A Novel Amino Acid Substitution Is Responsible for Spectral Tuning in a Rodent Violet-Sensitive Visual Pigment†

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ABSTRACT: Cone short-wave (SWS1) visual pigments can be divided into two categories that correlate with spectral sensitivity, violet sensitive above 390 nm and ultraviolet sensitive below that wavelength. The evolution and mechanism of spectral tuning of SWS1 opsins are proving more complex than those of other opsin classes. Violet-sensitive pigments probably evolved from an ancestral ultraviolet-sensitive opsin, although in birds ultraviolet sensitivity has re-evolved from violet-sensitive pigments. In certain mammals, a single substitution involving the gain of a polar residue can switch sensitivity from ultraviolet to violet sensitivity, but where such a change is not involved, several substitutions may be required to effect the switch. The guinea pig, *Cavia porcellus*, is a hystricognathous rodent, a distinct suborder from the Sciurognathi, such as rats and mice. It has been shown by microspectrophotometry to have two cone visual pigments at 530 and 400 nm. We have ascertained the sequence of the short-wave pigment and confirmed its violet sensitivity by expression and reconstitution of the pigment in vitro. Moreover, we have shown by site-directed mutagenesis that a single residue is responsible for wavelength tuning of spectral sensitivity, a Val86Phe causing a 60 nm short-wave shift into the ultraviolet and a Val86Tyr substitution shifting the pigment 8 nm long wave. The convergent evolution of this mammalian VS pigment provides insight into the mechanism of tuning between the violet and UV.

Visual pigments are members of the large superfamily of G protein coupled receptors that function through the activation of a guanine nucleotide binding protein, the G protein. They are based on a common basic structure of a seven transmembrane (TM)[1] opsin protein covalently attached to a chromophore via a Schiff base linkage to a conserved lysine residue in the seventh TM region. Each pigment shows a characteristic peak of maximal absorbance (λ_{max}), the precise location of this peak depending on interactions between the chromophore and the opsin protein. In vertebrates, a single rod class of visual pigment is generally present with up to four cone classes: a long-wave or LWS class with a λ_{max} of 490−570 nm, a middle-wave or Rh2 class with a λ_{max} of 480−530 nm, and two short-wave or SWS classes, with λ_{max} values of 355−450 nm for SWS1 and 410−490 nm for SWS2 (reviewed in ref *1*). In species with all four cone pigments, this provides the possibility for tetrachromatic color vision. Tetrachromacy has, however, been lost in eutherian mammals, probably during an early nocturnal evolutionary phase, by the loss of the SWS2 and Rh2 classes. Most mammals are therefore dichromats although trichromacy has re-evolved in old world primates through gene duplication of the LWS gene (*2*).

The SWS1 class of pigments falls into two groups, ultraviolet sensitive (UVS) with λ_{max} below 390 nm and violet sensitive (VS) with λ_{max} between about 390 and 450 nm. UVS pigments are relatively common in the animal kingdom and almost certainly represent the ancestral form of the SWS1 class (*3*). In birds, however, the UVS pigments appear to have evolved secondarily from a VS pigment (*3*, *4*), and the data of Odeen and Hastad (*5*) indicate that this may have occurred a number of times in avian evolution (*6*). The shift in avian pigments between VS and UVS is achieved by a single amino acid substitution, a Ser to Cys change at site 90 (bovine rod opsin number), although other mechanisms may be present (*6*).

The mechanism of tuning in mammalian SWS1 pigments is more varied. Unlike in avian pigments, site 90 is not the primary site as it is invariably occupied by Ser in all mammalian VS and UVS pigments reported to date (*6*, *7*). The tuning of primate VS pigments has been examined in detail by Yokoyama and Shi (*7*). Chimeric opsins combining TM regions from human VS and mouse UVS pigments, when expressed and regenerated with retinal, identified TM regions 2 and 3 of the opsin protein as important for violet spectral shifts in primates. Moreover, the simultaneous replacement of the residues present at seven sites (sites 46, 49, 52, 86, 93, 114, and 118) in mouse UVS with those in human VS pigment (*7*) and vice versa produced a corresponding exchange in ultraviolet/violet sensitivity. Overall, therefore, these substitutions achieve the spectral shift between mouse UVS and human VS pigments, although their effects must be synergistic since single substitutions had no effect on λ_{max}. Subsequently, Shi et al. (*8*) have shown that

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[1] Abbreviations: PBS, phosphate-buffered saline; cDNA, complementary DNA; CNBr, cyanogen bromide; λ_{max}, peak of maximal absorbance; LWS, long wave sensitive; mRNA, messenger RNA; RACE, rapid amplification of cDNA ends; SWS, short wave sensitive; PDB, protein data base; TM, transmembrane; UVS, ultraviolet sensitive; VS, violet sensitive.

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Table 1: Oligonucleotide Primers Used

primer	sequence (5′−3′)[a]	use
GPF1b	ATGTCAGAGGAAGANGAGTTTTA	PCR
GPF2	TGGGATGGGCCTCAGTACCAC	PCR
GPR	TCAGTGAGGGCCAACTTTGCTAG	PCR
MusBlue481+	GGGGTGTCCATCCCACCCTT	PCR
MusBlue520−	TGAACCTGCTCCAGCCAAAA	PCR
MusBlue1041−	TCAGTGAGGGCCAACTTTGCT	PCR
GPSP2	AAGGCTGCCTGAAGGCGGAAG	RACE
GPSP3	GTGGTACTGAGGCCCATCCCA	RACE
GPexpF	**GCGCGAATTCCACC**ATGTCAGAGGAAGAGGAGTTTTA	cloning
GpexpR	**CGGCGTCGACGC**GTGAGGGCCAACTTTGCTAG	cloning
C49F+	GGGCATTGTCTTCT*T*TATAGGGACACCAC	mutagenesis
C49F−	GTGGTGTCCCTATA*A*AGAAGACAATGCCC	mutagenesis
V86F+	GGGGCTTTCTCT*T*CTGCATATTCTC	mutagenesis
V86F−	GAGAATATGCAGA*A*GAGAAAGCCCC	mutagenesis
V86F/A93T+	GGGCTTTCTCT*T*CTGCATATTCTCTGTCCTTACTGTCTTCATCG	mutagenesis
V86F/A93T−	CGATGAAGACAG*T*AAGGACAGAGAATATGCAGA*A*GAGAAAGCCC	mutagenesis
A93T+	CTCTGTCCTT*A*CTGTCTTCATCGCC	mutagenesis
A93T−	GGCGATGAAGACAG*T*AAGGACAGAG	mutagenesis
V86Y+	GGGGCTTTCTCT*A*CTGCATATTCTC	mutagenesis
V86Y−	GAGAATATGCAG*T*AGAGAAAGCCCC	mutagenesis
V86Y/A93I+	GGGCTTTCTCT*A*CTGCATATTCTCTGTCCTTA*T*TGTCTTCATCG	mutagenesis
V86Y/A93I−	CGATGAAGACA*AT*AAGGACAGAGAATATGCAG*T*AGAGAAAGCCC	mutagenesis
F203Y/A204T+	CGCAGCGAGTATTAC*A*CCTGGTTCCTTTC	mutagenesis
F203Y/A204T−	GAAAGGAACCAGG*T*G*T*AATACTCGCTGCG	mutagenesis

[a] Underlined sequences are restriction enzyme sites, sequences in bold are not homologous to the opsin sequence, and nucleotides in italics are the targets for mutagenesis.

the murine pigment is shifted into the violet by substitution with the residues present in human at only four of these (sites 86, 93, 114, and 118). The amino acid present at site 114, however, is not conserved across other primate species and is therefore unlikely to be important.

In other groups of mammals, notably the even-toed ungulates (*9*, *10*) and a marsupial, the tamar wallaby (*11*), substitution at just a single site would appear to be sufficient for the generation of VS pigments. This is site 86, occupied by Tyr in these latter species compared to Phe in all known UVS pigments except those in birds. Moreover, site-directed mutagenesis has shown that a Tyr86Phe substitution into bovine VS pigment short wave shifts the λ_{max} into the UV whereas a Phe86Tyr substitution into goldfish and mouse UVS pigments long wave shifts the λ_{max} into the violet region of the spectrum (*9*, *10*).

The order Rodentia forms the single largest groups of mammals. It is divided into two suborders, the Sciurognathi with 11 families and the Hystricognathi with 18 families. The guinea pig, *Cavia porcellus*, is a South American hystricognathous rodent with a much higher cone density than many sciurognathous rodents (*12*). Typical for a mammal, it is a dichromat with a violet-sensitive short-wave pigment (*13*) that has been shown by microspectrophotometry to have a λ_{max} around 400 nm (*14*). This contrasts with the UV sensitivity found in another hystricognathous caviomorph rodent, the Chilean degu, *Octodon degus* (*15*, *16*). UVS pigments are also present in the mouse, *Mus musculus*, and the rat, *Rattus norvegicus*, members of the Sciurognathi, although other members of this suborder, the gray squirrel, *Sciurus carolinensis* (*17*), and two species of ground squirrel, *Spermophilus* sp. (*18*), have sensitivity peaks around 440 nm. Different species of rodents therefore possess either UVS or VS pigments. Since both suborders have species that have retained the ancestral UV sensitivity (*3*, *6*), it is probable that the violet sensitivity of the guinea pig and squirrel evolved quite separately. In this study, we have focused on the guinea pig SWS1 pigment in order to establish the amino acid sequence of the opsin protein and to identify the amino acid substitutions responsible for the VS tuning.

EXPERIMENTAL PROCEDURES

Production of Genomic DNA and Retinal cDNA. Guinea pig RNA was extracted from freshly dissected, dark-adapted retinae using a Quickprep total RNA or mRNA purification kit (Amersham). Single-stranded cDNA was synthesized from an oligo-d(T) anchor primer using the AMV kit (Roche).

PCR, Cloning, and Sequencing. All primers used are listed in Table 1. Guinea pig single-stranded cDNA was used as the template for PCR reactions (BioLine *BioTaq* polymerase) using primers designed to extant vertebrate SWS1 sequences. For 5′-RACE, single-stranded DNA was synthesized from total RNA using a gene-specific primer. This was used as a template for further reactions using a gene-specific primer and an anchor primer (RACE kit, Roche). All amplified fragments were cloned into the pGEM-T easy cloning vector (Promega) and fully sequenced. In all cases, sequences from several reactions were compared to eliminate PCR incorporation errors.

Generation of Guinea Pig SWS1 Expression Constructs and Production of Mutations. Wild-type SWS1 opsin from guinea pig was amplified from retinal cDNA using a proofreading polymerase (*Pfu*) and primers with added restriction enzyme sites designed to amplify the entire coding region (Table 1). The resulting fragment, after restriction enzyme digestion, consisted of an *Eco*RI−*Sal*I fragment that could be directionally inserted into the vector pMT4 in the correct reading frame for subsequent expression. pMT4 is a derivative of the pMT2 mammalian expression vector that additionally encodes the C-terminal 1D4 epitope and stop codon from bovine rod opsin which is placed downstream

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guinea pig  ---MSEEEEFYLFKNASSVGPWDGPQYHVAPVWAFRLQAAFMGIVFCIGTPLNGIVLVATLLYKKLRQPLNYILVNVSLGGFLVCIFSVL  87(92)
mouse       ---..G.DD....Q.I...........L.............F..FV.....AI......H.....................F.....F  87
human       MRK..-.........I...........I......Y.......T..L..F...AM......R.................F....L.....F  89
bovine      MSK.......L....I.L.........L......H...V...F..FV.....AT......R.R.................IY.....F  90

guinea pig  AVFIASCYGYFIFGRHVCALEGFLGSVAGMVTGWSLAFLAFERYLVICKPFGNFRFSSKHALIVVLATWVIGIGVSIPPFFGWSRYMPEG  177(182)
mouse       T......H...L.........A.......L.............V.......SI..N.....M......I...............FI...  177
human       P..V...N...V.............T...L.............I.................T......T...............FI...  179
bovine      I...T......V.........A...CT..L.............II................M..V...T...............FV...  180

guinea pig  LQCSCGPDWYTVGTKYRSEYFAWFLFIFCFIVPLSLICFSYCQLLRTLRTVAAQQQESATTQKAEREVSRMVVVMVGSFCVCYVPYAALA  267(272)
mouse       ....................YT.........I.........S.......A...................H..........L.........  267
human       ...................SYT...................T....A.KA.....................................F.  269
bovine      ................Y...YT.......Y...........S...GA..A.........S.........H..........L..T......  270

guinea pig  MYIVNNRNHGLDLRLVTIPAFFSKSSCIYNPIIYCFMNKQFRACIMELVCRKPMADESDMSTSQKTEVSAVSSSKVGPH  346(351)
mouse       ..M.......................V.................L.M...........V.G.......T.........  346
human       ..M...............S.....A...............Q....KM..G.A.T....TCS.......T...TQ...N  348
bovine      ..........V.............A.V...................M..G...T...EL.S.......T....Q...N  349
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FIGURE 1: Multiple alignment of the guinea pig SWS1 opsin protein sequence with those of mouse, human, and bovine. Only mismatches are shown for clarity. A match is denoted by a dot, and gaps are denoted by dashes. Residues mutated in this study are shown in bold type. Transmembrane regions are denoted by a heavy line. Bovine rod numbering is shown in parentheses.

of the inserted SWS1 coding sequence (*19*). The resulting construct was sequenced through the entire insert including the cloning sites to ensure fidelity of the opsin sequence. Mutations were introduced into the opsin sequence after cloning into pMT4 using the QuikChange site-directed mutagenesis kit (Stratagene). Double mutants had all changes introduced during a single round of mutagenesis using the primers shown in Table 1. The accurate introduction of all mutations was checked by sequencing.

Expression of Wild-Type and Mutant Pigments. HEK 293T cells were transiently transfected with the pMT4 expression vector using GeneJuice (Merck). Thirty 90 mm plates were used per experiment. Cells were harvested 48 h posttransfection and 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 cells in PBS (pH 7.0) and incubating them with 40 μM 11-*cis*-retinal in the dark (*20*). The pigment was solubilized from cell membranes and purified by immunoaffinity chromatography using an anti-1D4 antibody coupled to a CNBr-activated Sepharose column following the methods of Molday and MacKenzie (*21*). Purified pigment was eluted from the column and stored on ice. Absorbance spectra were recorded in the dark using a Spectronic Unicam UV500 dual-beam spectrophotometer. The sample was then treated with hydroxylamine to 56 mM or hydrochloric acid to 26 mM to create the retinal oxime or acid-denatured pigment, respectively. The spectra were recorded again. The post-hydroxylamine or acid treatment spectra were subtracted from the dark absorbance spectra to produce difference spectra.

Estimation of λ_{max} of Expressed Pigments. Visual pigment templates (*22*) were fitted using the Solver add-in to MS Excel to vary the λ_{max} to obtain the best fit. Difference spectra were fitted with Govardovskii templates with the appropriate template to represent the retinal oxime or acid-denatured product subtracted. Apparent λ_{max} values for comparison were estimated by fitting a trendline through the top of the curve and taking the wavelength at the maximum for the trendline. Values quoted in the text are taken from difference spectra as the most accurate estimation, absorbance spectra being distorted by the underlying absorbance and scatter of the protein.

Modeling of the Spectral Tuning Residues onto the Opsin Protein Structure. The guinea pig SWS1 opsin sequence was overlayed onto the human SWS1 VS pigment model of Stenkamp et al. (*23*), deposited in the PDB as version 1KPN. Manipulations of the structure produced were performed with DeepView/Swiss-PdbViewer v3.7 (*24*−*26*).

RESULTS

Sequence of SWS1 Opsin. The guinea pig SWS1 opsin sequence was obtained from retinal cDNA using a strategy of heterologous PCR followed by 5′ RACE. The sequence has been submitted to GenBank and assigned the accession number AY552608. The deduced amino acid sequence is shown in Figure 1, aligned with the murine, human, and bovine SWS1 sequences. The sequence shows 86%, 86%, and 85% nucleotide identity and 88%, 86%, and 84% amino acid identity to murine, human, and bovine sequences, respectively. Phylogenetic analysis of SWS1 pigment sequences (Figure 2) places the sequence for the guinea pig, a member of the suborder Hystricognathi, within the rodent SWS1 clade but in a separate lineage from the mouse, rat, and Dzhungarian hamster (*Phodopus sungorus*), all members of the suborder Sciurognathi.

Key residues for all opsins are, in the guinea pig sequence, Lys at 291 and Glu at 108 that provide respectively the Schiff base nitrogen for the binding of the chromophore and the negative counterion for the protonated Schiff base. The equivalent residues in bovine rod opsin are at positions 296 and 113. To compare the residues at particular sites in the different opsins, the bovine rod opsin numbering system will be used hereinafter, which means that 5 should be subtracted to obtain the actual position in the guinea pig sequence. Previous studies have shown that key sites for the tuning of VS and UVS pigments are 86 for mammals and 90 for birds. Site 90 is occupied by Ser, as found for all mammalian SWS1 pigments. However, site 86 is uniquely occupied by Val, which contrasts with Tyr found in ungulate and Leu in primate VS pigments.

Expression of Guinea Pig SWS1 Opsin. Expression of the full-length wild-type guinea pig opsin sequence in transiently transfected mammalian cells and subsequent purification and reconstitution with 11-*cis*-retinal yielded a pigment with a

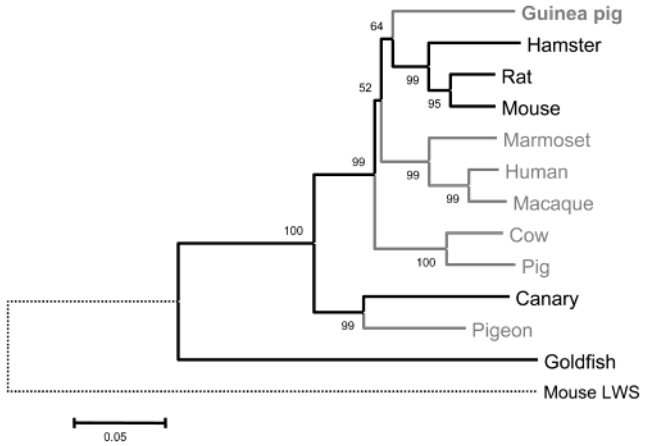

FIGURE 2: Phylogenetic tree of UVS/VS cone opsins. Amino acid sequences were aligned by Clustal W, and the tree was generated by the neighbor-joining method (*32*). The bootstrap confidence values are shown for each branch. The mouse LWS sequence was used as an outgroup (dashed line). The scale bar is equal to 0.05 substitution per site. UVS opsins are denoted in black and VS opsins in gray. Sequences were obtained from Genbank: hamster, accession number AY029603−6; rat, NM031015; mouse UVS, BC026021; marmoset, L76201; human, NP001699; macaque, AF158977; cow, NP776992; pig, AAM13692; canary, CAB91993; pigeon, AAD38035; goldfish, P32310; mouse, LWS NM008106.

λ_{max} of 420 nm (Figure 3), confirming therefore the violet sensitivity of this pigment. This value was obtained by fitting to the difference spectrum a visual pigment template curve (*22*), with the appropriate retinal oxime curve subtracted. The 390−500 nm region of the spectrum is problematic, since it is overlapped greatly by the acid-denatured absorbance curve and partly by the retinal oxime product (see Figure 3A) where hydroxylamine is used. These overlaps distort difference spectra and displace the apparent λ_{max} away from the true λ_{max} of the native pigment for any pigment below 500 nm when using hydroxylamine (for a discussion, see ref *27*) or in the 390−550 range when using acid. As a consequence, an accurate measure of λ_{max} can only be obtained by using such a template fitting process (see Table 2 for differences in true and apparent λ_{max}).

Candidate Sites for Spectral Tuning. Comparison of the guinea pig sequence with those from other mammals identified five candidate sites within TM regions with unique residues in the guinea pig pigment: Cys49, Val86, Ala93, Phe203, and Ala204. Sites 49, 86, and 93 have all been implicated in the UVS−VS transition between the mouse and human pigments (*7*, *8*). Site 49 generally contains a nonpolar aromatic or aliphatic residue, while site 93 varies more widely and can contain either polar or nonpolar residues. Val86 is uniquely present in the guinea pig pigment, and since Tyr86 is responsible for the long-wave shift seen in ungulate VS pigments, this substitution is a good candidate for the generation of the VS pigment in the guinea pig. Phe203 and Ala204 are unique to the guinea pig pigment, with all known mammalian pigments possessing Tyr and Thr, respectively, at these sites.

Table 2: Summary of λ_{max} of Expressed Pigments Obtained from Difference Spectra

pigment		true λ_{max}	apparent λ_{max}
guinea pig	wild type	420	428
	Cys49Phe	419	427
	Ala93Thr	419	427
	Phe203Tyr/Ala204Thr	415	420
	Val86Tyr	428	433
	Val86Tyr/Ala93Ile	430	437
	Val86Phe	367	366
	Val86Phe/Ala93Thr	367	364
goldfish	wild type	358	358
	Phe86Val	359	362

The importance of these five sites for spectral tuning was therefore investigated by replacement of the guinea pig residues at each site with those present in other pigments. A Cys49Phe mutation (the residue found in mouse) had little or no effect, yielding a pigment with a λ_{max} of 419 nm (Figure 4A, Table 2). The double mutation of Phe203Tyr/Ala204Thr yielded a pigment with a λ_{max} of 415 nm (Figure 4B, Table 2), some 5 nm shorter than the native pigment. Val86Phe and Ala93Thr, replacing the guinea pig residues with those found in mouse, were examined both as double and as single mutants. The Ala93Thr mutant yielded a pigment with a λ_{max} of 419 nm (Figure 4C, Table 2), little different from the wild-type pigment. However, the double Val86Phe/Ala93Thr mutant yielded a pigment with a λ_{max} of 367 nm (Figure 4D, Table 2), fully shifted into the UV, as did the single Val86Phe mutant, yielding a pigment with a λ_{max} of 367 nm (Figure 4E, Table 2), identical to the double mutant. A Val86Tyr/Ala93Ile double mutant, replacing residues in the guinea pig pigment with those found in the bovine pigment, was also generated, and this long wave shifted the λ_{max} of the pigment to that of the bovine pigment (*9*) at 430 nm (Figure 4F, Table 2). This shift is largely attributable, however, to Val86Tyr since when this single mutation was made, a λ_{max} of 428 nm was obtained (Figure 4G, Table 2, and in comparison with wild type and Phe203Tyr/Ala204Thr in Figure 4H), essentially identical to the double mutant.

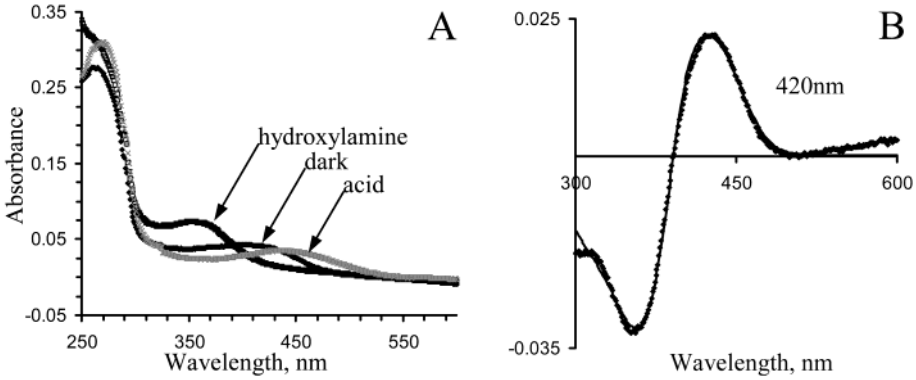

FIGURE 3: Spectra of the wild-type guinea pig SWS1 pigment. (A) Guinea pig wild-type dark, acid-denatured (gray), and hydroxylamine-treated spectra. (B) Difference spectrum (points) after hydroxylamine treatment. The value shown is the λ_{max} of the fitted template (line).

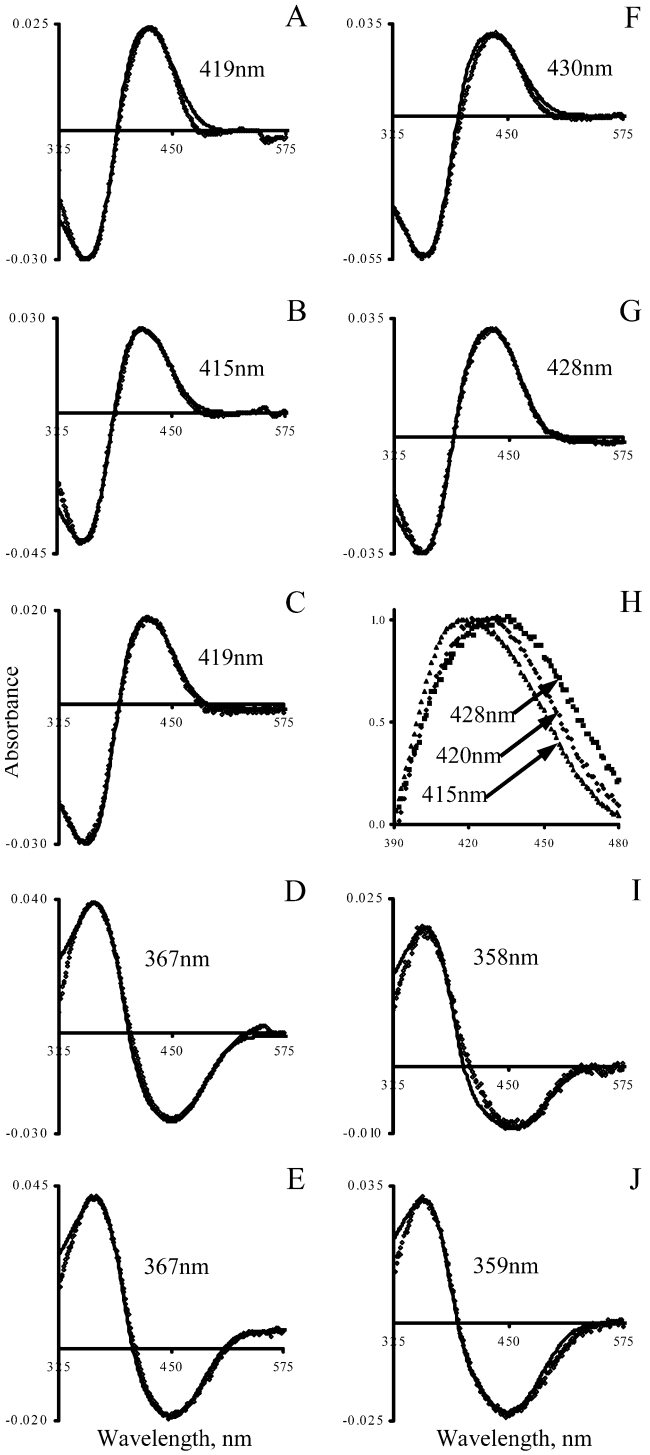

FIGURE 4: Spectra of guinea pig and goldfish SWS1 mutated opsins. Difference spectra of mutants/wild type (points) with fitted templates (lines): (A) guinea pig Cys49Phe; (B) guinea pig Phe203Tyr/Ala204Thr; (C) guinea pig Ala93Thr; (D) guinea pig Val86Phe/Ala93Thr; (E) guinea pig Val86Phe; (F) guinea pig Val86Tyr/Ala93Ile; (G) guinea pig Val86Tyr; (H) comparison of normalized spectra of, in order of increasing λ_{max}, guinea pig Phe203Tyr/Ala204Thr mutant (triangles), wild type (diamonds), and Val86Tyr mutant (squares); (I) goldfish Phe86Val; (J) goldfish wild type. Panels A−C show difference spectra of guinea pig mouse-like mutant opsins after hydroxylamine treatment. Panels D and E show difference spectra of guinea pig mouse-like mutant opsins after acid treatment. Panels F and G show difference spectra of guinea pig cow-like mutant opsins after hydroxylamine treatment. Panels I and J show difference spectra of goldfish guinea pig-like mutant opsin and wild type after acid treatment. Values shown are the λ_{max} of fitted templates.

To examine whether Val86 will long-wave shift a UVS pigment, a Phe86Val substitution was introduced into the UVS pigment of the goldfish, *Carassius auratus*. Expression and reconstitution of this mutant opsin yielded a pigment with a λ_{max} of 358 nm (Figure 4I, Table 2), essentially identical to the wild-type UVS pigment (Figure 4J, Table 2). Therefore, the replacement of Phe by Val at site 86 is not sufficient by itself to long-wave shift the λ_{max} of the goldfish UVS pigment.

Modeling of the Spectral Tuning Residues onto the Opsin Protein Structure. Stenkamp et al. (*23*) have recently modeled the human SWS1 VS pigment onto a refined version of the original bovine rhodopsin crystal structure. Overlaying the guinea pig SWS1 opsin sequence onto this model places Cys49 on the far side of helix I, blocked from the chromophore binding pocket by helix II, and Ala93 on the far side of helix II, blocked from the binding pocket by helix III (Figure 5A). This topography can account for the lack of effect of substitutions at these two sites. In contrast, the sites where substitution does affect λ_{max} are within the chromophore binding pocket, flanking the ionone ring of retinal in the case of sites 203 and 204, and adjacent to the Schiff base/counterion for site 86.

Substitutions of Phe86 in the UVS pigment of goldfish by three different nonpolar amino acids, Val (this study), Leu, and Met (*9*), present at site 86 in the VS pigments of guinea pig, primates, and *Xenopus* (*28*), fail to shift the λ_{max} to longer wavelengths. Likewise, Phe86Leu has no effect on the λ_{max} of mouse UVS pigment (*7*). In contrast, Tyr86 generates a long-wave shift into the violet in both mouse and goldfish UVS pigments (*9*, *10*). This latter residue places a polar hydroxyl group in the vicinity of the Schiff base linkage and the counterion residue Glu113 (separated by approximately 2.83 Å), with an estimated distance from the hydroxyl group to the Schiff base nitrogen of 2.69 Å and to the counterion of 3.26 Å (Figure 5B), sufficiently close, therefore, to form hydrogen bonds to either residue. In contrast, the nonpolar residue Val86, as well as Met86 and Leu86, would appear to be unable to exert a direct effect on the Schiff base. However, rotation of Ser90, which is already known to play an important role in the tuning of avian VS and UVS pigments, into a different conformation brings its polar side chain closer to the Schiff base and into a position (estimated distance 2.36 Å) to hydrogen bond with the counterion residue (Figure 5C).

DISCUSSION

The guinea pig possesses a SWS visual pigment with a λ_{max} around 400−405 nm (*14*). In this study, we have obtained the sequence of an opsin, expressed in the retina, that shows sequence homology and phylogenetic identity with other vertebrate SWS1 sequences. Expression of this opsin in vitro and regeneration with 11-*cis*-retinal resulted in a pigment with a λ_{max} at 420 nm, which is 15−20 nm longer than the native pigment as determined by microspectrophotometry. We are, however, confident that the cloned and expressed opsin is the same as the 400−405 nm pigment identified by Parry and Bowmaker (*14*), since differences of this magnitude have been reported previously between native and in vitro expressed pigments. In particular, the S cones in the pigeon retina have a λ_{max} around 409 nm, as

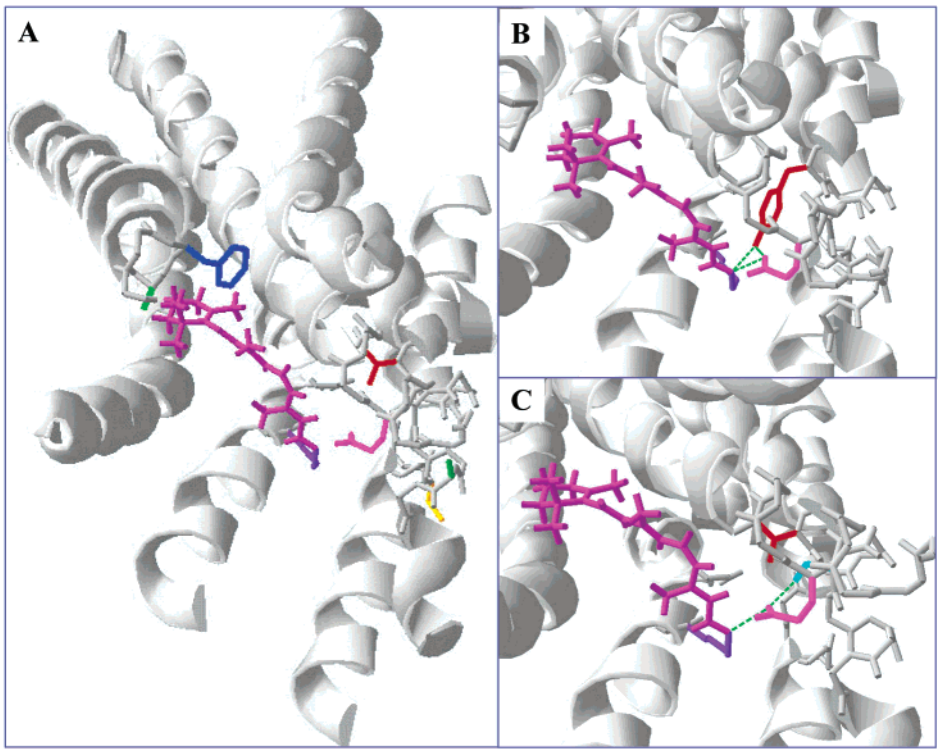

FIGURE 5: Structure of guinea pig VS opsin. The structure was modeled using SwissModel (*24*−*26*) and based upon the SWS1 template (*23*). Helices are shown as gray ribbons except where they have been cut away to allow a view of the retinal binding pocket, when the backbone is shown in gray. The chromophore, Schiff base, and counterion have been shown in shades of purple; bright green dashes represent hydrogen bonds. (A) Native pigment showing residues investigated in this study: Cys49 (yellow), Val86 (red), Ala93 (dark green), Phe203 (dark blue), Ala204 (green). (B) Close-up of the retinal binding pocket, showing Tyr (red) substituted for Val at site 86, as found in ungulate VS pigments. Tyr could affect tuning via hydrogen bonding either to the Schiff base or to the counterion as proposed for Ser90 in the native guinea pig pigment. (C) Close-up of the retinal binding pocket of the native pigment, showing the potential tuning residues discussed: Val86 (red); Ser90 (cyan). Ser90 has been rotated to the orientation discussed and may be involved in tuning via a hydrogen bond to the counterion.

determined by microspectrophotometry (*29*), whereas the in vitro expressed opsin when regenerated with 11-*cis*-retinal yields a λ_{max} at 393 nm (*30*). The explanation for these differences is not entirely clear but may relate either to the different environments of detergent for the in vitro expressed pigments and lipid disk membrane for the native pigment or to differences in posttranslational modification between the native and in vitro expressed pigments.

Five residues, Cys49, Val86, Thr93, Phe203, and Ala204, were identified in the opsin protein as candidate sites for tuning the guinea pig pigment into the violet region of the spectrum. However, substitutions by site-directed mutagenesis of Cys49Phe and Ala93Thr were without effect, and the double mutation of the Phe203Tyr/Ala204Thr produced only a small 5 nm shift toward the UV. Only the Val86Phe substitution had a significant effect on spectral absorbance, fully shifting the λ_{max} of the pigment to 366 nm in the UV. It would appear therefore that Val86 is having a similar effect in the guinea pig pigment to Tyr86 in the cow and pig, previously shown to be responsible for tuning these VS pigments (*9*, *10*). Interestingly, a Val86Tyr substitution into the guinea pig pigment long wave shifts the λ_{max} by 8 nm toward that of the bovine pigment, indicating that different residues at this site differentially tune the pigment. However, whereas a Phe86Tyr substitution into goldfish UVS pigment shifts the λ_{max} into the violet, the equivalent Phe86Val substitution is without effect; a Phe86Val substitution is not sufficient by itself to shift the λ_{max} of a UVS pigment into the violet.

All mammalian UVS pigments possess Phe at site 86. In cow, pig, and wallaby, the λ_{max} of the SWS1 pigment is in the violet region of the spectrum with Phe replaced by Tyr, whereas in the VS pigments of guinea pig and primates, this site is occupied by a nonpolar aliphatic amino acid. A mutant guinea pig pigment, Val86Phe, has its sensitivity fully shifted to the UV, as does Tyr86Phe in the bovine pigment (*9*). In contrast, a Phe86Val substitution in the goldfish UVS pigment and Phe86Leu substitutions in both goldfish and mouse UVS pigments all fail to produce a long-wave shift (*8*−*10*). On the other hand, a Phe86Tyr substitution in the goldfish pigment shifts the sensitivity into the violet region of the spectrum. In summary, therefore, Phe86 results in a UVS pigment, Tyr86 gives a VS pigment, but while Val86 and Leu86 are naturally present in VS pigments, their substitution into a UVS pigment is not sufficient by themselves to effect a shift to VS. This is partly explained by the results of Shi et al. (*8*), where the synergy of multiple substitutions is required to produce the full spectral shift. This strongly suggests a local structural change between UVS and VS pigments, perhaps moving helix II slightly further from the binding pocket in UVS pigments, thus preventing an interaction required for tuning to the violet. Phe, Leu, and Val are all nonpolar and as such are unlikely to have a direct effect on tuning the λ_{max} of a pigment, but in a VS structure, Leu and Val could allow the required tuning interaction where Phe does not. Our proposed candidate residue for such an interaction is Ser90. From the SWS1 model, it is located in a position such that it could form a

hydrogen bond to the counterion, which would shift the λ_{max} of the pigment. This residue is conserved in all known SWS1 pigments except avian UVS pigments where a mutation to Cys is solely responsible for the shift to UV, demonstrating that the residue at this site does indeed interact in the binding pocket. A similar Ser90 interaction has also been implicated in the tuning of the VS pigment of *Xenopus*, which also has a nonpolar residue, Met, at site 86, although it has Asp rather than Glu as the counterion, changing the geometry of the binding pocket. We propose that Tyr86 would cause violet sensitivity by interaction with the counterion, even if Ser90 were excluded from the binding pocket by Tyr86 or by structural differences in UVS pigments, hence the minimal effect caused by a Ser90Cys substitution in the bovine pigment (*10*). Thus the particular residue at sites 86, 90, and 113 (the counterion) and their positioning may be responsible for tuning SWS1 pigments from all classes of vertebrates, however disparate their tuning mechanisms may appear at first.

The selective advantage that is conferred by violet sensitivity of the SWS1 pigment in the guinea pig is uncertain. Guinea pigs have a greater cone density than many mammals (*12*), and although they exhibit coexpression of their LWS and SWS1 opsins in a percentage of cones (*14*, *31*), they are behaviorally dichromatic (*13*). The distribution of violet and ultraviolet sensitivity within terrestrial mammals is not straightforward and appears not to follow either phylogeny or behavioral ecology. Ultraviolet sensitivity is more common in small nocturnal rodents but is also found in the diurnal Chilean degu, a close relative of the guinea pig (*15*, *16*), which is more crepuscular. The significance of the various spectral locations of the SWS1 pigments in mammals has yet to be determined. Nevertheless, the fact that violet sensitivity in the guinea pig is conferred by a single residue, the replacement of which can either fine tune within the violet or short-wave shift the pigment to the ultraviolet, provides important insight into the mechanism of spectral tuning in SWS1 pigments.

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