Conserved Proline Residue at Position 189 in Cone Visual Pigments as a Determinant of Molecular Properties Different from Rhodopsins†

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ABSTRACT: To identify the amino acid residue(s) responsible for the difference in the molecular properties between rod and cone pigments, we have prepared chicken green mutants where each of the residues (Val77, Gly144, and Pro189) completely conserved in the cone pigments was replaced with the residue in the rod pigment rhodopsin. Among the mutants, the P189I mutant showed an expression level in cultured HEK293 cells and a thermal stability higher than did the wild-type chicken green. The mutation caused a reduced decay rate of the meta II intermediate, while the mutation of the wild-type chicken rhodopsin at position 189 (I189P) resulted in an increased decay rate. The additional mutation at position 122, the previously reported site where the amino acid residue is one of the determinants of the meta II decay rate, converted the meta II decay rate into that observed in the wild-type chicken rhodopsin. These results suggest that the difference in the meta II decay rate between the chicken green and rhodopsin is due to the difference in the amino acid residues at positions 189 and 122. The completely conserved nature of proline at position 189 could provide a clue to the molecular evolution of the pigments.

The visual transduction process in photoreceptor cells begins with photon absorption by a visual pigment, which is a member of the family of G-protein-coupled receptors and contains 11-*cis*-retinal as the light-absorbing chromophore (*3*). In most vertebrates, different types of visual pigments are present in the rod and cone photoreceptor cells, where they mediate vision under twilight and daylight conditions, respectively. On the basis of the similarity in the primary structure, vertebrate visual pigments are classified into four groups of cone visual pigments and a single group of rod visual pigment rhodopsins (*4*). Phylogenetic analysis indicated that an ancestral pigment first diverged into four groups of cone visual pigments and the rhodopsins diverged later from one of the groups of the cone visual pigments including the chicken green-sensitive pigment (*4*). Biochemical investigations revealed that the cone visual pigments exhibit a common motif of molecular properties different from that of the rhodopsins (*5*−*7*). Therefore, identification of the amino acid residue(s) responsible for the difference in the molecular properties between the rod and cone visual pigments is important for furthering our understanding of the functional diversity of the visual pigments.

There are at least two criteria for identifying the amino acid residues. One is based on the chemical characteristics of the amino acid residues, and the other is based on the conserved nature in the primary sequences. On the basis of the facts that the cone visual pigments have many basic amino acid residues while the rhodopsins have acidic residues, we previously selected the amino acid positions where the amino acid residues in the cone visual pigments have an electric property different from those in the rhodopsins (*8*). Mutational experiments revealed that the glutamic acid at position 122 is one of the determinants of the molecular properties unique to rhodopsins (Figure 1). That is, the replacement of E122 in the rhodopsin with the corresponding residues in the cone pigments accelerated the decay rate of the meta II and the regeneration rate of pigment from 11-*cis*-retinal and opsin, which would be related to the difference in the photosensitivity and dark adaptation of the photoreceptor cells, respectively (*9*). However, complete conversions of the molecular properties were not achieved in these experiments (*8, 10*), and one of the possibilities was that other residues responsible for the difference in the molecular properties are present.

In the present study, we have tried to determine the amino acid residue(s) from the other criterion based on the conserved nature in the primary sequences. For this purpose, we selected the amino acid positions where all the known cone visual pigments have the same amino acid residues that are different from those of the rhodopsins. Because three positions at 77, 144, and 189 meet this criterion, we designed and expressed the site-directed mutants of chicken green where each of the residues (Val77, Gly144, and Pro189) was replaced with the corresponding residue in rhodopsin. The results clearly showed that the proline residue at position 189 is responsible for the unstable properties of the chicken green opsin and meta intermediates of the chicken green. From these findings, the molecular mechanism underlying the difference in the molecular properties between the rod and cone pigments and the role of the proline at this position on the functional diversity will be discussed.

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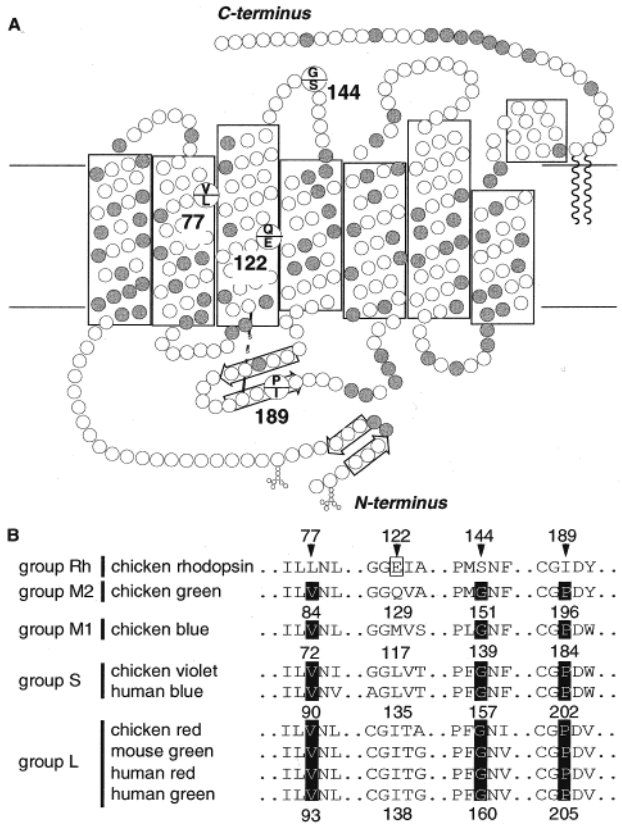

FIGURE 1: Comparison of amino acid sequences of chicken green and rhodopsin. (A) The secondary structure of chicken green and rhodopsin. The transmembrane topology is based on the 3D-structure of bovine rhodopsin (*21*). Residues common to chicken green and rhodopsin and different between them are denoted by the white and gray circles, respectively. Residues of chicken green and rhodopsin that were mutated in this study are denoted by bovine rhodopsin numbering system in the upper- and bottom-sides of the circle, respectively. (B) The amino acid sequences in rod and cone visual pigments at certain residues discussed in this paper. The conserved residues in all the known cone pigments are shown as white characters on a black background. The residue at position 122 has previously been reported as the residue responsible for the different molecular properties between the rod and cone visual pigments (*8*). The residue numbers of each pigment are denoted above the sequence (human red and green numbering are below the sequence), respectively. The classification of visual pigments according to the previous study (*4*) is shown at the left side.

MATERIALS AND METHODS

Sample Preparation. The wild-type and mutants of chicken green were expressed in the HEK293 cell lines (*11*, *12*) as previously reported (*13*). For purification, the cDNA of chicken green and rhodopsin were tagged by the monoclonal antibody Rho 1D4 epitope-sequence (ETSQVAPA) (*14*). The resulting cDNA was fully sequenced before introducing it into the expression vector (*15*). The method for the construction of the P189I mutant gene of the chicken green was previously described (*8*). To reconstitute a photoreactive pigment, the expressed protein was incubated with 11-*cis*-retinal overnight at 4 °C. The pigment was then extracted in 1% DM[1] or 0.75% CHAPS/0.8 mg/mL PC in the buffer (50 mM HEPES, 140 mM NaCl, and 1 mM $MgCl_2$, pH 6.5) and purified with an antibody-conjugated column. The purification method under the conditions where the pigment was extracted with 1% DM was previously reported (*16*). A similar method was applied under the conditions where the pigment was extracted with 0.75% CHAPS/0.8 mg/mL PC.

Spectrophotometry. The UV−vis absorption spectra were recorded using a Shimadzu Model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. The system for the measurements of the absorption spectra was previously reported (*17*). The sample was irradiated with light from a 1-kW tungsten halogen lamp (Rikagaku Seiki) which had been passed through a glass cutoff filter (VO56, VO54, and VY52; Toshiba).

Estimation of Expression Level. The regenerated pigments were extracted by 1% DM. The amounts of the extracted pigments were estimated from the absorbances at the maxima in the difference spectra before and after irradiation (>500 nm) for 5 min in the presence of 10 mM neutralized hydroxylamine.

Thermal Stability of Expressed Opsin. The membrane fraction containing the expressed opsin was prepared from HEK293 cells by a standard sucrose flotation method (*18*). To monitor the thermal stability of the expressed opsin, the membrane fraction was incubated at 37 °C for 24 h, followed by regeneration of the opsin by adding an ethanol solution of 11-*cis*-retinal in the fraction. The regenerated pigment was then extracted with 1% DM, and the amount of opsin functionally reconstituted with 11-*cis*-retinal was estimated by the method similar to that used for the estimation of the expression level of opsin.

Decay Rates of Meta II. To monitor the decay rates of meta II at 2 °C, the pigment sample purified in the buffer containing 0.02% DM was used. To monitor the decay rates at −8 °C, the sample purified in the CHAPS/PC mixture was used after the addition of glycerol at a final concentration of 56%. The decay rate of meta II was monitored by continuous measurements of the absorption spectra after irradiation of the sample with an orange light for 30 s.

RESULTS

We first expressed the V77L, G144S, and P189I mutants of chicken green using the HEK293 cell line. All the mutant proteins were successfully expressed and bound to 11-*cis*-retinal to form pigments with absorption maxima similar to the wild-type chicken green. During the course of the experiments, we noticed there were various expression levels between the wild-type and mutant proteins. The expression yield of chicken green was 2−3 times lower than that of the chicken rhodopsin (Figure 2A). It was frequently reported that cone visual pigments exhibited a less efficient expression than the rhodopsins in a cultured cell system (*18*). Among the three mutants of chicken green, V77L and G144S exhibited expression yields less than that of the wild-type, which was often seen in the expression experiments of the mutant proteins. On the other hand, P189I showed a yield about 2.5 times greater than that of the wild-type, and it was comparable to that of the wild-type chicken rhodopsin. There could be several mechanisms that regulate the expression

[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; DM, dodecyl β-D-maltoside; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CHAPS; 3-{(3-cholamidopropyl)dimethylammonio}-1-propanesulfonate; PC, L-α-phosphatidylcholine.

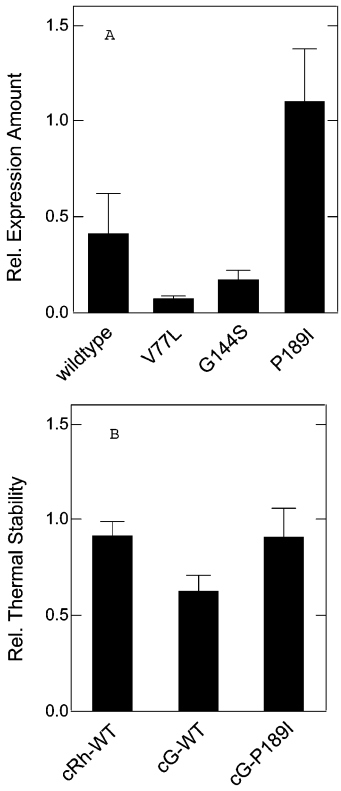

FIGURE 2: Expression level and thermal behavior of the chicken green mutants. (A) Normalized expression level of chicken green. Expressed pigments are reconstituted with 11-*cis*-retinal followed by extraction with 1% DM. The amount of the extracted pigments was estimated by the absorbances at the absorption maxima in the difference spectra before and after irradiation (>480 nm) for 5 min. The expression levels are normalized to that of the simultaneously expressed chicken rhodopsin. (B) Thermal stabilities of chicken green and rhodopsin. Membrane fractions of the cells containing expressed pigments were collected by standard sucrose flotation methods (*18*). The fractions were incubated for 24 h at 37 °C and then reconstituted with 11-*cis*-retinal followed by extraction with 1% DM. The amount of each extracted pigment was normalized to that before incubation.

yield of visual pigments. One of the mechanisms is that the expression yield could be regulated by thermal stability of the protein moiety, because protein moieties of cone visual pigments including chicken green are less stable than those of rhodopsins (*19*). To examine whether the thermal stability of chicken green is enhanced by the P189I mutation, we estimated the amount of the regenerated pigments from the opsins of the wild-type and P189I mutant of chicken green and the wild-type of rhodopsin after their incubation at 37 °C for 24 h (Figure 2B). As is the case of the wild-type rhodopsin, the amount of the regenerated P189I mutant did not change after the incubation, whereas that of the wild-type chicken green decreased to about 60%. These results suggest that the proline residue at this position affects the thermal stability of the chicken green.

Because the proline residue at this position regulated one of the properties of the cone pigments, we further examined the role of the proline residue in the other properties of the cone pigments different from the rhodopsins. In a previous study, we examined the decay rate of meta II of rhodopsin, which would be related to the photosensitivity of the photoreceptor cells and found that the mutation at position 122 caused the rapid decay of meta II. Thus, we examined if the proline residue fulfils the role similar to the residue at position 122 on the thermal decay rate of meta II. We prepared Q122E and Q122E/P189I double mutants of chicken green as well as P189I (Figure 3). All the mutants were normally expressed and bound to the retinal, while the absorption spectra were altered by the mutation. Namely, the λ_{max} value of P189I shows a slight blue shift, while Q122E shows a 20 nm red shift. The Q122E/P189I double mutant shows a slight blue shift compared to Q122E, suggesting the additive effects of the mutant sites on the absorption spectrum of the chicken green. Using this sample, we examined the decay rates of meta II of these mutants by monitoring the changes in the absorbance at 380 nm due to the conversion of meta II to meta III (Figure 4). The meta II of the wild-type chicken green was converted to meta III at a rate constant 70 times greater than that of the wild-type rhodopsin. The mutation at position 122 (cG-Q122E) decelerated the decay several times; however, this change was not so as prominent compared to the mutation at position 189. Namely, the single mutation at the position 189 (cG-P189I) remarkably altered the decay rate (27 times slower than that of the wild-type green). Double mutations at both positions 122 and 189 showed almost the same decay rate of meta II to the wild-type rhodopsin. Therefore, the residues at positions 122 and 189 change the decay rate of meta II of the chicken green closer to that of rhodopsin. The mutations at positions 77 and 144 of the chicken green caused no significant change in the decay rate of meta II (Table 1).

It is of interest whether the decay rates of meta II in the rhodopsin mutants are changed, if the residues at positions 122 and 189 were replaced with the corresponding residues present in the chicken green. From the experimental results obtained from the chicken green mutants, we expected that the mutant exhibited a decay of meta II faster than the wild-type rhodopsin, when it was irradiated under conditions similar to those of the chicken green. In fact, a single mutant at position 189 (cRh-I189P) shows about a two times faster decay of meta II than the wild-type rhodopsin. While this is not as prominent compared to the mutation at position 122 (cRh-E122Q) that shows about a 20 times faster decay, the decay rate in the E122Q/I189P double mutant is interesting. However, irradiation of the mutant at −8 °C caused the formation of a mixture mainly containing meta I, and it was difficult to precisely monitor the decay process to meta III due to the overlap of the spectrum between meta I and meta III. That is, replacements of the residues at positions 122 and 189 shifted the equilibrium between meta I and meta II toward meta I so that only a small amount of meta II was present in the irradiated sample, thereby resulting in less spectral shift during the conversion to meta III. Therefore, we searched for the experimental conditions where the decay process from meta II to meta III was precisely observed. It is well-known that the detergent DM shifts the equilibrium between meta I and II toward meta II (*20*), so we tried to observe the meta II decay process using the sample solubilized in this detergent (Figure 5). Under this condition, mutants E122Q and I189P showed a meta II decay rate about eight and five times faster than that of the wild-type meta II, respectively. Furthermore, the mutants at both positions (cRh-double) exhibited a decay rate 22 times faster than that of the wild-type. These results clearly showed that the amino acid residues at both positions regulate the decay rate of meta

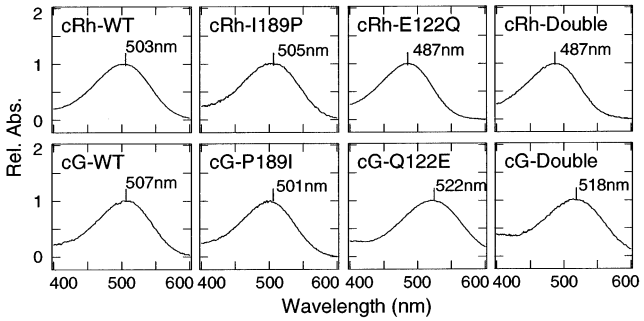

FIGURE 3: Absorption spectra of chicken green and rhodopsin mutants. UV−visible absorption spectra were recorded after the purification in the buffer containing 0.02% DM (chicken rhodopsin and its mutants) and in 0.75% CHAPS (chicken green and its mutants). The λ_{max} values are indicated for each spectra.

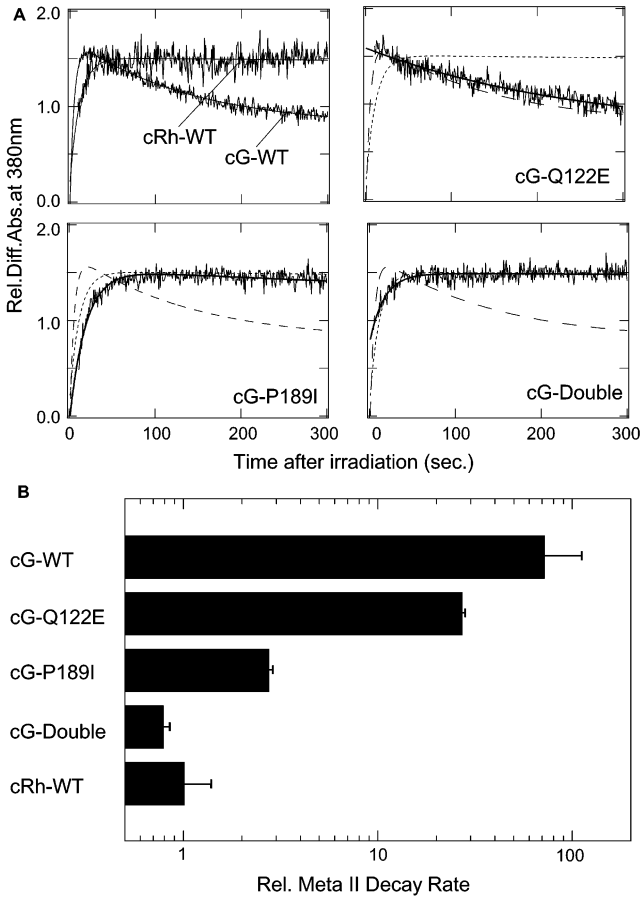

FIGURE 4: Thermal conversion rates of meta II to meta III of chicken green. (A) Purified chicken green and its mutants in the buffer containing 0.75% CHAPS as a detergent were cooled to −8 °C, followed by irradiation with an orange light for 6 s. The absorption spectra were then continuously recorded at the same temperature. Absorbance changes at 380 nm are plotted versus the incubation time after irradiation. Lines are the single-exponential curves with time constants of 100 (cG-WT), 210 (cG-Q122E), 4100 (cG-P189I), 6700 (cG-Q122E,P189I), 31000 (cRh-WT) s. The broken and dotted lines in each mutants show the chicken green and rhodopsin wild-types, respectively. (B) Rate constants of wild-type green and its mutants are represented relative to wild-type rhodopsin. Error bars represent the standard deviations estimated from three independent experiments using different sample preparations.

II even in rhodopsin. However, the decay process of meta II of the chicken green was too fast to be monitored under the conditions, indicating that the replacements of the amino acid residues at these position are insufficient for the complete conversion of rhodopsin into the chicken green.

Table 1: Parameters of the Chicken Green and Rhodopsin Mutants

sample[a]	λ_{max} (nm)	Meta II decay rate constant[c]
cG-WT	505 (1)[d]	70 (42)
cG-V77L	505 (2)	97 (11)
cG-G144S	505 (2)	92 (4.5)
cG-P189 I	501 (1)	2.7 (0.19)
cG-Q122E	523 (1)	27 (1.2)
cG-Double	518 (1)	0.63 (0.22)
cRh-WT	503 (1)	1.0 (0.39)
sample[b]	λ_{max} (nm)	Meta II decay rate constant
cRh-WT	503	1.0 (0.73)
cRh-I189P	505 (1)	5.0 (1.2)
cRh-E122Q	487 (1)	8.3 (1.1)
cRh-Double	485 (2)	22 (2.9)

[a] Samples are purified in the buffer containing CHAPS/PC, and the meta II decay rates are measured at −8 °C after the addition of glycerol at a final concentration of 56%. [b] Samples are purified in the buffer containing 0.02% DM, and the meta II decay rates are measured at 2 °C. [c] The relative decay rates of meta II were calculated as the initial rate of the meta II decay, and the decay rates for wild-type rhodopsin under the same condition were normalized to 1. [d] Values in parentheses are calculated standard deviations.

DISCUSSION

In the present study, we examined the roles of the three residues at positions 77, 144, and 189, which are completely conserved in cone visual pigments, in order to determine the residue(s) responsible for the different molecular properties between cone pigments and rod pigments (rhodopsins). Replacement of the proline at position 189 with the corresponding residue in rhodopsin affected the thermal stability of the protein moiety of the chicken green and the decay rate of meta II, whereas those of the valine and the glycine at positions 77 and 144, respectively, had no effect on the decay rate of meta II. The double mutation of 122 and 189 caused a remarkably slow decay of meta II of the chicken green, while the meta II decay of chicken rhodopsin was accelerated by the reciprocal mutation. Thus, it is clear that at least one residue out of the three plays a role in regulating the different molecular properties between the rod and cone visual pigments. Now it is significant to discuss the regulating mechanism of these molecular properties as well as the physiological and evolutional role of the site during the course of visual pigment evolution.

According to the three-dimensional structure of bovine rhodopsin (*21*), the residues S186 to D190 including I189 form one of the strands of an antiparallel β-sheet with that constituted by the residues R177 to E181, and this β-sheet covers the retinal chromophore from the extracellular side. Our recent study using FTIR spectroscopy with the aid of site-directed mutagenesis also showed that P189 is situated near the chromophore even in chicken green (*22*). The FTIR band location suggested that the C═O group of G188 forms a strong hydrogen bond that would be included in the β-sheet structure. Therefore, the environment of the residues at position 189 is similar between the chicken green and rhodopsin in the presence of 11-*cis*-retinal as a chromophore. This conclusion is supported by the fact that the spectroscopic properties of chicken green are similar to that of rhodopsin (*6*). In chicken green, the replacement of V77 and G144 with

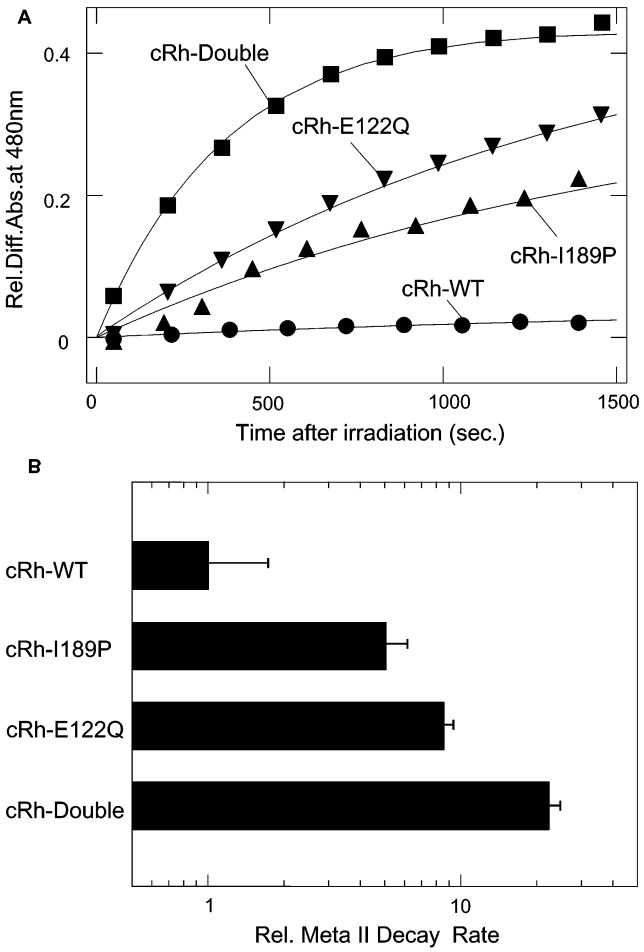

FIGURE 5: Thermal conversion rates of meta II to meta III of chicken rhodopsin. (A) Purified chicken rhodopsin and its mutants in the buffer containing 0.02% DM as a detergent were cooled to 2 °C, followed by irradiation with an orange light for 30 s. Absorption spectra were then continuously recorded at the same temperature. Absorbance changes at 480 nm of the sample are plotted versus the incubation time after irradiation. Lines are the single-exponential curves with time constants of 1600 (cRh-WT), 1700 (cRh-I189P), 1560 (cRh-E122Q), 380 (cRh-E122Q,I189P) s and different amplitudes of 0.07 (cRh-WT), 0.33 (cRh-I189P), 0.43 (cRh-E122Q), and 0.52 (cRh-E122Q,I189P) reflecting the shift in the thermal equilibrium between meta II and meta III. Note that time constants of the meta II decay of the wild-type and single mutants I189P and E122Q in the buffer containing 0.75% CHAPS are 2400, 950, and 110 s, respectively. (B) Rate constants of rhodopsin and its mutants are represented relative to wild-type rhodopsin. Error bars represent the standard deviations estimated from three independent experiments using different sample preparations.

the corresponding residues of rhodopsin (L and S) does not show significant change in the absorption spectrum. In the 3D structure of bovine rhodopsin, L77 locates in the cytoplasmic half of helix 2 and does not interact with the residues presented in the other helices by a hydrogen bonding network or hydrophobic interaction. S144 is situated in the second cytoplasmic loop which is part of the putative interaction region with transducin (*23*). Thus, both residues probably do not show any effect directly or indirectly on the chromophore configuration in the chicken green as well as rhodopsin. On the other hand, the replacement at position 189 in the chicken green and rhodopsin, the absence of proline, or the presence of isoleucine causes a slight but significant blue shift in the absorption spectrum of both pigments. In bovine rhodopsin, the CG1 carbon atom of the I189 is located at 3.8 Å from the 9-methyl group of the retinal chromophore and the peptide amide of I189 is located at 4.6 Å from the 9-methyl group. Thus, the replacement of the amino acid residue at position 189 may change the environment around the 9-methyl of the chromophore by a steric interaction, which perturbs the chromophore configuration or its electronic state. In a previous study, we reported that the replacement of the residue at the site of 122 between chicken green and rhodopsin induced an ~20 nm shift in the absorption spectrum (*8*). Because the δ-carboxyl of E122 is situated 3.5 Å from the β-ionone ring of the retinal chromophore, this would be due to the effect on the electronic state of the chromophore by the electrostatic interaction. These facts suggest that the mutation at position 189 induces less, if any, effect on the chromophore configuration or its electronic state than the mutant at position 122.

While the protein structure near the chromophore is similar between the chicken green and rhodopsin in the presence of the chromophore, it may be different in the absence of chromophore. For example, in the absence of the chromophore, the structure near the 122 and 189 positions may be different between the chicken green and rhodopsin. In fact, the regeneration rates of the chicken green and rhodopsin are affected by the replacements of these residues, but the effects of the mutations at these residues are different between the chicken green and rhodopsin (Kuwayama et al., manuscript in preparation). It has been reported that the 4−5 loop region containing I189 of bovine rhodopsin is important for protein folding, since deletion of the mutant at the positions 189, 190 and various mutants of this region cannot correctly fold (*24*). In our system, the expression level of the chicken green mutant P189I is higher than that of wild-type. On the other hand, that of the chicken rhodopsin mutant I189P is lower than that of the wild-type rhodopsin (data not shown). As shown in Figure 2, we demonstrated that the apoprotein of the chicken green becomes more stable by the mutation at this position. Thus, it is likely that the change of the thermal stability of the expressed pigment is one of the mechanisms that regulates the expression level, although other mechanisms based on mRNA stability (*25*), rate of protein translation (*26*), correct protein folding (*27*, *28*), and protein transport (*28*) are also important. In this context, it is interesting to discuss whether the presence of proline or absence of isoleucine destabilizes the protein moiety of the chicken green and rhodopsin. Because replacement of the isoleucine for alanine or valine caused no change in the expression level, it is likely that the presence of proline is crucial for the destabilization of the chicken green and rhodopsin I189P mutant. The proline residue destabilizes the β-sheet structure if it is present in the β-sheet structure (*29*). Because it is likely that the 4−5 loop of the chicken green opsin forms a β-structure (*22*), P189 could destabilize the structure, resulting in instability of the protein moiety. It should be noted that the stabilities of the protein moieties of chicken green and rhodopsin did not change apparently if they have the 11-*cis*-retinal chromophore with them. This fact suggests that the structure around the chromophore binding site in the presence of the chromophore is somewhat different from that in the absence of the chromophore. Namely, the effect of the amino acid residue at position 189

is larger in the absence of chromophore than in the presence of chromophore. It is likely that the local structure of opsin around this 4−5 loop region in the absence of chromophore is looser than that in the presence of chromophore. Thus, the opsin in the absence of chromophore would be more susceptible to the mutation at position 189. The detailed investigation of the mechanism by which the residue at position 189 regulates the molecular properties of the visual pigments would provide a clue to the elucidation of the role of β3 and β4 structure discovered in the crystal structure of rhodopsin.

We estimated the decay rates of meta II of rhodopsin by monitoring the changes in absorbance at 480 nm due to the formation and decay of meta III. This is because the meta II decomposes directly into opsin and all-*trans*-retinal in addition to the conversion to meta III, so that the changes in absorbance at 380 nm would be rather complicated reflecting the formation and decay of at least two species, the meta II and the extricating retinal. Because the reaction scheme under investigation contains three components (meta II, meta III, and opsin plus all-*trans*-retinal), the changes in absorbance at 480 nm after the irradiation of rhodopsin were fit by two sequential single-exponential curves. The two apparent rate constants estimated from the fitted curves are the combination of the microscopic rate constants for meta II-to-opsin plus all-*trans*-retinal conversion, meta II-to-meta III conversion and meta III-to-opsin plus all-*trans*-retinal conversion (*30*). Meta II decays with the larger rate constant, which is the sum of the microscopic rate constants for the conversion from meta II to opsin plus all-*trans*-retinal and that from meta II to meta III. Meta III forms with the larger rate constant and decays with the smaller one, which is the microscopic rate constant for the conversion from meta III to opsin plus all-*trans*-retinal. Formation of opsin and all-*trans*-retinal can be expressed by these two constants. In this model, these apparent rate constants can be monitored as absorbance change at any wavelengths. Thus, we can estimate the apparent decay rate of meta II from the fitted curve of the changes in absorbance at 480 nm. The decay process of meta II can be expressed mainly by the larger apparent rate constant, even if a back-reaction from meta III to meta II is considered in the reaction scheme (*31*). In fact, the larger apparent rate constant estimated from the absorbance change at 380 nm, which reflects mainly the decay of meta II and formation of all-*trans*-retinal, and that at 480 nm, which reflects the formation and decay of meta III, are not so significantly different from each other. In addition, we applied an acid denaturation method to directly monitor the formation process of opsin in rhodopsin and its mutants (*32*). The results showed that some fractions of meta II in both wild-type and mutants decay into opsin and all-*trans*-retinal before the decay of meta III. The apparent rate constants for the change from meta II to opsin estimated by this method are similar to those estimated by the absorbance changes at 480 nm (Kuwayama et al., manuscript in preparation).

In this study, we have showed that the residues at positions 122 and 189 regulate the decay rates of meta II in both chicken green and rhodopsin. We have previously discussed the regulation mechanism of the meta II decay rate by the residue at position 122 as an interaction with His 211 that was confirmed in the crystal structure of bovine rhodopsin (*8*, *21*). Thus, it is of interest as to what is the main factor for the regulation of the decay rate of meta II by the residue 189. Isoleucine is more bulky than proline, so that it tends to sterically interact with nearby residues. On the other hand, proline has an imide group instead of the amide group that is common in other amino acids, so that it does not form a hydrogen bond with the carbonyl group of the nearby amino acid upon forming secondary structures such as the α-helix and β-sheet. These characteristics of the amino acid residues may have some influence on the structure of meta II. There are many rhodopsins that have a valine instead of isoleucine at position 189, and valine has a volume more similar to proline than isoleucine (*33*). In the chicken green, the P189V mutant shows a slower decay of meta II than that of the wild-type, similar to that of the P189I mutant (data not shown). In addition, the replacement of isoleucine at position 189 of the chicken rhodopsin with valine or a smaller amino acid alanine caused no change in the decay rate of meta II (data not shown). Thus, it is unlikely that the bulkiness at the site of 189 is the main factor, and a specific feature of the proline residue may be the main factor for regulation of the fast meta II decay rate of the chicken green.

It should be noted that the effects of residues 122 and 189 on the decay rate of meta II are different between the chicken green and rhodopsin. That is, in the chicken green, the decay rate of meta II is 27 times slower compared to the wild-type by the mutation at position 189, while it is only 2.7 times slower by the mutation at position 122 (Table 1). On the other hand, the effect of replacement at position 189 is less than that of 122 in the chicken rhodopsin. Therefore, it is suggested that the structures around residues 122 and 189 are different between the chicken green and rhodopsin at the meta II stage, which would be one reason that the effects of these residues on the decay rate of meta II are different between the chicken green and rhodopsin. Although the effect would be different between the chicken green and rhodopsin, the double mutant at positions 122 and 189 in the chicken green shows a dramatically slower meta II decay, about 60 times slower than wild-type and almost equal to the chicken rhodopsin. In chicken rhodopsin, the meta II decay rate of the double mutant also changes much faster than that of the wild-type rhodopsin, and close to the chicken green. These results show that two amino acid residues, 122 and 189, are mainly responsible for the different meta II decay rates between the chicken green and rhodopsin.

Our results on the proline mutant could provide some insight into the molecular properties of vertebrate ancestral pigment. Previous studies have suggested that the incorporation of a glutamic acid at position 122 is one of the key steps in the divergence into rod and cone visual pigments (*8*). However, it is difficult to speculate on the residue at position 122 of the ancestral pigment, because the residue diverged even in the cone visual pigments (Figures 1 and 6). These facts hampered us in speculating about the molecular properties of the ancestral pigment and, therefore, to provide functional evidence that rhodopsins have evolved

[2] Pinopsin, the chicken pineal photoreceptive molecule, shows a slow decay of the meta II intermediate though it has a proline residue at the corresponding position (*1*). The residues responsible for the slow decay were recently identified at different positions in the same region, indicating that pinopsin has a mechanism for the thermal behavior of the meta II-intermediate different from the rod and cone visual pigments (*2*).

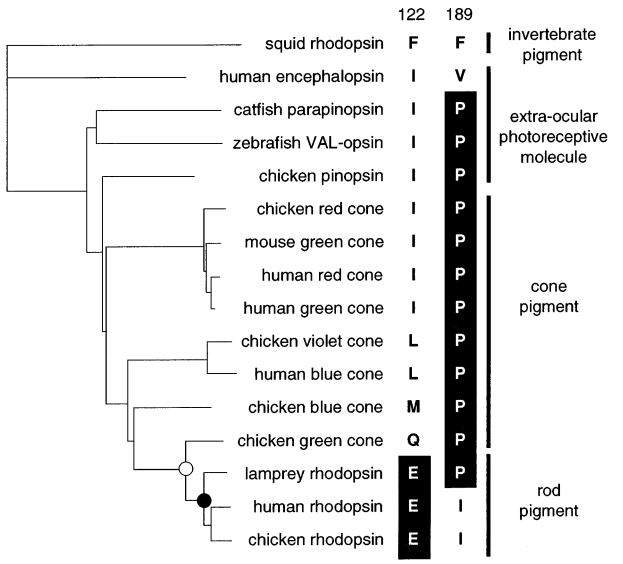

FIGURE 6: Phylogenetic relationship of vertebrate visual pigments. The phylogenetic tree of a representative sample of vertebrate visual pigments with some extraocular photoreceptive molecules and invertebrate pigment (*4*, *34*, *37*−*43*) constructed by the neighbor-joining method based upon the amino acid identity. Amino acid residues at position 122 and 189 are also denoted on the right side of the figure. The open circles represents the point that E122 has been achieved, and the filled circle represents the point that P189 has been lost.

out of the cone visual pigments (*4*). On the other hand, P189 is widely conserved in cone pigments and some extraocular photoreceptive molecules (*34*), suggesting that the vertebrate ancestral pigment has the proline at this position. The present study clearly showed that the proline residue is one of the determinants responsible for the molecular properties of the chicken green which is different from those of the rhodopsins. In addition, our results showed that the replacements of P189 in mouse green (*35*) and crab-eating monkeys (*Macaca fascicularis*) red-sensitive (*12*) cone pigments, which belong to the group L of the cone visual pigments (see Figure 1, chicken green belongs to the group M2), caused the slower decay of meta II than that of the wild-type (Kuwayama et al., manuscript in preparation). Because the pigments exhibit amino acid identity about 40% to that of chicken green or rhodopsin and the identity between them is smaller than any other identity between cone pigments, therefore, it is likely that the residue is functional not only in chicken green but also in the other types of cone pigments. Thus, the vertebrate ancestral pigment, the common ancestor of the rod and cone visual pigments, probably contains proline at position 189, and it would have a cone pigmentlike nature in regard to the molecular properties, such as the lifetime of meta II.[2] Interestingly, the rhodopsin of lamprey, one of the most primitive vertebrates, contains both the rod determinant E122 and the cone determinant P189 (*36*). We can speculate that P189 might have been lost in vertebrates higher than the lamprey, and that E122 might have been achieved when the rod visual pigment diverged from the cone visual pigment. In other words, the ancestral rhodopsin might first achieve E122 to express the molecular properties of "rhodopsin" and next lose P189 to enhance them. Thus, further mutational studies in addition to the studies of the intermediate pigments such as lamprey rhodopsins could provide a clue to the elucidation of the mechanism of functional diversity in the rod and cone visual pigments.

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