Spectral Tuning and Evolution of Short Wave-Sensitive Cone Pigments in Cottoid Fish from Lake Baikal†

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ABSTRACT: The cottoid fishes of Lake Baikal in eastern Siberia provide a unique opportunity to study the evolution of visual pigments in a group of closely related species exposed to different photic environments. Members of this species flock are adapted to different depth habitats down to >1000 m, and both the rod and cone visual pigments display short wave shifts as depth increases. The blue-sensitive cone pigments of the SWS2 class cluster into two species groups with λ_{max} values of 450 and 430 nm, with the pigment in *Cottus gobio*, a cottoid fish native to Britain, forming a third group with a λ_{max} of 467 nm. The sequences of the SWS2 opsin gene from *C. gobio* and from two representatives of the 450 and 430 nm Baikal groups are presented. Approximately 6 nm of the spectral difference between *C. gobio* and the 450 nm Baikal group can be ascribed to the presence of a porphyropsin/rhodopin mixture in *C. gobio.* Subsequent analysis of amino acid substitutions by site-directed mutagenesis demonstrates that the remainder of the shift from 461 to 450 nm arises from a Thr269Ala substitution and the shift from 450 to 430 nm at least partly from Thr118Ala and Thr118Gly substitutions. The underlying adaptive significance of these substitutions in terms of spectral tuning and signal-to-noise ratio is discussed.

Lake Baikal in eastern Siberia is one of the world's oldest lakes and, with depths in excess of 1600 m, the deepest freshwater lake. However, even at such depths, oxygenation levels are at 75−80% of surface levels (*1*). This has created a unique environment which has become inhabited by many endemic species, including a flock of cottoid fish that occupy different depth habitats. This imposes certain problems for the visual system of the deeper water species. First, downwelling sunlight diminishes rapidly with increasing depth, with the limit of scotopic vision occurring at ∼1000 m in clear water (*2*). Second, sunlight is selectively attenuated with depth so that after a few hundred meters (substantially less in some bodies of water), light primarily consists of a narrow band of radiation between 470 and 480 nm (*3*−*5*).

In deep sea fish, adaptations to photon-limited environments include the acquisition of multiple banks of photoreceptors, the loss of cone photoreceptors, an unusually large amount of visual pigment in the photoreceptors (*6*−*8*), large photoreceptor size (*9*), and the λ_{max}[1] of rod visual pigments short wave-shifted to around 480 nm to match the wavelength of downwelling light (*7*, *8*). Many of these adaptations are also present in the deeper-dwelling Baikal cottoids (*10*, *11*). The shallow water littoral species possess three cone classes (LWS, MWS, and SWS) with λ_{max} values around 546, 525, and 450 nm, respectively, and a single rod class with a λ_{max} around 515 nm (*10*). However, as the habitat depth increases, LWS cones are discarded, followed by SWS and finally MWS cones to give a rod-only retina. Commensurate with this, the λ_{max} values of the pigments in the MWS and SWS cone photoreceptors, and in the rod photoreceptors, show stepwise shifts to shorter wavelengths. For the MWS cone pigments, there are three shifts from around 525 to 510 to 500, and finally, to 495 nm, for the SWS cone pigments, a single shift from around 450 to 430 nm, and for the rod pigments, four shifts from around 516 to 505 to 495 to 490 to 484 nm. The key amino acid substitutions in the opsin protein underlying the spectral shifts in the rod pigments have been previously identified (*11*). This study focuses on the SWS opsins of these cottoid fishes.

Visual pigments are G-protein-coupled receptors and consist of an apoprotein, opsin, joined covalently via a Schiff base linkage to the chromophore retinal, an aldehyde of vitamin A. Opsins consist of seven hydrophobic transmembrane α-helices joined by hydrophilic extracellular and cytoplasmic loops which fold to form a central binding pocket for the chromophore. In birds and mammals, this chromophore is 11-*cis*-retinal, forming the visual pigment rhodopsin. In fishes, however, a second chromophore, 11-*cis*-3,4-dehydroretinal, may also be present forming porphyropsin pigments (*12*−*14*). The replacement of retinal with 3,4-dehydroretinal results in an additional double bond in the β-ionone ring and acts to shift the λ_{max} to longer wavelengths (*15*). Marine fishes tend to have pure rhodopsin-based visual pigments, whereas euryhaline fish generally have a rhodopsin/porphyropsin mixture. Studies on the cottoid fish of Lake Baikal indicate that they have pure rhodopsin visual pigments, suggesting a marine origin for this species flock (*10*).

The SWS pigments of vertebrates are divided into two distinct phylogenetic clades: the SWS2 group, which

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[1] Abbreviations: MWS, middle wave-sensitive; LWS, long wave-sensitive; SWS, short wave-sensitive; λ_{max}, wavelength of maximal absorbance.

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includes fish, avian, reptilian, and amphibian blue-sensitive pigments (*16*−*19*) with λ_{max} values generally between 430 and 470 nm, and the SWS1 group, which includes fish, avian, reptilian, amphibian, and mammalian violet- and ultraviolet-sensitive pigments (*16*−*18*, *20*, *21*) with λ_{max} values ranging from around 360 to 430 nm. In vitro expression studies within the SWS1 group have identified key sites responsible for the short wave shift between avian violet and ultraviolet pigments (*22*, *23*). In contrast, the mechanism of spectral tuning within the SWS2 group has yet to be studied in any group of animals. Here we report the first study of spectral tuning within the SWS2 group of visual pigments and assess the evolutionary changes which have led to the short wave shift in λ_{max} with increasing depth of habitat in the cottoid fishes of Lake Baikal.

EXPERIMENTAL PROCEDURES

Collection of Fish. Baikal cottoid fish were collected either by trawling at night at various depths down to 1200 m or by local fishermen who netted inshore species in the early hours of the morning. Liver tissue was removed, shredded, and stored in ethanol. However, it was not logistically possible to isolate mRNA from retinal tissues of these fish. Specimens of *Cottus gobio* were collected from a freshwater stream in southern England.

Microspectrophotometry of C. gobio Visual Pigments. Fish were dark-adapted for a minimum of 2 h. The eyes were then enucleated under dim red light (Kodak Safelight 2) and the cornea and lens removed. Tissue for microspectrophotometry was prepared as described previously (*24*). Absorption spectra of individual photoreceptors were determined using a computer-controlled modified Liebman dual-beam microspectrophotometer (*15*, *25*, *26*). By means of an infrared converter, the measuring beam (normally a cross section of $\sim$2 μm^2) was aligned to pass transversely through a given photoreceptor outer segment, while the reference beam passed through a clear space adjacent to the photoreceptor. Spectra were scanned from 750 to 350 nm in 2 nm steps and back from 351 to 749 nm. To estimate the wavelength of maximum absorbance (λ_{max}) of each outer segment, a standardized computer program was employed. Only records that passed rigid selection criteria (see ref *26*) were used for analysis. A detailed description of the experimental procedures and methods of analysis has been published previously (*26*, *27*).

cDNA Cloning and Sequencing. mRNA was extracted from freshly dissected, dark-adapted retinas of *C. gobio* using a Pharmacia Quickprep mRNA purification kit. Single-stranded cDNA was synthesized using an oligo-d(T) anchor primer; the product was then used in a 3′ RACE reaction with an anchor primer and an oligonucleotide primer designed from a known goldfish SWS2 opsin sequence, GFB1+ (5′-ACAACCTCTCAGCCTACAGC-3′). A secondary PCR was then performed using two goldfish SWS2 specific primers (5′-TGGCTGGTCATTTGCAAACC-3′ and 5′-CGTCACCTCCCTCTCTGCCT-3′). The resulting product was inserted into the pGEM-T easy cloning vector (Promega) and fully sequenced.

Genomic DNA Cloning and Sequencing. Genomic DNA was isolated from all species using a standard phenol/chloroform method. The 5′ end of the SWS2 opsin gene from the Lake Baikal cottoid, *Cottus kessleri*, was obtained using a walking PCR technique (*28*). For the primary walk, the primer pairs 5′-CACAGGAGCACTGCAGGCC-3′ and 5′-TTTTTTTTTTGTTTGTTGTGGGGGTGT-3′ were used. A secondary walk was then performed using the nested primers 5′-GGGCTTGAAAACAAAGGTA-3′ and 5′-TTTTTGTTTGTTGTGGG-3′. The resulting product was inserted into the pGEM-T easy cloning vector and sequenced.

Sequence data from both the 3′ RACE and walking PCR were then used to design gene specific primers to amplify the complete coding sequences of the SWS2 opsin gene from *C. gobio* and four Lake Baikal cottoids, *C. kessleri*, *Cottocomephorus inermis*, *Limnocottus eurystomus*, and *Batrachocottus nicolskii*.

Generation of Opsin Mutants and Construction of the Opsin Expression Vector. Wild-type SWS2 opsin from *C. gobio* was amplified from retinal cDNA using *Pfu* polymerase and the following primers which contain restriction site tags for *Eco*RI and *Sal*I: 5′-GCGCGAATTCCACC**ATGAAGCACGGTCGTGTCACGGAG**-3′ and 5′-CGGCGTCGACGC**AGCAGGCCCAACTTTGGAGAC**-3′. The underlined sequences are the *Eco*RI restriction site in the + primer and *Sal*I site in the − primer, respectively. Sequences homologous to *C. gobio* SWS2 opsin are shown in bold. The resulting fragment containing the entire *C. gobio* SWS2 opsin sequence was then directionally inserted, as the *Eco*RI−*Sal*I fragment, into an expression vector, pMT4. pMT4 is a derivative of the pMT2 mammalian expression vector and additionally carries the sequence of the bovine 1D4 epitope, including the stop codon, downstream of and in frame with the *Sal*I site (*29*). The sequence of the *C. gobio* SWS2 opsin was then checked using gene specific primers.

Mutations at sites 215 and 269 were introduced into the SWS2 opsin while inserted into the vector pGEM-T easy (Promega) using a QuikChange site-directed mutagenesis kit (Stratagene). Primers used to create the Pro215Gly and Thr269Ala mutations were as follows: 215Gly+, 5′-CCTGCCACTTTACTGTTGGCCTCTTAATCCTTG-3′; 215Gly−, 5′-CAAGGATTAAGAGGCCAACAGTAA-AGTGGCAGG-3′; 269Ala+, 5′-GCTGGATGCCTTACGC-CTGCTTTGCGC-3′; and 269Ala−, 5′-GCGCAAAGCAG-GCGTAAGGCATGGAGC-3′. Site-directed mutagenesis at site 118 was performed using an Altered Sites II kit (Promega) with the sequence inserted into the vector p-ALTER-1. The following primers were used: 5′-GAA-GGTTTTTTAGCAGGACTTGGCGGTATG-3′ for the Thr118Gly mutation and 5′-GTTTTTTAGCAGCACTTG-GCG-3′ for the Thr118Ala mutation. The underlined nucleotides indicate the mutagenic substitutions that have been introduced. Mutated fragments were transferred into pMT4 as described previously and resequenced.

Expression of Wild-Type C. gobio and Mutant SWS2 Pigments. Clones were transiently expressed in HEK 293T cells following transfection with Lipofectamine (Invitrogen). Thirty 90 mm plates were used per sample. Cells were harvested 48 h post-transfection and washed four times with PBS (pH 7.0), and the cell pellets were stored at −80 °C prior to generation of the pigments. Pigments were generated by suspending cells in PBS (pH 7.0) and incubating them with 40 μM 11-*cis*-retinal in the dark (*30*). The pigment was solubilized from cell membranes and purified by immunoaffinity chromatography using an anti-1D4 antibody coupled to a CNBr-activated Sepharose column following the meth-

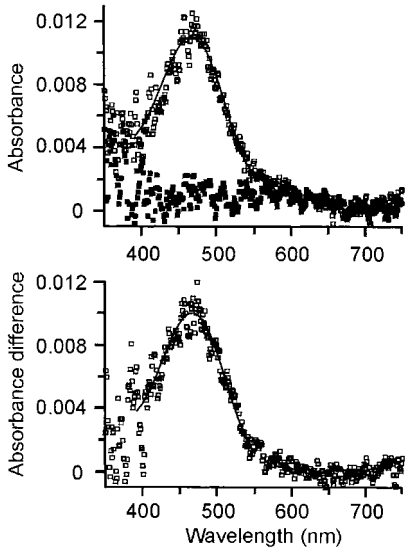

FIGURE 1: Absorbance and difference spectra of the SWS cones in *C. gobio*. The spectra are the means of records obtained from five individual cone outer segments. In the top panel, white squares represent the dark spectrum and black squares the bleached spectrum. The bottom panel shows the difference spectrum. The full curves in both panels are a pigment template with a λ_{max} at 470 nm composed of 35% rhodopsin and 65% porphyropsin.

ods of Molday and MacKenzie (*31*). Purified pigment was eluted from the column and stored on ice. Absorption spectra were recorded in the dark using a Spectronic Unicam UV500 dual-beam spectrophotometer. The sample was then photobleached for 5 min using white light from a fluorescent bulb and the spectrum recorded again. The λ_{max} value of the pigment was determined by subtracting the photobleached spectrum from the dark spectrum to produce a difference spectrum.

Table 1: Spectral Groups of Selected Baikal Cottoids

	no. of cone types	life style	habitat depth (m)	λ_{max} (nm) of the SWS2 pigment[a]
450 nm group				
C. kessleri	3	littoral	1−5	449.0 ± 2.7
Co. inermis	2	pelagic, sublittoral	50−450	450.4 ± 2.5
430 nm group				
B. nicolskii	2	benthic, abyssal	300−1000	428.2 ± 2.6
L. eurystomus	2	benthic, supra-abyssal	100−500	427.6 ± 4.5

[a] Data from ref *10*.

RESULTS

The λ_{max} values of the SWS pigments from 10 species of Baikal cottoids were originally determined by microspectrophotometry (*10*). These species fell into two spectral groups, with λ_{max} values around 450 nm and around 430 nm. For this study, two representatives from each spectral group were selected, *C. kessleri* and *Co. inermis* from the 450 nm group and *L. eurystomus* and *B. nicolskii* from the 430 nm group. The λ_{max} values for their SWS2 pigments, together with their life styles and depth habitats, are listed in Table 1.

Logistical difficulties prevented the collection of retinal tissue from these species for mRNA isolation. Therefore, to facilitate the amplification and cloning of the Baikal cottoid SWS2 opsin genes, the cDNA sequence of the corresponding SWS2 opsin gene was determined for a non-Baikal cottoid, *C. gobio*, native to the rivers of Britain. For completeness, the λ_{max} of the SWS2 pigment from this species was also determined by microspectrophotometry.

λ_{max} of C. gobio SWS2 Pigment. The retina of *C. gobio* contains rods, double cones, and single cones. The mean absorbance and difference spectra from the single cones are shown in Figure 1. The spectra can be best fitted by a mixed

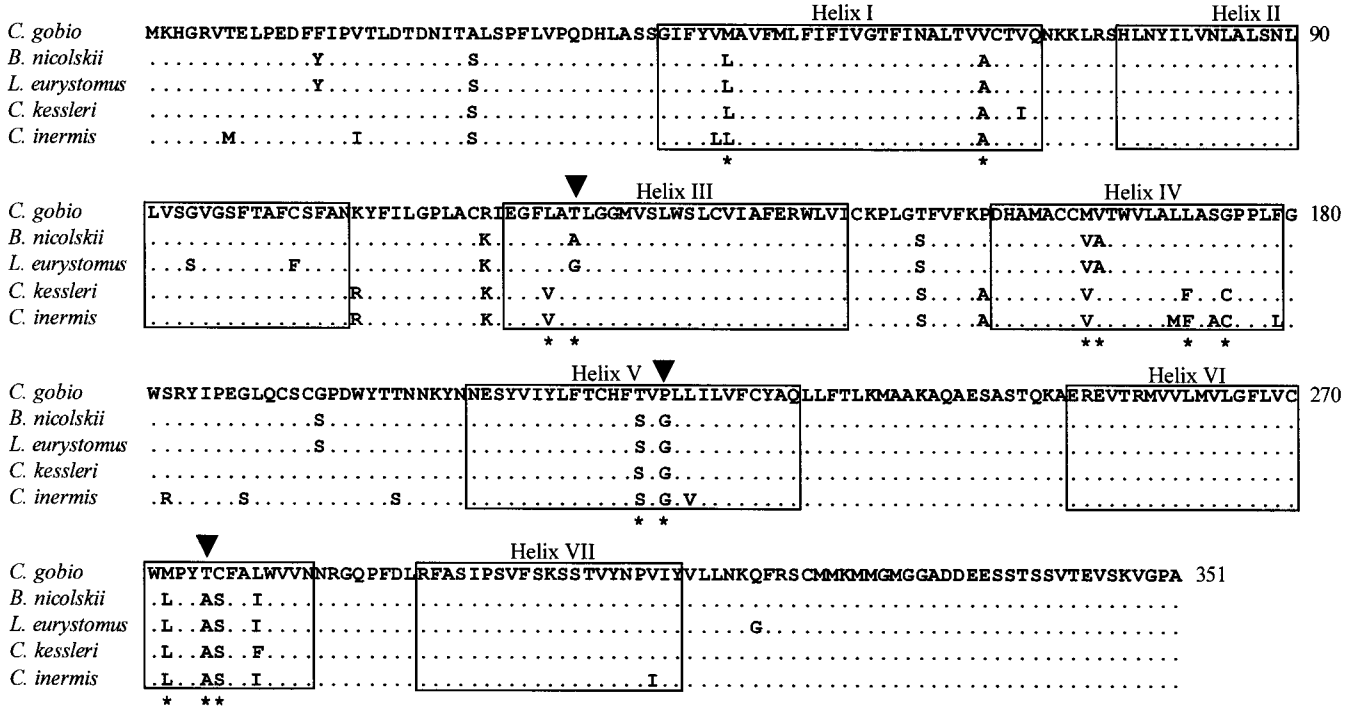

FIGURE 2: Alignment of deduced amino acid sequences of cottoid SWS2 opsins. The positions of helices I−VII are highlighted. Asterisks denote the 13 sites showing amino acid differences between the spectral groups, and arrowheads denote the positions of the three candidate tuning sites. Note that for comparative purposes, the residue positions mentioned in the text are based on the bovine rod numbering system (i.e., cottoid SWS2 amino acid position minus 6). EMBL accession numbers AJ430469, AJ430474, AJ430484, and AJ430489.

Table 2: Peak Absorbance of Wild-Type and Mutant SWS2 Pigments Expressed in HEK 293T Cells[a]

	λ_{max} (nm)	shift from the wild-type pigment (nm)
wild type (*C. gobio*)	463	
Thr118Ala	458	−5
Thr118Gly	452	−11
Pro215Gly	465	+2
Thr269Ala	453	−10

[a] Shifts from the wild type are shown as negative for short wave shifts and positive for long wave shifts.

rhodopsin/porphyropsin template with a λ_{max} of 467 ± 3 nm incorporating 35% rhodopsin. This would imply a visual pigment pair with the λ_{max} of the rhodopsin and porphyropsin at ~461 and ~470 nm, respectively (*32*). The SWS cone pigment of *C. gobio* therefore forms a spectral group distinct from the two Baikal classes. In *C. gobio*, both members of the double cones contained a LWS pigment with a λ_{max} at ~560 nm, whereas the rods possessed a pigment with a λ_{max} close to 520 nm. Both the 560 and 520 nm pigments also appear to be mixtures of rhodopsins and porphyropsins.

Cottoid SWS2 Opsin Gene Sequences. The cDNA sequence of the opsin from *C. gobio* was obtained by 3′ RACE using an anchor primer and gene-specific primers to obtain most of the coding sequence, including the 3′ end, followed by gene walking using genomic DNA to obtain the 5′ end. The resulting sequence exhibited the highest level of homology to the goldfish SWS2 pigment (*16*). The SWS2 sequences from the four Baikal cottoids were then obtained from genomic DNA using a combination of conventional PCR and gene walking with primers designed from the *C. gobio* coding sequence.

The deduced amino acid sequences for the SWS2 opsins of *C. gobio*, *C. kessleri*, *Co. inermis*, *L. eurystomus*, and *B. nicolskii* are shown in Figure 2. The sequences from the three spectral groups (461, 450, and 430 nm) were compared and sites likely to be involved in spectral tuning identified according to two basic criteria. First, the amino acid site should be in one of the transmembrane helices and face into the retinal binding pocket (*33*), and second, the amino acid change should be nonconservative, e.g., a change in charge or the gain/loss of a hydroxyl group. Amino acid differences between the spectral groups occur at 13 sites within the helices, although only four substitutions at three sites, Thr118Ala, Thr118Gly, Pro215Gly, and Thr269Ala (using the bovine rod numbering system), fulfill the above criteria.

Location of Tuning Sites within the Chromophore-Binding Pocket. The model proposed by Baldwin et al. (*33*, *34*) utilizes data from approximately 500 members of the G-protein-coupled receptor family together with electron cryomicroscopy data of frog rhodopsin (*35*) to produce a three-dimensional image model which can be applied to all opsins. This has been further refined (*36*, *37*) and supported by X-ray crystallographic data (*38*) and shows that the transmembrane helices are tilted to different degrees and interact to form the retinal-binding pocket. When the candidate spectral tuning sites are mapped onto this model, only sites 118 and 269 are close to the chromophore (Figure 3). Site 118 lies toward the luminal side of helix III and is adjacent to the polyene chain of the chromophore, and site 269 is located on helix VI close to the β-ionone ring. Each

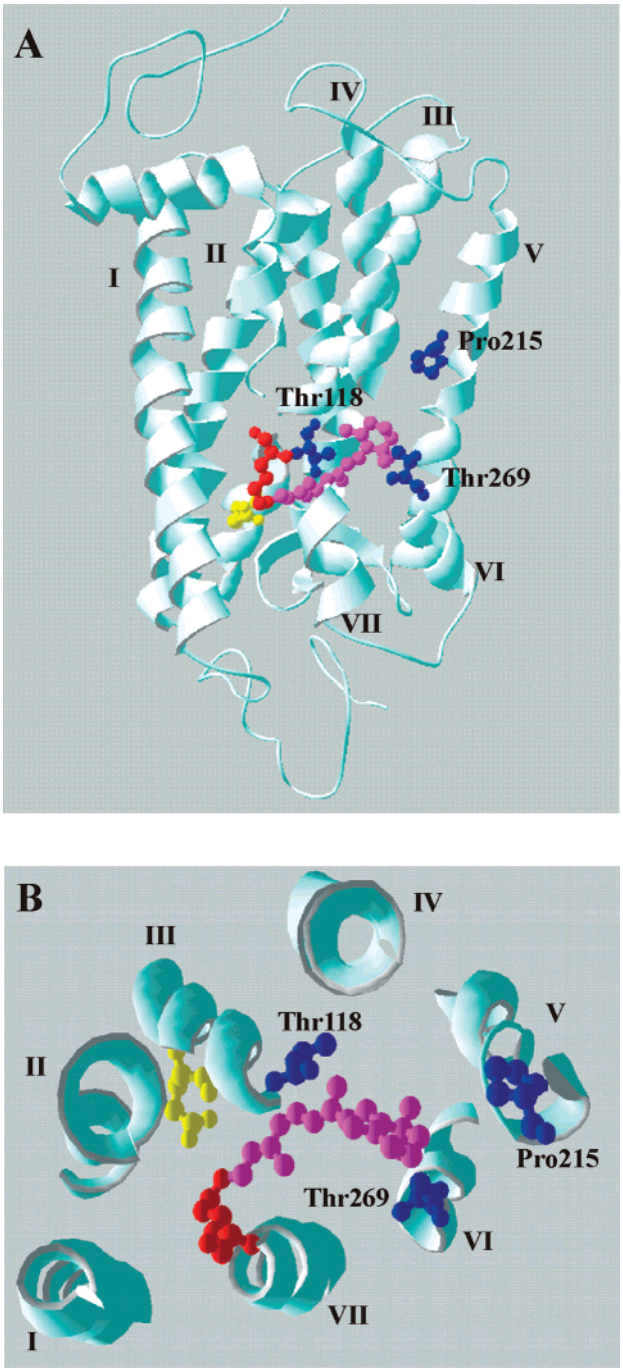

FIGURE 3: Structural model of the *C. gobio* SWS2 pigment. (A) Side view across the membrane showing the complete molecule but with portions of helices III and V−VII cut away to reveal the chromophore. (B) Partial (slab) view from the cytoplasmic side of the membrane at the level of the chromophore. The 11-*cis*-retinal chromophore is in pink, Lys296 in red, and the Glu113 counterion in yellow. Spectral tuning sites Thr118, Pro215, and Thr269 are in blue. The model was created using Swiss Model (*55*) and is based on the crystal structure of the bovine pigment (*38*).

of these substitutions results in the loss of a hydroxyl group. In contrast, the location of the Pro215Gly substitution on helix V means that it is unlikely to interact directly with the chromophore.

Spectral Properties of Wild-Type and Mutant SWS2 Pigments. Site-directed mutagenesis was used to introduce the Thr118Ala, Thr118Gly, Pro215Gly, and Thr269Ala substitutions into the wild-type *C. gobio* SWS2 opsin. The wild-type and mutant cDNAs were then expressed in HEK

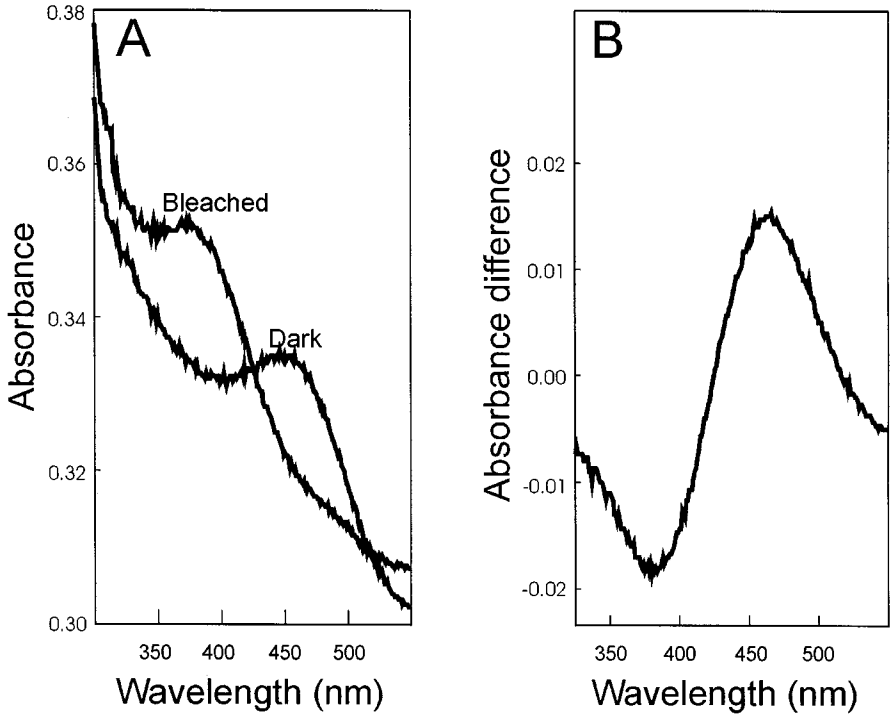

FIGURE 4: Absorbance spectra for the wild-type (*C. gobio*) SWS2 pigment expressed in HEK 293T cells. (A) Dark and bleached spectra. (B) Difference spectrum.

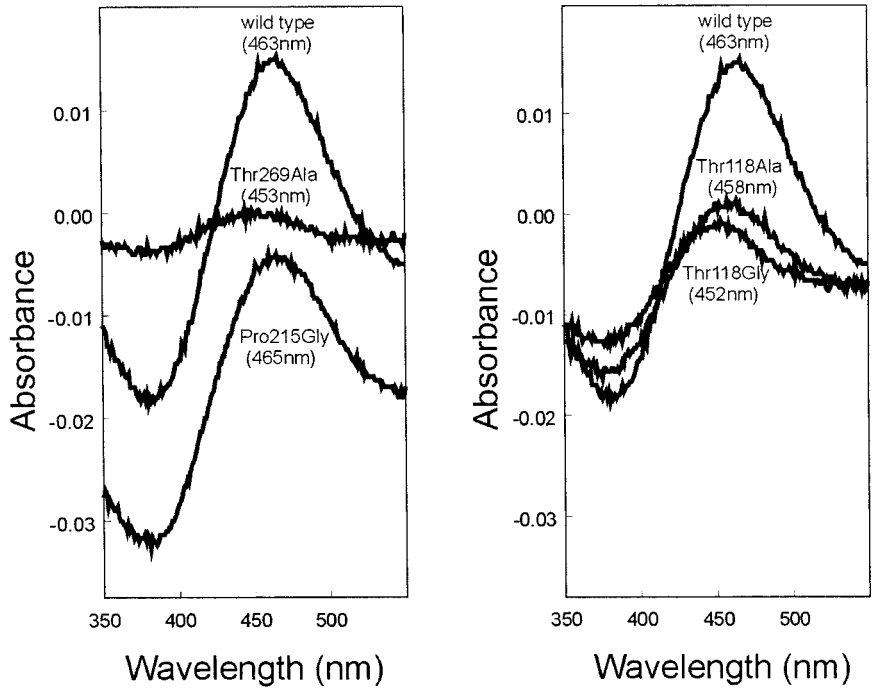

FIGURE 5: Absorbance difference spectra for wild-type and mutant SWS2 pigments expressed in HEK 293T cells. The λ_{max} values derived from these spectra are given in parentheses.

293T cells, the pigments reconstituted with 11-*cis*-retinal, and the proteins purified as described previously. Absorbance spectra were obtained for the wild type and four mutant pigments. The dark and photobleached spectra for the wild-type pigment are shown in Figure 4, together with the difference spectrum. Difference spectra for the wild type and for each of the mutants are shown in Figure 5; these spectra were used to determine the λ_{max}. In repeat experiments, the Thr269Ala mutant consistently gave a weak peak absorbance, indicating that this pigment may be less stable than the others.

The recombinant wild-type *C. gobio* pigment produced in this expression system has a λ_{max} of 463 nm, similar therefore to the estimated value of 461 nm for the native pigment (after correcting for 65% 3,4-dehydroretinal). The Thr118Ala substitution resulted in a 5 nm short wave shift to 458 nm, the Thr118Gly substitution in an 11 nm shift to 452 nm, and the Thr269Ala substitution in a 10 nm shift to 453 nm (Table 1). In contrast, the Pro215Gly substitution produced little or no shift. The shift from 461 nm of the *C. gobio* pigment to the 450 nm group of Baikal cottoids can

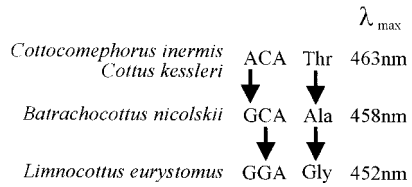

FIGURE 6: Nucleotide substitutions and the resulting amino acid replacement at site 118 in SWS2 pigments from Baikal cottoids.

be ascribed therefore to the Thr269Ala substitution, whereas the short wave shift to the 430 group would appear to be only partly explained by the substitutions at site 118.

The microspectrophotometry data reported previously (*10*) did not show any difference in the λ_{max} of the SWS2 pigments in the two 430 nm group species (see Table 1), whereas the expression data clearly show that the pigment with Gly118 is more short wave shifted than the Ala118 pigment. As shown in Figure 6, the most parsimonious explanation for the evolution of these two pigments is that the Thr118Ala substitution preceded an Ala118Gly change, requiring sequential mutations in the first and second positions of the codon, respectively, to generate the pigment present in *L. eurystomus*.

DISCUSSION

The data presented here form the first study of spectral tuning in the SWS2 class of opsins. Five freshwater species of cottoid fish have been studied, four endemic to Lake Baikal and the fifth, *C. gobio*, native to Britain. Microspectrophotometry places the λ_{max} of the SWS2 cone pigment of *C. gobio* at around 467 nm, whereas the pigments of the Baikal cottoids are short wave-shifted either to around 450 nm or to around 430 nm (*10*). Species belonging to the 450 nm spectral group are largely littoral or sublittoral, living at depths down to 450 m, whereas the 430 nm group consists of abyssal species living at depths down to 1000 m. The hypsochromic shift is therefore related to depth of habitat but not directly correlated to the wavelength of penetrating light since attenuation of shorter wavelengths with depth will selectively eliminate light at wavelengths of <470 nm.

Unlike the pigments of Lake Baikal cottoid fishes, the SWS2 pigment of *C. gobio* contains a chromophore mix of 35% retinal and 65% 3,4-dehydroretinal, the latter generating a long wave shift of approximately 6 nm (*32*) in comparison to the expressed pigment regenerated with retinal alone. The presence of 3,4-dehydroretinal accounts in part therefore for the 15−20 nm shift between the 450 nm group of Lake Baikal cottoids and *C. gobio*. To account for the remainder of the shift, the role of candidate amino acid substitutions at two sites, 215 and 269, was evaluated in vitro. While the Pro215Gly substitution appears to have little or no effect, the Thr269Ala substitution short wave shifts the pigment by 10 nm, thereby accounting for the remaining difference between the *C. gobio* pigment and the pigments of the 450 nm group of Baikal cottoids. Thr/Ala substitutions at this site are a common mechanism for spectral tuning in vertebrate rod and cone opsins (*39*−*44*), and expression studies have shown that the Thr269Ala substitution results in a 14−15 nm shift in primate and fish MWS and LWS cone pigments (*45*, *46*) and in mammalian rod pigments (*47*). Ala269 is also present in all SWS1 pigments sequenced to date (*17*, *21*, *22*, *44*, *48*−*50*), providing further evidence for the importance of this residue in short wave-shifted visual pigments.

Substitution at site 118 would appear to be the main candidate for the spectral shift between the SWS2 pigments of the 450 and 430 nm groups of Baikal cottoids. However, the two species that have been examined, *B. nicolskii* and *L. eurystomus*, have Ala118 and Gly118 substitutions, respectively, which give spectral shifts of 5 and 11 nm. As shown in Figure 6, sequential substitutions at positions 1 and 2 in codon 118 would appear to the most likely evolutionary mechanism. The failure to demonstrate this interspecies difference in λ_{max} in our previous report (*10*) may be attributed to the error associated with the estimates of λ_{max} by microspectrophotometry. The short wave shifts arising from these substitutions however only partly account for the spectral shift between the 450 and 430 nm groups. The only other site that has a nonconservative substitution in both species of the 430 nm group involves a Gly169Cys change in helix IV. Although a contributory shift arising from substitution at this site cannot be ruled out, it would however appear unlikely as, according to the Baldwin model (*33*), this site is on the membrane face of the helix. An alternative explanation is that the magnitude of the shifts achieved by the site 118 substitutions depends on other amino acid differences between the pigments of *C. gobio* and the Baikal cottoids. Substitution at site 215 may be relevant to this since proline at this site is highly conserved across the opsins and other G-protein-coupled receptors (*33*), although it is not associated with any marked distortion in the helix in bovine rhodopsin (*38*). Nevertheless, it may act synergistically with substitutions at site 118 to increase the magnitude of the short wave spectral shifts. The Gly215 substitution appears to be unique to Lake Baikal cottoid SWS2 opsins and must have occurred before the species divergence, as it is present in all four of the species that were studied.

Site 118 was previously identified as part of the tuning mechanism of avian UV- and violet-sensitive SWS1 pigments (*18*, *24*). From an adaptive standpoint, the short wave shifts arising from substitutions at this site in the Baikal cottoids are more difficult to understand as they shift the λ_{max} of the pigment away from the peak wavelength of downwelling light penetrating to the deeper regions of the lake. The concept has been advanced (*51*) that there may be a relationship between the spectral properties of a pigment and the energy barrier for its activation such that pigments with maximal sensitivities at longer wavelengths are thermodynamically less stable. It is possible therefore that the pigments in the Baikal cottoids have become short wave-shifted to reduce background noise and thus enhance the sensitivity of the visual system. Experimentally, the only demonstration of this phenomenon is where spectral shifts between pigments arise from the replacement of retinal with 3,4-dehydroretinal (*52*), and there is no evidence that the SWS classes of pigments are inherently less stable than the LWS class (*53*). The latter observation may however reflect the limitations of comparing between different pigment classes where evolutionary selection has already acted to stabilize spectrally distinct pigments. In the present case, the short wave shifts are in pigments of the same class with the same chromophore; such shifts may result therefore in a reduced level of thermal decomposition as the energy required for activation becomes greater. In deep waters where there is very little light, such a mechanism may be more effective in enhancing visual sensitivity over tuning toward the peak wavelength of downwelling light.

We have previously shown that adaptive changes in the spectral sensitivity of rod photoreceptors of Baikal cottoids can be traced to amino acid substitutions in rod opsin (*54*). In this case, the relationship between the λ_{max} of the rod pigment and depth of habitat would appear to reflect tuning of photoreceptor sensitivity to the peak wavelength of downwelling light. For the SWS2 cone pigments, exposure to the same evolutionary pressure of a photon-limited environment has again resulted in spectral shifts to shorter wavelengths, but in this case away from the peak wavelengths of downwelling light. Our explanation for this is that these short wave-shifted pigments have an increased signal-to-noise ratio and that this is the adaptive mechanism used to maximize photoreceptor sensitivity.

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