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Mechanism of Spectral Tuning in the Dolphin Visual Pigments†

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ABSTRACT: The absorption maxima of both rod and cone visual pigments of the bottlenose dolphin (*Tursiops truncatus*) are blue-shifted relative to those of terrestrial mammals. A comparison of the sequence of the dolphin rod photopigment gene with that of the bovine rod suggests that, of the 28 nonidentical amino acids, three amino acid substitutions at positions 83, 292, and 299 in the dolphin rod pigment are responsible for the 10 nm blue shift in absorption maxima. A similar comparison of the dolphin long-wavelength sensitive (LWS) cone photopigment gene with those of the human LWS cones suggests that a single substitution at position 292 (using the convention of rhodopsin numbering) in the dolphin LWS cone pigment results in a blue shift in absorption maxima. A mutagenesis study reveals that the combination of the three dolphin specific substitutions in the bovine rod pigment (^{83}D to ^{83}N, ^{292}A to ^{292}S, and ^{299}A to ^{299}S) causes a blue shift from the wild-type λ_{max} of 499 nm to 489 nm. The single substitution in the dolphin LWS cone pigment (^{292}S to ^{292}A) causes a red shift from the wild-type λ_{max} of 524 nm to 552 nm. The interactions of the three amino acids identified in the rod pigment with the chromophore may be a general mechanism for blue shifting in rod visual pigments. Furthermore, the single substitution in the dolphin LWS opsin gene is a novel mechanism of wavelength modulation in mammalian LWS pigments.

Mammalian visual pigments are comprised of a protein opsin covalently bound through a Schiff's base to the vitamin A-derived chromophore 11-*cis*-retinal. The wavelength of maximal absorption (λ_{max}) of different visual pigments can vary along the wavelength axis from the UV[1] to the far-red. The interaction between the chromophore and the opsin protein modulates the λ_{max} of a particular visual pigment. Most mammals possess two cone classes: a SWS class absorbing maximally in either the UV, violet, or blue regions of the spectrum and a LWS class absorbing maximally in either the green or red region (*1*). These two cone classes are the basis for the typical mammalian form of dichromatic color vision. The bottlenose dolphin (*Tursiops truncatus*) does not have this form of dichromatic color vision, and instead possesses only a rod and a LWS cone pigment with λ_{max} values of 488 and 524 nm, respectively (*2*). Although the dolphin also possesses a SWS opsin gene, it is not expressed in vivo and has accumulated a number of deletions, including a frame shift mutation which introduces a premature stop codon in the deduced opsin protein (*2*). The λ_{max} values of the dolphin visual pigments are blue-shifted when compared to those of the pigments of terrestrial mammals (see ref *1* for a review). Most terrestrial mammals studied to date have a single rod pigment with a λ_{max} of around 500 nm and at least one LWS pigment with a λ_{max} falling somewhere between 530 and 560 nm (*1*).

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[1] Abbreviations: UV, ultraviolet; SWS, short-wavelength sensitive; LWS, long-wavelength sensitive; PBS, phosphate-buffered saline; KO, knockout.

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Previous investigations of the wavelength modulation in vertebrate visual pigments have shown that most of the amino acid changes affecting λ_{max} of visual pigments are found in the transmembrane regions, and involve either a gain or loss of a hydroxyl group, or a change in charge (*3*−*9*). Blue-shifted pigments tend to have charged or polar residue substitutions toward the protonated Schiff base end of the retinal chromophore preferentially stabilizing the ground state over the excited state of the chromophore (*10*).

When the transmembrane regions of the deduced dolphin rod opsin sequence are compared to those of the domestic cow, only three amino acids in the dolphin, ^{83}N, ^{292}S, and ^{299}S, result in nonconservative substitutions of either non-charged-for-charged or hydroxyl-bearing-for-nonhydroxyl-bearing residues. Although the dolphin rod pigment is most similar to the bovine rod pigment in amino acid sequence (*2*), the λ_{max} of the dolphin pigment is more similar to those of the rod pigments of the deep-dwelling fishes than to those of terrestrial mammals. In comparative molecular studies of the rod visual pigments of deep-sea fish and abyssal cottoid fish of Lake Baikal, amino acid substitutions ^{83}D to ^{83}N and ^{292}A to ^{292}S were shown to be consistently found in the rod opsins of the deep-dwelling fish and correlated with blue-shifted λ_{max} values (*11*−*13*). However, these studies did not test the role of these positions in mutagenesis experiments.

Comparison of the deduced dolphin LWS cone opsin sequence with the human LWS opsin sequences indicates that the dolphin LWS opsin has the amino acid found in either human green or red opsin at each of the seven amino acid positions responsible for the spectral shifts between the two human LWS visual pigments (*5, 7, 9, 14*−*16*). This leads to the prediction that the dolphin cone pigment would have a λ_{max} value somewhere between 530 and 560 nm. On the basis of the complement of the dolphin amino acids at these seven sites, a back-propagation neural net model that calculates absorption maxima of chimeric human red/green visual pigments predicts a λ_{max} of 552 nm for the dolphin LWS pigment (*17*). Because the dolphin cone pigment has a λ_{max} of 524 nm, we hypothesized that an amino acid(s) other than those involved in the wavelength modulation between the human LWS pigments must be involved in the blue shifting of the dolphin cone pigment.

Unlike other mammalian opsin proteins, both the dolphin rod and LWS cone pigment opsins incorporate a serine at position 292 (cone opsins are numbered using the numbering system of the bovine rod opsin),[2] with the dolphin rod pigment also possessing an asparagine at position 83. All terrestrial mammalian rod pigments studied to date, as well as the human LWS cone pigments, have alanine and aspartate residues at positions 292 and 83, respectively. Experimental data support the idea that position 83 and, possibly to a greater extent, position 292 are playing a significant role in the spectral tuning of mammalian visual pigments. Site-directed mutagenesis experiments involving the mutant A292D in bovine rhodopsin resulted in a blue shift from 498 to 488 nm (*6*) with mutant D83N resulting in either no shift (*6*) or a blue shift of either 6 or 8 nm (*5, 18*). Sun et al. (*19*) have recently and independently identified a serine substitution at position 292 in the mouse LWS cone opsin that contributes to a blue shift. No experiments have been done to determine the contribution of position 299 to the spectral tuning of rhodopsin. In studies of deep-sea fish rod pigments, there is either a serine or alanine at position 299 and there appears to be little correlation between the identity of the amino acid at 299 and blue shifting of the pigments (*13*). In fact, the serine at position 299 in the dolphin rod pigment is commonly found in other mammalian rod opsins. The bovine rod opsin, however, has an alanine at position 299, and for this reason, we examined the contribution of this position.

In our study, we have constructed and expressed mutants of bovine rod visual pigment cDNA, as well as dolphin LWS cone visual pigment cDNA, to determine the mechanism underlying the blue shifting in the dolphin rod and cone pigments. To determine the contributions made by positions 83, 292, and 299 to the spectral tuning of the dolphin rod pigment, we have constructed single, double, and triple mutants in bovine rod opsin cDNA by substituting the respective dolphin amino acids. To determine the contribution made by position 292 to the spectral tuning of the dolphin LWS cone pigment, we have constructed a single mutant in dolphin LWS cone opsin cDNA by substituting the respective human LWS opsin amino acid.

EXPERIMENTAL PROCEDURES

Materials. Sources of materials used have been previously reported (*2*). The peptide used to elute pigments from the immunoaffinity resin as described below was synthesized by Macromolecular Resources (Fort Collins, CO). Oligonucleotide synthesis was carried out on an Millipore Expedite 8900 nucleic acid synthesizer.

Construction of Rod and Cone Opsin Mutants. Mutations were introduced into the synthetic bovine rhodopsin (*20*) and dolphin LWS cone opsin (*2*) genes by the Transformer Site-Directed Mutagenesis Kit v. 2 (Clontech). The wild-type opsin genes were first directionally transferred as the *Eco*RI−*Not*I fragment into a derivative of the mammalian expression vector pMT2 (*21*). The following oligonucleotides were synthesized for mutagenic experiments in the bovine rod opsin: ^{83}D to ^{83}N, 5′-CAACCTGGCCGTGGCAAACCTCT-TCATGGTCTTCGGTGGC-3′; ^{292}A to ^{292}S, 5′-GACCATC-CCGTCTTTCTTTGCC-3′; and ^{299}A to ^{299}S, 5′-CTTTC-TTTGCCAAAACGTCTTCCGTCTACAACCC-3′. The following synthetic oligonucleotide was synthesized for mutagenic experiments in the dolphin LWS cone opsin: ^{292}S to ^{292}A, 5′-CGCCCTGCCAGCCTACTTCGC-3′. Underlined nucleotides are mutagenic substitutions. The selection oligonucleotides used in these experiments are as follows: trans oligonucleotide *Ssp*I/*Eco*RV, 5′-CTTCCTTTTTC-GATATCATTGAAGCATTT-3′; and KO *Sal*I, 5′-GAC-GAGGCGTCAACCACCGTC-3′. The underlined portion of the sequence represents the selection restriction enzyme site after site conversion. The nucleotide sequence at the site of mutagenic oligonucleotide annealing was confirmed by the dideoxy method of sequencing on miniprep purified plasmid (Qiagen).

Expression and Purification of Mutant Opsins. Clones were transiently expressed in COS-7 cells following trans-

[2] Position 292 in the rod opsin numbering system corresponds to position 308 in the human red/green numbering system. The convention of the rod numbering system is used throughout the paper except for Table 3.

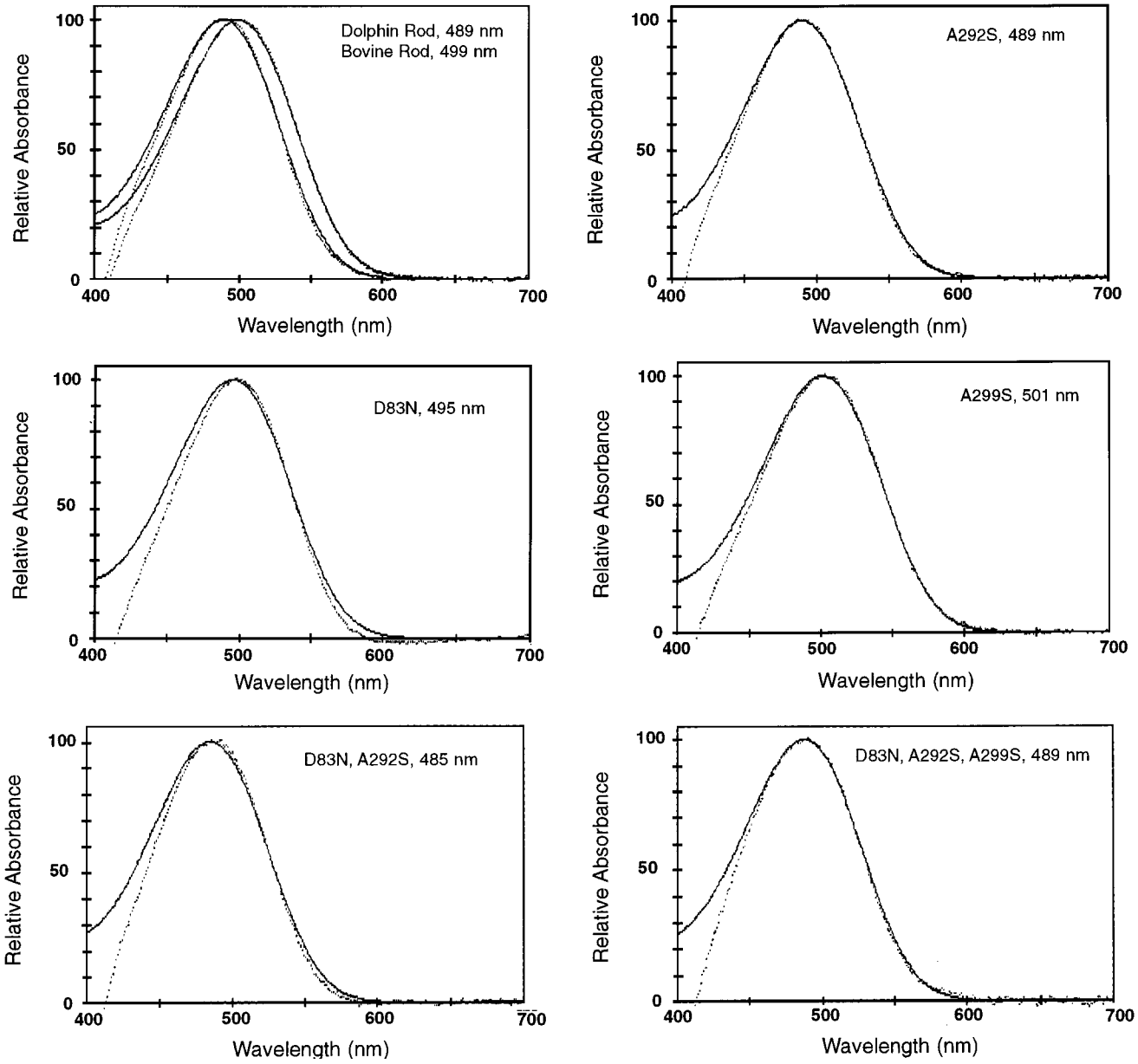

FIGURE 1: Normalized difference spectra of purified expressed bovine rod opsin, bovine opsin mutants, and dolphin rod opsin. Spectra were determined in the presence of hydroxylamine (10 mM, pH 7.0) and were compared with a template spectrum for rhodopsin. In each spectrum, the positive component of the curve is due to the formation of recombinant pigment and the negative component (λ_{max} = 367 nm, not shown) is due to the release of *all-trans*-retinal upon bleaching. The λ_{max} values of bovine and dolphin rod pigments are 499 and 489 nm, respectively. The λ_{max} values of all five mutants are presented in Table 1.

fection with Lipofectamine (Life Technologies, Gaithersburg, MD). Dolphin LWS cDNA and the LWS mutant S292A were appended with the epitope tag for the monoclonal antibody 1D4 prior to expression. Cells were harvested 48 h posttransfection for reconstitution and purification of the pigment. Cells were resuspended in PBS and incubated with 40 μM 11-*cis*-retinal in the dark (*20*, *22*). Proteins were solubilized from cell membranes as described by Okano et al. (*23*). Protein was purified by immunoaffinity chromatography using a bovine rhodopsin monoclonal antibody, 1D4 (*22*).

Characterization of Spectral Properties of Wild-Type and Mutant Opsins. Purified pigments eluted from the immunoaffinity matrix were maintained at 6 °C in a water-jacketed cuvette holder. A Hitachi model U-3300 dual-path spectrophotometer was used to record absorption spectra before and after bleaching with a 175 W fiber optic light source. Pigment samples were bleached for 10 min in the presence of 10 mM hydroxylamine (pH 7.0). Difference curves were then calculated from pre- and postbleach spectra. To assign λ_{max} values, difference spectra were compared with template spectra for rhodopsin using a least-squares procedure (*24*, *25*).

RESULTS

In the experiments reported here, we have constructed mutants in both the synthetic bovine rod opsin cDNA (*20*) and the dolphin LWS cone opsin cDNA to elucidate the mechanism underlying the blue shift of the dolphin visual pigments. Five site-directed mutants were constructed in the bovine rod opsin cDNA to test the involvement of sites 83, 292, and 299 in blue shifting of the dolphin rod visual pigment. The following single amino acid substitutions were constructed: D83N, A292S, and A299S. (Mutants are

Table 1: Rod and Cone Opsin Mutants Designed to Mimic Naturally Occurring Substitutions

mutation	λ_{max} (nm)[a]	shift from the wild-type pigment value (nm)[b]
D83N	495	−3
A292S	489	−10
A299S	501	+2
D83N/A292S	485	−14
D83N/A292S/A299S	489	−10
S292A (LWS cone)	552	+28

[a] λ_{max} was assigned to difference spectra when compared with template spectra (see Experimental Procedures). The precision is estimated to be ±1 nm. The λ_{max} behavior of bovine and dolphin rod pigments purified from COS cells were 499 and 489 nm, respectively. [b] λ_{max} shifts from that of bovine rod or dolphin LWS cone pigments are expressed as either positive or negative integers representing red or blue shifts, respectively. All mutants bound 11-*cis*-retinal to form a pigment.

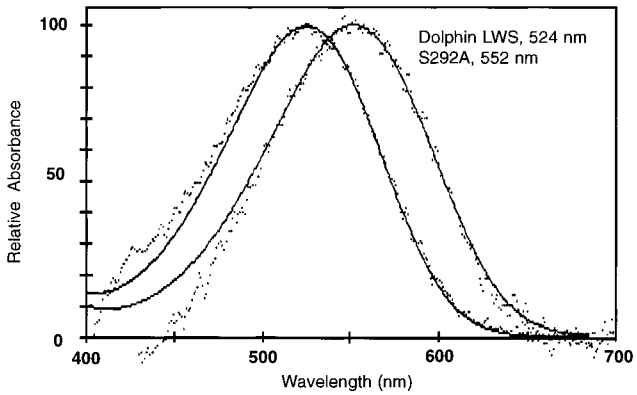

FIGURE 2: Normalized difference spectra of purified expressed dolphin LWS opsin, and the mutant, S292A. Spectra were determined as described in the legend for Figure 1. The λ_{max} values of dolphin LWS and the dolphin mutant pigment S292A are 524 and 552 nm, respectively.

referred to by the single-letter amino acid designation of the wild-type residue followed by its position number followed by the single-letter amino acid designation of the introduced residue.) One double mutant (D83N/A292S) and one triple mutant (D83N/A292S/A299S) were also constructed. Only a single mutant was made in the dolphin LWS cone opsin cDNA (S292A) by replacing the serine found at position 292 in the dolphin LWS cone opsin with the alanine found at position 292 in the human green and red cone opsins.[2]

The opsin genes were transiently expressed in COS-7 cells, reconstituted with 11-*cis*-retinal, and protein opsins purified as described (*2*). Upon incubation with 11-*cis*-retinal, all opsins produced photolabile pigments with absorbance spectra similar in shape to that of wild-type rhodopsin (see Figure 1). The wild-type bovine rod pigment produced in this expression system has a λ_{max} of 499 nm, identical with that for rhodopsin isolated from bovine retinas (data not shown), while the wild-type dolphin rod pigment has a λ_{max} of 489 nm. In the bovine rod pigment, mutating ^{83}D to ^{83}N shifts the λ_{max} to 495 nm, mutating ^{292}A to ^{292}S shifts the λ_{max} to 489 nm, and mutating ^{299}A to ^{299}S shifts the λ_{max} to 501 nm. The double mutant in the bovine rod pigment at positions 83 and 292 shifts the λ_{max} to 485 nm, while the triple mutant at positions 83, 292, and 299 shifts the λ_{max} to 489 nm (see Table 1).

The experimental data shown in Figure 2 demonstrate that position 292 is also an important site in blue shifting the dolphin LWS cone pigment. The expressed and reconstituted wild-type dolphin LWS cone pigment has a λ_{max} of 524 nm. When the serine at position 292 is changed to an alanine, there is a dramatic 28 nm red shift, resulting in a λ_{max} of 552 nm.

Table 2: Comparison of Positions 83, 292, and 299 in Mammalian and Marine Fish Rod Pigments

		residue		
	λ_{max} (nm)	83	292	299
mammals[a]				
cow	499	D	A	A
sheep	NA	D	A	S
human	498	D	A	A
dolphin	489	N	S	S
marine fish[b]				
Pomatoschistus minutus	501	D	A	A
Hoplostethus mediterraneus	483	N	S	A
Histiobranchus bathybius	477	N	S	S
freshwater eel	502	D	A	S
deep-sea eel	482	N	S	A

[a] The sequences were obtained from GenBank with the following accession numbers: cow (P02699), sheep (P02700), human (P08100), and dolphin (submitted to the GenBank). The λ_{max} values of cow and dolphin were determined in this lab using reconstituted expressed pigments. The λ_{max} value of human is from Bowmaker and Dartnall (*29*). [b] From Hope et al. (*12*).

DISCUSSION

The mutations made in the bovine rod pigment and their effect on spectral shifting of λ_{max} are summarized in Table 1. It appears that the bovine rod pigment mutant A292S is capable by itself of blue shifting the λ_{max} 10 nm to that of the dolphin rod pigment (489 nm). The single mutant D83N blue shifts the pigment 4 nm, while the single mutant A299S red shifts the pigment 2 nm. The double mutant D83N/A292S blue shifts the pigment 14 nm. Finally, the triple mutant D83N/A292S/A299S has a λ_{max} of 489 nm, identical with that of the dolphin rod pigment, and it appears that the effect of these three positions is not strictly additive. Although the bovine rod pigment has an alanine at position 299, the consensus at this position in other mammalian rod pigments is serine (data not shown). ^{83}N and ^{292}S in the dolphin rod pigment, however, are not found in any other mammalian rod pigment studied to date. In molecular studies of the rod pigments of some deep-sea fish and abyssal cottoid fish of Lake Baikal, both ^{83}N and ^{292}S are present in all blue-shifted pigments with position 299 possessing either alanine or serine in a manner not correlated with blue shifting (*11*−*13*). The experiments we have presented here suggest that serine at position 292 is sufficient to blue shift a rod pigment 10 nm. However, comparisons of the substitution pattern of wild-type dolphin and deep-dwelling fish rod opsins with those of terrestrial mammals and surface-dwelling fish reveal that positions 83 and 292 are associated in a manner whereby the consensus is either ^{83}N and ^{292}S, resulting in a blue-shifted pigment, or ^{83}D and ^{292}A, resulting in a pigment with a λ_{max} near 500 nm (see Table 2). It is unclear why this association between these two consensus pairs occurs in nature. Since a single mutation at either site 83 or 292 results in a stable pigment in our expression system (see Table 1), it is unlikely that stability of the pigment is the reason for the association of certain amino acids at

Table 3: Comparison of the 15 Amino Acids That Differ between the Human Green and Red Cone Pigments and Similarities of the human Pigment and Dolphin LWS Cone Pigment[a]

	residue														
pigment	65	111	116[b]	153	180[b]	230[b]	233[b]	236	274	275	277[b]	279	285[b]	298	309[b]
human red (λ_{max} = 563 nm[c])	T	I	S	L	S	I	A	M	I	F	Y	V	T	A	Y
human green (λ_{max} = 532 nm[c])	I	V	Y	M	A	T	S	V	V	L	F	F	A	P	F
dolphin LWS (λ_{max} = 524 nm[d])	I	V	Y	M	A	I	S	V	I	F	Y	L	T	A	Y

[a] Please note the numbering in this table is that of the human red opsin and the convention used by Asenjo et al. (*14*). To convert from the cone opsin amino acid position to the rod opsin numbering, 16 is subtracted from the cone opsin position. [b] The seven amino acids responsible for the wavelength modulation between the human green and red LWS cone pigments (*14*). [c] λ_{max} values of the human LWS pigments purified from COS cells (*14*). [d] λ_{max} value of the dolphin LWS pigment purified from COS cells (*2*).

positions 83 and 292. The similarities between the dolphin and deep-dwelling fish rod pigments at positions 83 and 292 and the blue shifting of the rod pigments of these distantly related vertebrates are examples of parallel evolution and indicate that visual pigments are subjected to strong environmental selective pressures (*26*).

Comparisons of the deduced dolphin LWS cone opsin sequence with the human green and red cone opsin sequences show the dolphin LWS opsin has an amino acid that is found in either the human red or green opsin at the seven amino acid positions responsible for the λ_{max} modulation between the two human visual pigments (*14*, *15*, *27*) as summarized in Table 3. The seven corresponding dolphin amino acids were entered as input into a back-propagation neural network designed to calculate the λ_{max} of chimeric human red/green visual pigments (*17*). The λ_{max} of the dolphin LWS cone pigment predicted by the neural network was 552 nm. This value is identical with that of the single mutant S292A constructed in the dolphin LWS cone pigment. Furthermore, the goat LWS cone class has a λ_{max} near 555 nm when measured with ERG flicker photometry (*1*). The amino acid sequence of the goat LWS opsin[3] is identical with the dolphin sequence at each of the seven modulating sites with an overall similarity at the amino acid level of 97.5%. The only nonconserved amino acid substitution in the transmembrane region facing the chromophore binding pocket of the goat LWS is an alanine, instead of a serine, at position 292. Because of the striking similarities between the λ_{max} of the dolphin LWS cone mutant S292A and the wild-type goat LWS cone class as well as the λ_{max} of the dolphin LWS cone pigment predicted by the neural network, we believe that the serine at position 292 in the dolphin LWS cone pigment is solely responsible for the almost 30 nm blue shift of the dolphin cone pigment. This finding is important in that, prior to this work, our understanding of the wavelength modulation and spectral tuning of the cone pigments was primarily derived from primate studies. It is known that primates rely on seven amino acid substitutions to modulate between the green and red LWS cone pigments (see Table 3 for summary) with each substitution contributing a portion to the total spectral shift. Furthermore, all seven amino acid substitutions must be present to achieve the entire 30 nm shift between the green and red cone pigments. The dolphin appears to have a different solution to the spectral tuning of its LWS cone pigment. It appears that the 30 nm spectral shift, requiring seven amino acid substitutions in primates, is achieved by a single amino acid substitution at position 292 in the dolphin LWS cone pigment, the same substitution responsible for the major blue shift of the dolphin rod pigment.

It has recently been reported that the mouse LWS cone pigment which has a maximum absorption of 508 nm also has a serine at position 292 (*19*). A serine at this position in the mouse LWS pigment is responsible for a blue shift of 18 nm relative to the human red pigment (*19*). The mouse also eliminates the chloride binding site at position 181 to account for an additional 28 nm blue shift. Mutagenesis experiments by Sun et al. (*19*) in both the mouse and human LWS pigments suggest that in order to form a stable pigment both the elimination of the chloride binding site and the ^{292}S substitution must occur concomitantly. However, the dolphin LWS pigment is a counter-example of this and suggests that a LWS pigment can have both a chloride binding site, with a histidine at 181, and a serine at 292. It is not clear why the human and mouse LWS pigments are unstable when there is a histidine at position 181 and a serine at position 292.

The serine at position 292 is positioned one helical turn below the pronated Schiff base linkage, and it appears, as our results have shown, that the orientation of this amino acid in both the dolphin rod and LWS cone pigments has a profound influence on stabilizing the ground state and the blue shifting of the pigments most likely through the interaction with serine's hydroxyl group and the protonated Schiff base nitrogen at position 296. The ability of position 292 to influence position 296 has previously been suggested in studies of the constitutive active mutant of human rhodopsin A292E in a patient with congenital stationary night blindness (*28*) and earlier mutagenesis experiments where the mutant A292D in bovine rhodopsin resulted in a shift from 498 to 488 nm (*6*).

In contrast to ^{292}S, the serine at position 299 is approximately one helical turn above the pronated Schiff base linkage and results in a 2 nm red shift rather than the predicted blue shift (*8*). ^{299}S may be positioned such that one of the serine's hydrogens has a positive dipole and is facing the retinal chain, instead of the protonated Schiff base nitrogen, causing an increase in electron localization along the polyene chain of the chromophore and decreasing the energy difference between the excited and ground state. The suggestion that ^{299}S may be in contact with the retinal chain is consistent with a recent model of rhodopsin proposed by Baldwin and co-workers, who suggest that alanine at position 299 in bovine rhodopsin is in contact with the retinal chain (*30*). The different effects of a serine at either position 292 or 299 suggest that wavelength modulation can be achieved

[3] S. Yokoyama, personnel communication.

by influencing the protonated Schiff base and the stability of the ground state.

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