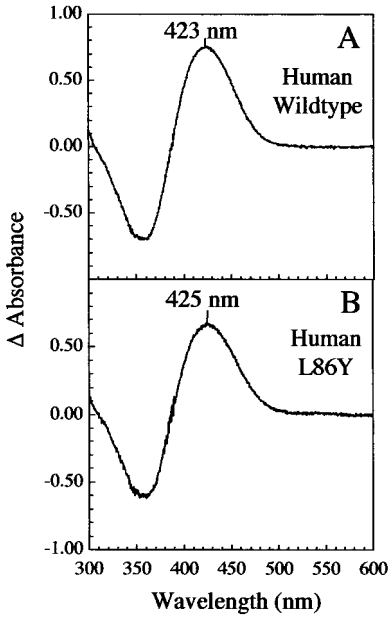

FIGURE 5: Difference spectra of the human blue wild-type pigment (A) and L86Y mutant (B). The position of the difference maximum is noted above each spectrum.

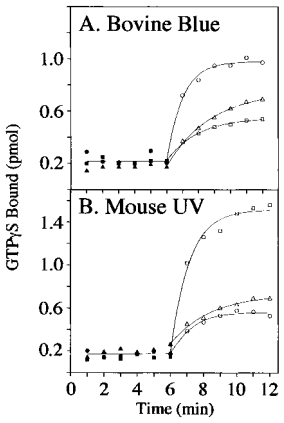

FIGURE 6: Light-dependent activation of bovine transducin by (A) bovine blue wild type (○), Y86F (□), and Y86F/I93T/T97A (△); and (B) mouse UV wild type (○), F86Y (□), and F86Y/T93I/A97T (△). Transducin activity was assayed by measuring the level of binding of [^{35}S]GTPγS using 15 nM purified pigment from transfected COS cells. The first six time points were taken from reactions carried out in the dark (filled symbols), while the last six time points were taken after reactions were exposed to light at t = 6.25 min (open symbols).

spectra were quite similar to that of Y86F (data not shown). However, the combination of Y86F, I93T, and T97A resulted in an additional 6 nm blue-shift (λ_{max} = 361 nm, Figure 4B, bottom) accounting for 96% of the total shift from wild type. Comparison of the absorption spectra of the bovine blue Y86F and Y86F/I93T/T97A mutants shows that the additional 6 nm blue-shift observed in the triple mutant may have resulted from the removal of an additional absorbance band that appears to be present in the long-wavelength arm of the single mutant spectrum.

To determine if Tyr86 has an effect on the absorption spectrum of the human blue pigment, we constructed the human Leu86→Tyr mutant. Difference spectra for the human blue wild type and L86Y mutant were generated and are shown in Figure 5. The difference maximum of the human blue wild-type pigment (Figure 5A; λ_{max} = 423 nm) is in good agreement with previously published results (*18*). The difference maximum of the human blue L86Y mutant (Figure 5B; λ_{max} = 425 nm) is red-shifted 2 nm from that of wild type. When compared to the 66 nm red-shift associated with the mouse F86Y mutant, it is clear that this mutation has little effect on the human blue pigment.

Transducin Activation by SWS1 Mutants. The bovine and mouse SWS1 wild-type pigments as well as the 86 and 86/93/97 mutants are functional in that they are capable of activating bovine rod transducin in a light-dependent manner, as shown in Figure 6. The mouse UV F86Y mutant showed greater activity than either the mouse UV wild type or the F86Y/T93I/A97T mutant, both of which had similar activities. The bovine blue Y86F and Y86F/I93T/T97A mutants had activities lower than bovine wild type and similar to those of the mouse UV wild type and F86Y/T93I/A97T mutant.

Mutagenesis of Glu113. Glu113 found on helix III (Figure 2) is a highly conserved residue in all visual pigments and has been shown to be the counterion to the protonated Schiff base in rhodopsin (*21*−*23*). Neutralization of this glutamate in the rhodopsin mutant E113Q results in a dramatic blue-shift of the λ_{max} from 500 to 380 nm as a consequence of deprotonation of the Schiff base nitrogen (*21*, *22*). To better understand the protonation state of the chromophore in the mammalian SWS1 cone pigments, we constructed a series of Glu113 mutants in both the mouse UV and bovine blue pigments. Although the bovine blue Glu113 mutants failed to regenerate with 11-*cis*-retinal, the mouse UV E113A and E113Q mutants resulted in spectra similar to that of the wild-type pigment as shown in Figure 7. The absorption spectra of the mouse E113A and E113Q mutants display modest shifts of 4 and 7 nm to the blue, respectively, when compared to wild type. The mouse E113Q spectrum is similar to those previously reported for this mutant (*9*, *24*).

The role of Glu113 in the human blue pigment (λ_{max} = 414 nm) was recently investigated by Yokoyama and co-workers (*9*). Because the human blue E113Q mutant failed to regenerate with 11-*cis*-retinal (see also ref *18*), Yokoyama and co-workers used instead a mouse UV mutant that contains seven amino acid substitutions from the human blue pigment, resulting in an absorption spectrum with a maximum at 411 nm. When Glu113 is changed to Gln in this mutant, the absorption maximum shifts from 411 to 369 nm, suggesting that the Schiff base nitrogen has deprotonated with the introduction of the Glu113→Gln mutation (*9*). Based on this result, and the fact that the absorption maxima of the human blue and mouse UV seven-site mutant are quite similar, Yokoyama and co-workers conclude that it is highly likely that the human blue pigment possesses a chromophore with a protonated Schiff base (*9*). To investigate the role of Glu113 in the bovine blue pigment, we conducted an experiment similar to that of Yokoyama and co-workers in which we introduced the Glu113→Gln mutation into the mouse UV F86Y mutant. Absorption spectra of the mouse UV F86Y/E113Q mutant are shown in Figure 8. At pH 6.0, the spectrum shows two absorption maxima, one at 351 nm and a second long-wavelength absorbance near 420 nm. When the pH was raised to pH 7.0, the long-wavelength maximum disappeared with a concomitant increase in absorbance at 351 nm resulting in a spectrum with a λ_{max} identical to that of the mouse UV E113Q mutant (see Figure

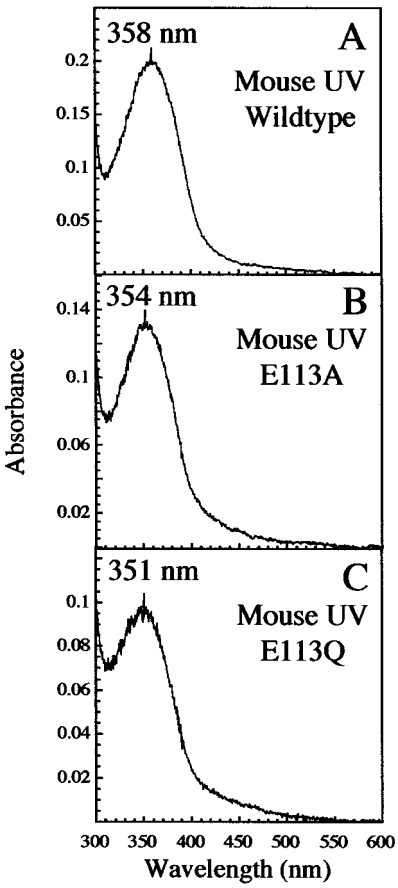

FIGURE 7: Absorption spectra of the wild-type mouse UV pigment and Glu113 mutants. The position of the absorption maximum is noted above each spectrum.

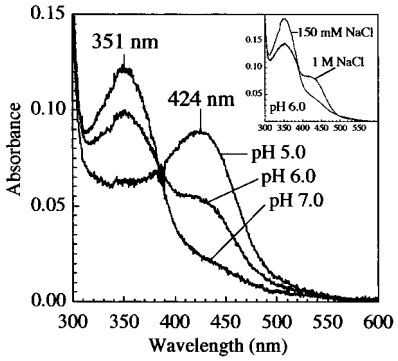

FIGURE 8: Effect of pH and NaCl concentration on the spectrum of the mouse UV F86Y/E113Q mutant. Each spectrum is labeled according to pH with absorption maxima noted above the pH 5.0 and 7.0 spectra. The inset shows mouse UV F86Y/E113Q spectra at pH 6.0 in the presence of 150 mM and 1 M NaCl. All spectra were normalized at 280 nm.

7C). When the pH was lowered to pH 5.0, the 351 nm maximum disappeared with a concomitant increase in the long-wavelength maximum of 424 nm resulting in a spectrum with a λ_{max} identical to that of the mouse UV F86Y mutant (see Figure 4A, middle). The mouse UV F86Y/E113Q spectra at pH 5.0, 6.0, and 7.0 all converted to a 440 nm absorbing species upon acid denaturation, characteristic of a covalently attached chromophore (not shown). These results suggest that the 424 nm absorbing species at pH 5.0 contains a protonated Schiff base while the 351 nm absorbing species at pH 7.0 contains an unprotonated Schiff base, with the pH 6.0 spectrum containing both the protonated and unprotonated forms. The protonated Schiff base species observed at both pH 5.0 and 6.0 are most likely associated with a chloride counterion recruited from solution. This conclusion is based on the fact that when the chloride concentration was increased from 150 mM to 1 M at pH 6.0, the concentration of the 424 nm species increased at the expense of the 351 nm species (Figure 8, inset).

DISCUSSION

The mouse and bovine SWS1 pigments are ideal candidates for identifying important residues involved in spectral tuning of the SWS1 pigments due to the 80 nm separation between absorption maxima and the 87% amino acid identity shared between the pigments. Large spectral shifts in absorption maxima resulting from the mouse/bovine SWS1 chimeric pigments narrowed our search to residues in helices I−III, with helices II and III being principal targets due to their proximity to the Schiff base region. Based on a site-directed mutagenesis study of helix II, we have shown that a single Phe/Tyr substitution at position 86 alone is responsible for a very large ~70 nm shift between the mouse UV and bovine blue absorption maxima. This single amino acid substitution accounts for the majority of the shift between the two pigments. The addition of two more amino acid substitutions at positions 93 and 97 is less important, but is involved in further shifts between these maxima. When the Tyr substitution at position 86 is introduced into the human blue pigment, the Leu86→Tyr mutation does not significantly alter the absorption spectrum.

Previous investigations have shown that in the avian blue cone pigments a Ser90→Cys substitution is responsible for an approximately 40 nm blue-shift from the visible to the UV region of the spectrum (*6*, *7*). Unlike the avian SWS1 opsins, all mammalian SWS1 opsins including the UV pigments have a Ser at position 90 (Table 1). Therefore, it is clear that the amino acid substitutions responsible for spectral tuning in the mammalian SWS1 pigments must be different from those found in birds (*6*, *7*), and we have shown here that a Ser90→Cys substitution has little effect on the spectrum of the bovine blue pigment.

Yokoyama and co-workers (*8*, *9*) have recently investigated the mechanisms of spectral tuning in the mouse UV and human blue cone pigments. They conclude that the ancestral SWS1 pigment was UV-sensitive and possessed the amino acids Phe46/Phe49/Thr52/Phe86/Ser90/Thr93/Ala114/Ser118. They also showed that the wavelength difference between the mouse UV and human blue cone pigments is accounted for by amino acids at eight sites: 46, 49, 52, 81, 86, 93, 114, and 118, as is shown in Table 1. Substitution of all eight amino acids is required to observe nearly the complete shift with five of these, at positions 52, 86, 93, 114, and 118, accounting for most of the shift (*8*). Interestingly, single amino acid substitutions at any of these eight positions fail to shift the absorption spectrum (*9*). Our finding that a single Phe/Tyr amino acid substitution at position 86 is responsible for most of the difference between the mouse UV and bovine blue cone pigments is at odds with the mutagenesis models proposed by Yokoyama and co-workers (*8*, *9*) for nonavian SWS1 pigments. In fact, reliance on a single amino acid substitution is more like the

avian SWS1 pigments. The human and bovine SWS1 pigments do not share any amino acids at the eight critical positions (Table 1), suggesting that the human blue pigment is not intermediate between the mouse UV and bovine blue pigments. This is further supported by the fact that the Leu86→Tyr substitution in human blue has little effect on the absorption spectrum. On the other hand, the mouse and bovine SWS1 pigments share five amino acids at these eight positions, a surprising fact considering the long evolutionary distance between these mammalian lineages (*25*). The simplest explanation for the variation in amino acid substitutions by which the bovine and human SWS1 pigments shift their absorption spectra away from the UV is that the mammalian blue pigments have evolved more than once. Because of the extensive evolutionary distance between the mouse and bovine lineages, we predict that other mammalian lineages also possess the Phe86→Tyr substitution, as described here, with the possibility that only the primates possess variations of the eight-site model as described by Yokoyama and co-workers (*9*). Of course, further sequence and spectral analyses of the SWS1 pigments from other mammalian orders will be required to determine if this is the case.

Substitution of the conserved glutamate at position 113 in the mouse UV pigment fails to shift significantly the absorption spectrum, suggesting that the wild-type mouse UV pigment has a chromophore with an unprotonated Schiff base (presented here as well as in refs *9* and *24*). In contrast, substitution of Glu113 in the mouse UV F86Y mutant dramatically shifts the absorption maximum from 424 to 351 nm at pH 7.0. Furthermore, the spectral properties of the mouse UV F86Y/E113Q mutant are pH-dependent. When the pH is dropped, there is a loss of the species with a UV maximum at 351 nm and a concomitant increase in a species with a long-wavelength maximum at 424 nm. This appears to result from protonation of the Schiff base nitrogen. Therefore, we conclude that the Schiff base in the mouse UV F86Y/E113Q mutant ($\lambda_{max} = 351$ nm) is unprotonated at neutral pH. Because the maximum of the mouse UV F86Y mutant (424 nm) is similar to that of the wild-type bovine blue pigment (430 nm) and because the mouse UV F86Y/E113Q maximum (351 nm) appears to result from a deprotonation event, we infer that the Schiff base in the wild-type bovine blue pigment is protonated. It is likely that the SWS1 cone pigments with absorption maxima greater than 414 nm result from protonation of the Schiff base nitrogen. This conclusion is based on several independent investigations of the human blue ($\lambda_{max} = 414$ nm) (*9*) and the *Xenopus* violet ($\lambda_{max} = 427$ nm) (*24*, *26*) pigments, both of which are believed to possess a protonated Schiff base chromophore. Based on our results, we conclude that the Phe86→Tyr substitution is responsible for protonating the Schiff base utilizing Glu113 as the counterion and shifting the absorption spectrum away from the UV region of the spectrum.

In summary, the results presented here suggest that the mouse UV pigment has an unprotonated Schiff base chromophore and the bovine blue pigment has a protonated Schiff base chromophore. While the human blue pigment appears to have a protonated Schiff base chromophore (*9*), it is clearly different from that of bovine in that the human blue pigment must rely strongly on perturbation from the opsin protein to achieve its 414 nm absorption maximum.

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