Molecular Basis of Spectral Tuning in the Newt Short Wavelength Sensitive Visual Pigment†

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ABSTRACT: Previously we reported the sequence of the member of the short wavelength sensitive 2 (SWS2) family of vertebrate visual pigments from the retina of the Japanese common newt, *Cynops pyrrhogaster* [Takahashi, Y. et al. (2001) *FEBS Lett. 501*, 151−155]. Now we have expressed the apopigment and regenerated it with A1 retinal. Its absorption maximum, 474 nm, is greatly red shifted compared to other known SWS2 pigments (418−455 nm). To determine the amino acid residues that control its spectral tuning, we replaced the residues that were near the chromophore and which differed between the newt and the bullfrog ($\lambda_{max} = 430$ nm) wild-type SWS2 pigments: Pro91Ser, Ser94Ala, Ile122Met, Cys127Ser, Ser211Cys, Tyr261Phe, and Ala292Ser. Each of these site-directed mutants led to blue shifts of the newt pigment with five of them causing substantial shifts; their sum was about equal to the difference between the absorption maximum of the bullfrog and newt pigments, 44 nm. The 32 nm shift of the absorption maximum of the multiple seven-residue mutant to 442 nm is fairly close to that of the wild-type bullfrog pigment. Thus, the seven amino acid residues that we replaced are the major cause of the red shift of the newt SWS2 pigment's spectrum. Two of the residues, 91 and 94, have not previously been identified as wavelength regulating sites in visual pigments. One of these, 91, probably regulates color via a new mechanism: altering of a hydrogen bonding network that is connected via a water to the chromophore, in this case its counterion, Glu113.

On the basis of their amino acid sequences, vertebrate visual pigments can be classified into five groups: rhodopsin (RH1),[1] rhodopsin-like (RH2), short wavelength sensitive 1 and 2 (SWS1, SWS2), and middle and long wavelength sensitive (M/LWS). Most vertebrate retinas contain several kinds of rod and cone photoreceptors, each often having different spectral sensitivities, since a distinct visual pigment is usually expressed in each type of photoreceptor cell (see review ref *2*, and for an exception ref *3*). Each member of any one of the five pigment groups has a very roughly similar absorption maximum within a broad range (*4*). There are about 31 amino acids located within 4.5 Å of the chromophore; these define the retinal binding pocket (*5*, *6*). Some of these residues are highly conserved for all visual pigment sequences, while others can vary from pigment to pigment and so are prime candidates for those residues that are involved in spectral tuning.

In general, there are three mechanisms for controlling the spectra of retinal pigments having protonated Schiff bases (discussed in ref *4*). You can (i) twist the retinal about single or double bonds, (ii) alter the distance between the protonated Schiff base and its counterion or alter other aspects of their environment, or (iii) place charged/polar residues near the retinal. Later, in the discussion, we will present what could be called a fourth mechanism. So far, there is no experimental evidence to suggest that alterations in twisting about single or double bonds of the chromophore, which would be caused by changes in the type of amino acid residues in the binding pocket, are used to control wavelength in visual pigments. With respect to the second mechanism, at present there seems to be only one or two types of mutations that probably act directly on the Schiff base environment (except for the addition of a chloride ion in place of the two polar residues near the Schiff base at positions 181 and 184 for the M/LWS pigments). This is a change initiated by the replacement of Ser292 by an alanine. Ser292 is one alpha helix turn down from the Schiff base lysine and seems to interact with it (*7*, *8*). Another candidate residue, Ser127, is investigated below. Thus, with these possible exceptions, the lack of variation of the Schiff base/counterion environment in visual pigments seems to be in distinction to what happens in a halobacterial retinal protein, where this mechanism has been implicated in explaining most of the large difference in the absorption maxima of bacteriorhodopsin (568 nm) and sensory rhodopsin II (500 nm) (*9*, *10*).

The third wavelength regulating mechanism relies on changes in the electrostatic environment of the chromophore by changing an amino acid residue in the binding pocket to another with different polarity and/or polarizability. A dozen

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[1] Abbreviations: For the five classes of vertebrate visual pigments: RH1 (rhodopsin), RH2 (rhodopsin like), SWS1 (short wavelength sensitive-1), SWS2 (short wavelength sensitive-2) and M/LWS (middle and long wavelength sensitive), PCR (polymerase chain reaction), HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), DM (β-D-dodecyl maltoside), PBS (phosphate buffer saline), PMSF (phenylmethylsulfonylfloride).

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or so examples of such amino acid residue changes are given in the Appendix. A specific example of this type of variation is the changing of three residues near the β-ionone ring of the chromophore of some M/LWS visual pigments from nonpolar to polar residues. The triad of changes, Ala164Ser, Phe261Tyr, and Ala269Thr, are largely responsible for the shifting of the spectrum of the human green cone pigment from 530 nm to that of the red cone pigment, ca. 560 nm (*11*−*13*). It is this type of change, involving the electrostatic environment of the retinal, which is most often invoked to explain differences in visual pigment spectra.

To understand the mechanisms of spectral tuning (wavelength regulation) of visual pigment spectra, many studies have been made of site-directed mutants of RH1 (rhodopsin) (e.g., refs *8*, *14*−*16*; see refs in Appendix), M/LWS (*7*, *12*), or SWS1 (*17*−*20*) pigments and some important spectral regulating residues were identified (summarized in Appendix). Until recently, there have been no studies on spectral tuning of members of the SWS2 family of visual pigments. Cowing et al. (*1*) have just reported results for the SWS2 pigments in fish from Lake Baikal. They proposed that the amino acid residues which shifted the absorption maxima among various species of Lake Baikal fish are at positions 118 and 269; however, these are conserved in other SWS2 pigments from most other animals (see Figure 3 and ref *4*), so there must be other amino acid residues that regulate the spectra in these species. The fish of Lake Baikal might evolve in their own way because of the very unique environment of this lake (*21*, *22*).

Previously we presented the sequence of the SWS2 pigment of the Japanese common newt, which is expressed in one particular type of single cone cell in its retina (*23*). In this paper, we report on the molecular basis for spectral tuning of the newt SWS2 pigment using site-directed single and multiple mutants.

EXPERIMENTAL PROCEDURES

Expression, Reconstitution, and Purification of SWS2 Pigments. The coding regions of the Japanese common newt and the bullfrog SWS2 pigments, to which is added the epitope sequence (ETSQVAPA) of the bovine rhodopsin monoclonal antibody mAb-1D4, are amplified with PCR and ligated between the HindIII and EcoRI sites of pBluescript KS+ (Stratagene) to confirm those sequences. The construct is then recut and religated into the same enzyme sites of the expression vector pcDNA3 (Invitrogen). The host cell line, HEK293T cells, which are derived from the human embryonic kidney cells, are incubated at 37 °C and 5% CO_2 in DMEM/F12 medium containing 10% fetal bovine serum (Invitrogen). Forty 10-cm diameter plates were used per trial. Using the calcium phosphate method (*15*), we transfected 10 μg of the plasmid DNA for transient expression in HEK293T cells. These cells were harvested 60−70 h post-transfection, washed with PBS, collected by centrifugation, and then stored in a deep freezer until used. The cell pellets were resuspended with ice cold 8.6% sucrose in HEPES buffer (50 mM HEPES (pH 6.5), 140 mM sodium chloride, and 3 mM magnesium chloride including 1 mM DTT (Sigma), 1 μg/mL aprotinin (Sigma), 1 μg/mL leupeptin (Sigma), and 100 μM PMSF (Sigma)) and homogenized by a glass homogenizer (Tenbroeck) on ice. Homogenized cell suspensions are layered on 40% sucrose in HEPES buffer, and the membrane fractions that floated to the interface were collected after centrifugation. The membranes were washed 2 times with 25 mL of HEPES buffer and resuspended with 500 μL of HEPES buffer. All subsequent steps were done in the dark or under dim red light. The membrane suspensions were then incubated with 11-*cis*-retinal in the dark in a 4 °C cold room for 3 h and then solubilized in a 1% solution of the detergent dodecyl maltoside (DM) in HEPES buffer for 1 h. The supernatants of the crude soluble fractions were separated by centrifugation (1000*g*, 5 min) and incubated with monoclonal anti-bovine rhodopsin antibody mAb-1D4 coupled to sepharose 4B in the dark in a cold room overnight as described by Oprian et al. (*24*). The bound pigment was washed with 0.02% DM in HEPES buffer and then eluted with 450 μL of 100 μM C-terminus peptide in 0.02% DM in HEPES buffer. The elutions were concentrated into about a 100 μL volume with Microcon-30 (Amicon) used as recommended by the manufacturer.

Analyses of Absorption Spectra. Absorption spectra were recorded at 20 °C with an AVIV model 14DS UV−Vis spectrophotometer interfaced with an IBM PC300-GL. Temperature was regulated by a RM6 LAUDA (Brinkmann) temperature controller. To determine their absorption maxima, we used at least two different independent preparations of each pigment. For the high absorbance spectra that have easily apparent peaks, we picked this point as λ_{max} and calculated the average from a couple of measurements on each sample. For the low absorbance noisier signals, for example, the Ile122Met mutant, we did a smoothing/fitting, moving average to determine λ_{max}, and then confirmed this value by a Gaussian fitting around the λ_{max}. The value via the Gaussian fitting gave us almost the same value within the error range ($\pm$2 nm).

Sequence Analysis of SWS2 Pigments. The amino acid sequences of several SWS2 pigments which include members of the bony fish (*25*, *26*), amphibian (*27*, *28*), reptilian (*29*), and avian families (*30*−*32*) have been reported (see the legend of Figure 2). We aligned these sequences along with that of the newt pigment (*23*) manually and shaded identical amino acids as black and similar amino acids as gray using the "Box-shade" program (Figure 2) (http://www.ch.embnet.org/software/BOX_form.html).

Mutagenesis of Newt SWS2 Pigments. Site-directed mutation at certain positions (91, 94, 122, 127, 211, 261, 292 according to bovine rhodopsin numbering) were made in the wild-type sequence using a QuickChange site-directed mutagenesis kit (Stratagene); for each mutation primers were designed as recommended by the manufacturer. The sequences of primers (Invitrogen) used to create the mutants (Pro91Ser, Ser94Ala, Ile122Met, Cys127Ser, Ser211Cys, Tyr261Phe, and Ala292Ser) were as follows:

newtSWS2−P91S−F, 5′-GGTCATCTTCATCGGCTC-TACCCTGTCCTTCTACTC-3′; newtSWS2−P91S−R, 5′-GAGTAGAAGGACAGGGTAGAGCCGATGAAGAT-GACC-3′; newtSWS2−S94A−F, 5′-CGGCCCTACCCTG-GCCTTCTACTCTAACTCCC-3′; newtSWS2−S94A−R, 5′-GGGAGTTAGAGTAGAAGGCCAGGGTAGGGCCG-3′; newtSWS2−I122M−F, 5′-GCCGCCACACTTGGAG-GTATGGTCGGTCTGTGG-3′; newtSWS2−I122M−R, 5′-CCACAGACCGACCATACCTCCAAGTGTGGCGGC-3′; newtSWS2−C127S−F, 5′-GGTATTGTCGGTCTGT-

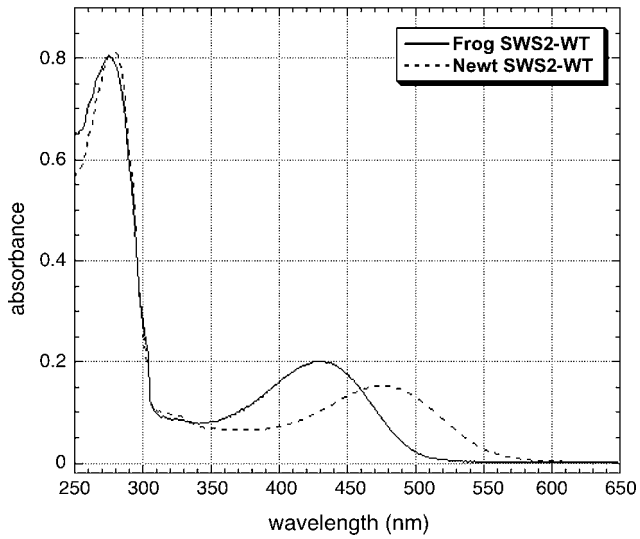

FIGURE 1: The absorption spectra of amphibian SWS2 pigments. The absorption spectra of the reconstituted bullfrog (solid line) and newt (broken line) SWS2 pigments made from expressed apopigment and A1 retinal. The absorption spectrum of the frog SWS2 pigment has its λ_{max} at 430 nm and the newt pigment is at 474 nm.

GGTCTTTAGCTGTGGTGGCC-3′; newtSWS2−C127S−R, 5′-GGCCACCACAGCTAAAGACCACAGACCGACAATACC-3′ newtSWS2−S211C−F, 5′-CTTCCTCTTCTGCTTCTGTTTTGGTGTCCCGCTC-3′; newtSWS2−S211C−R, 5′-GAGCGGGACACCAAAACAGAAGCAGAAGAGGAAG-3′; newtSWS2−Y261F−F, 5′-GTCATGGTGCTCGGCTTCCTGATCTGCTGGAGC-3′; newtSWS2−Y261F−R, 5′-GCTCCAGCAGATCAGGAAGCCGAGCACCATGAC-3′; newtSWS2-A292S−F, 5′-GGATGGCTTCCATCCCATCCATTTTCTCAAAGTCG-3′; newtSWS2-A292S−R, 5′-CGACTTTGAGAAAATGGATGGGATGGAAGCCATCC-3′.

Underlined nucleotides indicate the mutagenic substitutions that have been introduced. The sequences of the site-directed mutant were confirmed by ABI-3700 Auto analyzer (ABI Prism), and then each of the coding regions was ligated into the expression vector, pcDNA3.

For making the multiple mutant (seven altered residues) of the newt pigment, we carried out each reaction separately to add each mutation. To create the initial double mutant at positions 91 and 94, since these two positions were too close to introduce these mutations using the above primers, we designed other primers. These sequences were as follows:

newtSWS2−91+94-F, 5′-CGGCTCTACCCTGGCCTTCTACTCTAACTCCC-3′; newtSWS2−91+94-R,5′-GGGAGTTAGAGTAGAAGGCCAGGGTAGAGCCG-3′.

RESULTS

Absorption Spectra of Reconstituted Amphibian SWS2 pigments. The SWS2 apopigments tagged with the mAb-1D4 epitope were expressed in HEK293T cells and reconstituted with 11-*cis*-retinal. The spectra of reconstituted and purified newt and bullfrog wild-type SWS2 pigments are shown in Figure 1. The absorption maximum of the reconstituted newt SWS2 pigment is at 474 nm, 19 nm more red shifted than the hitherto known SWS2 champion, the chicken P455 pigment (*33*).[2] The bullfrog SWS2 pigment is expressed in its so-called "green rods" (*28*), and its absorption maximum as purified from bullfrog retinas (*34*) or as reconstituted with the "green rod" opsin and A1 retinal, is at 430 nm (Figure 1). The λ_{max} of our expressed bullfrog SWS2 pigment, reconstituted with A1 retinal (Figure 1), is in good agreement with this report. Some amphibians and reptiles utilize the A2 retinal chromophore in their natural environment (*35*, *36*); at present we do not know if the Japanese common newt uses A1, A2, or a combination of these retinals in nature.

Identification of Candidate Residues for Spectral Tuning of the Newt SWS2 Pigment. Figure 2 shows the alignment of the SWS2 pigment sequences for which an absorption spectrum has been obtained with A1 retinal as the chromophore (see figure caption). There are several amino acid residues at or near the retinal binding pocket as defined in the introduction (see Figure 3) that are unique for the newt SWS2 pigment compared to the other SWS2 pigments: Ser94, Ile122, Cys127, Ser211, and Tyr261 (Figure 2). The binding pocket residues are marked in Figure 2, along with two other residues known to influence visual pigment spectra, 90 and 292. To these, we add Ser/Pro91, slightly outside the 4.5 Å distance from the chromophore originally used to define the chromophore binding pocket. Recently, Okada et al. (*37*) have shown that in bovine rhodopsin the amide of the serine residue at position 91 is hydrogen bonded to a water which is hydrogen bonded in turn to Glu113. Glu113 is of course in the binding pocket and is the counterion for the protonated Schiff base. In the Discussion, we examine if these seven residues are in similar positions in the newt SWS2 pigment as in the bovine RH1 pigment by energy minimizations (*38*). We expected that these residues could have a role in red shifting the newt pigment compared to the bullfrog pigment, which absorb at 430 nm (with an retinal A1 chromophore), and so each residue was mutated.

Absorption Spectra of the Newt SWS2 Site-Directed and Multiple Mutant Pigments. One at a time, the Pro91Ser, Ser94Ala, Ile122Met, Cys127Ser, Ser211Cys, Tyr261Phe, and Ala292Ser substitutions were introduced into the wild-type newt SWS2 pigment. In each case, the newt residue was changed to the residue in the bullfrog sequence. It seems that the nucleotide changes to make these mutations are plausible, because all were obtained by a single nucleotide substitution (see primer sequences in Experimental Procedures). Each mutated DNA was transfected into HEK293T cells using the calcium phosphate method (*15*). We then carried out reconstitution and purification following the same procedures as for the wild-type pigments.

The dark and photobleached spectra for the site-directed mutants are shown in Figure 4; each absorption maximum was calculated by the average of 2−4 independent samples. All of the site-directed mutations caused blue shifts. Mutating amino acid residues at positions 91, 94 122, 261, and 292 to those of bullfrog led to comparatively large blue shifts. The Pro91Ser substitution resulted in a 10-nm blue shift to 464 nm (Figure 4A), and the Ser94Ala substitution resulted in a 14-nm blue shift to 460 nm (Figure 4B). The Ile122Met

[2] It has been suggested that the reconstituted retinal A1 SWS2 pigment of the fish *Cottus gobio* absorbs at 461−463 nm (*1*). However, the data were presented as difference spectra, in which the absorption maximum would be red-shifted to considerably longer wavelengths compared to the actual absorption maxima. We estimate that the absorption maximum of the *C. gobio* SWS2 pigment is at around 450 nm, and believe that chicken SWS2 pigment is the most red-shifted SWS2 pigment before our report.

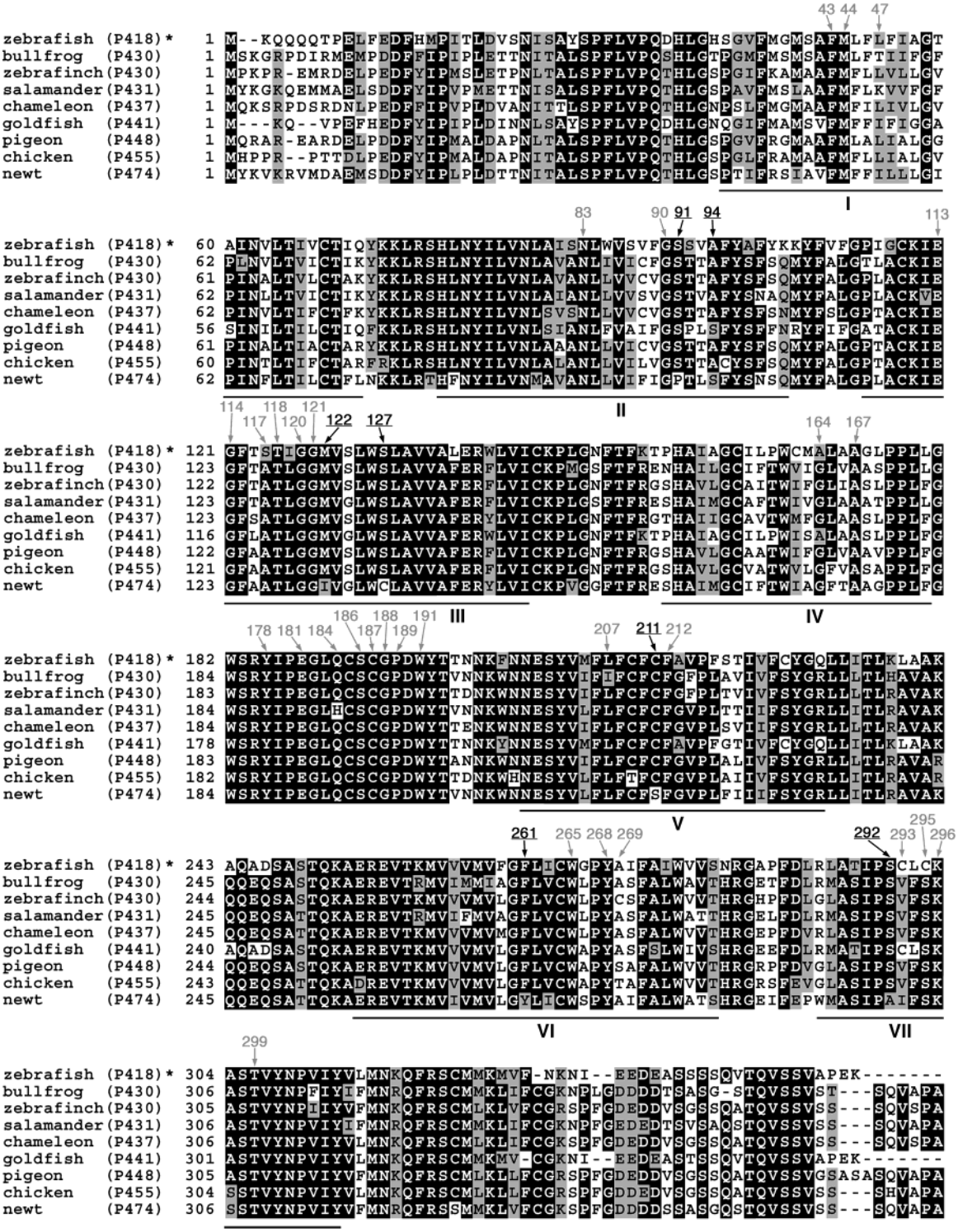

FIGURE 2: Alignment of amino acid sequences of SWS2 pigments. The alignment of the sequences of those SWS2 pigments whose absorption spectrum has been determined by reconstitution with the A1 retinal chromophore. The number in parentheses indicates their reported λ_{max} with an A1 retinal chromophore. Roman numbers I−VII indicate the seven transmembrane regions. These Arabic numbers colored as gray and black indicate important amino acids that are located around retinal binding pocket. The underlined black numbers indicated the seven-amino acid residues that differ between the newt and frog sequences: 90, 94, 122, 127, 211, 261, and 292. All of the amino acid residue numbering followed the bovine rhodopsin numbering. Genebank accession numbers for the respective sequences are zebrafinch SWS2, AF222332; tiger salamander SWS2, AF038946; bullfrog SWS2, AB010085; American chameleon SWS2, AF133907; goldfish SWS2, L11864; zebrafish (danio) SWS2, AF109372; pigeon SWS2, AF149238; chicken SWS2, M92037; newt SWS2 AB040148. *The absorption maximum of the zebrafish SWS2 pigment reconstituted with A1 retinal was at 418 nm (Kono, M., unpublished data).

substitution resulted in a 6-nm blue shift to 468 nm (Figure 4C), while the Tyr261Phe substitution resulted in a 5-nm blue shift to 469 nm (Figure 4D). The Ala292Ser substitution resulted in an 8-nm blue shift to 466 nm (Figure 4E). The other mutants Ser211Cys and Cys127Ser showed comparatively small shifts. The Ser211Cys substitution resulted in a 2-nm blue shift to 472 nm (Figure 4F), and the Cys127Ser substitution also resulted in a 2-nm blue shift to 472 nm (Figure 4G). The Cys127Ser and Ser211Cys mutants do not have a large effect on wavelength regulation.

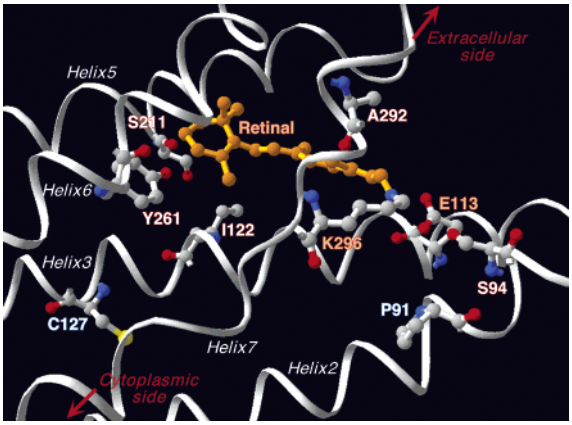

FIGURE 3: Location of some residues in the retinal binding pocket of the newt SWS2 pigment. This figure was created using "Swiss model" and is based on the crystal structure of the bovine rhodopsin (PDB 1L9H). According to this model, Pro91, Ser94, and Ala292 were close to Schiff base or the counterion. Ile122, Ser211, and Tyr261 were located around the β-ionone ring.

A multiple mutant, consisting of the replacement of all seven amino acid residues, had its absorption maximum shifted by 32 nm, to 442 nm (Figure 5). This value is fairly close to the λ_{max} of the bullfrog SWS2 wild-type pigment (430 nm). These results suggested that the seven amino acid residues which are replaced are largely responsible for the large red shift in the newt SWS2 pigment compared to the bullfrog pigment.

DISCUSSION

In this paper, we investigated the molecular basis of spectral tuning for the newt SWS2 visual pigment. Initially, we expected that the absorption maximum of the newt SWS2 pigment would be close to that of the other amphibian and reptilian SWS2 pigments, 430−440 nm, but when the newt SWS2 pigment was expressed and reconstituted with A1 retinal, the resulting pigment was at 474 nm, greatly red shifted from our expectation.

The SWS2 family of vertebrate visual pigments is especially interesting since many members of this family have their absorption spectrum close to that of the model compound for most visual pigments—a protonated Schiff base of retinal, which absorbs at ca. 440 nm. Most members of other visual pigment families do not absorb as closely to 440 nm, and thus it seems like the SWS2 family is a natural place to start when trying to fashion an understanding of the molecular basis for all visual pigment spectra (*4*). For the newt pigment, we wanted to find out what amino acid residues were responsible for shifting the spectrum of the newt pigment, 474 nm, down to that of a more typical amphibian pigment, bullfrog, ca. 430 nm. Other similar pigments are salamander (*Ambystoma tigrinum*, 431 nm, (*3*)), American chameleon (*Anolis carolinesis*, 437 nm, (*29*)), and, although they have not been sequenced nor expressed and regenerated with retinal A1, several amphibian "green" rod pigments whose absorption spectra have been measured using microspectrophotometry: frog (*Rana pipiens*, 432 nm, (*39*)), (*Rana temporania*, 437 nm, (*40*)), (*Rana ridibunda*, 432 nm, (*41*)), and toad (*Bufo marinus*, 433 nm, (*42*)), (*Bufo bufo*, 432 nm, (*41*)). Until the newt SWS2 pigment, the furthest red shifted SWS2 pigment known was that of chicken (λ_{max} = 455 nm).[2] For the chicken pigment, the major source of its red shift from ca. 440 nm is probably the substitution of threonine for alanine at position 269, the same residue that is responsible for much of the human green cone to red cone absorption spectrum change. This replacement led to about a 10−15 nm red shift in RH1 or M/LWS group pigments (see Appendix), so probably this same underlying mechanism is causing the red shift (*4*). However, this substitution cannot explain the shift of newt SWS2 pigment, because it has an alanine residue at position 269, just like the bullfrog SWS2 pigment. So we must look for other candidates for the residues that cause the large red shift.

To examine how the absorption maximum of the pigment is influenced by changing amino acid residues in the retinal binding pocket of the newt SWS2 pigment, we first examined the structure of the binding pocket itself. We took the chromophore binding pocket of bovine rhodopsin and asked if there are any alterations in the position of the backbone of the binding pocket residues in going from bovine rhodopsin, for which the X-ray structural determination was done (*5*, *37*), to that of the newt or bullfrog SWS2 pigments. To do this, we threaded the newt and bullfrog SWS2 pigment's sequences into the bovine rhodopsin structure and then performed a molecular dynamics simulation leading to an energy minimization (*38*), to see if the newt and bullfrog pigments had any major structural differences compared to each other or to bovine rhodopsin. All differences between the newt and bullfrog backbone residues which made up the binding pocket were quite close to that of bovine rhodopsin, except for Pro91 in the newt pigment (Atkinson, H., Ebrey, T. G., and Parson, W. unpublished simulations). So, taking the backbone structures of the binding pockets as similar, we examined which amino acid residues in or near the binding pocket were changed in going from the amphibian SWS2 pigments to the newt one. In all, we have identified seven candidate residues that were suspected of being able to change the absorption maxima of the newt SWS2 pigment to that of bullfrog. They were identified by three different sorts of criteria: (a) one replacement (Ala292Ser) which is known to affect the spectrum of other visual pigments and which probably acts by varying the environment near Lys296. A second change (Cys127Ser) might alter helix−helix interactions and so possibly alter counterion/Schiff base interactions (see Figure 5C of ref *5*). (b) Four residues are part of the retinal binding pocket and are changed in going from newt to the bullfrog pigment (Ser94Ala, Iso122M, Ser211Cys, and Tyr261Phe); and (c) one residue (Pro91) disrupts the usually present hydrogen bond to a water, which in turn is hydrogen bonded to Glu113, the counterion to the chromophore's Schiff base (*37*). To evaluate their effect on the spectral shift, these seven amino acid residues from the newt SWS2 sequence were substituted with the residues found in the bullfrog (and salamander/anolis) SWS2 sequence.

Discussing our results in the order of the amino acid residue numbers: In the newt the replacement of Pro91 to Ser91 introduces a hydrogen bond from the peptide amide to a water that is in turn hydrogen bonded to Glu113. Probably the 10-nm blue shift caused by the Pro91Ser mutant is attributable to this creation of a hydrogen bonded network from this residue to Glu113. A proline at this position would not be able to have its amide participate in the hydrogen bonding. The creation/disruption of such hydrogen bonded networks from amino acid residues to the Schiff base/

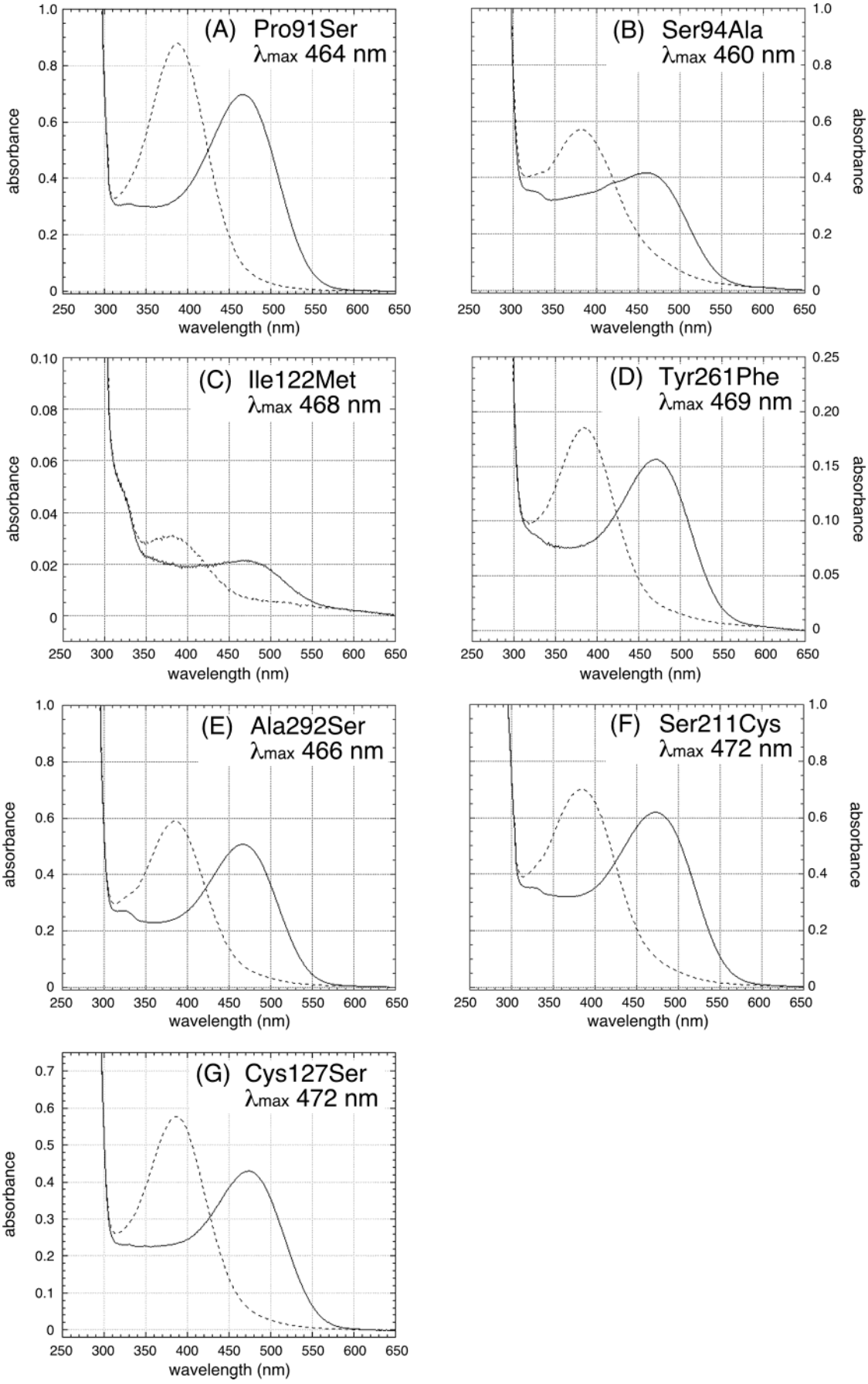

FIGURE 4: The absorption spectra of site-directed mutants of the newt SWS2 pigment. The solid line indicates the absorption spectrum in the dark and the broken line shows the bleached pigment spectra. (A) Pro91Ser, (B) Ser94Ala, (C) Ile122Met, (D) Tyr261Phe, (E) Ala292Ser, (F) Ser211Cys, and (G) Cys127Ser.

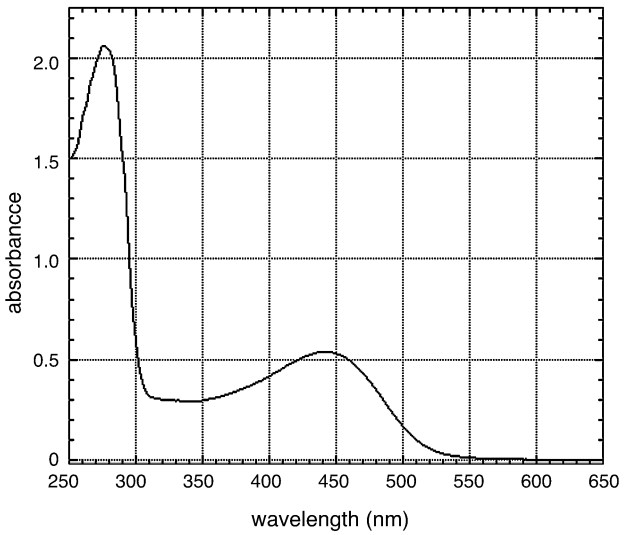

FIGURE 5: The absorption spectrum of the newt SWS2 pigment with seven residues mutated. This figure shows the absorption spectrum of the reconstituted newt SWS2 multiple mutant in which seven residues have been replaced: Pro91Ser, Ser94Ala, Ile122Met, Cys127Ser, Ser211Cys, Tyr261Phe, and Ala292Ser. The absorption spectrum of this multiple mutant has its λ_{max} at 442 nm.

counterion region which incorporate water molecules can be considered a new type of mechanism for regulating wavelength in visual pigments.

The amino acid residue at position 94 is located very close to counterion and Schiff base (see Figure 6 of refs *5* and *6*), and its substitution to an alanine leads to a 14-nm blue shift. The effect of this substitution is probably via a direct electrostatic effect on the chromophore. The absorption spectrum of the Ser94Ala mutant was not only blue shifted it also showed a slightly wider bandwidth compared to other mutants. However, after bleaching, the difference spectrum had a similar bandwidth to the other mutants (data not shown). It may be that there are two or more components perturbing the environment around Schiff base or counterion because of the replacement of Ser94 by alanine. The effect of the Ser94Ala substitution is the first report of a visual pigment using the residue at position 94 to regulate its absorption spectrum.

The Ile122Met mutant showed a 6-nm blue shift, a smaller effect on spectral tuning compared to the changes at positions 91 and 94. It has been proposed that the amino acid residue at position 122 forms a salt bridge with the residue at position 211 (*43*). Although this may be possible with bovine rhodopsin, the quite different electrostatic properties of the residues in these positions in the newt and indeed in all the SWS2 pigments, make the existence of any crucial salt bridge linkage between these two groups unlikely.

It may be that a usually conserved serine residue at position 127 can alter the spectrum by exerting an influence at a distance like Ser292Ala, by altering the environment of or the distances between the Schiff base and its counterion. Ser127 (helix III) makes internal hydrogen bonds connecting it to Helix-II (via Asn78 and Asp83), and Helix-VII (via the carbonyl of Ala299) (*5*); changes in the Ser127 contribution could alter distances between helices III (Glu113) and VII (Lys296). The replacement of Cys127 by serine in the newt SWS2 pigment had a just minor effect on spectral tuning, and so we conclude that its alteration does not modulate the chromophore.

Table 1: Summary of the Spectral Shift of the Newt SWS2 Mutants[a]

pigment name	λ max (nm)	$\Delta\lambda$ max	Opsin shift (cm^{-1})
newt WT	**474 ± 2**		0
newt − P91S	464 ± 0	−10	460
newt − S94A	460 ± 1	−14	640
newt − I122M	468 ± 2	−6	270
newt − C127S	472 ± 0	−2	90
newt − S211C	472 ± 1	−2	90
newt − Y261F	469 ± 1	−5	230
newt − A292S	466 ± 2	−8	370
total shift		**−47**	**2150**
bullfrog WT	**430 ± 1**	**−44**	**2160**
newt multiple	**442 ± 1**	**−32**	1530

[a] The absorption maxima and calculated opsin shift of the wild-type, site-directed and multiple mutant pigments.

In the X-ray structure of bovine rhodopsin, His211 is located near retinal ring, but its side chain faced away from the retinal (*5*). The substitution of Ser211 by cysteine in the newt SWS2 pigment has only a minor effect on the spectrum, 2 nm, perhaps because of the direction of the side chain. The substitution of Tyr261 by phenylalanine in bovine rhodopsin (RH1) or some members of the M/LWS group of pigments leads to about a 7−10 nm blue shift (data collected in Appendix). In the newt SWS2 pigment, we found a similar result: the λ_{max} of the Tyr261Phe mutant is blue shifted by 5 nm. The substitution of Ala292 by serine in bovine rhodopsin (RH1) or the M/LWS group of pigments leads to about a 10−20 nm blue shift (see Appendix, refs *7*, *8*, *44*). In the newt SWS2 pigment, we found a similar result: the λ_{max} of the Ala292Ser mutant is blue shifted by 8 nm. Since our Tyr261Phe and Ala292Ser mutants showed similar sized blue shifts as found in previous studies of other pigment families, probably similar mechanisms underlie their effects on spectral tuning in the SWS2 pigment.

Other considerations are that the highly conserved, within visual pigment families, of the amino acid residue at positions 122 may affect the efficiency of activating transducin and the rate of regeneration of a visual pigment (*45*); alligator rhodopsin, which has a very similar binding pocket to bovine rhodopsin except for residue 127, shows a much faster regeneration rate compared to bovine rhodopsin (*46*). The newt SWS2 pigment has unique residues at both these positions, so that some of its properties, for example, regeneration rate, may be quite different when compared with other SWS2 pigments.

There was a 44-nm difference, equivalent to a 2160 cm^{-1} opsin shift, between the newt and the bullfrog SWS2 pigment. The total opsin shift taken as the sum of the changes at the seven positions is 2150 cm^{-1} (see Table 1). This deduced value is very close to the size of the opsin shift between newt and bullfrog. We note that the seven residue mutant of the newt SWS2 pigment has a smaller blue shift (λ_{max} 442 nm; opsin shift 1530 cm^{-1}) than the sum of the seven individual changes, suggesting that the spectral shift mechanisms are not quite as simple as just the sum of the individual site-directed mutant shifts. To obtain the full spectral shift, it probably is necessary to mutate additional residues even if by themselves they probably would not give a large spectral shift; this was shown necessary in obtaining the full spectral shift from a green human cone pigment to a red one (*12*).

Finally, it is of interest to ask whether the changes at positions 91 and 94 have been used to regulate the spectra

of other visual pigments. Among the SWS2 pigments, that of goldfish also has an alanine at position 94; its red shift from most of the amphibian pigments from ca. 435 to 441 nm can plausibly be explained by this substitution. Three of the visual pigment families have a conserved residue at position 94 (RH1, Thr; M/LWS, Ser; and SWS1, Val) and so do not seem to use this residue to modulate the absorption spectrum. With respect to Pro91, an examination of all the available visual pigment sequences finds that the newt SWS2 pigment is unique in having a proline at this position; all other sequenced SWS2 pigments have a serine. There is also conservation within the other visual pigment families. They either have a valine or isoleucine (SWS1), phenylalanine (RH1 and RH2), or a serine (M/LWS) at this position. A plausible speculation is that the hydrogen bonding of the water is essential for the stability or efficient function of visual pigments. It would be quite interesting to know the physiological effect, if any, of this proline being present in the newt SWS2 pigment. In any case, in the newt pigment, we propose that a new mechanism of visual pigment absorption spectrum regulation has been introduced—the alteration of the hydrogen bonding patterns of a water which is itself hydrogen bonded to the counterion of the Schiff base.

APPENDIX

Mutations of visual pigments that can cause significant spectral shifts are given in Table 2.

Table 2: Mutations of Visual Pigments that Can Cause Significant Spectral Shifts[a]

mutation	visual pigment family	spectral shift (nm)	ref	mutation	visual pigment family	spectral shift (nm)	ref
D83N	RH1	±0	Nakayama & Khorana (*47*)	E122Q	RH1	−16	Nakayama & Khorana (*47*)
D83N	RH1	−6	Nathans (*15*, *48*)	E122Q	RH1	−20	Sakmar et al. (*60*)
D83N	RH1	−2	Nagata et al. (*49*, *50*)	E122Q	RH1	−17	Nagata et al. (*49*)
D83N	RH1	−6	DeCaluwe et al. (*51*)	E122Q	RH1	−15	Nagata et al. (*50*)
D83N	RH1	±0	Zhukovsky & Oprian (*52*)	E122Q	RH1-chicken	−21	Imai et al. (*45*)
D83N	RH1	−9	Fasick & Robertson (*44*)	Q122E	RH1−Coelacanth	+10	Yokoyama et al. (*61*)
D83G	RH1	+3	Nathans (*15*)	Q122E	RH2−Coelacanth	+13	Yokoyama et al. (*61*)
F86Y	SWS1(UVS)[b]	+66	Fasick et al. (*17*)	L125T	RH1	+7	Andres et al. (*62*)
F86Y	SWS1(UVS)[b]	+60	Cowing et al. (*18*)	L125D	RH1	+5	Andres et al. (*62*)
Y86F	SWS1(VS)[b]	−71	Fasick et al. (*17*)	L125N	RH1	−5	Andres et al. (*62*)
Y86F	SWS1(VS)[b]	−75	Cowing et al. (*18*)	L125P	RH1	+8	Andres et al. (*62*)
F86L	SWS1(UVS)[b]	±0	Cowing et al. (*18*)	W126L	RH1	−8	Nakayama & Khorana (*47*)
F86M	SWS1(UVS)[b]	+38	Cowing et al. (*18*)	W126A	RH1	−13	Nakayama & Khorana (*47*)
V87D	RH1	−9	Nathans (*48*)	S164A	M/LWS	−7	Asenjo et al. (*12*)
C90S	SWS1(UVS)[b]	+38	Yokoyama et al. (*19*)	A164S	RH1	+2	Chan et al. (*14*)
C90S	SWS1(UVS)[b]	+35	Wilkie et al. (*53*)	A164S	M/LWS	+2	Asenjo et al. (*12*)
S90C	SWS1(VS)[b]	−46	Yokoyama et al. (*19*)	A164S	M/LWS	+4	Merbs & Nathans (*63*)
S90C	SWS1(VS)[b]	−34	Yokoyama et al. (*19*)	A164S	M/LWS	+6	Neitz et al. (*11*)
S90C	SWS1(VS)[b]	−7	Fasick et al. (*17*)	E181Q	RH1	+4	Nathans (*48*)
S90G	SWS1(VS)[b]	−7	Fasick et al. (*54*)	H181Y	M/LWS	−28	Sun et al. (*7*)
G90S	SWS1(VS)[b]	−11	Lin et al. (*8*)	E197Q	RH1	−4	Nathans (*48*)
G90S	RH1	−13	Janz & Farrens (*55*)	L207M	RH2	+6	Yokoyama et al. (*61*)
G90D	RH1	−18	Kaushal & Khorana (*56*)	H211C	RH1	−6	Nathans (*15*)
G90D	RH1	−17	Rao et al. (*57*)	F261Y	RH1	+10	Chan et al. (*14*)
G90D	RH1	−16	Zvyaga et al. (*58*)	F261Y	M/LWS	+6	Asenjo et al. (*12*)
A117G	RH1	−4	Lin et al. (*8*)	F261Y	M/LWS	+9	Neitz et al. (*11*)
A117G	RH1	−4	Han et al. (*59*)	Y261F	RH1	−8	Yokoyama et al. (*64*)
A117F	RH1	−8	Nakayama & Khorana (*47*)	Y261F	M/LWS	−7	Merbs & Nathan (*63*)
A117W	RH1	+4	Han et al. (*59*)	Y261F	M/LWS	−10	Asenjo et al. (*12*)
T118S	RH1	−13	Nagata et al. (*50*)	W265Y	RH1	−15	Nakayama & Khorana (*47*)
T118C	RH1	−16	Nagata et al. (*50*)	W265Y	RH1	−15	Lin et al. (*8*, *65*)
T118G	RH1	−26	Nagata et al. (*50*)	W265Y	RH1	−17	Nagata et al. (*50*)
T118V	RH1	−15	Nagata et al. (*50*)	W265F	RH1	−18	Nakayama & Khorana (*47*)
T118I	RH1	−12	Nagata et al. (*50*)	W265F	RH1	−19	Lin & Sakmar (*65*)
T118A	RH1	−18	Nagata et al. (*50*)	W265F	RH1	−20	Nagata et al. (*50*)
T118A	RH1	−16	Janz & Farrens (*55*)	W265A	RH1	∼ −28	Nakayama & Khorana (*47*)
A118T	SWS1(UVS)[b]	+3	Wilkie et al. (*53*)	Y265W	SWS1(VS)[b]	+10	Fasick et al. (*54*)
G120P	RH1	−10	Nagata et al. (*50*)	Y268F	RH1	−5	Nakayama & Khorana (*47*)
G121S	RH1	−3	Han et al. (*59*)	A269T	RH1	+14	Chan et al. (*14*)
G121T	RH1	−17	Han et al. (*59*)	A269T	M/LWS	+15	Neitz et al. (*11*)
G121V	RH1	−23	Han et al. (*59*)	A269T	M/LWS	+10	Asenjo et al. (*12*)
G121I	RH1	−25	Han et al. (*59*)	T269A	M/LWS	−16	Asenjo et al. (*12*)
G121L	RH1	−25	Han et al. (*59*)	T269A	M/LWS	−15	Merbs & Nathans (*66*)
G121P	RH1	+7	Nagata et al. (*50*)	A292S	RH1	−10	Sun et al. (*7*)
E122A	RH1	−25	Zhukovsky & Oprian (*52*)	A292S	RH1	−10	Fasick & Robertson (*44*)
E122A	RH1	−22	Nakayama & Khorana (*47*)	A292S	RH1	−9	Lin et al. (*8*)
E122L	RH1	−5	Lin et al. (*8*)	A292S	RH1	−10	Janz & Farrens (*55*)
E122L	RH1	−3	DeCaluwe et al. (*51*)	S292A	RH1	+8	Yokoyama et al. (*61*)
E122D	RH1	−22	Nakayama & Khorana (*47*)	S292A	M/LWS	+18	Sun et al. (*7*)
E122D	RH1	−25	Sakmar et al. (*60*)	S292A	M/LWS	+28	Fasick & Robertson (*44*)
E122D	RH1	−23	Janz & Farrens (*55*)	S292A	SWS1(VS)[b]	±0	Fasick et al. (*54*)
E122D	RH1-chicken	−20	Imai et al. (*45*)	A292D	RH1	−10	Nakayama & Khorana (*47*)
E122Q	RH1	−20	Zhukovsky & Oprian (*52*)	A295S	RH1	−5	Lin et al. (*8*)
E122Q	RH1	−19	Nathans (*15*, *48*)	A295S	RH1	−2	Janz & Farrens (*55*)

[a] This table was updated from Table 3 of ref *4*. Copyright 2001 Valdenmar Publishing Company. A significant shift is taken as at least one study of the pigment finding an absorption shift in the mutant of more than 2 nm. [b] SWS1(UVS; UV−sensitive) and SWS1(VS; violet (or visible region)-sensitive) pigments belong to same SWS1 group, but we gave these names to make differentiate between them in this table.

NOTE ADDED IN PROOF

After we submitted our paper, a paper by Ramon et al. ((2003) *J. Biol. Chem. 278*, 6427−6432) appeared which reported that the Thr94IIe mutant of bovine rhodopsin was blue shifted to 478 nm compared with the WT. And in a very interesting paper Yokoyama and Tada ((2003) *Gene 306*, 91−98) identified several other residues that can control spectral tuning in SWS2 pigments.

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REFERENCES

1. Cowing, J. A., Poopalasundaram, S., Wilkie, S. E., Bowmaker, J. K., and Hunt, D. M. (2002) *Biochemistry 41*, 6019−6025.
2. Ebrey, T., and Koutalos, Y. (2001) *Prog. Retinal Eye Res. 20*, 49−94.
3. Ma, J., Znoiko, S., Othersen, K. L., Ryan, J. C., Das, J., Isayama, T., Kono, M., Oprian, D. D., Corson, D. W., Cornwall, M. C., Cameron, D. A., Harosi, F. I., Makino, C. L., and Crouch, R. K. (2001) *Neuron 32*, 451−461.
4. Ebrey, T. G., and Takahashi, Y. (2001) in *Photobiology for the 21st Century* (Coohill, T., and Valenzeno, D., Eds.) pp 101−33, Valdenmar Publishing Company, Overland Park, Kansas.
5. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science 289*, 739−745.
6. Menon, S. T., Han, M., and Sakmar, T. P. (2001) *Physiol. Rev. 81*, 1659−1688.
7. Sun, H., Macke, J. P., and Nathans, J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8860−8865.
8. Lin, S. W., Kochendoerfer, G. G., Carroll, K. S., Wang, D., Mathies, R. A., and Sakmar, T. P. (1998) *J. Biol. Chem. 273*, 24583−24591.
9. Ren, L., Martin, C. H., Wise, K. J., Gillespie, N. B., Luecke, H., Lanyi, J. K., Spudich, J. L., and Birge, R. R. (2001) *Biochemistry 40*, 13906−13914.
10. Hayashi, S., Tajkhorshid, E., Pebay-Peyroula, E., Royand, A., Landau, E. M., Navarro, J., and Schulten, K. (2001) *J. Phys. Chem. B 105*, 10124−10131.
11. Neitz, M., Neitz, J., and Jacobs, G. H. (1991) *Science 252*, 971−974.
12. Asenjo, A. B., Rim, J., and Oprian, D. D. (1994) *Neuron 12*, 1131−1138.
13. Yokoyama, R., and Yokoyama, S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9315−9318.
14. Chan, T., Lee, M., and Sakmar, T. P. (1992) *J. Biol. Chem. 267*, 9478−9480.
15. Nathans, J. (1990) *Biochemistry 29*, 937−942.
16. Archer, S., and Hirano, J. (1996) *Proc. R. Soc. London B Biol. Sci. 263*, 761−767.
17. Fasick, J. I., Applebury, M. L., and Oprian, D. D. (2002) *Biochemistry 41*, 6860−6865.
18. Cowing, J. A., Poopalasundaram, S., Wilkie, S. E., Robinson, P. R., Bowmaker, J. K., and Hunt, D. M. (2002) *Biochem. J. 5*, 129−135.
19. Yokoyama, S., Radlwimmer, F. B., and Blow, N. S. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 7366−7371.
20. Hunt, D. M., Wilkie, S. E., Bowmaker, J. K., and Poopalasundaram, S. (2001) *Cell Mol. Life Sci. 58*, 1583−1598.
21. Bowmaker, J. K., Govardovskii, V. I., Shukolyukov, S. A., Zueva, L. V., Hunt, D. M., Sideleva, V. G., and Smirnova, O. G. (1994) *Vision Res. 34*, 591−605.
22. Hunt, D. M., Fitzgibbon, J., Slobodyanyuk, S. J., and Bowmaker, J. K. (1996) *Vision Res. 36*, 1217−1224.
23. Takahashi, Y., Hisatomi, O., Sakakibara, S., Tokunaga, F., and Tsukahara, Y. (2001) *FEBS Lett. 501*, 151−155.
24. Oprian, D. D., Molday, R. S., Kaufman, R. J., and Khorana, H. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8874−8878.
25. Johnson, R. L., Grant, K. B., Zankel, T. C., Boehm, M. F., Merbs, S. L., Nathans, J., and Nakanishi, K. (1993) *Biochemistry 32*, 208−214.
26. Vihtelic, T. S., Doro, C. J., and Hyde, D. R. (1999) *Vision Neurosci. 16*, 571−585.
27. Xu, L., Hazard, E. S., 3rd, Lockman, D. K., Crouch, R. K., and Ma, J. (1998) *Mol. Vision 4*, 10.
28. Hisatomi, O., Takahashi, Y., Taniguchi, Y., Tsukahara, Y., and Tokunaga, F. (1999) *FEBS Lett. 447*, 44−48.
29. Kawamura, S., and Yokoyama, S. (1998) *Vision Res. 38*, 37−44.
30. Yokoyama, S., Blow, N. S., and Radlwimmer, F. B. (2000) *Gene 259*, 17−24.
31. Kawamura, S., Blow, N. S., and Yokoyama, S. (1999) *Genetics 153*, 1839−1850.
32. Okano, T., Kojima, D., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5932−5936.
33. Okano, T., Fukada, Y., Artamonov, I. D., and Yoshizawa, T. (1989) *Biochemistry 28*, 8848−8856.
34. Makino-Tasaka, M., and Suzuki, T. (1984) *Vision Res. 24*, 309−322.
35. Crim, J. W. (1975) *Comp. Biochem. Physiol. A 52*, 719−720.
36. Provencio, I., and Foster, R. G. (1992) *Vision Res. 32*, 2201−2208.
37. Okada, T., Fujiyoshi, Y., Silow, M., Navarro, J., Landau, E. M., and Shichida, Y. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 5982−5987.
38. Lee, F. S., Chu, Z. T., and Warshel, A. (1993) *J. Comp. Chem. 14*, 161−185.
39. Liebman, P. A., and Entine, G. (1968) *Vision Res. 8*, 761−775.
40. Bowmaker, J. K., and Harosi, F. I. (1977) *Vision Res. 17*, 17−23.
41. Govardovskii, V. I., Fyhrquist, N., Reuter, T., Kuzmin, D. G., and Donner, K. (2000) *Vision Neurosci. 17*, 509−528.
42. Harosi, F. I. (1975) *J. Gen. Physiol. 66*, 357−382.
43. Pogozheva, I. D., Lomize, A. L., and Mosberg, H. I. (1997) *Biophys. J. 72*, 1963−1985.
44. Fasick, J. I., and Robinson, P. R. (1998) *Biochemistry 37*, 433−438.
45. Imai, H., Kojima, D., Oura, T., Tachibanaki, S., Terakita, A., and Shichida, Y. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2322−2326.
46. Smith, W. C., Adamus, G., Van Der Wel, H., Timmers, A., Palczewski, K., Ulshafer, R. J., Hargrave, P. A., and McDowell, J. H. (1995) *Exp. Eye Res. 61*, 569−578.
47. Nakayama, T. A., and Khorana, H. G. (1991) *J. Biol. Chem. 266*, 4269−4275.
48. Nathans, J. (1990) *Biochemistry 29*, 9746−9752.
49. Nagata, T., Terakita, A., Kandori, H., Shichida, Y., and Maeda, A. (1998) *Biochemistry 37*, 17216−17222.
50. Nagata, T., Oura, T., Terakita, A., Kandori, H., and Shichida, Y. (2002) *J. Phys. Chem. A 106*, 1969−1975.
51. DeCaluwe, G. L., Bovee-Geurts, P. H., Rath, P., Rothschild, K. J., and de Grip, W. J. (1995) *Biophys. Chem. 56*, 79−87.
52. Zhukovsky, E. A., and Oprian, D. D. (1989) *Science 246*, 928−930.
53. Wilkie, S. E., Robinson, P. R., Cronin, T. W., Poopalasundaram, S., Bowmaker, J. K., and Hunt, D. M. (2000) *Biochemistry 39*, 7895−7901.
54. Fasick, J. I., Lee, N., and Oprian, D. D. (1999) *Biochemistry 38*, 11593−11596.
55. Janz, J. M., and Farrens, D. L. (2001) *Biochemistry 40*, 7219−7227.
56. Kaushal, S., and Khorana, H. G. (1994) *Biochemistry 33*, 6121−6128.
57. Rao, V. R., Cohen, G. B., and Oprian, D. D. (1994) *Nature 367*, 639−642.
58. Zvyaga, T. A., Fahmy, K., Siebert, F., and Sakmar, T. P. (1996) *Biochemistry 35*, 7536−7545.
59. Han, M., Lin, S. W., Minkova, M., Smith, S. O., and Sakmar, T. P. (1996) *J. Biol. Chem. 271*, 32337−32342.
60. Sakmar, T. P., Franke, R. R., and Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8309−8313.
61. Yokoyama, S., Zhang, H., Radlwimmer, F. B., and Blow, N. S. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 6279−6284.

62. Andres, A., Kosoy, A., Garriga, P., and Manyosa, J. (2001) *Eur. J. Biochem. 268*, 5696−5704.
63. Merbs, S. L., and Nathans, J. (1992) *Nature 356*, 433−435.
64. Yokoyama, R., Knox, B. E., and Yokoyama, S. (1995) *Invest. Ophthalmol. Vision Sci. 36*, 939−945.
65. Lin, S. W., and Sakmar, T. P. (1996) *Biochemistry 35*, 11149−11159.
66. Merbs, S. L., and Nathans, J. (1993) *Photochem. Photobiol. 58*, 706−710.

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