Evolutionary replacement of UV vision by violet vision in fish

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The vertebrate ancestor possessed ultraviolet (UV) vision and many species have retained it during evolution. Many other species switched to violet vision and, then again, some avian species switched back to UV vision. These UV and violet vision are mediated by short wavelength-sensitive (SWS1) pigments that absorb light maximally (λmax) at approximately 360 and 390–440 nm, respectively. It is not well understood why and how these functional changes have occurred. Here, we cloned the pigment of scabbardfish (Lepidopus fitchi) with a λmax of 423 nm, an example of violet-sensitive SWS1 pigment in fish. Mutagenesis experiments and quantum mechanical/molecular mechanical (QM/MM) computations show that the violet-sensitivity was achieved by the deletion of Phe-86 that converted the unprotonated Schiff base-linked 11-cis-retinal to a protonated form. The finding of a violet-sensitive SWS1 pigment in scabbardfish suggests that many other fish also have orthologous violet pigments. The isolation and comparison of such violet and UV pigments in fish living in different ecological habitats will open an unprecedented opportunity to elucidate not only the molecular basis of phenotypic adaptations, but also the genetics of UV and violet vision.

SWS1 Gene in the Scabbardfish. Since all fish studied to date are known to use UV vision at least during certain stages of their lives, it is likely that scabbardfish also has an additional UV opsin gene. In our attempt to isolate the UV opsin gene, we constructed a scabbardfish genomic DNA library and screened it by using the full-length SWS1 opsin cDNAs of scabbardfish and human sequentially. After screening approximately 1 × 106 recombinant plaques by using the scabbardfish opsin cDNA, we obtained five positive clones. Screening a similar number of plaques with the human opsin cDNA, we obtained two additional positive clones. These seven clones represented the same SWS1 gene. This gene (Fig. 2B) encodes the amino acid sequence of SCABBARD opsin (Fig. 1). Furthermore, when a Southern blot containing the scabbardfish genomic DNA digested with HindIII was hybridized with its own SWS1 opsin cDNA under nonstringent conditions (see Materials and Methods), we could detect only two hybridizing bands (1.2 and 1.8 kb) (Fig. 2C). These bands correspond exactly to the restriction map of the cloned scabbardfish SWS1 gene (Fig. 2B). Taken together, these results strongly suggest that the ancestral UV opsin gene was replaced by the violet opsin gene during scabbardfish evolution.

Molecular Evolution of SCABBARD. How did this functional differentiation occur? We obtained an important clue by comparing...
the violet-sensitive SCABBARD with the LAMPFISH and another UV-sensitive pigment of bluefin killifish (*Lucania goodei*) (KILLIFISH with a λ_max of 354 nm) (19), which diverged about the same time (7). The sequence comparison shows that 1 aa in the transmembrane (TM) helix II is missing from SCABBARD (Fig. 1).

It is most likely that the SWS1 pigment of the vertebrate ancestor (*pigment a*) had amino acids Phe-86, Cys-87, and Ile-88 (Phe-86/Cys-87/Ile-88) (5) (Figs. 1 and 2 D), where the amino acid site numbers are standardized by those of bovine rhodopsin (20). Pigment a has a λ_max of 361 nm and is UV-sensitive (5). A simple visual inspection of the amino acid sequences of SCABBARD, LAMPFISH, and KILLIFISH suggests that their common ancestor (*pigment b*) had Phe-86/Val-87/Thr-88 (Fig. 2 D). Although GOLDFISH and ZEBRAFISH have Phe-86/Asp-87/Thr-88, the predicted amino acids can also be found in most other fish, including tilapia, medaka, halibut, rainbow trout, and different cichlid and salmon species. Hence, the amino acid deletion in SCABBARD could have occurred at any one of sites 86, 87, and 88 with amino acid substitutions Val87Ala/Thr88Met, Phe86Ala/Thr88Met, and Phe86Ala/Val87Met, respectively. Among these, Phe86Ala requires two steps of amino acid substitutions, whereas the others require only one-step changes, suggesting that site 86 is deleted (Fig. 2 D). Interestingly, several amino acid changes at this site, including Phe86Tyr (9, 12, 21) and Phe86Ser (5, 11), are known to increase the λ_max by 20–70 nm. Because of the functional importance of this site, it is unclear whether a pigment with the deletion of Phe-86 can actually form a stable visual pigment. Despite this uncertainty, we hypothesize that the deletion of Phe-86 caused the dramatic λ_max-shift of SCABBARD.

Assuming that Phe-86 has been deleted during scabbardfish evolution and by using a computer program PAML (22), we inferred the amino acids between sites 22 and 322 of various ancestors of SCABBARD, LAMPFISH, KILLIFISH, and 14 additional SWS1 pigments (see Materials and Methods). The result shows that most of the amino acids inferred for pigment b have posterior probabilities >0.95 and are highly reliable; indeed, pigment b seems to have had Phe-86/Val-87/Thr-88, confirming the prediction based on the visual inspection (Fig. 1). When the amino acids between sites 22 and 322 are compared, pigment a and pigment b differ at 50 sites, whereas pigment b differs from KILLIFISH, LAMPFISH, and SCABBARD at 27–50 aa sites (Fig. S1).

### Mutagenesis Experiments

To test whether the deletion of Phe-86 in SCABBARD shifted the λ_max from 360 nm to 423 nm, we first...
become violet-sensitive, but

\[ \text{Hence, LAMPFISH and KILLIFISH with Phe-86-deletion} \]

shifted the pigment a... among these, the Phe-86- and Cys-87-deletions deleted individually Phe-86, Cys-87, and Ile-88 from the ancestral pigment a. Among these, the Phe-86- and Cys-87-deletions shifted the \( \lambda_{max} \) by 19 and 5 nm, respectively, whereas the Ile-88-deletion caused no \( \lambda_{max} \)-shift (Table 1). When we deleted Phe-86 from LAMPFISH and KILLIFISH, the deletion pigments increased their \( \lambda_{max} \) by 37 and 58 nm, respectively (Table 1). Hence, LAMPFISH and KILLIFISH with Phe-86-deletion become violet-sensitive, but pigment a with the same deletion is still UV-sensitive. On the other hand, when Phe-86 or Cys-87 were introduced into SCABBARD, only the former change made the pigment UV-sensitive (Table 1). Therefore, the Phe-86-deletions in LAMPFISH and KILLIFISH and the Phe-86-deletion in pigment a does not. The paradox can be resolved by combining these mutagenesis results with the quantum chemical computations. The quantum chemical analyses explore the fundamental chemical bases of the \( \lambda_{max} \)-shifts in various visual pigments.

**Quantum Chemistry of SCABBARD and Its Ancestors.** UV and violet pigments have been proposed to have unprotonated and protonated Schiff base-linked 11-cis-retinals (SBRs), respectively (9, 15, 23). By using a QM/MM method (24), we first obtained the three-dimensional structures and ground-state energies of pigment a, pigment b, LAMPFISH, KILLIFISH, and SCABBARD with unprotonated and protonated SBRs separately. The results show that in the first four pigments with unprotonated SBRs, water molecules W1 and W2 generate five H-bonds in the pigment, whereas those with protonated SBR have three H-bonds (for pigment b, see Fig. 3 A and B). This difference makes the pigment a, pigment b, LAMPFISH, and KILLIFISH with unprotonated SBR energetically more stable than the corresponding pigments with protonated SBRs by 2.0–6.2 kcal/mol (Table 1), showing that they have unprotonated SBRs. In SCABBARD, the side chain OH moiety of Ser-90 as well as W1 and W2 move toward the space created by the deletion of Phe-86 (Fig. 3 C and D), and W2 cannot form any H-bond with Tyr-265 and Ser-295. Moreover, W1 is attracted by the deprotonated Glu-113 to form a H-bond for the protonated SBR, but it cannot form the H-bond with protonated Glu-113 of the pigment with unprotonated SBR due to the repulsion of their hydrogens. Consequently, SCABBARD with unprotonated and protonated SBRs have 2 and 3 H-bonds, respectively (Fig. 3 C and D), making the latter pigment more stable than the former pigment by 1.3 kcal/mol (Table 1). Hence, SCABBARD has a protonated SBR.

By the same token, when Phe-86 is deleted, pigment b, LAMPFISH, and KILLIFISH have protonated SBRs, whereas when Phe-86 is introduced into SCABBARD, it has an unprotonated SBR (Table 1). These results show that LAMPFISH, KILLIFISH, and their immediate ancestor, pigment b, with Phe-86-deletion have very similar chemical characteristics near their SBRs. However, pigment a with Phe-86-deletion still has an unprotonated SBR (Table 1). It should be noted that the pigment a mutant with protonated and unprotonated SBRs both form H-bonds only between W1 and Ser-90 and between W1 and W2; however, the two H-bonds in the pigment with an unprotonated SBR are shorter than those in the pigment with a protonated SBR by 0.06 Å, making the phe86-deleted analog of pigment a...
more stable with unprotonated SBR. Hence, the QM/MM and mutagenesis analyses of pigment a, LAMPFISH, KILLIFISH, and SCABBARD FISH establish that UV and violet pigments indeed have unprotonated and protonated SBRs, respectively. The analyses also show that the chemical properties of pigment a and pigment b (and LAMPFISH and KILLIFISH) with Phe-86-deletion are fundamentally different.

By using the QM/MM method, we can also evaluate the $\lambda_{\text{max}}$ of visual pigments. It should be noted that the absolute $\lambda_{\text{max}}$ calculated by using this method often deviate from the comparable experimental values, but the $\lambda_{\text{max}}$-shifts generally match with the experimental values (24). Here, the visual pigments with unprotonated and protonated SBRs have $\lambda_{\text{max}}$ of 391–417 and 441–463 nm, respectively, which are significantly higher than the comparable experimental values (Table 1). Importantly, however, the estimated magnitudes of $\lambda_{\text{max}}$-shifts caused by the protonation of SBR are reasonably close to the experimental values. That is, the increased $\lambda_{\text{max}}$-shifts for LAMPFISH (38 nm) and KILLIFISH (50 nm) are similar to the corresponding experimental results of 37 and 58 nm (Table 1). Similarly, the calculated $\lambda_{\text{max}}$-shift of 73 nm caused by the deprotonation of SBR in SCABBARD is similar to the experimental value of 60 nm (Table 1). Since the pigment a and its Phe-86-deletion analog both have unprotonated SBRs, the calculated $\lambda_{\text{max}}$ of pigment a with and without Phe-86-deletion are similar. However, when Phe-86 is deleted from pigment b, it increases the $\lambda_{\text{max}}$ by 31 nm, strongly suggesting that it is violet-sensitive.

These QM/MM results and the corresponding mutagenesis results together show that SCABBARD has acquired its violet-sensitivity from pigment b by converting the unprotonated SBR into a protonated form. This chemical change was triggered by the deletion of Phe-86 that increased the number of H-bonds near the SBR.

Discussion

It was 20 years ago that vision scientists began to appreciate the existence of UV vision in a wide range of vertebrates (4), and the genetic analyses of UV vision started <10 years ago (13, 14). Among fish, the absorption spectra of SWS1 pigments have been determined only for lamprey (17), goldfish (25), zebrafish (26), bluefin killifish (19), tilapia (27), and some cichlids (28). Genetically engineered ancestral SWS1 pigments strongly suggest that early vertebrate ancestors used UV vision, and many species have retained it during evolution (5). In the absence of UV light, UV vision becomes unnecessary and is expected to disappear, as exemplified by coelacanth (29) and many nocturnal mammals (30). However, despite being exposed to abundant UV light, many species have switched from UV vision to violet vision; however, living in poor light conditions, some nocturnal animals, such as voles (2) and bats, (31) have retained UV vision. Moreover, despite their exposure to UV and/or violet (or blue) light, the SWS1 genes in various mammals, including whales, dolphins, and seals, have mysteriously become nonfunctional (30, 32). Some of these changes may be explained (4), but the actual causes of the evolutionary switches between UV and violet vision are difficult to pinpoint.

It is very likely that evolutionary biologists infer the possibility of adaptive evolution of various genes by using computer programs, which compare the nonsynonymous and synonymous nucleotide substitutions per site (22, 33). However, these analyses not only predict a significant number of false-positives but also fail to predict many positively selected sites; consequently, the positively selected amino acid changes inferred by the statistical methods must be tested by using experimental methods (34). Under natural selection, the phenotypes must have adapted to specific environments. For UV and violet vision, one critical selective factor is whether or not SWS1 pigments are exposed to UV light. The water environment is conveniently easy to quantify; shallow water receives a broad spectrum of visible light between 360 and 650 nm from the sun, but as we go deeper, the spectrum becomes narrower at approximately 480 nm (35). When we have a sufficiently large number of UV- and violet-sensitive SWS1 pigments in fish, we can check the correlation between the UV and violet vision and their ecological environments and test whether these phenotypes have undergone adaptive evolution (7).

SCABBARD is the only currently known fish violet-sensitive SWS1 pigment. This gives a hope that if we broaden our search for violet-sensitive SWS1 pigments, then we should be able to isolate additional violet pigments from other fish species as well. In some fish species, small juveniles use UV vision, but UV vision disappears in adults (36–41). The disappearance of UV vision is often associated with a habitat shift from shallow waters to deeper waters and with a shift from a diet of small zooplanktons to larger crustaceans and small fish (41); the deeper habitats also protect fish from severe skin damage (42). The causes of these vertical migrations during fish development suggest that fish living at the depths of 20–100 m may be good candidates to isolate violet-sensitive SWS1 pigments.

It is not possible to test the adaptive evolution of UV and violet pigments at this time, but we can still look into possible adaptive significances of SCABBARD and LAMPFISH. Scabbardfish spend a significant portion of their life at depths of 25–100 m, where intensity of UV light is reduced to a much greater extent than violet light (35), and feed mainly on smaller fish rather than zooplanktons (www.fishbase.org). These behavioral activities of scabbardfish might have been improved by replacing UV vision by violet vision. In contrast, despite their activities at much deeper depths, lampfish have retained UV vision. Feeding near the surface in twilight, lampfish can catch their major food source, copepods (43), which are almost transparent in visible light but are readily visible in UV light (44). Hence, the acquisition of violet vision in scabbardfish and the maintenance of UV vision in lampfish are associated with their foraging behaviors, suggesting possible adaptation of their respective short wavelength-sensitivities.

Materials and Methods

Samples and PCR Cloning of SWS1 Genes. High-molecular-weight DNAs of Northern lampfish (Stenobrachius leucopsaricus) and scabbardfish (Lepidopus fitchi) have been isolated from their body tissues by using a standard phenol-chloroform extraction procedure (48). The nucleotide sequences between exon 3 and exon 4 of scabbardfish and lampfish SWS1 genes were first cloned by the PCR (PCR) method by using degenerate forward primer (F: 5′-CAGTGTCTNCGTGGHCWGACTGGTAC-3′) and reverse primer (R: 5′-ACCATBAVACACCATCTTGTTGACAGATC-3′). The PCR products in the pBluescript SK(+) vector were sequenced by using a LI-COR 4300 automated DNA sequencer (LI-COR). To clone the remaining coding regions of the scabbardfish gene, inverse PCR was performed by using F: 5′-GTCACTGTGTGTGATGCTG-3′ and R: 5′-TGAAAGCTAAGCTT-3′. For the lampfish gene, F: 5′-GTTCCATGGTTGATGTGTG-3′ and R: 5′-GCAAGCAGGTGCATGATGGCTGGT-3′, as well as F: 5′-GCGATTACCATGGTTGATGTATG-3′ and R: 5′-CACGCTCACCCCTAATTTCGAC-3′ were used.

Genomic DNA Library Screening and Southern Hybridization. A genomic library of scabbardfish was constructed with Xhol-digested fIXIII vector and Sau3AI partially digested scabbardfish genomic DNA (10–20 kb). This library was screened by using the full-length SWS1 cDNA of scabbardfish and human (GenBank M13295).

For the Southern hybridization, approximately 5 μg per lane of HindIII-digested scabbardfish genomic DNA was electrophoresed on a 0.5% agarose gel and blotted onto a Hybond-n + nylon membrane (Amersham). The hybridization probe was first PCR-amplified from the entire scabbardfish SWS1 cDNA and then labeled with [α-32P]dATP. Hybridization was carried out at 55 °C, by using commercial protocol for Hybond-n + membrane. The hybridized membrane was washed at 55 °C in 1xSSC/0.1% SDS.
Spectral Analyses of SWS1 Pigments. The contiguous full-length SWS1 opsins for scabbardfish and lampfish were obtained by RT-PCR amplification by using species-specific primers: (i) F: 5'-TCCACACCAAGTTACATCTGGGGAAGGACTTCCACTT-3' and R: 5'-GCCGGCGCGCCACCTGACAGACCCACCTACAGTCTGGCTGCCG-3' (for scabbardfish); and (ii) F: 5'-AACAAGATCCATCCTAGGGGAAGGACTTCCACTT-3' and R: 5'-ACAGGGTGGAGAACTGAGATACTGATATACCCC-3' (for lampfish). These opsins were cloned into the vector expression plasmid pMT5 and were expressed in COS1 cells by transient transfection (17). The SWS1 pigments were regenerated by incubating the opsins with 11-cis-retinal (Storm Eye Institute) and purified by using immobilized 1D4 (The Culture Center) in buffer W1, 50 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 6.6), 140 mM NaCl, 3 mM MgCl₂, 20% (v/v) glycerol and 0.1% dodecyl maltoside. UV visible spectra were recorded at 20 °C by using a Hitachi U-3000 dual beam spectrophotometer. The SWS1 pigments were bleached for 3 min by using a 60 W standard light bulb equipped with a Kodak Wratten #3 filter at a distance of 20 cm. Data were analyzed by using Sigmaplot software (Jandel Scientific) (18).

Statistical Analyses. We considered 17 representative SWS1 pigments from a wide range of species: GOLDFISH (Carassius auratus: GenBank accession no. D85863), ZEBRAFISH (Danio rerio: AB087810), KILLIFISH (Lucania goodei: AF296735), LAMPFISH, SCABBARD, FROG (Xenopus laevis: U23463), SALAMANDER (Ambystoma tigrinum: AF390894), CHAMELEON (Anolis carolinensis: AF131492), GECKO (Gekko gekko: YA024356), PIGEON (Columba livia: AF149234), CHICKEN (Gallus gallus: M92039), ZEBRA FINCH (Gallus gallus: M92039), ZEBRA FINCH (Gallus gallus: M92039), CHAMELEON (Chamaeleo calyptratus: AF103968), coelacanth (AF131253), frog (L07770), chameleon (L31503) and bovine (M21606), we first constructed a rooted phylogenetic tree of the 17 SWS1 pigments. By using the NJ tree and others based on other molecules (7, 47), as well as morphological data (48, 49), we then constructed a composite tree topology (Fig. S1). Based on the composite tree topology, we inferred the amino acids of ancestral SWS1 pigments by using the computer program, PAML (22).

ONIOM (QM/MM) Analyses of LAMPFISH and Its Mutant. The coordinates of the amino acids, 11-cis-retinal, and water molecules of LAMPFISH and its Phe-86-deleted analog were obtained by applying homology modeling method (Modeller 9v7, www.salilab.org/modeller) to bovine rhodopsin (pdb code: 1U19) (50). The geometries were optimized at pure AMBER96 force field level (http://ambermd.org) after including H atoms and were further refined by hybrid quantum mechanical/molecular mechanical (QM/MM) calculations in the ONIOM (Our own N-layer Integrated molecular Orbital + molecular Mechanic) electronic embedding scheme (QM = B3LYP; MM = AMBER) (24). The QM portion consists of the 11-cis-retinal, Schiff-base N-H moiety, and Glu-113 along with hydrogen link atoms. In the gas phase, the protonated SBR in the QM portion is unstable and Glu-113 is thus protonated (Fig. S2). However, the protonated SBR becomes more stable under the electrostatic and polarizing influence of MM portion. However, the MM portion is more stable with deprotonated SBR. To evaluate the relative stabilities of unprotonated and protonated SBRs, we performed B3LYP/AMBER ground-state energy calculations (24). The B3LYP/AMBER pigments were evaluated from the TD-B3LYP/AMBER excitation energies at their B3LYP/AMBER-optimized structures (24).
Supporting Information
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Fig. S1. A composite tree topology of 17 vertebrate SWS1 pigments. The numbers besides branches show the total numbers of amino acid substitution that led contemporary KILLIFISH, LAMPFISH, and SCABBARD.
Fig. S2. The quantum mechanical (QM) model that was used for ONIOM (Our own N-layer Integrated molecular Orbital + molecular Mechanics) calculations. The circled H is bonded to (A) the carboxylic O of Glu-113 (unprotonated SBR) or (B) the Schiff-base N (protonated SBR).