Evolution of Dim-Light and Color Vision Pigments

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Annu. Rev. Genomics Hum. Genet. 2008. 9:259–82

First published online as a Review in Advance on June 10, 2008

The *Annual Review of Genomics and Human Genetics* is online at genom.annualreviews.org

This article's doi:
10.1146/annurev.genom.9.081307.164228

1527-8204/08/0922-0259$20.00

Key Words

spectral tuning, adaptive evolution, ancestral pigments, mutagenesis, protein engineering

Abstract

A striking level of diversity of visual systems in different species reflects their adaptive responses to various light environments. To study the adaptive evolution of visual systems, we need to understand how visual pigments, the light-sensitive molecules, have tuned their wavelengths of light absorption. The molecular basis of spectral tuning in visual pigments, a central unsolved problem in phototransduction, can be understood only by studying how different species have adapted to various light environments. Certain amino acid replacements at 30 residues explain some dim-light and color vision in vertebrates. To better understand the molecular and functional adaptations of visual pigments, we must identify all critical amino acid replacements that are involved in the spectral tuning and elucidate the effects of their interactions on the spectral shifts.

Molecular adaptation: the process by which molecules are positively selected by natural selection

TM: transmembrane

λ_{max}: the wavelength of maximal absorption of a visual pigment

Spectral tuning: the phenomenon in which a chromophore attains different absorption spectra when attached to different opsins

Visual pigments: light-sensitive molecules that consist of a chromophore (either 11-*cis*-retinal or 11-*cis*-3,4-dehydroretinal) and an opsin

In vitro assay: a cell culture–based method of measuring the absorption spectra of visual pigments

Functional adaptation: the process by which a protein's function is positively selected by natural selection

INTRODUCTION

Many vertebrates use vision as a principal means to interpret their environments and have consequently evolved diverse visual systems (8, 40, 71). The extensive data collected by vision scientists suggest strongly that this diversity is a result of organisms' adaptations to various photic environments and to their new lifestyles (8, 40, 71, 81, 83, 94). Did animals really modify their visual systems to adapt to different environments? If so, how did they do it? These questions touch on a remarkably difficult problem of molecular adaptation in evolutionary biology as well as on a central unsolved problem of phototransduction in vision science.

In most vertebrates, rod photoreceptors are responsible for highly sensitive dim-light vision, whereas cone photoreceptors mediate color discrimination and high visual acuity at higher light intensities (6, 60, 75). The nocturnal Tokay gecko (*Gekko gekko*) and the diurnal American chameleon (*Anolis carolinensis*) provide interesting oddities in that they have pure-rod retinas and pure-cone retinas, respectively (81).

The light-sensitive molecules, visual pigments, in these photoreceptor cells consist of an integral transmembrane (TM) protein, opsin, and a chromophore, either 11-*cis*-retinal or 11-*cis*-3,4-dehydroretinal. The chromophore is covalently bound to an opsin via a Schiff base linkage to a conserved lysine at residue 296 (K296) (70). The 11-*cis*-retinal in solution absorbs light maximally (λ_{max}) at 440 nm (36); however, by interacting with various opsins, it detects a wide range of λ_{max}s between 360 and 560 nm, which is known as spectral tuning in visual pigments (37).

Animals live in diverse light environments that range from the darkness at the bottom of the ocean to bright light on land. A strong association exists between the types of visual pigments animals possess and the environments they live in. For example, humans have a total of four types of visual pigments and their λ_{max}s range from 414 nm to 560 nm (51); zebrafish, living in shallow water, have a total of nine types of visual pigments and their λ_{max}s range from 360 to 560 nm (14), which corresponds to the wide range of light available to them. Compared with these species, coelacanths, living at a depth of 200 m, have only two types of visual pigments whose λ_{max}s are very close to the maximal wavelength of downwelling sunlight at 480 nm (94) (**Figure 1**).

Molecular genetic analyses of spectral tuning in visual pigments became feasible when the bovine rhodopsin gene was cloned (49) and when an in vitro assay (**Figure 2**) became available (46, 47, 54). Thanks to these developments, virtually any opsins in vertebrates can now be manipulated, expressed in cultured cells, reconstituted with 11-*cis*-retinal, and the λ_{max} of the resulting visual pigments can be measured (82). The recent crystal structural analyses of bovine rhodopsin (**Figure 3**) also lay a solid foundation for studying the chemical basis of spectral tuning (53, 56). Despite these developments, the molecular basis of spectral tuning in visual pigments is still not well understood. Analyses of molecular adaptations in vertebrates are also fraught with major problems because it is remarkably difficult not only to detect minute selective advantages caused by molecular changes in nature (38), but also to find genetic systems where evolutionary hypotheses can be tested (79).

To study the possible functional adaptation of visual pigments, we must understand how the spectral tuning in visual pigments works. To understand the molecular basis of spectral tuning, we must identify amino acid changes that shift the absorption spectra of visual pigments. To identify such amino acid changes, we must know how animals modified their visual pigments in the past (79). Hence, the evolutionary biology and vision science approaches are closely intertwined and share an important common goal of elucidating why and how organisms modified their visual pigments to live in their new environments (79, 81, 83).

With the possibility of many adaptive events, the availability of the in vitro assay, and the crystal structure of the bovine rhodopsin, dim-light and color vision provide an opportunity

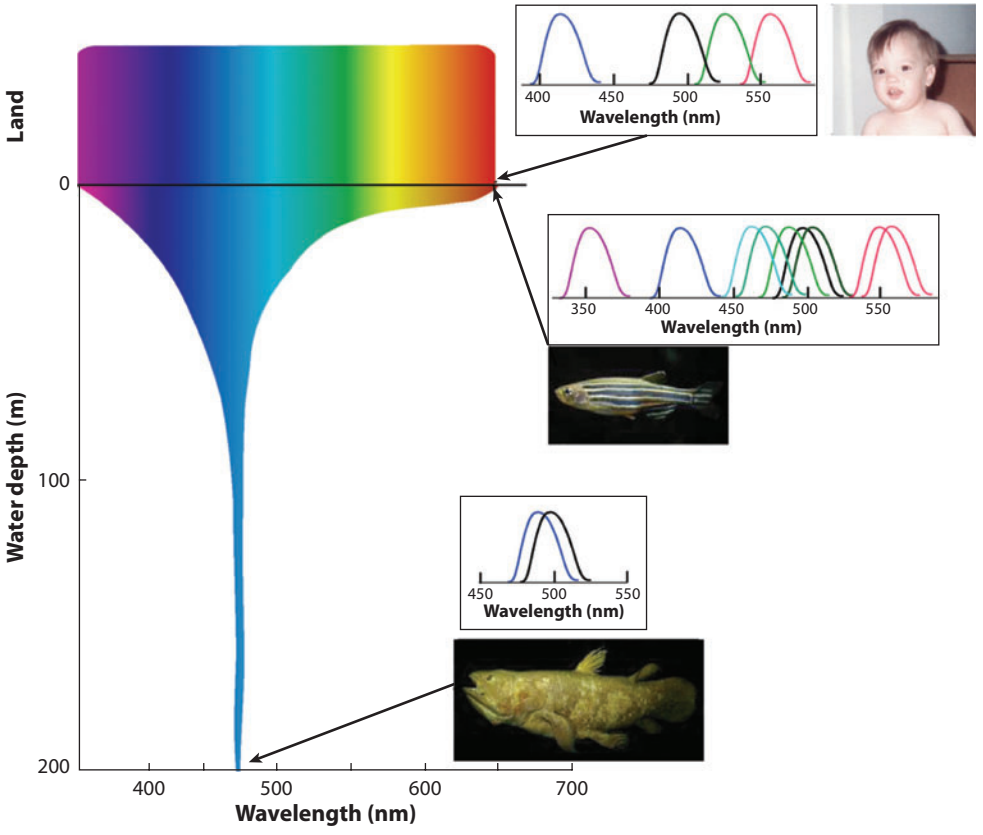

Figure 1

Photic environments and the visual pigments of human (51), zebrafish (14), and coelacanth (94). The pictures of zebrafish and coelacanth were taken by M. Noren and JJ Photo, respectively. See **http://www.fshbase.org**, version 09/2006.

to explore not only why and how molecular and functional adaptations of visual pigments occurred, but also how various visual pigments modulate their light sensitivities. Mutagenesis analyses of visual pigments have improved our understanding of the molecular bases of spectral tuning in visual pigments (25, 81). Consequently, visual pigments became one of a small number of model systems in the exploration of molecular adaptations in vertebrates (19, 29, 79, 81, 83, 93). In fact, the molecular analyses of the origin and evolution of color vision produced arguably "the deepest body of knowledge linking differences in specific genes to differences in ecology and to the evolution of species" (10).

Here I review recent developments in the functional differentiations of visual pigments that have generated red-green color vision, UV-violet vision, and dim-light vision in ancestral as well as contemporary species. The key ingredient in these analyses is mutagenesis experiments. A survey of mutagenesis results of visual pigments reveals that the direction and magnitude of the spectral shift caused by a certain amino acid change can be affected strongly by the amino acid composition of a specific visual pigment under study. Consequently, the specific mutagenesis result may not apply even to the identical mutation in other pigments, making it difficult not only to derive a general principle of the spectral tuning of visual pigments, but also to elucidate the molecular mechanism of functional adaptation of visual pigments. The seemingly conflicting mutagenesis results can

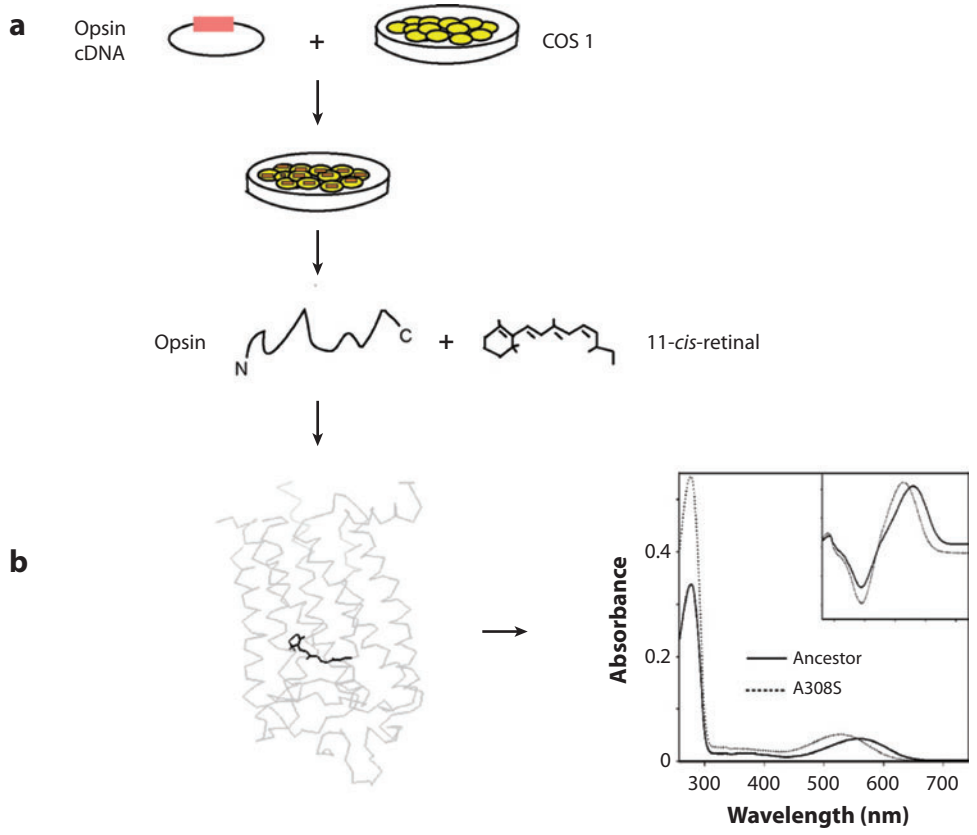

Figure 2

In vitro assay of the absorption spectra of the ancestral mammalian middle and long wavelength-sensitive (M/LWS) pigment and its mutant, containing A308S. (*a*) The opsin cDNAs are expressed in an expression vector, pMT5, in COS1 cells after transient transfection. The opsins are then reconstituted with 11-*cis*-retinal. The resulting visual pigments are purified by immunoaffinity chromatography with the monoclonal antibody 1D4 Sepharose 4B. (*b*) The absorption spectra (dark spectra) of the visual pigments are recorded in the dark using a spectrophotometer. The amino acid change A308S was made by site-directed mutagenesis. The absorption spectra in the inset show the difference spectra by subtracting a spectrum measured after photobleaching from a spectrum evaluated before light exposure.

"make sense only in the light of evolution" (21) and the way we conduct mutagenesis experiments must be re-evaluated.

Rhodopsins (RH1): visual pigments that are expressed typically in rods

RH1-like (RH2) pigments: visual pigments whose amino acid sequences are most closely related to those of RH1 pigments

EVOLUTION OF VISUAL PIGMENTS

Since the first molecular clonings of the bovine and human opsin genes (49–51), more than 470 opsin genes from ~180 vertebrate species have been characterized. For 126 of these genes, not only have the complete nucleotide sequences of the coding regions been determined but also the λ_{max}s of the corresponding visual pigments have been measured with in vitro assays. The phylogenetic analyses of these pigments show that they are divided into five groups: rhodopsins (RH1 pigments), RH1-like (RH2) pigments, short wavelength–sensitive type 1 (or SWS1) pigments, SWS type 2 (or SWS2) pigments, and middle and long wavelength–sensitive (M/LWS) pigments (79, 81, 83). These pigment groups have a tree topology of (M/LWS, (SWS1, (SWS2, (RH2, RH1)))), and

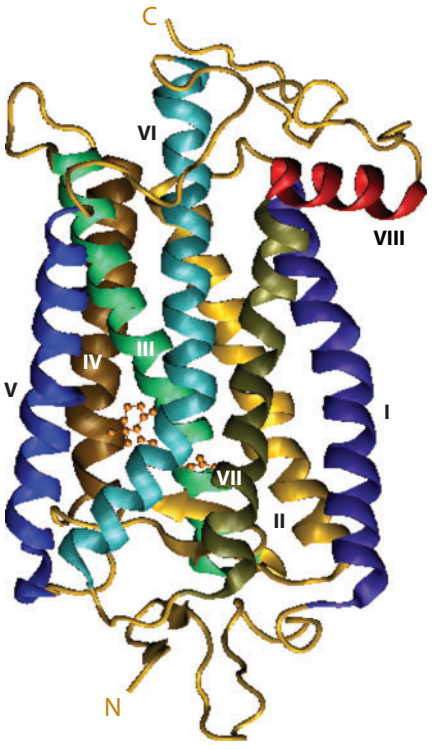

Figure 3

The seven α helices and chromophore of bovine rhodopsin (Protein Data Bank ID 1U19).

reveal two major characteristics: First, the RH1 group includes pigments from a wide range of vertebrate species, from fish to mammals, showing that the early vertebrate ancestor already possessed all five types of visual pigments. Second, humans lack RH2 and SWS2 pigments. In fact, no placental mammals have RH2 and SWS2 pigments, but platypus has SWS2 pigment (18). Hence, the RH2 gene seems to have become nonfunctional in the mammalian ancestor, whereas the SWS2 gene in the placental mammals became nonfunctional after the divergence between placental mammals and marsupials. The λ_{max}s of RH1 pigments (~480–510 nm), RH2 pigments (~450–530 nm), SWS1 pigments (~360–440 nm), SWS2 pigments (~400–450 nm), and M/LWS pigments (~510–560 nm) can overlap among different groups. These λ_{max}s have been measured in two different ways: dark spectra, which were measured in the dark, and/or difference spectra, which were measured by subtracting a spectrum measured after photobleaching from a spectrum evaluated before light exposure (dark spectrum) (**Figure 2**).

Considering representative visual pigments of the five pigment groups, I now discuss what types of amino acid replacements generated the variable λ_{max}s in contemporary pigments. In these analyses, the amino acid sequences of ancestral visual pigments were inferred using a computer program, PAML (74). Unless stated otherwise, the amino acid residues of RH1, RH2, SWS1, and SWS2 pigments are standardized by the corresponding amino acid positions of the bovine RH1 pigment and the residues of the M/LWS pigments are standardized by the corresponding amino acid positions of the human M/LWS pigments.

Several ancestral RH1 pigments were engineered by introducing all necessary amino acid changes into contemporary pigments or into engineered ancestral pigments (S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt, unpublished data). The in vitro assays of these pigments show that the RH1 pigments of early ancestors had λ_{max}s of ~500 nm (**Figure 4*a***). The ancestral RH1 pigment in the archosaur, the major branch of the diapsid reptiles, was also engineered and its λ_{max} was estimated to be 508 nm (13). Studies with various mutations introduced into the engineered ancestral pigments show that a total of nine amino acid replacements explain the λ_{max}s of most ancestral and contemporary RH1 pigments in **Figure 4*a*** (S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt, unpublished data). Interestingly, D83N, E122Q, F261Y, and A292S occurred seven, two, two, and seven times, respectively (**Table 1**); in particular, D83N/A292S occurred on five separate occasions and caused similar functional changes (**Figure 4*a***).

A limited number of ancestral RH2 pigments has been engineered and the evolutionary mechanisms of λ_{max} shifts of many RH2 pigments are still largely unknown. However, molecular bases of spectral tuning in the four

SWS1 pigments: short wavelength–sensitive type 1 pigments

SWS2 pigments: short wavelength–sensitive type 2 pigments

M/LWS pigments: middle and long wavelength–sensitive pigments

zebrafish pigments (15), coelacanth 2 (P478) (94), chameleon 2 (P478), gecko 2 (P467), and medaka 2-A (P452) (68) have been examined by mutagenesis experiments (**Figure 4*b***). E122Q occurred on four separate occasions and seems to be the most common amino acid replacement that caused the major λ_{max} shifts of RH2 pigments. As in the case of the coelacanth RH1 pigment, E122Q/A292S explains the λ_{max} shift in medaka 2-A (P452) (68). However, the amino acid replacements at residues 49, 52, 83, 86, 97, and 164 explain only 65% of the λ_{max} difference between chameleon 2 (P495) and gecko 2 (P467) (68).

The λ_{max}s of engineered SWS1 pigments at various stages of vertebrate evolution (62) show that those in early ancestors had λ_{max}s of ~360 nm and were UV sensitive (**Figure 4*c***). Hence, most UV pigments in contemporary species, including fish and mouse, have inherited UV vision directly from the early ancestors. An interesting exception is the avian lineage, where the common ancestor developed violet vision with a λ_{max} of 393 nm, but a lineage of descendants, such as zebra finch and budgerigar, reinvented UV vision with a single amino acid replacement, S90C (**Figure 4*c***). This

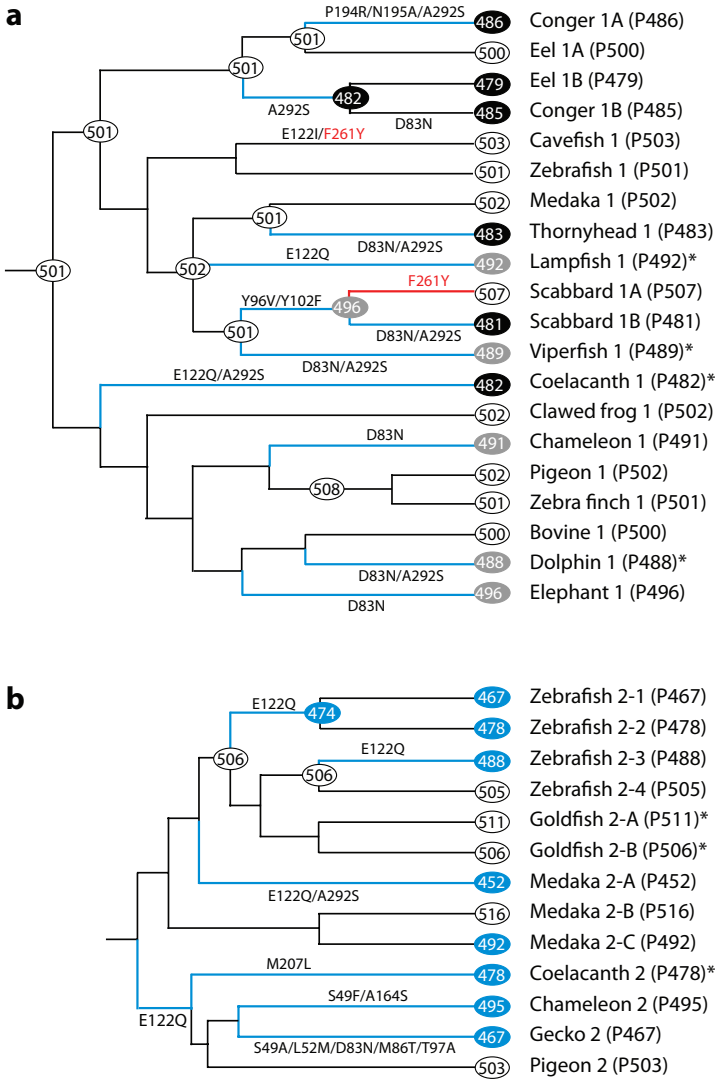

Figure 4

Phylogenetic trees of visual pigments and amino acid replacements that caused λ_{max} shifts. The numbers after P refer to dark and difference (*) spectra. The decrease (*blue line*) and increase (*red line*) of λ_{max}s are shown. (*a*) Rhodopsin (RH1) pigments: conger, eel, lampfish, scabbard fish pigments (S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt, unpublished data); medaka pigment (41); thornyhead pigment (88); elephant pigment (91); and others (82). The ancestral pigment of the pigeon and zebra finch pigments and others are from 11 and S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt (unpublished data), respectively. The ovals denote surface (*white*), intermediate (*gray*), and deep-sea (*black*) pigments. (*b*) RH1-like (RH2) pigments: zebrafish pigments (14), goldfish pigments (34), medaka pigments (41), and others (82). The ovals indicate the ancestral pigment (*white*) and its descendant pigment with a blue-shifted λ_{max} (*blue*) (15). (*c*) Short wavelength–sensitive type 1 (SWS1) pigments: bluefin killifish pigment (92), bovine pigment (28), elephant pigment (91), wallaby pigment (20), and others (82). The ovals indicate UV pigments (*purple*) and violet pigments (*blue*) (62). (*d*) SWS type 2 (SWS2) pigments: bluefin killifish pigments (92), medaka pigments (41), frog and newt pigments (66), platypus pigment (18), and others (82). The white oval indicates the ancestor of the goldfish and zebrafish pigments (16). (*e*) Middle and long wavelength–sensitive (M/LWS) pigments: zebrafish pigments (14), medaka pigments (41), wallaby pigment (20), platypus pigment (18), and others (82). The ovals indicate LWS (*red*) and MWS pigments (*green*). Data for the ancestral pigments are taken from 83.

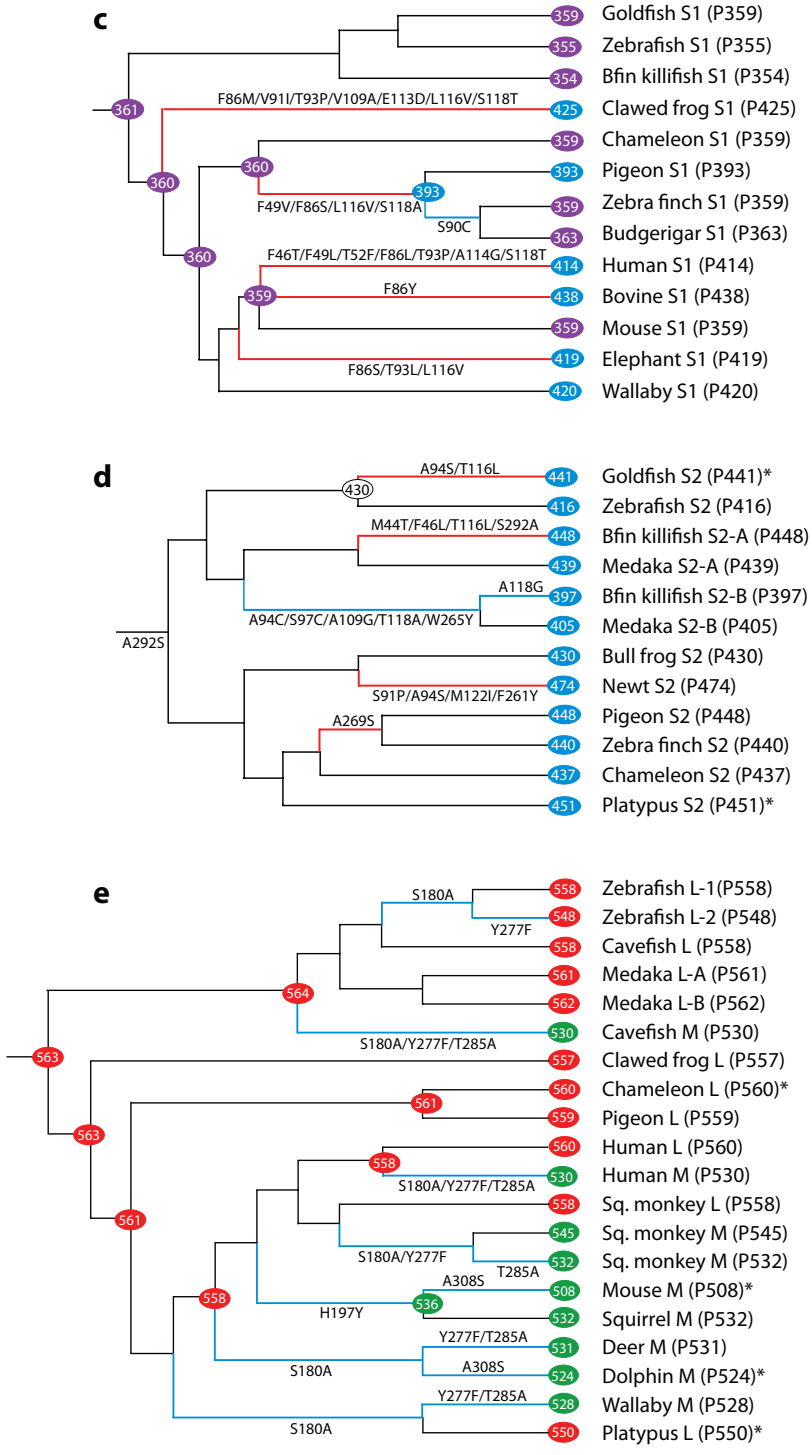

Figure 4

(*Continued*)

Table 1 Critical amino acid changes that shift λ_{max}s of visual pigments

Site[1]	Mutation	RH1	RH2	SWS1	SWS2	M/LWS	All
44	M44T				1		1
46	F46T			1			1
	F46L				1		1
49	S49F		1				1
	S49A		1				1
	F49V			1			1
	F49L			1			1
52	L52M		1				1
	T52F			1			1
83	D83N	7	1				8
86	M86T		1				1
	F86M			1			1
	F86S			2			2
	F86L			1			1
	F86Y			1			1
90	S90C			1			1
91	V91I			1			1
	S91P				1		1
93	T93P			2			2
	T93L			1			1
94	A94S				2		2
	A94C				1		1
96[2]	Y96V	1					1
97	T97A		1				1
	S97C			1			1
102[2]	Y102F	1					1
109	V109A			1			1
	A109G				1		1
113	E113D			1			1
114	A114G			1			1
116	L116V			3			3
	T116L				2		2
118	S118T			2			2
	S118A			1			1
	T118A				1		1
	A118G				1		1
122	E122I	1					1
	E122Q	2	4				6
	M122I				1		1
164	S164A					6	6
	A164S		1				1

(*Continued*)

Table 1 (*Continued*)

Site[1]	Mutation	RH1	RH2	SWS1	SWS2	M/LWS	All
181	H181Y					1	1
194[2]	P194R	1					1
195[2]	N195A	1					1
207	M207L		1				1
261	F261Y	2			1		3
	Y261F					6	6
265	W265Y				1		1
269	A269S				1		1
	T269A					5	5
292	A292S	7	2		1	2	12
	S292A				1		1

[1]Sites 164, 181, 261, 269, and 292 correspond to 180, 197, 277, 285, and 308 of middle and long wavelength–sensitive pigments (M/LWS) pigments, respectively. H211C and A295S in bovine 1 (P500) also cause λ_{max} shifts (32, 39, 46).
[2]S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt (unpublished data).

interpretation is also supported by mutagenesis results showing that the zebra finch and budgerigar UV pigments become violet-sensitive by the mutation C90S (73, 86) and the chicken and pigeon violet pigments become UV-sensitive by S90C (86).

Despite extensive mutagenesis analyses (27, 61, 62, 67), the molecular basis of spectral tuning in the SWS1 pigments is still poorly understood. This is simply because, with the exception of some amino acid changes at residues 86 and 90, extremely strong interactions exist among different amino acid residues. The functional differentiations of various violet pigments in some species from the ancestral UV pigment were caused by different sets of amino acid replacements. The λ_{max}s of human S1 (P414) (61) and clawed frog S1 (P425) (67) each can be explained by seven amino acid replacements, in which T93P and S118T were shared (**Figure 4*c***). Intriguingly, all these 12 amino acid changes cause no λ_{max} shift individually and the λ_{max}s of two pigments have been modified only through synergistic interactions of the seven amino acid replacements in each lineage (61, 67, Yokoyama & N. Takenaka, unpublished result). Conversely, F86Y in bovine S1 (P438) (27; see also 11, 17) and F86S in the ancestral avian pigment and in elephant S1 (P419) (61, 91) increased the λ_{max} significantly individually. In the SWS1 pigments, F86S, T93P, L113V, and S118T occurred more than once (**Table 1**).

For the SWS2 pigments, only the common ancestor of goldfish S2 (P441) and zebrafish S2 (P416) has been engineered (16). Because this ancestral pigment had a λ_{max} of 430 nm and bluefin killifish S2-B (P397) decreased its λ_{max} to 397 nm (**Figure 4*d***), the ancestral SWS2 pigment probably had a λ_{max} of 400–430 nm. This relatively low λ_{max} seems to have been caused by A292S that occurred in the ancestral SWS2 pigment (**Figure 4*d***). Mutagenesis analyses suggest that goldfish S2 (P441) (16), bluefin killifish S2-A (P448) (92), and newt S2 (P474) (66) increased their λ_{max}s, whereas bluefin killifish S2-B (P397) decreased it (**Figure 4*d***). The mutagenesis results of goldfish S2 (P441) (87) also suggest that A269S increased the λ_{max} of the common ancestor of pigeon S2 (P448) and zebra finch S2 (P440). In this group, A94S and T116L occurred twice (**Table 1**).

The engineered ancestral M/LWS pigments reveal that early vertebrate ancestors used LWS pigments with λ_{max}s of ~560 nm (85). A wide range of species, including fish, amphibians, reptiles, birds, and mammals have kept LWS pigments, whereas others have switched from the ancestral LWS pigments to MWS pigments (**Figure 4*e***). The ancestral LWS

pigments had a specific amino acid composition of S180, H197, Y277, T285, and A308, from which variable λ_{max}s of contemporary M/LWS pigments have been created by certain combinations of S180A, H197Y, Y277F, T285A, and A308S. The ancestral rodent pigment is unique and reduced its λ_{max} to 536 nm by H197Y (63). One striking aspect of these amino acid replacements is that the identical changes S180A/Y277F/T285A occurred independently in cavefish M (P530), human M (P530), squirrel monkey M (P532), deer M (P531), and wallaby M (P528). In addition, the gecko MWS pigment with a λ_{max} of 527 nm also incorporated S180A/Y277F/T285A (N. S. Blow & S. Yokoyama, unpublished data). The probability that these six sets of S180A/Y277F/T285A occurred by chance alone is on the order of 10^{-28}. In addition, H198Y occurred once and S180A/A308S three times during vertebrate evolution, and the chance of the parallel replacements under neutral evolution is on the order of 10^{-9}. These rare parallel changes strongly suggest that the switches from the ancestral LWS pigment to the six MWS pigments were caused by adaptive evolution (90).

When the five groups of visual pigments are considered together, a certain pattern of amino acid replacements emerge. That is, amino acid replacements, such as D83N and A292S, occurred repeatedly in different pigment groups. In particular, A292S occurred on at least 12 separate occasions (**Table 1**). A292S offers an important lesson in understanding the molecular basis of spectral tuning in various pigments. A292S often decreases the λ_{max} of visual pigments by ~10 nm (**Figure 4*a*,*b*,*e***). However, A292S that occurred in the ancestral pigment of conger 1A (P486) does not decrease the λ_{max}; conversely, the reverse mutation, S292A, in conger 1A (P486) increases the λ_{max} by 12 nm (S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt, unpublished data). Furthermore, the reverse mutation, S292A, in human S1 (P414) does not increase the λ_{max} at all (26). These mutagenesis results suggest that synergistic interactions can occur among different amino acid residues. Such synergistic effects of different amino acids have significant implications in the analyses of spectral tuning, but they have been paid little attention.

As discussed below, critical amino acid changes that cause significant λ_{max} shifts are localized to a total of 30 residues, most of which are located near the N terminus of the TM segments. Because the chromophore is also located near the N-terminal tail (luminal face) (**Figure 3**), these amino acid changes are expected to interact with the chromophore and modify the λ_{max} of various visual pigments. However, residues 102, 194, and 195 in RH1 pigments and 197 in M/LWS pigments, which is equivalent to site 181 in the bovine RH1 pigment, are located in the luminal face, which is outside of TM segments. In particular, residues 194 and 195 are ~20 Å away from the chromophore (S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt, unpublished data). At present, the molecular structural bases of such amino acid interactions at long distance are not known.

EVOLUTION OF DIM-LIGHT AND COLOR VISION

Dim-Light Vision

One of the critical times for the survival of animals in shallow water and on land is at twilight, during which the most abundant light wavelengths are 400–500 nm (45). In this environment, a majority of animals use RH1 pigments (referred to as surface rhodopsins) with λ_{max}s of 500–507 nm. In contrast, in deep water, the distribution of downwelling sunlight is narrower at ~480 nm (33), and many deep-sea fish use RH1 pigments (deep-sea rhodopsins) with λ_{max}s of ~480–485 nm. The other RH1 pigments with λ_{max}s of ~490–495 nm can be classified as intermediate rhodopsins. On the basis of considerations of their light environments, lifestyles, and the types of RH1 pigments they use, vertebrate dim-light visions are classified mainly as surface, intermediate, and deep-sea vision (S. Yokoyama, T. Tada, N. Takenaka, H. Zhang & L. Britt, unpublished

data). The transitions among the three types of dim-light visions have occurred on 12 separate occasions (**Figure 4*a***), strongly suggesting that dim-light vision has undergone adaptive evolution. The evolution of dim-light vision reveals two characteristics. First, natural selection can be subtle and selective force can differentiate even 5 nm of λ_{max} differences of RH1 pigments. Second, many transitions show the ancestral surface vision → intermediate vision (represented by lampfish and viperfish) or surface vision → intermediate vision → deep-sea vision [represented by scabbard 1B (P481)]. However, the lineage of scabbard 1A (P507) shows the transition of surface vision → intermediate vision → surface vision. Similarly, certain lineages of squirrelfish have switched back to their ancestral surface and intermediate vision from intermediate and deep-sea vision, respectively (89). In addition, as described below, a certain lineage of birds has experienced a UV vision → violet vision → UV vision transition. These observations show that evolution of dim-light vision and color vision can be reversible. To detect such reverse changes correctly, we must engineer ancestral pigments at different stages of vertebrate evolution.

Red-Green Color Vision

Vertebrates achieve red-green color vision using not only M/LWS pigments but also RH2 pigments. Some fish and primates, including humans, use LWS and MWS pigments with typical λ_{max}s of ~560 and ~530 nm, respectively, for their red-green color vision. To achieve red-green color vision, other species have modified their visual pigments and photoreceptor cells. That is, many fish species, birds, and reptiles do not have typical MWS pigments, but they can still achieve red-green color vision using 11-*cis*-3,4-dehydroretinal or colored oil droplets. For example, goldfish 2-A (P511) with 11-*cis*-retinal has a λ_{max} of 511 nm; however, when its 11-*cis*-retinal is replaced by 11-*cis*-3,4-dehydroretinal, the pigment achieves a λ_{max} of 537 nm (55). Conversely, the chicken RH2 pigment with 11-*cis*-retinal has a λ_{max} of 508 nm (52), but because it has a green oil-droplet in its photoreceptor cell, the chicken cones with the RH2 pigments actually have λ_{max}s of 533 nm (9).

Having neither 11-*cis*-3,4-dehydroretinal nor colored oil droplets, the red-green color vision of mammals is mediated solely by their M/LWS pigments. In higher primates, red-green color vision evolved in two separate ways. Hominoids and Old World monkeys use LWS and MWS opsins, encoded by two duplicated X-linked loci (51). Most New World monkeys have one M/LWS locus with three alleles (8, 31); for example, the squirrel monkey has three M/LWS alleles with λ_{max}s of 532, 545, and 558 nm (81). In such species, all males are red-green color blind, whereas females are either color blind or have red-green color vision, depending on their genotypes.

Because the molecular basis of spectral tuning in RH2 pigments is not well understood, I consider the subgroup of red-green color vision that is based only on M/LWS pigments with 11-*cis*-retinal. As noted earlier, cavefish, gecko, human, squirrel monkey, deer, and wallaby switched their LWS pigment into an MWS pigment independently. Furthermore, because of the extremely low chance of the occurrence of S180A/Y277F/T285A in the six lineages, the switches from the ancestral LWS pigment to MWS pigments in these species seem to have undergone adaptive evolution (90). This conclusion comprises one surprise; that is, the positively selected MWS pigments are found in animals with red-green color vision (cavefish, human, and squirrel monkey) and also in red-green color blind animals (gecko, deer, and wallaby). This finding contradicts a widely accepted notion that animals with red-green color vision have a selective advantage over those with color blindness (64), but it is compatible with the observation that the majority of mammalian species and many other species are red-green color blind (31, 71).

Evidence is rather scant and is sometimes controversial, but at least two observations suggest that animals with red-green color blindness can have a selective advantage over those

with red-green color vision. First, colorblind people can detect color-camouflaged objects much better than those with red-green color vision (44, 57). Second, color-blind individuals of capuchin monkey, crab-eating monkeys, and chimpanzees are capable of discriminating color-camouflaged stimuli, but those with red-green color vision failed to do so (58). However, in another survey no advantage was detected between female tamarins with red-green color vision and males without red-green color vision (23). Clearly, more analyses are needed to determine whether the ability of decoding color camouflage gives a selective advantage to color blind individuals over those with red-green color vision. The decoding of color-camouflage may be only one facet of selective advantage of red-green color blindness over red-green color vision. In the future, the other causes for the selective advantage of red-green color blindness over red-green color vision may be discovered.

UV and Violet Vision

UV and violet (or blue) vision are mediated by SWS1 and SWS2 pigments, which have λ_{max}s of 360–440 and 400–450 nm, respectively. Hence, with the exception of UV pigments in the SWS1 group, the λ_{max}s of the two groups of visual pigments are indistinguishable, but the molecular mechanisms of functional differentiation of the two groups of pigments are very different (**Figure 4*c*,*d***; **Table 1**). At present, the molecular basis of spectral tuning in the SWS1 pigments is better understood than that in the SWS2 pigments. Therefore, I consider the subgroup of UV-violet vision that is based on SWS1 pigments.

The engineered ancestral pigments show that early vertebrate ancestors had UV vision (**Figure 4*c***). Because UV vision works under UV light, organisms are expected to switch from UV vision to violet vision or simply shut off the function of the *SWS1* gene in the absence of UV light. However, given abundant UV light in their environments, many organisms also switched from UV vision to violet vision. Two major causes for these changes can be considered (30). First, UV light can damage retinal tissues, and the yellow pigments in the lenses or corneas in many species, including humans, are devised to obviate most UV light from reaching the retina. In such cases, UV pigments are of no use. Second, by achieving violet vision, organisms can improve visual resolution and subtle contrast detection.

In the avian lineage, the ancestor lost UV vision, but some of its descendants restored it (**Figure 4*c***). The reinvention of UV vision seems to have been related to avian migration. For migratory birds, the pineal gland senses changes in day length and releases hormones that initiate migration (1). UV vision is also essential in orientation based on the sun (7). Surprisingly, the mouse, a nocturnal animal, also uses UV vision (**Figure 4*c***). Voles mark their runway with urine and feces, which reflect UV light and are used as a method of communication (69). Furthermore, UV pigments are the major visual pigments expressed in the third eye (or parietal eye) of chameleon (35). Clearly, UV detection through this organ is important in addition to UV vision.

Thus, the use of UV pigments and UV vision by organisms is strongly associated with their light environments and behaviors. Compared with organisms with violet vision, those with UV vision have an advantage of recognizing certain UV-reflecting objects much more quickly, but they lack precision in viewing their surroundings and are subjected to a higher chance of developing retinal damage caused by UV light. Whether or not organisms use UV vision or violet vision must depend on the relative importance of these and other conflicting characteristics associated with UV vision to them (61). To appreciate the evolution of UV-violet vision in nature, we must study the roles of UV and violet pigments of many species in various light environments.

SPECTRAL TUNING

The Problem

Certain amino acid changes at a total of 26 residues were known to have generated variable

Table 2 Forward and reverse mutations that shift λ_{max}s of visual pigments

Site	RH1	RH2	SWS1	SWS2	M/LWS
83	D83N (−6)[a]	–	–	–	–
	N83D (2)[b]	–	–	–	–
86	–	–	F86Y (66)[c]	–	–
	–	–	Y86F (−75)[d]	–	–
	–	–	F86S (17)[e]	–	–
	–	–	S86F (−52)[b]	–	–
90	G90S (−13)[f]	–	S90G (−7)[g]	–	–
			S90C (−7)[c]		
			C90S (38)[h]		
93	–	–	T93I (0)[c]	–	–
	–	–	I93T (−6)[b]	–	–
113	E113D (7)[i]	–	E113D (−4)[j]	–	–
	–	–	D113E (−12)[k]	–	–
116	–	–	L116V (0)[j]	–	–
	–	–	V116L(−3)[l]	–	–
118	T118A (−16)[f]	–	A118T (3)[m]	–	–
122	E122Q (−20)[i]	Q122E (10)[n]	–	–	–
	Q122E (10)[n]	–	–	–	–
164	A164S (2)[o]	–	–	–	S164A (−7)[p]
	–	–	–	–	A164S (6)[b]
261	F261Y (10)[o]	–	–	Y261F (−5)[q]	Y261F (−10)[p]
	Y261F (−8)[r]	–	–	–	F261Y (6)[p]
265	W265Y (−15)[s]	–	Y265W (10)[g]	–	–
269	A269T (14)[o]	–	–	A269T (6)[t]	A269T (10)[p]
					T269A (−16)[p]
292	A292S (−10)[f]	–	S292A (0)[g]	A292S (−8)[q]	S292A (28)[u]
	S292A (8)[n]				

[a]46; [b]91; [c]28; [d]17; [e]62; [f]32; [g]26; [h]86; [i]96; [j]67; [k]5; [l]90; [m]73; [n]94; [o]12; [p]4; [q]66; [r]76; [s]39; [t]87; [u]27.

λ_{max}s of visual pigments in vertebrates (92). Amino acid replacements in **Figure 4*a–e*** cover changes at 24 residues. **Table 1** also lists amino acid changes at four additional residues that are involved in the spectral tuning of RH1 pigments. Therefore, amino acid changes at a total of 30 residues are now known to cause significant λ_{max} shifts individually and synergistically.

Mutagenesis results reveal three characteristics of spectral tuning of visual pigments (**Table 2**). First, mutations in opposite directions do not necessarily shift the λ_{max} to opposite directions. For example, G90S in a RH1 pigment decreases the λ_{max} by 13 nm, but the reverse change, S90G, in a SWS1 pigment also decreases the λ_{max} by 7 nm. Similarly, E113D and D113E in two different SWS1 pigments both decrease the λ_{max}s. Second, identical amino acid changes may cause different magnitudes of λ_{max} shifts. For example, S292A in a SWS1 pigment does not shift the λ_{max}, but the same mutation in a MWS pigment increases the λ_{max} by 28 nm. Although it is not clear from **Table 2**, the λ_{max} shifts caused by S90C in different SWS1 pigments range between −46 and 0 nm (24, 27, 61, 62, 86). Third, even when the forward and reverse mutations shift the λ_{max} to opposite directions, the magnitudes of λ_{max}

shifts can differ significantly. For example, pairs of F86Y and Y86F, F86S and S86F, S90C and C90S, T118A and A118T, E122Q and Q122E, A269T and T269A, and A292S and S292A shift λ_{max}s to opposite directions, but the difference in the magnitudes of λ_{max} shifts for each pair is more than 10 nm. As more mutagenesis results accumulate, the list of these examples is expected to grow.

Hence, λ_{max} shifts caused by forward mutations that actually occurred in nature should not be inferred from those of the identical amino acid changes or corresponding reverse mutations in contemporary pigments. As the next two examples illustrate, even if we are interested in understanding the molecular basis of spectral tuning only, the actual evolutionary process cannot be ignored.

The Human M/LWS Pigments

An extensive mutagenesis analysis has been conducted using human L (P560) and human M (P530), whose difference spectra are given by 563 nm and 531 nm, respectively (4). S180A/Y277F/T285A in human L (P560) decrease the λ_{max} by 33 nm and explain fully the λ_{max} difference between the two pigments. However, the reverse changes A180S/F277Y/A285T in human M (P530) increase the λ_{max} only by 23 nm and do not explain the λ_{max} of human L (P560). In this case, not only A180S/F277Y/A285T but also Y116S/T230I/S233A/F309Y are needed to explain the λ_{max} difference between the two pigments (4). Therefore, depending on which pigment we choose to mutate, we end up with two different molecular mechanisms of spectral tuning! If we are not satisfied with two different answers, then how can we resolve the problem? One natural way is to try to understand the molecular mechanism of spectral tuning that actually occurred in the past (78).

We have seen that the engineered ancestral pigment of human L (P560) and human M (P530) had a λ_{max} of ~560 nm (**Figure 4*e***). The ancestral LWS pigment had the amino acid composition of S180/Y277/T285, and S180A, Y277F, and T285A occurred in the past. With a possible exception of S233A, it is highly unlikely that any of Y116S, A180S, T230I, F277Y, A285T, and F309Y occurred in the ancestral pigment (85). S233A decreases the λ_{max} of human L (P560) by 3 nm (4), but its actual effect on the λ_{max} shift in the ancestral pigment is unknown. In fact, when 180A/Y277F/T285A were introduced into the ancestral mammalian LWS pigment that was engineered previously (pigment d in 83), the mutant pigment had a λ_{max} of 532 nm (S. Yokoyama & H. Yang, unpublished data). Hence, the three forward mutations explain fully the spectral tuning in the human M (P530) and the effect of S233A on the λ_{max} shift is negligible. Therefore, the evolutionary interpretation of the mutagenesis results is simple: The λ_{max} of human M (P530) was achieved by S180A/Y277F/T285A, whereas human L (P560) inherited its λ_{max} directly from the ancestral pigment without any critical amino acid changes. Hence, the seven reverse amino acid changes in human M (P530) describe a mostly hypothetical situation and are unrealistic.

The Clawed Frog SWS1 Pigment and Its Ancestor

Two sets of chimeras of different SWS1 pigments (27, 62) suggested that the λ_{max} differences between pairs of UV and violet pigments were generated by amino acid differences at residues in TM I–III. Consequently, the search for amino acids that caused variable λ_{max}s among SWS1 pigments has been focused in that region. To date, a total of 13 amino acid residues in that region have been shown to be involved in the λ_{max} shift of SWS1 pigments (**Table 1**). However, considering the chimeric pigments between clawed frog S1 (P425) [or simply, frog S1 (P425)] and its ancestral amphibian pigment with a λ_{max} of 359 nm [pigment (P359)], an entirely different picture of the molecular basis of spectral tuning of SWS1 pigments has emerged (67).

The regions of interest in the two pigments were distinguished into four segments:

Table 3 The effects of transmembrane domain (TM) exchanges on the λ_{max} shift in frog S1 (P423) and its ancestor, pigment (P359)[a]

TM	Forward	Reverse
I	0	−5
II	24	−19
III	51	−15
IV–VII	1	−1
I × II	6	5
I × III	7	1
I × IV–VII	1	4
II × III	−13	−28
II × IV–VII	20	14
III × IV–VII	−7	−18
I × II × III	−12	−2
I × II × IV–VII	−3	−11
I × III × IV–VII	−8	−6
II × III × IV–VII	−17	3
I × II × III × IV–VII	14	14
Total	64	−64

[a]Data from 65.

TM I (residues 31–66), TM II (residues 67–98), TM III (residues 99–151), and TM IV–VII (residues 152–311). The amino acids at the N and C termini of the two pigments were replaced by those of chameleon S1 (P359). Then, all single and multiple combinations of these four segments were constructed (67). Considering the evolution of frog S1 (P425) from pigment (P359), the magnitudes of the λ_{max} shift caused by replacing the TM I (θ_I), TM II (θ_{II}), TM III (θ_{III}), and TM IV–VII (θ_{IV-VII}) of pigment (P359) by the corresponding segments of frog S1 (P425) and those of their synergistic effects $\theta_{I\times II}$, $\theta_{I\times III}$, $\theta_{I\times IV-VII}, \ldots$, and $\theta_{I\times II\times III\times IV-VII}$ on the λ_{max} shift were evaluated (**Table 3**). The results show that TM II and TM III have significant individual effects in the spectral tuning of frog S1 (P425) and, at the same time, TM IV–VII reveal significant interactions with the other TM segments. However, the overall effect of TM IV–VII on the λ_{max} shift ($\theta_{IV-VII} + \theta_{I\times IV-VII} + \theta_{II\times IV-VII} + \theta_{III\times IV-VII} + \theta_{I\times II\times IV-VII} + \theta_{I\times III\times IV-VII} + \theta_{II\times III\times IV-VII} + \theta_{I\times II\times III\times IV-VII}$) is only 1 nm. This negligible overall effect and negligible θ_{IV-VII} give an impression that the spectral tuning in frog S1 (P425) is determined exclusively by amino acid changes in TM I–III.

By considering the change from frog S1 (P425) to pigment (P359), we can also evaluate the effects of amino acid changes in the opposite direction (**Table 3**). In this case, TM II and TM III also cause significant λ_{max} shifts, but their impacts are much smaller than those of the forward changes; in particular, the decrease in the λ_{max} caused by TM III of pigment (P359) is 36 nm smaller than the expected value from the λ_{max} shift caused by that of frog S1 (P425). In fact, the absolute values of the corresponding θ_{III}, $\theta_{II\times III}$, $\theta_{III\times IV-VII}$, $\theta_{I\times II\times III}$, and $\theta_{II\times III\times IV-VII}$ values between the forward and reverse TM exchanges differ by 10 nm or more. For the reverse changes, the overall effect of TM IV–VII on the λ_{max} shift is −1 nm and is again negligible.

The analyses of the chimeric pigments reveal three main features of spectral tuning of SWS1 pigments. First, amino acid changes not only in TM I–III but also in TM IV–VII are involved in the spectral tuning in clawed frog S1 (P425), where the critical amino acids in TM IV–VII remain to be discovered. Second, the effects of forward and reverse TM changes and amino acid changes on the λ_{max} shift can be very different. Third, despite a significant amount of interaction between TM I–III and TM IV–VII, the overall effect of amino acid changes in TM IV–VII on the λ_{max} shift is negligible. The cause and implications of the last observation are not immediately clear.

A Solution

For RH1, RH2, SWS1, and SWS2 pigment groups, we do not have sufficient information on the effects of forward amino acid changes and their interactions. However, we have a significant amount of data to study the molecular basis of spectral tuning in the M/LWS pigments. In 2001, applying multiple regression analysis to all M/LWS pigments that were known at that time, various combinations of

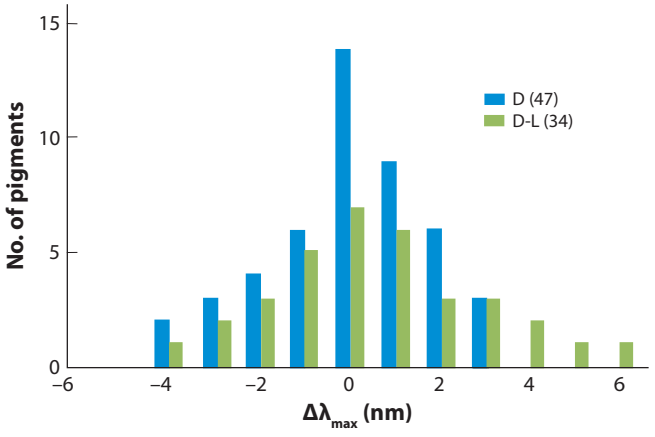

Figure 5

The distribution of the differences ($\Delta\lambda_{max}$) between the expected λ_{max} based on the five-sites rule and actual λ_{max} of middle and long wavelength–sensitive pigments (M/LWS) pigments; dark (D) (47 pigments) and difference (D-L) (34 pigments) spectra were evaluated separately.

λ_{max} shifts caused by S180A (−7 nm), H197Y (−28 nm), Y277F (−8 nm), T285A (−15 nm), A308S (−27 nm), and S180A/H197Y (11 nm) were suggested to have generated the variable λ_{max}s of M/LWS pigments (85). In the analyses, the effects of forward amino acid replacements S180A, H197Y, Y277F, and T285A on the λ_{max} shift were evaluated experimentally, but those of A308S and S180A/H197Y were not. Recently, A308S, S180A/A308S, and H197Y/A308S were introduced into the ancestral mammalian LWS pigment. The results show that the respective mutant pigments have λ_{max}s of 527, 525, and 516 nm (S. Yokoyama & H. Yang, unpublished data).

At present, the dark spectra of a total of 9 ancestral and 38 contemporary M/LWS pigments and the difference spectra of 9 ancestral and 25 contemporary pigments are available (4, 42, 43, 64, 83–85) (**Figure 5**). Applying multiple regression analysis to the λ_{max}s and the amino acid compositions at residues 180, 197, 277, 285, and 308 of these pigments, the effects of the individual and synergistic effects of the five amino acid replacements on the λ_{max} shift were evaluated. The results show that the λ_{max}s of M/LWS pigments are determined mainly by λ_{max} shifts caused by S180A (−6 nm), H197Y (−26 nm), Y277F (−10 nm), T285A (−16 nm), A308S (−33 nm), H197Y × A308S (15 nm), and S180A × H197Y × A308S (−8 nm). However, the effects of S180A × H197Y (2 nm), S180A × Y277F (2 nm), S180A × T285A (1 nm), S180A × A308S (3 nm), H197Y × T285A (−2 nm), Y277F × T285A (−1 nm), and S180A × Y277F × T285A (0 nm) on the λ_{max} shift are much smaller and are negligible. Hence, the effect of S180A × H197Y is now negligible; instead, the effects of interactions H197Y × S308A and S180A × H197Y × S308A become important. As suspected, the results depend strongly on the data set used.

Only the rodent and dolphin pigments have incorporated H197Y and A308S (**Figure 4*e***). If we exclude them, the absorption spectra of M/LWS pigments in a wide range of vertebrate species are explained mostly by the additive effects of S180A, Y277F, and T285A, and a so-called three-sites rule holds (77, 80). If we exclude only the rodent pigments from consideration, then the λ_{max}s of M/LWS pigments are modulated mostly by the additive effects of S180A, Y277F, T285A, and A308S.

The ancestral pigments with S180, H197, Y277, T285, and A308 have dark and difference spectra of 560 ± 2 and 561 ± 2 nm, respectively (S. Yokoyama & H. Yang, unpublished data). Theoretically, the λ_{max}s of all visual pigments can be evaluated by the λ_{max}s of the ancestral pigment and θs. Hence, the expected λ_{max}s based on the new five-sites rule can be compared with the corresponding observed values of M/LWS pigments. The differences between the expected and observed λ_{max}s of M/LWS pigments were evaluated for the dark and difference spectra separately (**Figure 5**). For the dark spectra, the λ_{max} differences are within 4 nm. Because the standard deviation of λ_{max}s of ancestral pigments is 2 nm, these λ_{max} differences are within the margin of experimental error. The majority of λ_{max} differences for the difference spectra are also within 4 nm, but the differences of two pairs of pigments are larger than 4 nm. Because even when they are not reliable, dark spectra are used in evaluating difference spectra, the deviations might have been

caused by inaccurate estimates of the difference spectra. Overall, therefore, the variable λ_{max}s of the currently known M/LWS pigments can be explained reasonably well by the new five-sites rule (S. Yokoyama & H. Yang, unpublished result).

ADAPTIVE EVOLUTION

By studying functional differentiations of ancestral RH1 and SWS1 pigments and relating them to the associated environmental changes of organisms' habitats and to new lifestyles, we have established that dim-light and UV-violet vision have undergone adaptive evolution. For the adaptive evolution of red-green color vision, a more probabilistic argument of parallel amino acid replacements in M/LWS pigments was used. Thus, surveying the amino acid sequences and λ_{max}s of various visual pigments, followed by mutagenesis analyses, amino acid replacements that generated a wide range of λ_{max}s in nature have been uncovered (81).

Without an available functional assay, molecular adaptations have often been inferred by identifying amino acid changes using statistical methods (65, 74, 95). The mutagenesis analyses of visual pigments establish five fundamental features of molecular evolution that cannot be learned from the standard statistical analyses of protein sequence data. First of all, mutagenesis experiments can offer critical and decisive tests of whether or not amino acid changes that are inferred as adaptive actually cause any functional changes (19). Second, as exemplified by several sets of mutations (**Table 2**), the same amino acid replacements do not always produce the same functional change but instead the change can be affected by the background amino acids of the opsin. Therefore, the probability of parallel amino acid replacements, which may or may not result in any functional change, can overestimate the actual chance that functional adaptive events occur. Third, similar functional changes can be achieved by different amino acid replacements. For example, D83N/A292S, P194R/N195A/A292S, and E122Q all decrease the λ_{max} by 14–20 nm (**Figure 4*a***). Thus, by simply looking for parallel replacements of specific amino acids, one can miss other amino acid changes that generate the same functional change, thereby underestimating the chance of finding functional adaptations.

Fourth, as stressed already, not only can the identical mutations in different pigments cause different magnitudes of λ_{max} shift, but also the effects of forward and reverse amino acid changes on the λ_{max} shift can differ significantly. Hence, if we are interested in elucidating the evolutionary mechanisms of functional and phenotypic changes, we must study the effects of forward mutations, not reverse mutations. As noted earlier, this evolutionary approach also simplifies our understanding of the molecular basis of spectral tuning.

Fifth, even when the phylogenetic position of a molecule is uncertain, its functional assay can clarify the molecular evolution of functional adaptation. For example, the phylogenetic position of lampfish 1 (P492) is uncertain (**Figure 4*a***). However, because the E122Q mutation that generated its λ_{max} is different from the other critical amino acid replacements in the closely related thornyhead 1 (P483), scabbard 1B (P481), and viperfish 1 (P489) proteins (**Figure 4*a***), we can easily establish an independent origin of the functional change in lampfish 1 (P492). Therefore, to explore the adaptive evolution of certain traits, both functional and molecular analyses of such traits are valuable (19). Analyses of functional adaption of visual pigments also demonstrate the importance of relating the functional changes to the environmental or behavioral changes that presumably caused the functional and phenotypic changes in the first place.

To fully appreciate how adaptive evolution of dim-light and color vision occurred, we must study the effects of critical forward amino acid replacements on the λ_{max} shift at the chemical level as well. For example, quantum chemical analyses of the effects of forward amino acid changes on the λ_{max} shift will improve significantly our understanding of the molecular

basis of spectral tuning in visual pigments (2, 3). The same analyses will, in turn, improve significantly our understanding of the chemical basis of the functional adaptations of dim-light vision and color vision.

CONCLUSIONS

Studies on the structure and function of bovine RH1 pigment by Doi and coworkers (22) and a series of subsequent papers by H. G. Khorana and his colleagues as well as other vision researchers (46, 48, 59, 72, 96) have improved dramatically our understanding of how key amino acids in visual pigments work. Unfortunately, most mutations considered in these biochemical studies are not found in nature, so their roles in the actual spectral tuning in various visual pigments are not immediately clear (79, 81). If we want to elucidate the mechanisms of spectral tuning that generated the λ_{max}s of contemporary visual pigments, then we must consider amino acid replacements that actually occurred in nature. Such changes can be inferred only by comparing the amino acid sequences of contemporary visual pigments, and the actual functional changes caused by the predicted amino acid changes can be evaluated using in vitro assays. To date, using this approach, certain amino acid replacements at a total of 30 residues have been shown to be involved in the spectral tuning of different visual pigments (**Table 1**).

Phylogenetic analyses of contemporary visual pigments show that early vertebrate ancestors already had RH1, RH2, SWS1, SWS2, and M/LWS pigments (25, 81, 93). Many contemporary species still use all five sets of visual pigments, and more recent gene duplications in some species generated additional variations in the λ_{max}s of visual pigments, whereas RH2 and SWS2 pigments have become nonfunctional in some lineages, including placental mammals, and their color vision has become more specialized (**Figure 4*a*–*e***). The engineered visual pigments show that the RH1, SWS1, and M/LWS in early ancestors had λ_{max}s of ~500, ~360, and ~560 nm, respectively.

Depending on the organisms' light environments, lifestyles, and the λ_{max}s of their RH1 pigments, dim-light vision of organisms can be distinguished into deep-sea, intermediate, and surface vision. The RH1 pigments of the respective groups have λ_{max}s of 480–485, 490–495, and 500–510 nm. Some species inherited the ancestral surface vision directly from the vertebrate ancestor, whereas others have switched to different types of dim-light vision. During vertebrate evolution, such transitions occurred on 12 separate occasions. As the λ_{max}s of the three types of dim-light vision indicate, natural selection can be subtle and selective force may differentiate even 5 nm of λ_{max} difference. These adaptive events were accomplished mostly by amino acid changes at nine residues, where D83N/A292S occurred seven times independently.

Many contemporary LWS pigments have maintained the ancestral λ_{max} of ~560 nm, whereas others have decreased their λ_{max}s by using various combinations of S180A, H197Y, Y277F, T285A, and A308S. In particular, identical amino acid replacements (S180A/Y277F/T285A) occurred on six separate occasions and shifted the λ_{max}s of M/LWS pigments in an additive fashion. In the lineage of rodent M/LWS pigments, H197Y occurred in their ancestral pigment, followed by A308S in some MWS pigments, and they decreased λ_{max}s individually and synergistically. Similarly, many contemporary SWS1 pigments inherited their UV sensitivities from the common ancestor, and others developed violet sensitivities using different sets of amino acid replacements, many of which remain to be discovered. Most of the currently known critical amino acid replacements modify the λ_{max} mainly through their synergistic effects, but some amino acid replacements at residues 86 and 90, including F86Y, F86S, and S90C, can cause significant λ_{max} shifts individually as well as synergistically (91).

Despite these advances, our understanding of the molecular bases of adaptive evolution and spectral tuning of visual pigments is still fragmental. This is because we still don't have much

information on how the chromophore and different amino acids interact with each other. In particular, amino acid changes in opposite directions do not shift the λ_{max} in the opposite direction by the same magnitudes. Or, when introduced into different pigments, even the identical amino acid replacements can cause different magnitudes of λ_{max} shifts. The most reasonable approach in resolving these seemingly contradictory observations is to consider amino acid changes that actually generated the variable λ_{max}s of contemporary visual pigments. Then, the functional adaptation and spectral tuning of visual pigments can be understood together by studying the mechanisms of adaptive evolution of visual pigments at the molecular and phenotypic levels.

To solve the problem, we must engineer ancestral pigments for the five groups of visual pigments at various stages of vertebrate evolution and introduce mutations into them. Such ancestral pigments at various stages of vertebrate evolution have been engineered for RH1, SWS1, and M/LWS pigments, but those for RH2 and SWS2 pigments remain to be engineered. By dissecting these and contemporary visual pigments at the molecular level and relating their λ_{max}s to organisms' light environments and lifestyles, we can start to learn why and how organisms adapted to their light environments.

SUMMARY POINTS

1. Visual pigments in vertebrates are classified into rhodopsins (RH1), RH1-like (RH2), short wavelength-sensitive type 1 (SWS1), SWS type 2 (SWS2), and middle and long wavelength–sensitive (M/LWS) groups with λ_{max}s of 480–510, 450–530, 360–440, 400–450, and 510–560 nm, respectively.
2. Dim-light vision is mediated by RH1 pigments and can be classified into three different types; the evolutionary switches among them occurred on 12 separate occasions.
3. Red-green color vision and color blindness mediated by M/LWS pigments were generated by certain combinations of amino acid changes (S180A, H197Y, Y277F, T285A, and A308S); S180A/Y277F/T285 occurred on six separate occasions.
4. The parallel replacements of S180A/Y277F/T285A in various vertebrate species suggest that both red-green color vision and color blindness have undergone adaptive evolution, but the selective advantage of color blindness over red-green color vision is still not well understood.
5. Many fish, reptile, and mammalian species inherited their UV vision from the vertebrate ancestor, but the bird ancestor achieved violet vision by F4V/F86S/L116V/S118A, and some of its descendants reinvented UV vision by S90C.
6. With the exception of some amino acid changes at residues 86 and 90, the molecular basis of spectral tuning in SWS1 pigments is characterized by strong interactions among amino acid residues.
7. Mutagenesis data show that mutations in opposite directions do not necessarily cause λ_{max} shifts to the opposite directions by the same magnitudes, implying that the molecular basis of spectral tuning in visual pigments should be understood by considering forward amino acid changes that actually generated the variable λ_{max} shifts of contemporary pigments.

8. Among the five pigment groups, the molecular basis of spectral tuning is best understood for the M/LWS group and the most recent data show that the ancestral M/LWS pigment had a λ_{max} of 560 nm. Significant λ_{max} shifts have been caused mostly by S180A (−6 nm), H197Y (−26 nm), Y277F (−10 nm), T285A (−16 nm), A308S (−33 nm), H197Y/A308S (15 nm), and S180A/H197Y/A308S (−8 nm).

FUTURE ISSUES

1. All amino acid replacements that generated the variable λ_{max}s of the five groups of contemporary visual pigments need to be identified.
2. Individual and synergistic effects of these forward amino acid changes on the λ_{max} shifts need to be evaluated.
3. The molecular bases of spectral tuning in various visual pigments need to be understood in terms of the individual and synergistic effects of the forward amino acid changes on the λ_{max} shifts.
4. The spectral tuning in visual pigments need to be understood at the chemical structural level, where quantum chemical computations of visual pigments at various stages of vertebrate evolution should be performed.
5. The molecular bases of functional adaptation of visual pigments need to be understood not only by studying the molecular basis of differentiation of visual pigments, but also by relating them to organisms' move to new photic environments or to new lifestyles.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The author's research was supported by a grant from the National Institutes of Health.

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