

The Molecular Evolution of Avian Ultraviolet- and Violet-Sensitive Visual Pigments

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The shortwave-sensitive SWS1 class of vertebrate visual pigments range in λ_{\max} from the violet (385–445 nm) to the ultraviolet (UV) (365–355 nm), with UV-sensitivity almost certainly ancestral. In birds, however, the UV-sensitive pigments present in a number of species have evolved secondarily from an avian violet-sensitive (VS) pigment. All avian VS pigments expressed *in vitro* to date encode Ser86 whereas Phe86 is present in all non-avian ultraviolet sensitive (UVS) pigments. In this paper, we show by site directed mutagenesis of avian VS pigments that Ser86 is required in an avian VS pigment to maintain violet-sensitivity and therefore underlies the evolution of avian VS pigments. The major mechanism for the evolution of avian UVS pigments from an ancestral avian VS pigment is undoubtedly a Ser90Cys substitution. However, Phe86, as found in the Blue-crowned trogon, will also short-wave shift the pigeon VS pigment into the UV whereas Ala86 and Cys86 which are also found in natural avian pigments do not generate short-wave shifts when substituted into the pigeon pigment. From available data on avian SWS1 pigments, it would appear that UVS pigments have evolved on at least 5 separate occasions and utilize 2 different mechanisms for the short-wave shift.

Introduction

The spectral sensitivities of visual pigments are subject to great variation across the animal kingdom and, in many cases, are linked to adaptive changes in the visual ecology of particular species. Of these, the spectral shifts seen in the shortwave-sensitive (SWS) pigments are amongst the largest, resulting in the loss or gain of sensitivity to ultraviolet (UV) light. Such changes are found in most vertebrate groups, with many instances of multiple independent occurrences, and represent therefore one of the major changes that has occurred in the evolution of the vertebrate visual system.

Visual pigments belong to the large family of G protein-coupled receptors that share a common structure of 7 α -helical transmembrane regions joined by cytoplasmic and luminal loops. They form a group of closely-related proteins (opsins) that bind retinal, a derivative of vitamin A. Five classes of pigment are recognised in vertebrates, a rod class and 4 different cone classes distinguished on the basis of spectral sensitivity and amino acid sequence of their respective opsins: longwave-sensitive (LWS) with λ_{\max} 500–570 nm, middlewave-sensitive (MWS) with λ_{\max} 480–530 nm, and 2 shortwave-sensitive classes, SWS2 with λ_{\max} 400–470 nm and SWS1 with λ_{\max} 355–445 nm. In eutherian mammals, this complement is reduced to a rod and 2 cone classes, LWS and SWS1. In all classes, the spectral tuning of the pigment arises from interactions between retinal and the amino acid residues that form the retinal-binding pocket of the opsin protein.

All visual pigments possess a Lys residue at site 296 (bovine rod opsin numbering) that is covalently linked to the chromophore via a Schiff base (SB) (reviewed in Hargrave 2001). In vertebrate pigments with λ_{\max} values >385 nm, the SB is protonated, with a negatively-charged residue at site 113 (Glu113) acting as a counterion to stabilize the proton of the SB (Nathans 1990). Absorption of light causes the isomerisation of the chromophore from 11-*cis*- to all-*trans*-retinal in a photobleaching sequence with

consequent conformational changes in the opsin protein leading to the activation of the G protein transducin by the activated form, metarhodopsin II.

The λ_{\max} values for SWS1 pigments in different vertebrate species range from the violet (385–445 nm) to the UV (355–365 nm), with UV-sensitive (UVS) pigments almost certainly ancestral (Hunt et al. 2001, 2004, 2007). Violet-sensitive (VS) SWS1 pigments are found, however, in all vertebrate classes except fishes; the generation of VS pigments has clearly occurred many times in vertebrate evolution and a number of different molecular mechanisms have been identified that tune the pigment from UVS to VS. In most cases, it is the amino acid present at site 86 that is key to the spectral sensitivity of the pigment (table 1). The ancestral pigment almost certainly had non-polar Phe at this site (Hunt et al. 2007) and this is replaced in the VS pigments of the cow and pig with polar Tyr (Cowing et al. 2002; Fasick et al. 2002). The same substitution is found in the VS pigment of the manatee (Newman and Robinson 2006), whereas the VS pigment of the elephant has polar Ser86 (Yokoyama et al. 2005). Tyr86 is again found in the VS pigments of the Tamar wallaby (Deeb et al. 2003) and quokka (Arrese et al. 2005) whereas other marsupials have UVS pigments that have retained Phe86 (Arrese et al. 2002, 2005; Strachan et al. 2004). VS pigments are also present in both suborders of the Rodentia, the Sciurognathi and the Hystricognathi. The mouse and rat, members of the Sciurognathi, both have UVS pigments as does the hystricognathous caviomorph rodent, the Chilean degu (*Octodon degus*) (Chavez et al. 2003; Jacobs et al. 2003). In contrast, VS pigments are found in the guinea pig (*Cavia porcellus*), a South American member of the Hystricognathi, and in gray (*Sciurus carolinensis*) and ground squirrels (*Spermophilus spp.*), members of the Sciurognathi (Jacobs 1976; Jacobs et al. 1976; Jacobs and Deegan 1994; Peichl and Gonzalez-Soriano 1994). The UVS pigments in the mouse and rat have retained Phe86 whereas the grey squirrel and guinea pig have Tyr86 and Val86 respectively (Parry et al. 2004; Carvalho et al. 2006). The other major mammalian group with VS pigments is the primates, where yet another substitution, Leu86, is found in Old and New World monkeys and in tarsiers, whereas either Cys86 or Ser86 is present in the closely related lemurs. In all primate pigments however, other residues, notably Pro93 and Val118, are

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Table 1
Amino Acid Present at Site 86 in Vertebrate UVS and VS SWS1 Pigments.

	Pigment	Amino acid at site 86
Agnathans		
Lamprey (<i>Geotria australis</i>)	UVS	Phe
Teleost fish		
Goldfish, Zebrafish	UVS	Phe
Amphibia		
Salamander	UVS	Phe
Frog	VS	Met
Reptilia		
Green anole	UVS	Phe
Eutheria		
Mouse, Rat	UVS	Phe
Cow, Pig	VS	Tyr
Elephant	VS	Ser
Manatee	VS	Tyr
Guinea pig	VS	Val
Squirrel	VS	Ty
Old and New World Primates		
Tarsiers	VS	Leu
Lemurs	VS	Cys/Ser
Metatheria		
Dunnart, Quenda	UVS	Phe
Wallaby, Quokka	VS	Tyr

Sequence accession numbers: Lamprey AY366495; Goldfish D85863; Zebrafish AB087810; Green anole AH007736; Xenopus BC084882; Salamander AF038948; Manatee AY228443; Cow U92557; Pig AY091587; Elephant AY686753; Coquerel's mouse lemur DQ191903; Coquerel's sifaka DQ191935; Brown lemur AB111464; Fat-tailed dwarf lemur DQ191908; Gray mouse lemur DQ191922; Greater dwarf lemur DQ191918; Red-tailed sportive lemur DQ191945; Ring-tailed lemur DQ191943; Woolly lemur DQ191894; Western tarsier DQ191949; Philippine tarsier DQ191954; Human NM_001708; Marmoset L76201; Mouse AF190671; Rat AF051163; Guinea pig AY552608; Tree squirrel DQ302163; Dunnart AY442173; Quenda AY726544; Quokka AY726545; Tamar wallaby AY286017.

also required for the long-wavelength (LW) shift (Shi et al. 2001; Hunt et al. 2004). Finally, the SWS1 pigments of amphibians may be either VS with Met86 in frogs (Takahashi and Yokoyama 2005), or UVS with Phe86 in the salamander (Ma et al. 2001).

In contrast to the other vertebrate classes, molecular phylogenetic analysis of UVS pigments in birds indicates that they have arisen secondarily from an ancestral avian VS pigment (Hunt et al. 2001) by the replacement of Ser90, present in all other vertebrates pigments, with Cys (Wilkie et al. 2000; Yokoyama et al. 2000). In birds, therefore, UVS pigments have been "re-invented". The mechanism for the generation of the avian VS pigments from an ancestral UVS pigment has, however, remained uncertain. VS pigments are relatively common in the Avia and the majority possess Ser86, making this substitution a good candidate for the original shift from UVS to VS, although the failure of a Phe86Ser substitution to LW shift the goldfish UVS pigment (Hunt et al. 2004) indicates that other substitutions may be required.

Cys86 has also been implicated in the generation of avian UVS pigments (Shi and Yokoyama 2003); a Ser86Cys substitution may be an alternative way therefore to generate a UVS pigment. Significantly, in a study of a small region of the avian SWS1 gene in 46 avian species distributed across 14 Orders, Ödeen and Håstad (2002)

have shown that Cys86 is paired with Ser90 in the pigments of 11 species and they also reported that either Ala86 or Phe86 is present in a number of other species. If all these substitutions generate short-wavelength shifts, it would mean that UVS pigments are much more widely distributed amongst the different avian Orders than presently thought.

In this study, we have used site-directed mutagenesis, *in vitro* expression and regeneration of wild type and mutant opsins to examine the molecular basis for spectral shifts in the evolution of VS pigments in birds and the effect of substitutions at site 86 as well as site 90 on the generation of avian UVS pigments.

Materials and Methods

cDNA synthesis

Poly(A)⁺ mRNA was extracted from pigeon, chicken, common cormorant and green anole retinae using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, distributed by GE Healthcare UK Ltd, Little Chalfont, UK). Single-stranded cDNA was synthesized using an oligo-d(T) anchor primer (Roche Diagnostics, Burgess Hill, UK).

Rapid amplification of cDNA ends (RACE)

An initial partial sequence of the common cormorant SWS1 opsin was obtained with primers designed to a conserved region of the budgerigar SWS1 gene (table 1). Species specific primers were then designed using this initial sequence for RACE reactions (table 1) and the full coding sequence (Accession number EF568933) was then obtained using the 3' / 5' RACE Kit 2nd Generation (Roche Diagnostics, Burgess Hill, UK).

Generation of expression vectors for wild type and mutant opsins

The polymerase chain reaction (PCR) amplification of the coding sequence of SWS1 opsins and their cloning into the pMT4 vector has been described previously (Wilkie et al. 2000; Cowing et al. 2002). The PCR primers used are listed in table 2. The Quikchange™ Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands) was used to introduce point mutations into the wild type sequences using oligonucleotides listed in table 2. Following mutagenesis, the clones were fully re-sequenced to check that the correct mutation had been made and that no other changes had been introduced.

Expression and regeneration of wild type and mutant pigments

The wild type and mutant expression constructs were used to transiently transfect HEK 293T cells using GeneJuice™ Transfection Reagent (Novagen, distributed by Merck, Nottingham, UK). In general, 12 × 14 cm tissue culture plates were used. Forty-eight hours post-transfection, the cells were harvested and washed 4 times with phosphate buffered saline (PBS) (pH 7.0). Cell pellets were stored at -80°C prior to regeneration of the pigment. The regeneration, solubilization and purification of the pigments

Table 2
Oligonucleotides for PCR and Site-Directed Mutagenesis

	Sequence (5'–3')	Substitution	Direction of primer	Species
1	GCGCGAATTCACCATGTCGGGTGACGAGGAGTTTAC		F	Pigeon
2	CGGCGTCGACGCGCTGGGGCTGACCTGGCTGGAGGA		R	Pigeon
3	GCGCGAATTCACCATGTCATCGGACGACGACTTC		F	Chicken
4	CGGCGTCGACGCGCTGGGGCTGACCTGGCTGGAGG		R	Chicken
5	GCGCGAATTCACCATGTCGGGCCAAGAAGACTTCTAC		F	Green anole
6	CGGCGTCGACGCGCTGGGGCTGACCTGGCTGGA		R	Green anole
7	GCGCGAATTCACCATGTCGGGTGAGGAGGAGTTCTAC		F	Cormorant
8	CGGCGTCGACGCGCTGGGGCTGACCTGGCTGGAGGA		R	Cormorant
9	CAGCGGCTTCATCTGCTGCATCTTCAGC	Ser86Cys	F	Pigeon
10	GCTGAAGATGCAGCAGATGAAGCCGCTG		R	Pigeon
11	GCGGCTTCATCTTCGCTGCATCTTCAGC	Ser86Phe	F	Pigeon
12	GCTGAAGATGCAGAAGATGAAGCCGC		R	Pigeon
13	CAGCGGCTTCATCGCTGCATCTTCAGC	Ser86Ala	F	Pigeon
14	GCTGAAGATGCAGGCGATGAAGCCGCTG		R	Pigeon
15	GGAGGCCTTCCTGGGGGCCACCGG	Val116Leu	F	Pigeon
16	CCGGTGGCCCCAGGAAGGCCTCC		R	Pigeon
17	CCTGCATCTTCGCTGCTTCACCGTC	Ser90Cys	F	Pigeon
18	GACGGTGAAGACGAGAAGATGCAGG		R	Pigeon
19	CGGATTCGCTTCGCTGCTCAG	Ser86Phe	F	Chicken
20	CTGAGCACGAGAAGACGAATCCG		R	Chicken
21	GGAGGCCTTCCTGGGCACCCACG	Val116Leu	F	Chicken
22	CGTGGGTGCCAGGAAGGCCTCC		R	Chicken
23	GCCGGTTCCTCTCCTGCACCTTCTCC	Phe86Ser	F	Green anole
24	GGAGAAGGTGCAGGAGAGGAACCCGGC		R	Green anole
25	GGAGGCCTTCGTTGGGATCGTGGC	Leu116Val	F	Green anole
26	GCCACCGATCCCACGAAGGCCTCC		R	Green anole

NOTE.—1–8. PCR primers for amplifying full length SWS1 coding sequence. *EcoRI* and *SalI* restriction enzyme sites for cloning into pMT4 are underlined and 5' and 3' coding sequence are shown in bold. 9–26. Mutagenesis primers for pigeon, chicken and green anole opsins. For the green anole double mutant the Phe86Ser mutation was created first, followed by the Leu116Val mutation.

followed the method of Molday and MacKenzie (1983). Briefly, pigments were regenerated by suspending cells in PBS (pH 7.0) containing 40 μ M 11-*cis*-retinal in the dark. The cells were then pelleted and re-suspended in a buffer containing 1% dodecyl-maltoside and 20 mg/ml phenylmethylsulfonyl fluoride (PMSF). Finally, the pigment was isolated by immunoaffinity chromatography using monoclonal antibody (bovine rhodopsin 1D4) coupled to a CNBr-activated Sepharose. The 1D4 epitope was present at the C-terminus of all opsins expressed from the pMT4 vector. Where no pigment was recovered, the experiment was repeated at least once to eliminate the possibility of experimental error.

Determination of the λ_{\max} of expressed pigments

The absorbance spectra of the purified pigments (dark spectra) were determined by UV/visible spectroscopy using a Spectronic Unicam UV500 dual-beam spectrophotometer. The pigments were then either acid denatured by the addition of 1M HCl to a pH < 2.0 or treated with hydroxylamine (56mM) and the spectrum measured again.

For each pigment, the spectrum after acid denaturation or hydroxylamine-treatment was subtracted from the dark spectrum to generate a difference spectrum. This gives a more accurate estimation of λ_{\max} since dark spectra can be distorted by underlying absorbance and scatter by the protein. The λ_{\max} was determined by fitting a standard visual pigment template (Govardovskii et al. 2000) to the difference spectra using a Solver add-in to Microsoft Excel which varies the λ_{\max} until the best fit to the template is found.

Opsin protein structure modeling

The SWS1 opsin protein structure was modeled using the human blue VS pigment model (PDB: 1KPN) of Stenkamp et al. (2002). All manipulations of the structure were performed using DeepView/Swiss-Pdb Viewer v3.7 (Guex and Peitsch 1997).

Results

Evolution of avian VS pigments

Role of Ser86 and Ser90

The SWS1 pigments of the pigeon (*Columba livia*) and chicken (*Gallus gallus*) with *in situ* λ_{\max} values in the violet region of the spectrum (Bowmaker et al. 1997; Yokoyama et al. 1998; Okano et al. 1992) were selected as starting points for the site-directed mutagenesis of avian VS pigments. These pigments have Ser at sites 86 and 90 as found for all avian VS pigments that have so far been fully sequenced. *In vitro* expression and reconstitution of these wild type pigments gave difference spectra with estimated λ_{\max} values at 388 nm for pigeon and 419 nm for chicken (fig. 1A and B). The λ_{\max} for the pigeon pigment is significantly shorter than the value of 409 nm obtained by microspectrophotometry of intact pigeon photoreceptors (Bowmaker et al. 1997), but similar to the value of 393 nm previously obtained by Yokoyama et al. (1998) for the *in vitro* expressed pigment. In contrast, the λ_{\max} value for chicken SWS1 pigment at 415 nm obtained by microspectrophotometry (Okano et al. 1992) is very similar to the value obtained here for the *in vitro* expressed pigment.

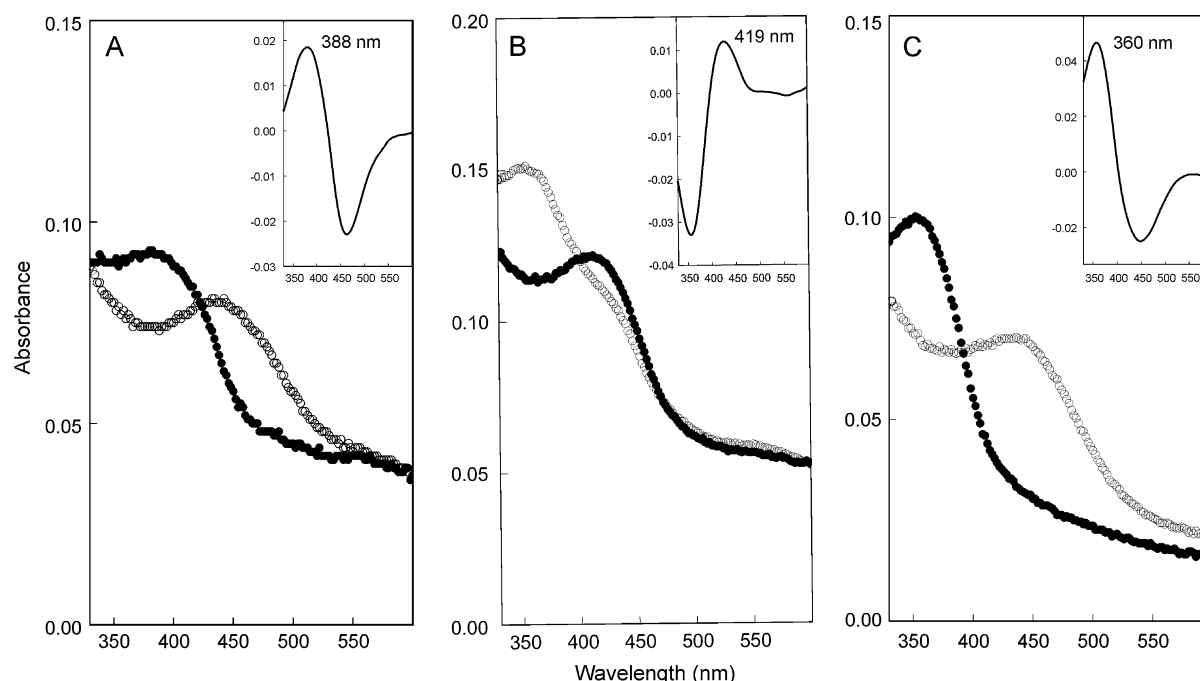


FIG. 1.—Absorbance spectra for (A) pigeon, (B) chicken and (C) green anole wild type SWS1 pigments. Solid symbols: dark spectra; open symbols: spectra after acid denaturation (A, C) or bleaching (B). Solid line in inset is the fitted template (Govardovskii et al. 2000).

Replacement of Ser90 with Cys in the pigeon wild type pigment by site-directed mutagenesis resulted as expected in the generation of a UVS pigment with a λ_{\max} at 359 nm (table 3), as previously reported by Yokoyama et al. (2000). Significantly, however, the replacement of Ser86 with Phe also resulted in short-wavelength (SW) shifts of 31 and 47 nm in pigeon and chicken VS pigments respectively to λ_{\max} values of 357 and 372 nm (fig. 2A). The replacement of polar Ser86 with non-polar Phe results therefore in substantial shifts in the λ_{\max} values of avian VS pigments, consistent with a role for Ser86 in the evolution of VS pigments in birds.

Since Cys90 is sufficient to generate a UVS pigment in birds, it is not possible to assess the effect of single substitutions elsewhere in the opsin protein on the λ_{\max} of

an avian UVS pigment. As expected therefore, an Ala86Ser substitution into the UVS pigment of the budgerigar, *Melopsittacus undulatus*, has essentially no effect on λ_{\max} (table 3) and our attempt to circumvent this effect by generating the double substitution of Ala86Ser and Cys90Ser (table 3) was unsuccessful as the mutant opsin failed to produce a functional pigment. The alternative approach is to use a non-avian UVS pigment as the starting point for mutagenesis: teleost fish are in evolutionary terms very distant from birds so we chose the green anole (*Anolis carolinensis*) from the Reptilia, the closest relatives of the Avia. This pigment has Phe86 and the λ_{\max} was confirmed as UVS by *in vitro* expression (fig. 1C). A Phe86Ser substitution generated a LW shift although it was small at 10 nm (figure 2B); this implies that other substitutions are required to move the λ_{\max} of this pigment fully into the violet region of the spectrum.

Table 3
Effect of Substitutions on λ_{\max} of Wild Type Pigments

Species	λ_{\max} of wild type SWS1 opsin	Amino acid substitution	Change in λ_{\max}
Goldfish	358 nm	Phe86Ser	+5 nm ^a
Pigeon	388 nm	Phe86Ser, Met116Val	No pigment formed
		Ser86Phe	-31 nm
		Val116Leu	-22 nm
Chicken	419 nm	Val116Met	-8 nm
		Ser86Phe	-47 nm
Budgerigar	360 nm	Val116Leu	0 nm
		Cys90Ser	+60 nm ^a
		Ala86Ser	+1 nm ^b
Green anole	360 nm	Ala86Ser, Cys90Ser	No pigment formed
		Leu116Val	No pigment formed
		Phe86Ser/Leu116Val	No pigment formed

^aHunt et al. (2004)

^bWilkie et al. (2000)

Role of Val116

From the experiments of Shi and Yokoyama (2003), we have previously inferred (Hunt et al. 2004) that Val116 may also be a key residue in the generation of avian VS pigments. Val116 is present in all avian VS pigments so far reported (Humboldt penguin, *Spheniscus humboldti*, Wilkie et al. 2000; pigeon, GenBank acc no. AJ238856; chicken, Okano et al. 1992) and in the VS pigment of the clawed frog, *Xenopus laevis* (Starace and Knox 1998). In order to examine the role of Val116 in more detail, we generated mutant pigeon and chicken VS pigments with Val replaced with Leu as found in mouse, rat and green anole UVS pigments. As shown in figure 3, the substitution results in a SW shift of 22 nm in the pigeon pigment but was without effect on the chicken pigment. This difference in

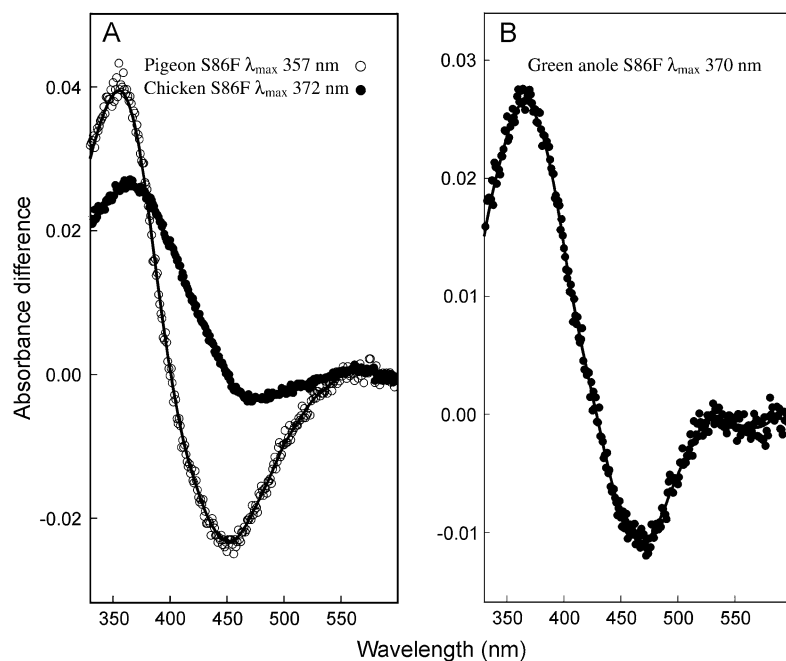


FIG. 2.—Difference spectra for site 86 substitutions. (A) Pigeon and chicken Ser86Phe mutants. (B) Green anole Phe86Ser mutant.

the behavior of the 2 pigments suggests that conformation differences must exist between the 2 pigments such that the replacement of Val116 by Leu abolishes protonation in the pigeon but not in the chicken. Single and double reverse mutations of Leu116Val and Phe86Ser/Leu116Val were then introduced into the green anole UVS opsin but neither opsin formed a functional pigment (table 3), so it was not possible to determine whether Val116 by itself or combined with Ser86 is able to shift the λ_{\max} of a UVS pigment into the violet region of the spectrum. The double mutation of Phe86Ser/Met116Leu in the goldfish UVS pigment also failed to generate a functional pigment (table 3).

Evolution of avian UVS pigments

In a study of the SWS1 pigments in 45 bird species distributed across 14 Orders, Ödeen and Håstad (2003) were able to deduce the amino acid residues present at sites 86 and 90 (fig. 4). The approach they took was to amplify a small fragment of the SWS1 gene that included the coding region for these sites. No spectral data were obtained for the corresponding pigments so the spectral sensitivity was inferred from the residue present at site 90: UVS for Cys and VS for Ser.

In addition to the substitutions at site 90, Ödeen and Håstad (2003) identified a number of different residues at site 86. Amongst the UVS pigments (with Cys90), site 86 is never occupied by Ser but may contain either Ala, Cys or Met. The residue at site 86 also varies in pigments with Ser90, with 8 species with Cys86, 5 species with Ala86, and 1 species with Phe86. A series of site-directed mutagenesis experiments was therefore undertaken to determine whether any of the latter 3 substitutions are capable of SW shifting the λ_{\max} of the pigeon pigment. As shown in figure

2, Ser86Phe substitutions into pigeon and chicken VS pigments generate SW shifts, so it is likely therefore that the single species with this substitution, the blue-crowned Trogon, *Trogon curucui*, possesses a UVS pigment. In contrast, a Ser86Ala substitution would not be expected to SW shift as a Cys90Ser substitution into budgerigar UVS pigment causes a LW shift even though it retains Ala86 (Wilkie et al. 2000). This was confirmed by an essentially unchanged λ_{\max} for a pigeon pigment with a Ser86Ala substitution and a Ser86Cys substitution likewise failed to generate a significant SW shift (fig. 5A), indicating that species with either of these residues at site 86 combined with Ser90 possess VS pigments. The effect of Cys86 was further explored by cloning and sequencing of the SWS1 opsin gene from the common cormorant, a species that naturally possesses a pigment with Cys86 and Ser90. When expressed *in vitro*, the regenerated pigment showed a λ_{\max} at 405 nm (fig. 5B).

Modeling of key amino acid differences in SWS1 pigments

In order to relate the key tuning sites to the retinal binding pocket and the SB, 3 structural models of SWS1 pigments with substitutions at these sites were generated. These models are based on a theoretical human blue VS pigment (PDB: 1KPN—Stenkamp et al. 2002). In each case, Lys296 with bound retinal, the counterion Glu113 and sites 86, 90 and 116 are shown (fig. 6).

Retinal lies deep within the protein structure with the residues at sites 86, 90 and 116 surrounding the retinal binding pocket (fig. 6A). This model sets the counterion to Schiff base separation at 2.83 Å, slightly less than the 3.3 to 3.5 Å separation derived from the crystal structure of bovine rod opsin (Palczewski et al. 2000). The ancestral vertebrate

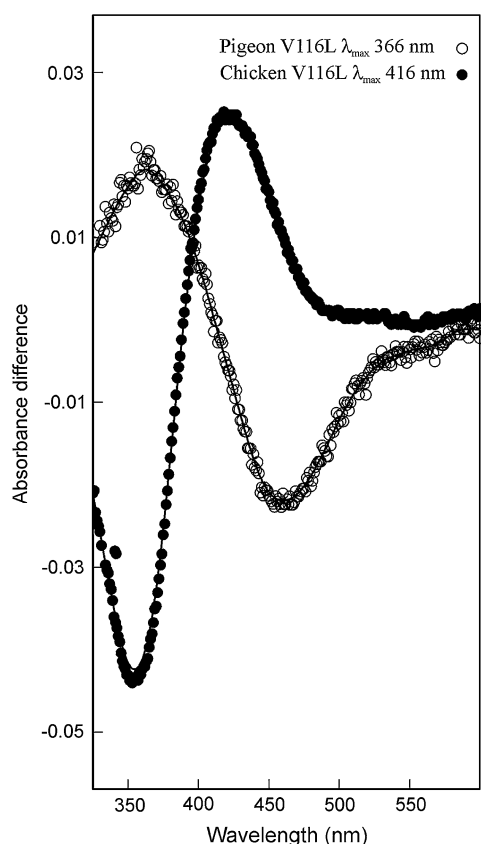


FIG. 3.—Difference spectra for pigeon and chicken Val116Leu mutants.

pigment (fig. 6A and B) is based on the lamprey UVS sequence which has Phe86, Ser90 and Leu116 (Davies et al. 2007). Figures 6C and D show avian VS and UVS pigments respectively, with substitutions at all 3 key sites. Although these models provide some structural information, they are nevertheless based on the structure of a rod pigment. The precise distances between residues may be very different in a cone pigment.

Discussion

Phylogenetic evidence indicates that the peak sensitivity of the ancestral SWS1 visual pigment in vertebrates was UVS and that VS pigments have evolved separately from this ancestral pigment in each of the major vertebrate lineages except fishes, largely as a result of substitutions at site 86. One of the objectives of the present study was therefore to identify the molecular basis for the evolution of the ancestral avian VS pigments. All avian SWS1 pigment genes that have been shown spectrally to encode a VS pigment specify Ser86 whereas Phe86 is present in all non-avian UVS pigments. In fact, Phe86 by itself can confer UV-sensitivity as demonstrated by the LW shift that results from its replacement with Tyr either naturally as in the VS pigments of ungulates (Cowing et al. 2002; Fasick et al. 2002), the grey squirrel (Carvalho et al. 2006), and the Tamar wallaby (Deeb et al. 2003), or by site-directed mutagenesis

in the goldfish UVS pigment (Cowing et al. 2002). Significantly, a Tyr86Ser substitution into bovine VS pigment maintains a violet λ_{\max} with a small SW shift from 435 to 420 nm (Hunt et al. 2004), so Ser86 is clearly able to maintain the violet sensitivity of an SWS1 pigment. Consistent with this, we have now shown that the substitution of Ser86 with Phe by site-directed mutagenesis of the pigeon and chicken VS pigments results in SW shifts in the λ_{\max} of the mutant pigments to 360 and 372 nm respectively, confirming that Ser86 is required in an avian VS pigment to maintain violet-sensitivity. The converse Phe86Ser substitution in the goldfish UVS pigment does not, however, generate a LW shift (Hunt et al. 2004) and when the same substitution was introduced into the SWS1 pigment of the green anole, it resulted in only a small 10 nm LW shift. It would appear, therefore, that substitution at site 86 must be paired with substitutions elsewhere in the opsin protein to shift the λ_{\max} of a reptilian UVS pigment fully into the violet.

One such site is 116 where Val would appear to be required in birds to generate a VS pigment. A previous study by Shi and Yokoyama (2003) has shown that Phe86Ser and Leu116Val, when substituted along with Phe49Val and Ser118Ala into a genetically engineered ancestral avian UVS pigment, LW shift the λ_{\max} from 360 to 393 nm. The latter 2 sites, however, cannot be critical for the LW shift, since Val49 is found in avian UVS but not in VS pigments and Ala118 is present in both avian VS and UVS pigments. Our results show that a Val116Leu substitution SW shifts the pigeon VS pigment by 22 nm, but is without effect on the λ_{\max} of the chicken VS pigment, and the reverse single substitution of Leu116Val or double substitution of Phe86Ser and Leu116Val in the green anole UVS pigment both failed to generate a functional pigment. This suggests that these substitutions generate conformational changes that require other compensatory changes elsewhere in the opsin protein to produce a functional pigment. Such changes may have been important in the evolution of the ancestral avian VS pigment but have yet to be identified.

Vertebrate visual pigments with λ_{\max} values >385 nm possess a protonated SB (Fasick et al. 2002) and the role of certain residues in the vicinity of the retinylidene SB linkage may be to stabilize this protonation. Stabilization of protonation does not occur in UVS pigments, presumably as a result of amino acid substitution at key sites, so the SB remains unprotonated. Examples are the Ser90Cys substitution in the evolution of avian UVS pigments (Wilkie et al. 2000; Yokoyama et al. 2000) and the Phe86Tyr substitution in the evolution of many VS pigments (Cowing et al. 2002; Fasick et al. 2002; Carvalho et al. 2006). Significantly, when Tyr86 is modeled on to the bovine rhodopsin template (Palczewski et al. 2000), it is sufficiently close to the SB base to influence its stability (Hunt et al. 2004). The evolution of the ancestral avian VS pigment from the ancestral vertebrate UVS required therefore the protonation of the SB. This was most likely achieved by the replacement of Phe86 with polar Ser86. Similar therefore to Tyr86, Ser86 in avian pigments may serve to facilitate the electrostatic stabilization of protonation in the generation of a VS pigment. In contrast, all the residues

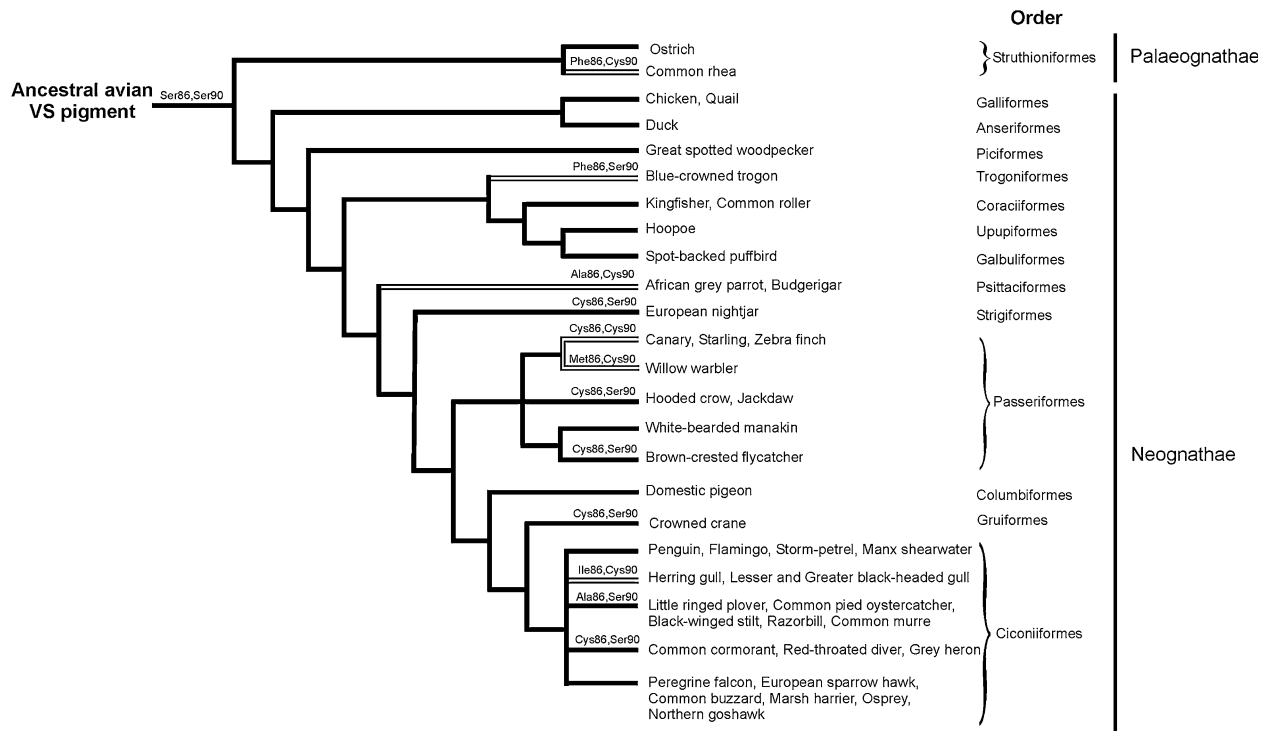


FIG. 4.—Phylogenetic relationships of the different avian Orders showing presence of VS and UVS SWS1 pigments in the species so far examined. Solid lines are lineages with VS pigments, open lines with UVS pigments. The ancestral avian VS pigment was most probably Ser86, Ser90. Substitutions at these sites are shown on the respective branches. The tree was generated from the DNA-DNA hybridization data of Sibley and Ahlquist (1990). The branch lengths do not reflect evolutionary distances.

at site 116 are non-polar, so the influence of this site on protonation may be indirect via a conformational change. The 3 amino acids, Val, Leu and Met, found at this site in natural pigments differ in amino group size. Val is the

least bulky, so the differential effect of these amino acids on the stabilization of protonation may arise from space constraints on conformation in the vicinity of the SB imposed by the more bulky residues (fig. 6).

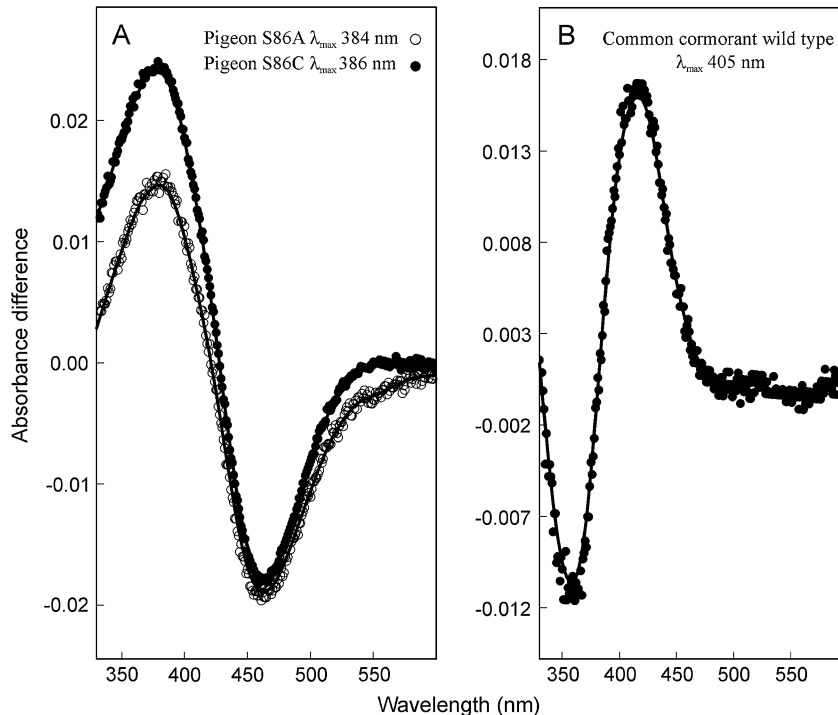


FIG. 5.—Difference spectra for cormorant wild type and mutant pigeon pigments. (A) Pigeon Ser86Ala and Ser86Cys. (B) SWS1 pigment of common cormorant (GenBank acc. EF568933).

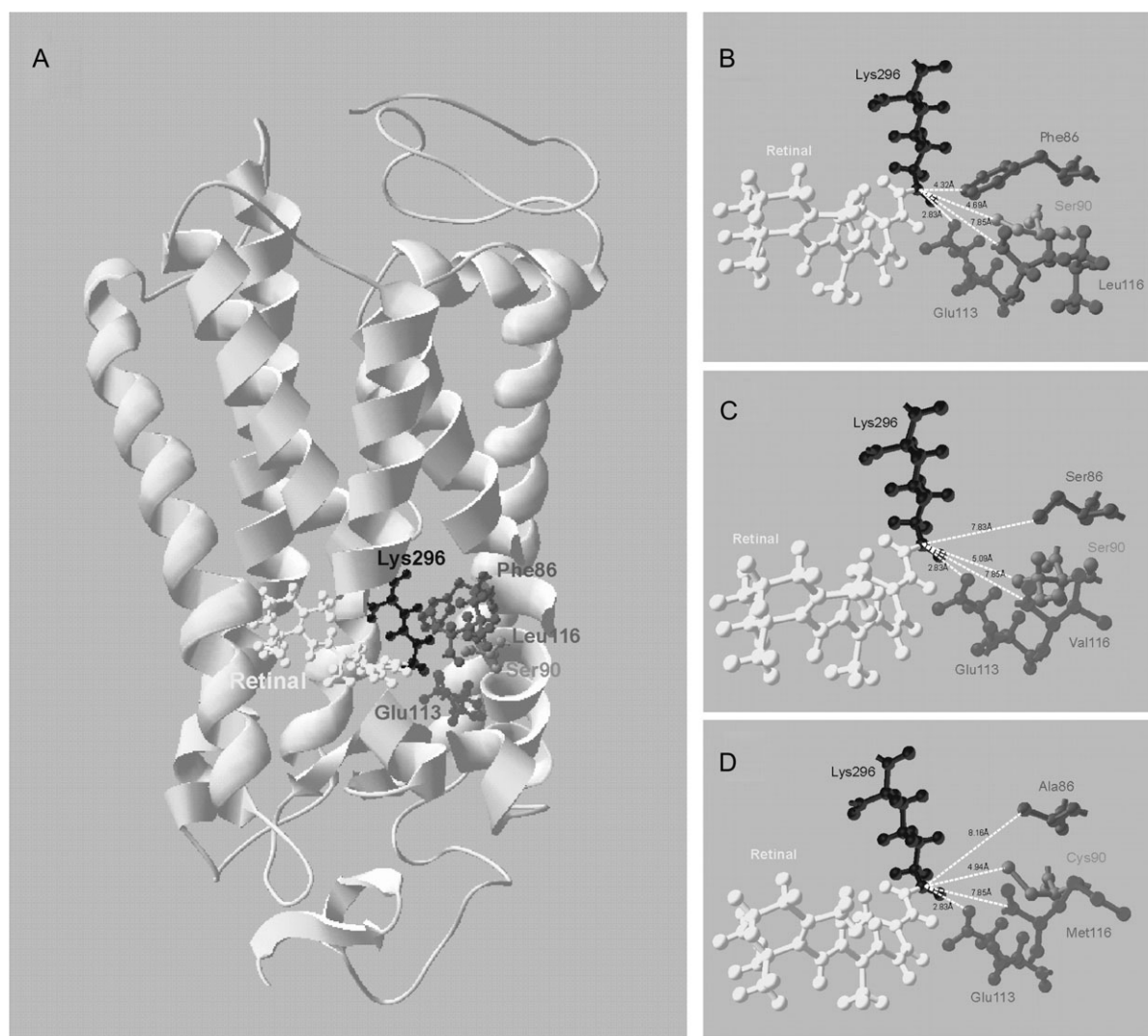


FIG. 6.—Structural model of SWS1 pigments. (A) Ancestral (lamprey) SWS1 showing the retinal binding pocket with surrounding amino acids Lys296, Glu113, Phe86, Ser90 and Leu116. Portions of helices III and V–VII have been cut away to reveal the retinal and key residues. (B) Enlarged view of the lamprey SWS1 pigment showing the relative positions of retinal, Phe86, Ser90, Glu113, Leu116 and Lys296. (C) Avian VS pigment with Ser86, Ser90 and Val116. (D) Avian VS pigment with Ala86, Cys90 and Met116. This figure is best viewed in full color and a full color version is available under supplementary materials.

The major mechanism for the evolution of avian VS pigments from an ancestral avian VS pigment is undoubtedly a Ser90Cys substitution, as originally reported by Wilkie et al. (2000) and Yokoyama et al. (2000). Amongst the 46 species of birds studied by Ödeen and Håstad (2003), 10 were shown to have this substitution. These species belong to 4 different avian Orders, the Struthioniformes, the Passeriformes, the Psittaciformes, and the Ciconiiformes, and, with the exception of the Psittaciformes, these Orders include species with VS pigments. It is probable, therefore, that the change to Cys90 occurred separately in each lineage. One other substitution that has been identified by the present study as conferring UV-sensitivity in an avian pigment is Phe86 as found in the blue-crowned trogon; it will be interesting to obtain spectral data to confirm UV-sensitivity in this species, since this is the only avian species so far identified with Phe86. The position of the blue-crowned trogon within the avian phylogeny would indicate, how-

ever, that this is almost certainly a back-mutation to Phe rather than the retention of the ancestral state.

Two other relatively common substitutions that are found in pigments with Ser90 are Ala86 and Cys86 (Ödeen and Håstad 2003). Ala86 is found in 5 species of ciconiiforms, whereas Cys86 is present in 3 species of passeriforms, 1 species of gruiforms, and 6 species of ciconiiforms. Neither substitution, however, results in a SW shift when substituted into an avian VS pigment, a conclusion consistent with a λ_{\max} at 405 nm for the pigment in the common cormorant that naturally has Cys86, and with the failure of Ala86 in the UVS pigment of the budgerigar to elicit a LW shift when a Cys90Ser substitution is introduced (Wilkie et al. 2000). The failure of Cys86 to tune the pigment into the UV contrasts with the observation of Shi and Yokoyama (2003) that a Ser86Cys substitution into a genetically engineered ancestral pigment SW shifts the λ_{\max} from 393 to 366 nm. From present and previous studies (Parry

et al. 2004), it is evident that the local environment of the residue present at site 86 is critical for its effect on spectral tuning and an important difference between the 2 studies is the nature of the pigment used for substitution. Cys86 would appear to SW shift in some pigments but, significantly, does not have this effect in an extant avian pigment. From this, we conclude that avian pigments with Cys86 are VS. Phe86 is unable to stabilize the protonation of the SB, whereas stabilization is maintained when either Ala86 or Cys86 is present in a VS pigment.

In summary therefore, from the data so far available, it would appear that UVS pigments in birds have evolved on at least 5 separate occasions from an ancestral avian VS pigment and that 2 different mechanisms have been used to achieve the shift back into the UV.

Supplementary Material

Supplementary figure 6 is available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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