The molecular mechanism for the spectral shifts between vertebrate ultraviolet- and violet-sensitive cone visual pigments

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The short-wave-sensitive (SWS) visual pigments of vertebrate cone photoreceptors are divided into two classes on the basis of molecular identity, SWS1 and SWS2. Only the SWS1 class are present in mammals. The SWS1 pigments can be further subdivided into violet-sensitive (VS), with $\lambda_{\max}$ (the peak of maximal absorbance) values generally between 400 and 430 nm, and ultraviolet-sensitive (UVS), with a $\lambda_{\max} < 380$ nm. Phylogenetic evidence indicates that the ancestral pigment was UVS and that VS pigments have evolved separately from UVS pigments in the different vertebrate lineages. In this study, we have examined the mechanism of evolution of VS pigments in the mammalian lineage leading to present day ungulates (cow and pig). Amino acid sequence comparisons of the UVS pigments of teleost fish, amphibia, reptiles and rodents show that site 86 is invariably

occupied by Phe but is replaced in bovine and porcine VS pigments by Tyr. Using site-directed mutagenesis of goldfish UVS opsin, we have shown that a Phe-86 → Tyr substitution is sufficient by itself to shift the $\lambda_{\max}$ of the goldfish pigment from a wild-type value of 360 nm to around 420 nm, and the reverse substitution of Tyr-86 → Phe into bovine VS opsin produces a similar shift in the opposite direction. The substitution of this single amino acid is sufficient to account therefore for the evolution of bovine and porcine VS pigments. The replacement of Phe with polar Tyr at site 86 is consistent with the stabilization of Schiff-base protonation in VS pigments and the absence of protonation in UVS pigments.

Key words: evolution, opsin, spectral tuning.

INTRODUCTION

Visual pigments are members of the superfamily of G-protein-coupled receptors which function through the activation of a G-protein and an effector enzyme. Visual pigments are based on a common basic structure of an opsin protein with a chromophore covalently attached via a Schiff-base linkage to a conserved Lys residue in transmembrane (TM) helix 7. Each pigment shows a characteristic $\lambda_{\max}$ (the peak of maximal absorbance), the precise location of the peak depending on interactions between the chromophore, 11-*cis*-retinal, and the opsin protein. In vertebrates, the opsin protein consists of a single polypeptide chain of 340–370 amino acids that forms seven α -helical TM regions connected by cytoplasmic and luminal loops [1,2]. In the tertiary structure, the seven α -helices form a bundle within the membrane creating a cavity on the luminal side for the chromophore.

In vertebrates, a single rod class of visual pigments is generally present with up to four cone classes as follows: a long-wave (LW)-sensitive class with a $\lambda_{\max}$ of 530–570 nm, a middle-wave-sensitive class with a $\lambda_{\max}$ of 480–520 nm and two short-wave-sensitive (SWS) classes (SWS1 and SWS2) with $\lambda_{\max}$ values of 430–470 nm for SWS2 and 355–435 nm for SWS1. The latter class shows some of the largest naturally occurring variations in $\lambda_{\max}$, ranging for example in mammals from around 430 nm in primates [3] to less than 370 nm in rodents [4], and in birds

from around 420 nm in chicken [5] and duck [6] to around 365 nm in canary [7] and budgerigar [8].

The amino acid changes responsible for the spectral shift between the violet-sensitive (VS) and ultraviolet-sensitive (UVS) SWS1 pigments of birds have now been identified. By comparing the amino acid sequences of SWS1 pigments from a number of avian species with $\lambda_{\max}$ values varying from 365 to 420 nm, Wilkie et al. [9,10] demonstrated by *in situ* mutagenesis and regeneration of the recombinant opsins *in vitro* that replacement at site 90 in TM 2 of Cys present in avian UVS pigments with Ser present in avian VS pigments produced a spectral shift of about 35 nm from 363 to 398 nm. The remaining shift to around 420 nm can be accounted for by the additive effects of substitutions at sites 93 and 118. The tuning of avian pigments into the UV has also been examined by Yokoyama et al. [11], who confirmed that the reverse substitution of Cys at site 90 into an avian VS pigment produced a short-wave (SW) shift of similar magnitude. The presence of Cys-90 in avian SWS1 pigments is responsible therefore for tuning into the UV. However, since the UVS pigments of all other vertebrate classes (teleost fish [12,13], amphibia [14], reptiles [15,16] and mammals [17,18]) retain Ser-90, the central role of Cys-90 in tuning into the UV would appear to be limited to avian UVS pigments. A different mechanism must be present therefore in non-avian species.

The first study of the mechanism of tuning of mammalian SWS1 pigments focused on differences between human rod and

Abbreviations used: $\lambda_{\max}$, peak of maximal absorbance; LW, long wave; SW, short wave; SWS, short-wave-sensitive; TM, transmembrane; UVS, ultraviolet-sensitive; VS, violet-sensitive.

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VS cone opsins [19]. Nine sites were identified which, when simultaneously substituted into the rod pigment, produced about 80 % of the SW shift from 500 nm of the human rod pigment to around 425 nm of human VS pigment. A subsequent study [20], however, highlighted an inherent problem of substituting across different classes of opsins. Starting with human VS opsin, the reverse mutation at one of the previously identified sites in the vicinity of the β -ionone ring [19] produced the expected shift whereas the reverse mutation at another of the sites near the Schiff base produced a shift in the wrong direction.

Such problems can be largely avoided by limiting the sequence comparisons to SWS1 pigments. A chimaeric opsin comprising TMs 1–3 from human VS and TMs 4–7 from mouse UVS, when expressed and regenerated with retinal, produced a pigment with a $\lambda_{\max}$ very close to the native human pigment [21]. Therefore the same region of the opsin protein (TMs 1–3) is important for UV/violet spectral shifts in mammals and birds. Moreover, the simultaneous replacement of the residues present at seven sites (Phe-46 $\rightarrow$ Thr, Phe-49 $\rightarrow$ Leu, Thr-52 $\rightarrow$ Phe, Phe-86 $\rightarrow$ Leu, Thr-93 $\rightarrow$ Pro, Ala-114 $\rightarrow$ Gly and Ser-118 $\rightarrow$ Thr) in mouse UVS with those in human VS produced a shift from 359 to 411 nm, and the reverse mutations in human VS shifted the $\lambda_{\max}$ to 360 nm [22]. Overall therefore, these substitutions go a long way towards achieving the full shift of 65 nm between mouse UVS and human VS pigments, although the effect must be synergistic since single substitutions had no effect on $\lambda_{\max}$.

The other group of mammals that possess VS SWS1 pigments are the Artiodactyla or even-toed ungulates [23]. However, in the two species (cow and pig) from this group of which the sequence of the SWS1 opsin is known [24] (accession numbers U92557 and AY091587), none of the seven sites detailed above are occupied in both pigments with the same amino acid residue present in the human VS pigment. The inevitable conclusion is that a different mechanism of spectral tuning occurs in these pigments. In the study reported in this paper we examined the molecular basis for the violet shift in the $\lambda_{\max}$ of these pigments from the ancestral UVS pigment [21,22,24].

EXPERIMENTAL

cDNA synthesis

Poly(A)⁺ mRNA was extracted from the retinae of freshly isolated, dark-adapted goldfish and frozen bovine eyes using a Quickprep Micro mRNA purification kit (Amersham Biosciences). Single-stranded cDNA was synthesized using an oligo-d(T) anchor primer (Boehringer Mannheim).

Generation of opsin mutants and construction of opsin expression vector

The entire coding sequences of wild-type goldfish UVS and bovine VS opsin were amplified from goldfish and bovine retinal cDNAs with *Pfu* DNA polymerase, using primer pairs containing restriction-site tags for *Eco*RI and *Sal*I (Table 1). The resulting PCR products were then directionally cloned as *Eco*RI/*Sal*I fragments into pGEM-T easy (Promega) and into a derivative of the mammalian expression vector pMT4 carrying the sequence of bovine rod opsin 1D4 epitope (including the stop codon) downstream of and in-frame with the *Sal*I site [25]. The sequences of the resulting opsin fragments were then checked using vector- and gene-specific sequencing primers.

The pGEM-T easy clones of the goldfish UVS and bovine VS coding sequences were used for generating mutants using the QuikChangeTM site-directed mutagenesis kit (Stratagene). Primers used to create the Phe-86 $\rightarrow$ Leu, Phe-86 $\rightarrow$ Met and

Table 1 Primers containing restriction enzyme tags for *Eco*RI and *Sal*I, used to amplify goldfish and bovine SWS1 opsins

Underlined sequences are the *Eco*RI sites in the +primers and *Sal*I sites in the –primers. Sequences homologous to the corresponding SWS1 opsins are shown in bold.

	Primer sequence (5' $\rightarrow$ 3')
Goldfish UVS +	GCGCGAATTCACCATGGACGCGTGGACCTATCAA
Goldfish UVS –	CGGCGTCGACGCTGCAGACACAGAGGAGGTTTC
Bovine VS +	GCGCGAATTCACCATGAGCAAGATGTCAGAGGAG
Bovine VS –	CGGCGTCGACGCGTTGGGGCCAACCTGGCTAGA

Table 2 Primer pairs used with the QuikChangeTM site-directed mutagenesis kit to introduce mutations into the goldfish and bovine SWS1 opsins

Underlined bases indicate the substitutions that were introduced.

	Primer sequence (5' $\rightarrow$ 3')
Goldfish 86Leu +	CCCTAGGAGGCTTTATTCTCGACACTTTTCTGTAAAGC
Goldfish 86Leu –	GCTTACAGAAAAAGTGTGAGAAATAAGCCTCCTAGGG
Goldfish 86Met +	CCCTAGGAGGCTTTATTATGACACTTTTCTGTAAAGC
Goldfish 86Met –	GCTTACAGAAAAAGTGTCCATAATAAGCCTCCTAGGG
Goldfish 86Tyr +	CCCTAGGAGGCTTTATTACGACACTTTTCTGTAAAGC
Goldfish 86Tyr –	GCTTACAGAAAAAGTGTGCTAAATAAGCCTCCTAGGG
Bovine 86Phe +	CTGGGGGGCTTCATCTTCTGCATCTTCTCTG
Bovine 86Phe –	CAGAGAAGATGCAGAGATGAAGCCCCCAG

Phe-86 $\rightarrow$ Tyr mutations in goldfish UVS opsin and the Tyr-86 $\rightarrow$ Phe mutation in bovine VS opsin are listed in Table 2. Following mutagenesis, the nucleotide sequences of the resulting opsin fragments were confirmed by sequencing using vector- and gene-specific primers. Mutated fragments were then inserted into the expression vector.

Expression, purification and characterization of wild-type and mutant pigments

Opsins were transiently expressed in HEK-293T cells following transfection with Lipofectamine reagent according to the manufacturer's instructions (Invitrogen). Thirty 90 mm plates were used per transfection. Cells were harvested 48 h post-transfection, washed four times with PBS, pH 7.0, and the cell pellets stored at -80°C prior to generation of the pigments. Pigments were generated by suspending the cells in either PBS, pH 7.0, or a Hepes buffer, pH 6.6 [50 mM Hepes, 140 mM NaCl, 3 mM MgCl_2 and 20 % (w/v) glycerol] and incubating with 40 μM 11-*cis*-retinal in the dark [26,27]. Pigment was solubilized from cell membranes by incubating with buffer containing 1 % (w/v) dodecyl-maltoside and 20 mg/ml PMSF and purified by immunoaffinity chromatography using a monoclonal antibody directed against the 1D4 epitope of bovine rod opsin coupled to a CNBr-activated Sepharose column [26].

Absorption spectra of purified pigments were recorded in the dark using a dual-path spectrophotometer (Spectronic Unicam, Cambridge, U.K.). UVS pigments were acid denatured and the spectra recorded after the addition of 1 M HCl to bring the pH down to less than 2. VS pigments were bleached by the addition of 56.7 mM hydroxylamine and exposure to fluorescent light for 10 min. The $\lambda_{\max}$ value of each pigment was determined by subtracting the acid-denatured or hydroxylamine-treated spectrum from the dark spectrum to produce a difference spectrum.

RESULTS

The ancestral vertebrate UVS pigment

The phylogenetic tree shown in Figure 1 is derived from the amino acid sequences of SWS1 opsins and includes representative species of all five vertebrate classes. The topology of the tree clearly indicates that the ancestral pigment was most likely UVS. The amino acids at three sites, 86, 90 and 93, have been placed on to the branch and end points of the tree. These sites generally show a consistency of substitution across species with UVS or VS pigments and are adjacent to the Schiff base linkage/Glu-113

counterion in the folded protein [9,19,28,29]. Phe-86 and Ala/Ser-90 have been retained from the ancestral into the UVS pigments of fish, amphibians, reptiles and rodents. The VS pigments of birds have a Phe-86 → Ser substitution, with UVS pigments secondarily arising in the avian lineage from a VS pigment in which a Ser-90 → Cys substitution has occurred [9,10,21,30]. In the bovine and porcine VS pigments, a Phe-86 → Tyr substitution is present whereas in primate VS pigments a double substitution (Phe-86 → Leu and Thr-93 → Pro) is found in all species. The amphibian VS pigment also has the Thr-93 → Pro substitution but possesses Phe-86 → Met. The consistent substitutions

Figure 1 Phylogenetic tree of SWS1 opsins based on amino acid divergence

The amino acid residues present at sites 86, 90 and 93 are shown at each point on the tree where substitution is inferred to have occurred. Redrawn with the addition of the porcine and gecko branches from [21]. GenBank accession numbers: bovine VS, U92557; budgerigar UVS, Y11787; canary UVS, AJ277922; chameleon UVS, AF134192; chicken VS, M92039; cichlid fish UVS, AF191219; clawed frog VS, U23463; gecko VS, M92035; goldfish UVS, D85863; human VS, NM001708; macaque VS, AF158976; marmoset VS, [34]; mouse UVS, AF190671; pigeon VS, AJ238856; porcine VS, AY091587; rat UVS, AF051163; salamander UVS, AF038948; squirrel monkey VS, U53875; zebra finch UVS, AF222331; zebrafish UVS, AF109373.

Figure 2 Absorbance spectra for goldfish wild-type and mutant SWS1 pigments

(A) Dark and acid-denatured spectra of the wild-type pigment. (B–D) Difference spectra for wild-type and mutant pigments. For details see the Experimental section.

in the VS pigments across the different species are therefore Phe-86 → Ser in birds, Phe-86 → Tyr in cow and pig, and Thr-93 → Pro in amphibia and primates.

Mutagenesis at site 86 in goldfish UVS and bovine VS pigments

Recombinant goldfish UVS pigments were generated by transient transfection of HEK-293T cells followed by regeneration of the expressed opsin with 11-*cis*-retinal. The wild type and two of the mutants (Phe-86 → Met and Phe-86 → Leu) produced a photopigment when regenerated at pH 7.0 whereas no photopigment was produced from the Phe-86 → Tyr mutant at this pH. The wild-type and Phe-86 → Tyr regenerations were repeated at pH 6.6 where both formed photopigments. The lower pH had no effect on the λ_{max} of the wild-type pigment. The dark and acid-denatured spectra for wild-type goldfish UVS pigment generated at pH 7.0 are shown in Figure 2(A), and the difference spectra of the wild-type and mutant pigments in Figures 2(B)–2(D). The

Figure 3 Absorbance difference spectra for bovine wild-type and mutant SWS1 pigments

wild-type pigment has a λ_{max} of 360 nm, in line therefore with the λ_{max} of the native pigment obtained by microspectrophotometry [31]. The Phe-86 → Met and Phe-86 → Leu mutants both regenerated to give pigments with λ_{max} values identical to the wild type. These substitutions are found respectively in amphibia and primates; the absence of any spectral shift in the mutant pigments indicates that neither is important for the spectral tuning of the corresponding pigments. In contrast, the Phe-86 → Tyr substitution produced a LW shift of 60 nm to give a λ_{max} of approx. 420 nm.

The difference spectra for wild-type and the Tyr-86 → Phe mutant bovine VS pigments generated at pH 7.0 are shown in Figure 3. The λ_{max} of the wild type is 435 nm, somewhat shorter therefore than the reported value of 451 nm for the native pigment obtained *in situ* by electroretinogram flicker photometry [23]. In contrast, the Tyr-86 → Phe mutant showed a SW shift to a λ_{max} of around 360 nm. The substitution therefore of Tyr-86 → Phe in bovine VS opsin and Phe-86 → Tyr in goldfish UVS opsin produces shifts in the regenerated pigments of very similar magnitudes but in opposite directions. Substitution at this site is sufficient by itself therefore to change a UVS into a VS pigment and vice versa. Since this substitution is shared by the cow and pig pigments, this would appear to be the evolutionary mechanism whereby the SWS1 pigments of the Artiodactyla have been LW-shifted to produce VS pigments.

Modelling of site 86 mutations

The alignment of the rod and SWS1 opsin sequences (Figure 4A) demonstrates the relatively low level of amino acid identity of

A

Bovine rod	MNGTEGPNFY	VPFSNKTGVV	RSPFEAPQYY	LAEPHQSML	AAVFLIML	GPIINFLTY	VIVQHKLRT	PLNYIILNLA	80
Bovine VS	MSKMSEEEF	LLFNISLV-	-GPWDGPOYH	LAPVWAFHQ	AVFMGFVFFV	GPIINATVLA	ATLRYRKLRO	PLNYILNVVS	
Goldfish UVS	MDAWTYQ---	--EQLSKI-	-SPFEGPOYH	LAPKWAELQ	AAFMGFVFFV	GPIINATVLF	VIMKYKLRQ	PLNYILNVIS	
Bovine rod	VADLFMVFGG	FTTLYTSLH	GYFVFGPTGC	NLEGFFATLG	SEIALWSLAV	LATERYVVC	KMSNFRHGE	NHAIMGVAFI	160
Bovine VS	LGGFIYCIFS	VFIVFITSY	GYFVFGRHVC	ALEAFLGCTA	GLVTGWSLAF	LAFERYIIC	KFFGNFRESS	KHALMVMVAT	
Goldfish UVS	LGGFIFDTFS	VSQVFFSALR	GYFFFGYTLC	AMEAAMGSIA	GLVTGWSLAV	LAFERYVVIC	KFFGSEKFGQ	SOALGAVAIT	
Bovine rod	WMALACAAF	PLVWSRYIF	EQMCCSGID	YYPHEETNN	ESFVIYMFV	HFIIPLVIF	FCYQOLFTV	KENAAQCES	240
Bovine VS	WTIGIGVSI	PFGWRSEVP	EGLQSCGHD	WYTVGTRYYS	EYTWFLFIF	CYIVPLSLIC	FSYQQLLAL	RAVAAQCES	
Goldfish UVS	WIIGIGCATP	PFGWSRYIF	EGIGTACGH	WYTKNEEYNT	ESYTYFLVS	CFMIPIMIT	FSYQQLLAL	RAVAAQCES	
Bovine rod	ASTQKAEKV	TRMVLIMIA	FLICNLFPYAG	VAFYLFTHQG	SDFGPIFMTI	PAFFAKTSAV	YNPVIYIMN	KQERNQVTT	320
Bovine VS	ASTQKAEKV	SRMVMVMS	FCLCYTPYAA	LAMYIVNNRN	HGVDLRLVTI	PAFFSKSACV	YNPIIYCFMN	KQFRACIMEM	
Goldfish UVS	ASTQKAEKV	SRMVMVMS	EVVICYGYAI	TALYFSYAE	SNKDYRLVAT	PSLFSKSSCV	YNPIIYAFMN	KQFNACIMEV	
Bovine rod	LCCKGNPLGD	DEASTTVSKT	ETSSVAPA						348
Bovine VS	VC-GK-PMTD	ESELSSSQKT	EVSTVSSSQV	GPN					
Goldfish UVS	VF-GK-KIDE	SSEVSS--KT	ETSSVSA

Figure 4 Sequence alignments and structural model of SWS1 pigments

(A) Clustal W alignment of bovine rod, bovine VS and goldfish UVS opsins. Amino acids showing identity across all three sequences are boxed. The TM regions are indicated by horizontal lines. (B) View of wild-type goldfish UVS pigment modelled with Swiss Model [31] on to the bovine rod opsin crystal structure [32]. The complete molecule is shown but with portions of helices 3 and 5 $\rightarrow$ 7 cut away to reveal retinal, Lys-296, the Schiff base linkage, Glu-113, and Phe-86, Ser-90 and Gln-93. (C) Enlarged view showing retinal and the key amino acid residues only, with the Phe-86 $\rightarrow$ Tyr substitution.

around 40% between rod and SWS1 cone opsin sequences. Nevertheless, the structures of the goldfish UVS and bovine VS opsins were successfully mapped using Swiss Model [32] on to the structure of bovine rod opsin derived from X-ray crystallographic data [29]. The model of the wild-type goldfish pigment, with Phe at position 86, and the Phe-86 $\rightarrow$ Tyr mutant are shown in Figures 4(B) and 4(C). The locations of Lys-296, Glu-113 and the residues at positions 90 (Ser) and 93 (Gln) are also indicated. In the wild type, Phe-86 approaches close to the Schiff-base nitrogen atom but both Ser-90 and Gln-93 are relatively remote. Hence, aside from Glu-113, there is no polar or charged group in the vicinity to stabilize the protonation of the Schiff-base linkage. Substitution of a Tyr residue at position 86, however, introduces a polar hydroxyl group adjacent to the Schiff-base linkage. The estimated distance between the Schiff-base nitrogen and the oxygen of Tyr-86 is 3.6 Å, which is sufficiently close to mediate stabilization of a proton on the Schiff base via an intervening water molecule. The other two mutants studied, Phe-86 $\rightarrow$ Met and Phe-86 $\rightarrow$ Leu, do not introduce a polar group at position 86

and are too remote to exert an influence on the Schiff-base linkage.

DISCUSSION

The UVS pigments of fish, reptiles, amphibia and rodents all possess Phe-86. This is replaced in the VS pigments of cow and pig by Tyr-86. The introduction of a Phe-86 $\rightarrow$ Tyr substitution by site-directed mutagenesis into goldfish UVS pigment LW-shifts the $\lambda_{\max}$ from 360 to 420 nm and the reverse Tyr-86 $\rightarrow$ Phe substitution in bovine VS pigment SW-shifts the $\lambda_{\max}$ from 435 to 360 nm. The single replacement of Phe by polar Tyr at site 86 therefore accounts for the evolution of the VS bovine and porcine pigments from an ancestral UVS pigment. In the VS pigment of primates [33,34] and the clawed frog [35], Leu and Met respectively are present at site 86. Substitution of these residues into the goldfish UVS pigment is, however, without effect on $\lambda_{\max}$, indicating that substitution at site 86 is not involved in the tuning of the primate and amphibian VS pigments.

There is little doubt that all vertebrate visual pigments with $\lambda_{\max}$ values > 400 nm possess a protonated Schiff base and the role of certain residues in the vicinity of the retinylidene Schiff-base linkage may be to stabilize this protonation. The replacement of Phe by Tyr at site 86 in the bovine and porcine VS pigments may serve therefore to facilitate the electrostatic stabilization of protonation in these pigments. The distance between the hydroxyl group of tyrosine and the Schiff-base nitrogen of 3.6 Å predicted from modelling the Phe-86 → Tyr mutant goldfish pigment on to the bovine rhodopsin template is consistent with this interpretation, although the interaction may require an intervening water molecule. In UVS pigments, the absence of Tyr-86 and lack of stabilization would lead to the loss of protonation and a SW shift in the $\lambda_{\max}$ of the pigment into the UV. In avian pigments, a similar mechanism may exist for the shift from VS to UVS. Ser-90 is present in all VS pigments and this may again serve to stabilize protonation. The Ser-90 → Cys substitution present in all avian UVS pigments may so alter the environment of the Schiff-base nitrogen and Glu-113 counterion that protonation is prevented.

One of the difficulties with the hypothesis that UVS pigments possess an unprotonated Schiff base is that they all retain Glu-113 and thereby the ability to provide a counterion to a Schiff-base proton. If this is not required, it might reasonably be expected that substitution at site 113 would have occurred in one or more instances during evolution with the loss of the acidic residue. The fact that this has not occurred would appear to be an argument in favour of the protonation of UVS pigments. However, there is now accumulating evidence that in mutant opsins that lack a Schiff-base counterion (e.g. Asp-113 → Gly/Asn/His mutants in the clawed frog [36]), the Meta I and Meta II photointermediates of the mutant pigments are substantially more stable than the wild type, thereby prolonging these steps in the photobleaching cycle [36]. The Schiff-base counterion may be required therefore even in UVS pigments for a normal rate of opsin hydrolysis.

In contrast to bird and ungulate SWS1 pigments, the evolution of primate VS pigments would appear to involve substitution at more than one site. Shi et al. [22] have identified seven sites that, when reciprocally substituted in human VS or mouse UVS pigment, resulted respectively in SW or LW shifts of around 65 nm. Examination of the residues present at these sites across other great apes, and Old and New World monkeys shows, however, that only Leu-49, Leu-86, Pro-93 and Thr-118 are conserved across all species. It would appear unlikely therefore that the other three sites, 46, 52 and 114, are important in the spectral tuning of primate VS pigments. Site 118 also shows substantial variation in pigments with similar $\lambda_{\max}$ values from other species, casting doubt on the role of this site in the spectral tuning of SWS1 pigments. All primate SWS1 pigments possess Leu-86 and Pro-93, although the Phe-86 → Leu substitution in goldfish UVS pigment and the Pro-93 → Thr substitution in the clawed frog VS pigment [28] do not alter the $\lambda_{\max}$ of the resulting pigments, and the simultaneous substitution of Phe-86 → Leu and Thr-93 → Pro in mouse UVS pigment is also without effect on $\lambda_{\max}$ [22]. Therefore the precise mechanism for the stabilization of protonation in primate SWS1 pigments remains unclear.

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