Adaptive evolution of color vision of the Comoran coelacanth
(Latimeria chalumnae)

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ABSTRACT The coelacanth, a “living fossil,” lives near the coast of the Comoros archipelago in the Indian Ocean. Living at a depth of about 200 m, the Comoran coelacanth receives only a narrow range of light, at about 480 nm. To detect the entire range of “color” at this depth, the coelacanth appears to use only two closely related paralogous RH1 and RH2 visual pigments with the optimum light sensitivities (Amax) at 478 nm and 485 nm, respectively. The Amax values are shifted about 20 nm toward blue compared with those of the corresponding orthologous pigments. Mutagenesis experiments show that each of these coadapted changes is fully explained by two amino acid replacements.

The coelacanth, Latimeria chalumnae, is the sole surviving species of the old lineage of crossopterygian fishes that were abundant during the Devonian period. The morphology of the coelacanth has changed very little over 400 million years (1–3). This “living fossil” lives at a depth of about 200 m near the coast of the Comoros archipelago in the western Indian Ocean (4, 5). Living at a depth of 200 m, the Comoran coelacanths receive only a narrow range of color, at about 480 nm (6). To see in this photic environment, the retina of the coelacanth consists mostly of rods, with a much smaller number of cones (7, 8). The coelacanth has changed very little over 400 million years (1–3).

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MATERIALS AND METHODS

Genomic DNA Library Cloning and DNA Sequencing.

Genomic DNA was isolated from the Comoran coelacanth, which was obtained from the American Museum of Natural History, NY (tissue loan no. AMNH 59196). A genomic library of this coelacanth was constructed with BamHI-digested λEMBL3 vector and Sau3AI partially digested coelacanth genomic DNA (10–20 kb). By using bovine RH1 and human SWS1 cDNAs as probes, we screened about 1.4 × 10⁸ and 10⁷ recombinant plaques, respectively. From the first screening, we isolated two clones, ALc16 and ALc58, which represent two distinct functional opsins genes. From the second screening, we isolated two nonoverlapping clones, ALc17 and ALc01, which represent a pseudogene. These genomic clones were subcloned into pBluescript SK(−), and the nucleotide sequences were determined for both strands by the dye-fiber-chain-termination method (15).

Creation of Minigenes and Site-Directed Mutagenesis.

The five exons of each of the two functional opsin genes were PCR-amplified by using five sets of oligonucleotides. For ALc16, we used 5′-CCAAGGGAAATTCACCATGAGAACAGGGGTCC-3′ (forward) and 5′-TTACCTCCTAGGGTCCAGAAATACTCTAAAT-3′ (reverse) for exon 1, 5′-CCCTCTTCAAGGTCATGTTGTCTATCTGTT-3′ (forward) and 5′-GAGCTATCGATACCCATCGGAACTGCAT-3′ (reverse) for exon 4, and 5′-CTGAAATCTCTAGATATCATCGCCAGGAGGTTCTGCA-3′ (forward) and 5′-ACAAAGCTCAAGACATCTTGACGACGACGACG-3′ (reverse) for exon 3. The underlined sequences indicate restriction enzyme sites for cloning purposes. The extra 5′–6 nt at the 5′ end are to facilitate restriction enzyme digestion. For ALc58, we used 5′-CAAAACGAATTTCCACATGAGCCACGCGAAGA-3′ (forward) and 5′-CTACCTCTAGGGTTCGAAGAAACCTGTC-3′ (reverse) for exon 1, 5′-TTCCTTCTAGGAGTCAAAGCTCTCTTG-3′ (forward) and 5′-AAACCTCATCGTCCGAGAAAAGAAGAA-3′ (reverse) for exon 2, 5′-TACCACTCTGCTCTGGTACGCTCCTGTTGCAA-3′ (forward) and 5′-CCCTCAGGACGACATCTGGACGACGACGAC-3′ (reverse) for exon 3. The underlined sequences indicate restriction enzyme sites for cloning purposes. The extra 5′–6 nt at the 5′ end are to facilitate restriction enzyme digestion.
forward) and 5'-AAGAGCATTCGGGAACACGGTTGT-
TCAACAACAC-3' (reverse) for exon 4, and 5'-TCTAGT-
CCTAGGACTGATGATCACCACGTTG (forward) and 5'-GAAGTGGTCGACGCGGGCGAAACCTGACTGG-
A-3' (reverse) for exon 5. PCR was performed by using 30
cycles at 92°C for 45 sec, 55°C for 60 sec, and 72°C for 90 sec.
At each cycle, the duration of the extension reaction was
progressively extended by 3 sec. The PCR products were
digested by appropriate restriction enzymes and ligated into
pBluescript SK. Nucleotide sequences of the entire regions
of the minigenes were determined. We selected clones that
code identical amino acid sequences to those of the corre-
sponding sequences deduced from the genomic sequences.

Mutants were generated by using QuikChange site-directed
mutagenesis kit from Stratagene. All DNA fragments that
were subjected to mutagenesis were sequenced to rule out
spurious mutations.

Absorption Spectra. The two coelacanth minigenes were
ligated into an expression vector pMT, expressed in cultured
COS1 cells, and regenerated with 11-cis retinal, and the
resulting visual pigments were purified as described (16, 17).
UV-visible spectra were recorded at 20°C by using a Hitachi
U-3000 dual beam spectrophotometer. Visual pigments were
bleached for 3 min by using a 60 W standard light bulb
equipped with a Kodak Wratten no. 3 filter at a distance of 20
cm. Data were analyzed with SIGMAPLOT software (Jandel, San
Rafael, CA).

Sequence Data Analyses. The amino acid sequences of the
coeelacanth visual pigments were compared with those of other
vertebrate pigments. The root of the phylogenetic tree of these
pigments was determined by using goldfish LWS (GenBank
accession no. L11867), African clawed frog LWS (U90895),
chicken LWS (M62903), and human MWS (K03490–K03497)
pigments as the outgroup. The number (K) of amino acid
replacements per site for a pair of sequences was estimated by
K = In (1 - p), where p is the proportion of different amino
acids per site. Topology and branch lengths of the phylogenetic
tree were evaluated by using the neighbor-joining method (18).
The reliability of the neighbor-joining tree topology was
evaluated by the bootstrap analysis with 1,000 replications
(19).

The amino acid sequences of ancestral pigments were
evolved by using likelihood-based Bayesian method (20) with a
modified version of the Jones, Taylor, and Thornton (JTT; ref.
Among these, opsins cDNA as a probe, we isolated 12 positive phage clones. but no LWS/MWS genes. Shown). Thus, the coelacanth has RH1 and SWS1-like genes (Fig. 2). The Southern analysis revealed strongly hybridizing bands to SWS1 (24), and human LWS (24) opsin cDNAs separately. To determine what types of opsin genes the Comoran coelacanths possess, we hybridized Southern blots containing the coelacanth genomic DNA, digested with various restriction enzymes, to bovine RH1 (25), human SWS1 (24), and human LWS (24) opsin cDNAs separately. The Southern analysis revealed strongly hybridizing bands to bovine RH1 (23), human RH1 (23), and the equal-input models (20) of amino acid replacements.

RESULTS AND DISCUSSION

Coelacanth Opsin Genes. To determine what types of opsin genes the Comoran coelacanths possess, we hybridized Southern blots containing the coelacanth genomic DNA, digested with various restriction enzymes, to bovine RH1 (23), human SWS1 (24), and human LWS (24) opsin cDNAs separately. The Southern analysis revealed strongly hybridizing bands to the first two probes, but no band to the third probe (data not shown). Thus, the coelacanth has RH1 and SWS1-like genes but no LWS/MWS genes.

After genomic DNA library screening using bovine RH1 opsin cDNA as a probe, we isolated 12 positive phage clones. Among these, αLc17 containing exons 1 and 2 and αLc01 containing exons 3 and 4, were found to represent a pseudogene, designated as ωsws1Lc. As previously noted, the coelacanth does not have any LWS/MWS opsin genes. From an extensive screening of genomic DNA library with human SWS1 opsin cDNA as a probe, we could isolate only one SWS1 pseudogene. This strongly suggests that the coelacanth has also lost a functional SWS2 opsin gene.

The RH1Lc and RH2Lc opsins, deduced from the DNA sequences of rh1Lc and rh2Lc consist of 355 aa (Fig. 2). The pseudogene ωsws1Lc contains premature stop codons because of nonsense and frameshift mutations (Fig. 2). When the coding regions of rh1Lc and rh2Lc are compared, the proportion of identical nucleotides is 70%. When rh1Lc and rh2Lc are compared with bovine rh1, the sequence similarities are given by 76% and 71%, respectively. Thus, rh1Lc is more closely related to bovine rh1 gene than to rh2Lc, suggesting that rh1Lc and rh2Lc were duplicated before the vertebrate radiation.

Evolution of Coelacanth Visual Pigments. Fig. 3 shows the rooted phylogenetic tree of RH1Lc, RH2Lc, ψβLc, deduced from ωsws1Lc, and RH1, RH2, SWS1, and SWS2 pigments in other vertebrates whose Amax values have been directly determined. The bootstrap values of forming RH1, SWS1, and SWS2 clusters are 99%, 100%, and 100%, respectively, and are highly reliable, but that for RH2 cluster is only 81%. Note that RH1 cluster includes a wide range of vertebrates, from lamprey to mammals, and furthermore, the bootstrap value of forming the cluster of RH1 and RH2 groups is 100%. Thus, RH1 and RH2 clusters consist of two sets of paralogous pigments. These observations show that RH1Lc, RH2Lc, and ψβLc belong to the RH1, RH2, and SWS1 clusters, respectively.

Within the SWS1 cluster, ψβLc is most closely related to mammalian opsins, but this “misclassification” is probably caused by the much faster evolutionary rate of ωsws1Lc, a characteristic of pseudogenes. Fig. 3 suggests that, in the RH1 cluster, American chameleon (P491) pigment is not closely related to chicken (P503) pigment. This unexpected tree topology arises because of an accelerated rate of amino acid replacements in the former pigment in the rodless American chameleon (13). In the RH2 cluster, the American chameleon (P495) pigment is more closely related to the chicken pigment than to the gecko pigment. This may be explained not only by the accelerated evolutionary rate of the gecko pigment but also by the decelerated rate of the chicken pigment. This can be seen by making pairwise comparisons of chicken (P508), gecko (P467), and American chameleon (P495) pigments with an appropriate outgroup. For example, although they are not statistically significant, chicken (P508) pigment has shorter branch lengths than gecko (P467) and American chameleon (P495) pigments (Table 1). The branch length, 0.149, for gecko (P467) pigment is significantly longer than 0.003 for American chameleon (P495) pigment (Table 1).

It is widely accepted that tetrapods originated from lobe-finned fish, which include the coelacanth (25). According to
this scenario, RH1Lc and RH2Lc pigments should be more closely related to tetrapod pigments than to ray-finned fish pigments within each orthologous group. Although the opsin sequences alone are not sufficient to resolve the divergence of the coelacanths, tetrapods, and bony fishes (Fig. 3), there is other considerable evidence to support a common ancestor for tetrapods and coelacanth (25). But, if the coelacanth is more closely related to tetrapods than to ray-finned fishes, then amino acid replacements that occurred in the early stages of the coelacanth evolution are expected to be shared more often with tetrapods than with ray-finned fishes. Such shared amino acid replacements can be traced by inferring the amino acid sequences of the ancestral RH1 and RH2 pigments. Applying the JTT model of amino acid replacements, we inferred the amino acid sequences of all ancestral RH1 and RH2 pigments. Then, by comparing the amino acids of RH1Lc and RH2Lc pigments with those at the corresponding sites of the orthologous ancestral pigments, 20 shared amino acid replacements can be identified (Fig. 4). Among these, 13 favor the closer relationship of the coelacanth and tetrapods, whereas 7 disfavor the relationship. Even when the coelacanth pigments are assumed to be more closely related to ray-finned fishes than to tetrapods, the ratio between the favorable and unfavorable amino acid replacements becomes 12:7. Similar ratios were obtained with the Dayhoff and equal-input models of amino acid replacements (data not shown). Although none of the ratios is significantly different from 1:1, they do suggest a closer relationship between the coelacanth and tetrapods. Of interest, the rh1 genes of the coelacanth and tetrapods contain four introns at identical positions, whereas those of ray-finned fishes contain no introns, having lost them during evolution (26). These observations are compatible with the notion that the coelacanth is more closely related to tetrapods than to ray-finned fishes.

Absorption Spectra of RH1Lc and RH2Lc Pigments. The spectral sensitivity of visual pigments usually is evaluated directly by expressing opsin cDNAs in cultured cells (16, 17). However, because the retina of the coelacanth is not available...
to us, the 5’- and 3’-flanking regions and the four intron segments of \( rh_{1Lc} \) and \( rh_{2Lc} \) were deleted by using recombinant DNA techniques (see Materials And Methods). The resulting minigenes containing only the five exon sequences were ligated into an expression vector pMT, expressed in COS1 cells, and reconstituted with 11-cis retinal. The \( RH_{1Lc} \) and \( RH_{2Lc} \) pigments constructed in this way were shown to have \( \lambda_{max} \) values of 485 ± 3 nm and 478 ± 1 nm, respectively (Fig. 5). The latter \( \lambda_{max} \) of the cone-specific pigment corresponds to the previously described pigment with a \( \lambda_{max} \) of 473 nm (9), suggesting that the coelacanth pigment described by Dartnall (9) might have been sampled from a rare cone photoreceptor cell.

With the exception of gecko (P467) pigment, most RH1 and RH2 pigments have \( \lambda_{max} \) values of about 500 nm (Fig. 3). Compared with them, the \( \lambda_{max} \) values of \( RH_{1Lc} \) and \( RH_{2Lc} \) pigments are about 20 nm blue-shifted. The fascinating part of the two \( \lambda_{max} \) values (478 nm and 485 nm) is that they correspond to the narrow range of light around 480 nm in the Comoran coelacanth’s natural photic environment (6). Thus, the \( RH_{1Lc} \) and \( RH_{2Lc} \) pigments have coevolved to detect two edges of the available light spectra so that the coelacanths can distinguish the entire range of “colors” available to them. Because the coelacanth retina has both rods and cones (7, 8), the finding of a rod-specific \( RH_{1Lc} \) pigment and at least one cone-specific pigment is expected. However, it was unexpected to find that the coelacanth uses RH2 pigment instead of SWS1 or SWS2 pigments for its blue vision. It has been demonstrated that color vision is possible with only rods and a single class of cones in cats. By using rods and blue-sensitive cones simultaneously, cats can distinguish wavelengths in the range 440–500 nm at twilight (27). Evidently, the coelacanths use rod-specific \( RH_{1Lc} \) pigments and cone-specific \( RH_{2Lc} \) pigments to visualize the entire, albeit limited, range of color available to them.

**Adaptive Mechanisms of \( RH_{1Lc} \) and \( RH_{2Lc} \) Pigments.** How did \( RH_{1Lc} \) and \( RH_{2Lc} \) pigments achieve the blue shifts? It is known that amino acid changes E122Q and A292S in the bovine (P500) pigment shift the \( \lambda_{max} \) of 485 nm (29) toward blue, respectively. Within the \( RH_{1Lc} \) cluster, the two amino acid replacements E122Q and A292S occurred in the coelacanth after its divergence from other organisms (Fig. 3, branch a). Our mutagenesis analysis shows that single mutants Q122E and S292A and a double mutant Q122E/S292A of \( RH_{1Lc} \) pigment have \( \lambda_{max} \) values of 495 ± 2 nm, 493 ± 1 nm, and 511 ± 2 nm, respectively (Fig. 5). Thus, E122Q and A292S together can cause a 25 nm blue-shift in the \( \lambda_{max} \) and fully explain the observed \( \lambda_{max} \) of \( RH_{1Lc} \) pigment.

The blue-shifted \( \lambda_{max} \) of \( RH_{2Lc} \) pigment also is explained by two amino acid replacements, E122Q and M207L. Q122 is shared by the RH2 pigments of coelacanth, gecko, chicken, and American chameleon, and the E122Q replacement occurred before the divergence of coelacanth and tetrapods (Fig. 3, branch b). Figs. 3 and 4 clearly show that amino acid replacements E122Q in \( RH_{1Lc} \) and in \( RH_{2Lc} \) occurred independently from each other. With the exception of \( RH_{2Lc} \) pigment, all RH1 and RH2 pigments have M207, whereas virtually all SWS1 and SWS2 pigments have L207. Thus, L207 is associated with the pigments with blue-shifted \( \lambda_{max} \) values and M207L is suspected to cause a blue-shift in the \( \lambda_{max} \) of \( RH_{2Lc} \) pigment. M207L occurred in \( RH_{2Lc} \) pigment after its divergence from the rest of RH2 pigments (Fig. 3, branch c). The inferred amino acids of the ancestral pigments support these amino acid replacements at branches b and c in Fig. 3. Mutants Q122E, L207M, and Q122E/L207M of \( RH_{2Lc} \) pigment have \( \lambda_{max} \) values of 491 ± 1 nm, 484 ± 1 nm, and 499 ± 1 nm, respectively (Fig. 5). Thus, E122Q and M207L can cause a 21 nm blue-shift in the \( \lambda_{max} \) and fully explain the adaptation of \( RH_{2Lc} \) pigment. As already noted, the gecko RH2 pigment has a \( \lambda_{max} \) value of 467 nm (Fig. 3). By constructing chimeric pigments, the cause of this blue-shift in the \( \lambda_{max} \) was previously localized somewhere between transmembrane domains I and III (30).

The present analysis strongly suggests that E122Q in transmembrane domain III (Fig. 2) is a major cause of the blue shift in the \( \lambda_{max} \) of the gecko RH2 pigment.

**CONCLUSIONS**

The molecular mechanisms of color vision in vertebrates may be elucidated in three steps: (i) cloning and characterization of the opsin genes; (ii) identification of amino acid changes that are potentially important in shifting the \( \lambda_{max} \) of the pigments by using phylogenetic inferences; and (iii) determination of the actual effects of these mutations identified in the second step on the shifts in the \( \lambda_{max} \) of visual pigments by using the in vitro assays (12–14). By so doing, we identified the potentially important amino acid replacements and tested specific hypotheses on the molecular mechanisms of adaptive evolution in the Comoran coelacanth.

The Comoran coelacanth evolved rod-rich retinas to adapt to a depth of about 200 m, where only a narrow range of colors at around 480 nm reaches from the surface. To visualize the entire range of available colors, coelacanths appear to have lost SWS1, SWS2, and LWS/MWS pigments and modified \( RH_{1Lc} \) and \( RH_{2Lc} \) pigments so that they can detect the two edges of the available light spectra, 478 nm and 485 nm. Our mutagenesis analyses based on this evolutionary hypothesis demonstrate that the coelacanth accomplished the coadaptations of \( RH_{1Lc} \) and \( RH_{2Lc} \) pigments to its unique habitat by two independent sets of amino acid replacements E122Q/A292S and E122Q/M207L, respectively.

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