Amino Acid Residues Responsible for the Meta-III Decay Rates in Rod and Cone Visual Pigments†

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ABSTRACT: Vertebrate retinas have two types of photoreceptor cells, rods and cones, which contain visual pigments with different molecular properties. These pigments diverged from a common ancestor, and their difference in molecular properties originates from the difference in their amino acid residues. We previously reported that the difference in decay times of G protein-activating meta-II intermediates between the chicken rhodopsin and green-sensitive cone (chicken green) pigments is about 50 times. This difference only originates from the differences of two residues at positions 122 and 189 (Kuwayama, S., Imai, H., Hirano, T., Terakita, A., and Shichida, Y. (2002) *Biochemistry 41*, 15245−15252). Here we show that the meta-III intermediates exhibit about 700 times difference in decay times between the two pigments, and the faster decay in chicken green can be converted to the slower decay in rhodopsin by replacing the residues in chicken green with the corresponding rhodopsin residues. However, the inverse directional conversion did not occur when the two residues in rhodopsin were replaced by those of chicken green. Analysis using chimerical mutants derived from these pigments has demonstrated that amino acid residues responsible for the slow rhodopsin meta-III decay are situated at several positions throughout the C-terminal half of rhodopsin. Considering that rhodopsins evolved from cone pigments, it has been suggested that the molecular properties of rhodopsin have been optimized by mutations at several positions, and the chicken green mutants at two positions could be rhodopsin-like pigments transiently produced in the course of molecular evolution.

Visual pigments are members of the family of G-protein-coupled receptors (GPCRs), which contain 11-*cis*-retinal as a light-absorbing chromophore (*1*). Previous studies indicated that ancestral visual pigments first evolved into four groups of cone pigments, and a group of rhodopsins diverged from one of these cone pigment groups, including chicken green-sensitive pigment (chicken green) (*2*, *3*). The divergence of these cone pigment groups could result in the acquisition of cone visual pigments with different absorption maxima. Divergence of the group of rhodopsins from the cone pigment group could result in the acquisition of different molecular properties, such as the thermal behavior of the intermediates, stabilities of pigments, and G protein activation efficiencies (*4*−*8*). These differences originated from the differences in amino acid residues among visual pigments. While amino acid residues responsible for the absorption maximum regulation are situated around the chromophore (*9*−*11*), those responsible for the differences in molecular properties, including interaction with G protein, are distributed throughout the protein moiety. To further understand the functional diversity of rod and cone pigments, the responsibilities of amino acid residues on the pigments need further investigation.

We conducted mutational analysis and found that the residue at position 122 acts as one of the determinants of the difference in the meta-II intermediate decay rate, the key state for activation of retinal G protein (*12*). However, only a partial conversion of the meta-II decay rate was achieved by replacing this residue. This indicates that there are other amino acid residues that can be responsible for the differences. Further study revealed that the amino acid residue at position 189 is another candidate of the determinant for the differences in protein thermal stability and meta-II decay rate between chicken green and rhodopsin (*13*) (Figure 1A). Replacements of the residue at this position and the residue at 122 caused almost the complete conversion of the meta-II decay rate between rhodopsin and chicken green (*13*).

In the present study, we extended our investigation on the identification of amino acid residues to the regulation of the meta-III intermediate decay rate. Meta-III is thought to be a physiological intermediate that follows the equilibrium between meta-I and meta-II, and it contributes to the prolongation of G protein activation by forming an equilibrium with meta-I and meta-II (*14*) (Figure 1B). In addition, some "leaking activities" resulting from the equilibrium were considered to be important for the generation of a light-adapted state when the photoreceptor cell is extensively bleached (*15*). It was also reported that there is a pathway

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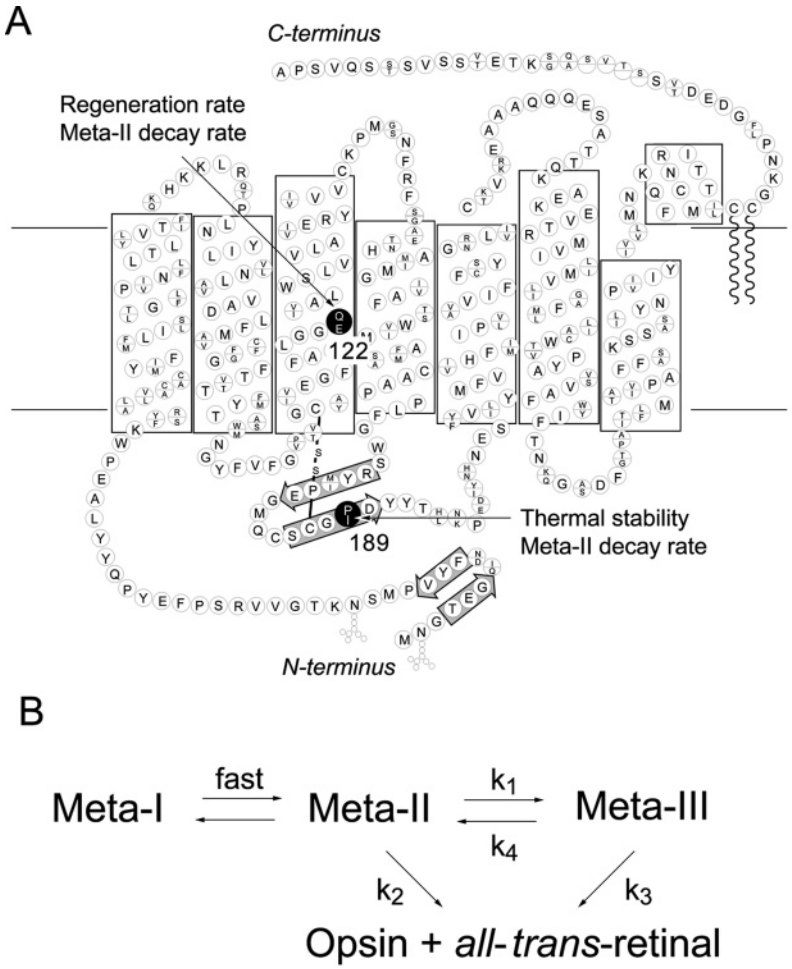

FIGURE 1: Comparison of amino acid sequences of chicken green and rhodopsin/the reaction scheme of visual pigments. (A) The secondary structure of chicken green and rhodopsin. The transmembrane topology is based on the 3D-structure of bovine rhodopsin (*35*, *36*). Residues of chicken green and rhodopsin are denoted in the upper and bottom sides of the circle, respectively. Residues responsible for the difference in molecular properties between cone and rod pigments are shown by the white characters on a black background. The molecular properties regulated by these residues are denoted in the figure. (B) The reaction scheme of visual pigments based on a previous study (*15*). Once pigments are irradiated, they decay to opsin plus *all-trans*-retinal via various intermediates such as meta-I, -II, -III. It is suggested that opsin forms via a dual pathway from meta-II and -III intermediates (*16*, *18*, *31*).

where meta-II directly decays into opsin plus *all-trans*-retinal, (*16*−*18*) and meta-III is produced directly from meta-I (*19*).

Current findings showed that the meta-III decay rate was about 700 times faster in chicken green than in rhodopsin. An interesting observation was that the meta-III decay rate of chicken green was decelerated to the point where it was almost equal to that of rhodopsin by replacing the residues with the corresponding rhodopsin residues, suggesting that the residues are also responsible for the meta-III decay rate. However, the replacements of the two residues in rhodopsin with those of chicken green did not accelerate the meta-III decay rate. Analysis using chimerical mutants derived from chicken green and rhodopsin demonstrated that amino acid residues responsible for the slow meta-III decay are situated in several positions throughout the C-terminal half of rhodopsin. On the basis of these results, the difference in regulation mechanism of molecular properties between rod and cone visual pigments and the diversity of visual pigments are discussed.

MATERIALS AND METHODS

Sample Preparation. The wild types and mutants of chicken green and rhodopsin were expressed in the HEK293 cell lines as previously reported (*20*, *21*). For purification, the cDNAs of chicken green and rhodopsin were tagged by the monoclonal antibody Rho1D4 epitope-sequence (ETSQVAPA) (*22*). The cDNAs of site-directed mutants of chicken green and rhodopsin were constructed following the method used in a previous report (*12*). The cDNAs of chimerical mutants derived from chicken green and rhodopsin were constructed by the SOE method (*23*, *24*), and each chimerical pigment is referred to here by an abbreviation that reflects the origin of the amino acid sequences. For example, N-H4(1−173)cG/cRh is a chimerical pigment that contains the chicken green sequence from N-terminus to helix IV and the rhodopsin sequence from the position at 174 to that at C-terminus. The wild type and mutant cDNAs were fully sequenced before being introduced into the expression vector (*25*). To reconstitute a photoreactive pigment, the expressed protein was incubated with 11-*cis*-retinal for more than 3 h at 4 °C. The pigments were extracted in buffer A [1% (w/v) dodecyl maltoside (DM[1]), 25 mM HEPES, 70

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

mM NaCl, and 500 μM $MgCl_2$, pH 6.5]. These extracted pigments were then purified with an antibody-conjugated column (*26*). They were finally eluted by buffer B (0.3 mg/mL 1D4 peptide, 0.02% DM, 50 mM HEPES, 140 mM NaCl, and 1 mM $MgCl_2$, pH 6.5) and were subjected to spectroscopy.

Spectrophotometry. The UV−vis absorption spectra were recorded using two types of spectrophotometers. First, a Shimadzu Model MPS-2000 spectrophotometer connected to an NEC PC-9801 computer was used for the "slow" (over 1 min) reaction of the intermediates. The system for measuring the absorption spectra was previously reported (*27*). The sample was irradiated with light from a 1-kW tungsten halogen lamp (Rikagaku Seiki) that had been passed through a glass cutoff filter (VO54 and VY52; Toshiba). The second one was a CCD spectrophotometer specially constructed by Hamamatsu Photonics Co. Ltd. that was used for the "fast" (within 1 min) reaction of the intermediates. This spectrophotometer is capable of continuously recording spectra (400 nm width, between 800 and 200 nm) with a wavelength resolution of 2 nm and intervals of 9.7 ms, where the latter is limited to the data acquisition rate of the camera after irradiation of the sample inside. The sample was photoexcited with light from a flash lump (~10 μs; Nissin Electronic Co. Ltd.) that had been passed through a glass cutoff filter (VO54 and VY52; Toshiba). The excitation was performed 100 ms after the monitoring started. Thermal reaction of the intermediates initiated by excitation of the samples was monitored by recording the absorption spectra at intervals of 9.7 ms up to 1 s or at suitable intervals until the reactions were nearly saturated. To record the spectra at a selected temperature, a cell-holder connected to a temperature controller (Neslab RTE-111) with an optical cell of 1-cm light path was placed into the sample compartment of the both spectrophotometers.

Estimation of Opsin Contents. Because photoexcited rhodopsin converts to opsin (and *all-trans*-retinal) not only through meta-III but also through meta-II, it is important to monitor the formation process of opsin independently from the meta-III decay process. In the present study, we applied two different methods to monitor the process. First, we applied the acid denaturation method (*28*). The pigment sample (250 μL, 1 μM) was irradiated for 6 s with an orange light (>520 nm) at 2 °C to convert all the pigments in the sample into their intermediate states that have *all-trans*-retinal chromophores. Then the remaining intermediates at selected time after the incubation was converted to the protonated Schiff base of *all-trans*-retinylidene opsins having absorption maximum at 440 nm (*28*) by the addition of 7 μL of 2 N HCl to the sample. The amount of opsin formed in the sample was therefore estimated by comparing the absorbance at 440 nm of the samples before and after the incubation. In the experiments on rhodopsin, the absorbance at 440 nm of the acidified samples did not change up to the 40 min incubation (Figure 2C), so that the amount of original rhodopsin was estimated from this absorbance. On the other hand, the intermediates formed from chicken green were relatively unstable so that it was difficult to estimate the original amount of pigment by recording the absorbance at 440 nm. Thus, we considered the absorbance value derived from the same amount of rhodopsin as the value of chicken green, because the extinction coefficient of protonated Schiff base

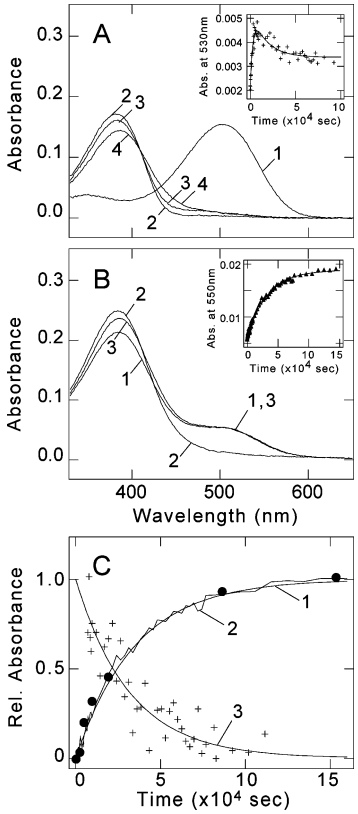

FIGURE 2: Comparison of the opsin formation rate and decay rate of the meta intermediate in chicken rhodopsin. (A) The absorption spectra of rhodopsin in the absence of additional 11-*cis*-retinal. Purified chicken rhodopsin (1 μM) in buffer B was cooled to 2 °C, followed by irradiation with an orange light (>520 nm) for 6 s. The spectra were recorded before irradiation (curve 1), immediately after irradiation (curve 2), and 40 min (curve 3) and 1280 min (curve 4) after irradiation. (Inset) The absorbance change at 530 nm is plotted (crosses) versus the incubation time after irradiation. The solid curve is a double-exponential curve with time constants of 2420 and 27 000 s, respectively. (B) The absorption spectra of rhodopsin in the presence of additional 11-*cis*-retinal. 11-*cis*-Retinal in ethanol (4.4 μL, 570 μM) was added to the purified chicken rhodopsin (1 μM) in buffer B (250 μL) to obtain the concentration of 10 μM. The sample was then cooled to 2 °C, followed by irradiation with an orange light (>520 nm) for 6 s. The spectra were recorded before irradiation (curve 1), immediately after irradiation (curve 2), and 2560 min after irradiation (curve 3). (Inset) The absorbance change at 550 nm is plotted (triangles) versus the incubation time after irradiation. The solid curve is a single-exponential curve with a time constant of 33 000 s. (C) The comparison of the kinetics of the opsin formation and the meta-III decay in chicken rhodopsin. The opsin formation process monitored by acid denaturation (circles) could be fitted by a single-exponential curve with a time constant of 29 000 s (curve 1). The noisy line shows the data of inset B normalized as the initial absorbance at 550 nm before irradiation to become 1 (curve 2). The decay of meta-III intermediate was monitored as the absorbance changes at 530 nm versus the incubation time after irradiation (crosses). The later stage of the fitted curve shown in inset A was normalized as the peak absorbance (0.0045) to become 1 and the final absorbance (0.003) to become 0. The fitted curve is a single-exponential curve with a time constant of 27 000 s (curve 3).

of *all-trans*-retinylidene opsins is expected to be identical whether it is formed from the intermediates of chicken green or rhodopsin.

Second, we monitored the amount of pigments regenerated from the irradiated pigments in the presence of 11-*cis*-retinal (*28*, *29*). Pigments (250 μL, 1−1.5 μM) were irradiated for 6 s (MPS-2000 spectrophotometer) or ~10 μs (CCD spec-

Table 1: Comparison of Time Constants for the Photobleaching Reactions between Wild Type Chicken Rhodopsin and Chicken Green[a]

	cRh-WT	cG-WT
meta-II decay	2350 (670)	0.96 (0.62)
meta-III decay	27000 (6100)	39.4 (13.4)
pigment + 11-*cis*-retinal	35200 (4700)	28.9 (0.4)
acid denature	29100 (5000)[b]	36.1 (9.3)[b]

[a] Values in parentheses are standard deviations calculated from at least three independent experiments, except where noted. [b] Values in parentheses are standard deviations of fitted curve.

trophotometer) with an orange light in the presence of 10-folds excess of 11-*cis*-retinal in buffer B at 2 °C. The increase in absorbance at 550 nm due to the regeneration of the pigments from 11-*cis*-retinal and opsin was monitored after the irradiation.

RESULTS

Decay Process of Meta-III of Rhodopsin. Figure 2A shows the spectral changes observed after rhodopsin irradiation with a >520-nm light for 6 s at 2 °C. Rhodopsin solubilized by DM (curve 1 in Figure 2A) was subjected to the experiments. The irradiation led to a shift in the maximum absorption, to about 380 nm, indicating the meta-II formation (curve 2 in Figure 2A). Subsequent incubation at this temperature resulted in an increase in absorbance at about 480 nm and a decrease in absorbance at about 380 nm. These changes reflect the formation of an equilibrium mixture containing meta-II and meta-III (and possibly meta-I) (*14*) (curve 3 in Figure 2A). Under our experimental conditions, the meta-III amount in the equilibrium is very small. Prolonged incubation caused an increase in absorbance at about 440 nm with a decrease in absorbance at about 380 nm (curve 4 in Figure 2A). These spectral changes reflect the dissociation of intermediates into opsin and *all-trans*-retinal, the latter of which forms a protonated Schiff base with lysine residues of the proteins or with amino groups present in lipids and others (*30*, *31*).

Meta-III has an absorption maximum at 480 nm (*7*), and it exhibits an absorbance at a wavelength longer than that of the protonated Schiff base in solution. In fact, absorbance at 530 nm as a function of the incubation time reflects the formation and decay process of meta-III (inset in Figure 2A) with time constants of 2350 ± 670 and 27 000 ± 6100 s, respectively, by simulating the processes with two exponential functions (Table 1).

Because of small changes in absorbance at 530 nm, we have attempted to monitor the decay process of meta-III by other methods. Opsin should be formed concurrently with the meta-III decay, so that we can estimate the decay process by monitoring the formation process of opsin. In this context, it should be noted that opsin can be formed from meta-II and its formation process could be fitted by two exponential functions.

When rhodopsin is irradiated in the presence of 11-*cis*-retinal, formed opsin is regenerated to rhodopsin. It was reported that the regeneration process of rhodopsin in the presence of an excess amount of 11-*cis*-retinal is much faster than the decay processes of both meta-II and meta-III, so that the formation kinetics of opsin from meta-II or meta-III can be monitored by the regeneration of rhodopsin under the conditions where an excess amount of 11-*cis*-retinal is present (*28*). Figure 2B shows the formation process of opsin by this method. An interesting observation is that the formation process of opsin could be fitted by a single-exponential function. The time constant was estimated to be 35 200 ± 4700 s, which is similar to that of the meta-III decay process (Table 1), indicating that opsin was formed only from meta-III under our experimental conditions.

We also estimated the formation process of opsin by the acid-denaturing method. The results showed that the formation time constant of opsin is 29 100 ± 5000 s, which is close to that of the decay time constant of meta-III (curve 1 in Figure 2C, Table 1). On the basis of these results, we concluded that the decay time constant of meta-III is about 30 000 s and that opsin can be formed only from meta-III under our experimental conditions.

Decay Process of Meta-III of Chicken Green. We then estimated the decay time constant of meta-III of chicken green by using similar methods. Figure 3A shows the spectral changes observed after excitation of chicken green with >520 nm light pulse (10 μs) at 2 °C. In reference to the previous results using the low-temperature spectroscopy (*7*), the initial shift of the spectrum (curve 2 in Figure 3A) results in the formation of meta-I of chicken green, and subsequent changes of the spectrum (curve 3 in Figure 3A) cause the formation of an equilibrium state among meta-I, -II, and -III. An increase in absorbance at about 380 nm concurrent with the decrease in absorbance at about 480 nm (curve 4 in Figure 3A) reflects the dissociation process of intermediates into opsin and *all-trans*-retinal. To estimate the time constants of the processes, we plotted the changes in absorbance at 480 nm as a function of time after excitation. These contacts were determined as 46 ms, 0.96 ± 0.62 s, and 39.4 ± 13.4 s, respectively, by fitting the curve with three exponential functions (inset in Figure 3A, Table 1).

Next, we irradiated chicken green in the presence of excess amount of 11-*cis*-retinal to estimate the formation process of opsin (Figure 3B). The formation process can be fitted by a single-exponential function with a time constant of 28.9 ± 0.4 s (inset in Figure 3B, Table 1). It should be noted that only 70% of the opsin was regenerated to chicken green, which is in contrast to the fact that complete regeneration was observed in the rhodopsin system. The fact that the formation process can only be fitted by a single exponential function strongly suggests that the regeneration of opsin was only observed from the opsin fraction that formed from a single-exponential process.

The formation processes of opsin were also estimated by acid denaturation methods. The process is a composition of two components: about 30% of total opsin was formed with a time constant of less than 10 s and the remaining 70% was formed with a time constant of 36.1 ± 9.3 s (curve 1 in Figure 3C, Table 1). On the basis of these results, we concluded that the decay time constant of meta-III of chicken green is about 40 s, which is about 700 times shorter than that of rhodopsin. In addition, there must be a pathway of the direct formation of opsin from meta-II in chicken green under our experimental conditions. An interesting observation is that the amount of opsin formed from meta-II is comparable to that of opsin not regenerated (see the Discussion).

Regulation Mechanism of the Difference in Meta-III Decay Rates between Chicken Green and Rhodopsin. Next, we

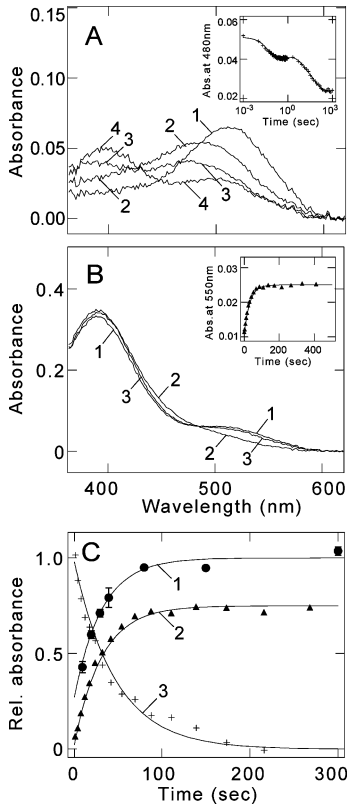

FIGURE 3: Comparison of the opsin formation and the decay rate of the meta intermediate in chicken green. (A) The absorption spectra of chicken green in the absence of additional 11-*cis*-retinal. Purified chicken green (1.5 μM) in buffer B (250 μL) was cooled to 2 °C, followed by irradiation with a yellow light (>500 nm) from flash lamp (~10 μs). The spectra were recorded before irradiation (curve 1), immediately after irradiation (curve 2), and 1 s (curve 3) and 3 min (curve 4) after irradiation. (Inset) The absorbance change at 480 nm is plotted (crosses) versus the incubation time after irradiation. The solid curve is a triple exponential curve with time constants of 46 ms, 1.4 s, and 46.8 s. (B) The absorption spectra of chicken green in the presence of additional 11-*cis*-retinal. 11-*cis*-retinal in ethanol (6.6 μL, 570 μM) was added to the purified chicken green (1.5 μM) in buffer B (250 μL) to obtain the concentration of 15 μM, and the sample was cooled to 2 °C, followed by irradiation with a yellow light (>500 nm) from flash lamp (~10 μs). The spectra were recorded before irradiation (curve 1), immediately after irradiation (curve 2), and 160 min after irradiation (curve 3). (Inset) The absorbance change at 550 nm is plotted (triangles) versus the incubation time after irradiation. The solid curve is a single-exponential curve with a time constant of 29 s. (C) The comparison of the kinetics of the opsin formation and the meta-III decay in chicken green. The opsin formation process monitored by acid denaturation (circles) could be fitted by a single-exponential curve with a time constant of 36.1 s (curve 1). The triangles and fitted curve show the data of inset of B normalized as the initial absorbance at 550 nm before irradiation to become 1 (curve 2). The decay of meta-III intermediate was monitored as the absorbance changes at 480 nm versus the incubation time after irradiation (crosses). The third component of the fitted curve shown in inset A was normalized as the peak absorbance (0.04) to become 1 and the final absorbance (0.024) to become 0 and is shown as a solid curve with a time constant of 46.8 s (curve 3).

attempted to identify amino acid residues responsible for the different meta-III decay rates in chicken green and rhodopsin. Because amino acid residues at positions 122 and 189 are the determinants of the meta-II decay rate, we first examined whether these residues are also responsible for the meta-III decay rate (Table 2).

Table 2: Relative Meta-III Decay Rate of Chicken Green and Rhodopsin Site-Directed Mutants[a]

chicken green	decay rate	chicken rhodopsin	decay rate
wild type	690 (350)	wild type	1.0 (0.3)
Q122E	100 (7.0)	E122Q	1.1 (0.16)
P189I	2.1 (0.54)	I189P	1.1 (0.33)
double	0.73 (0.15)	double	2.5 (0.78)

[a] The decay rate for wild-type rhodopsin under the same condition was normalized to 1. The values in parentheses are standard deviations calculated from at least three independent experiments.

Figure 4, parts A and B, demonstrates the changes in the meta-III decay rate caused by the mutation of chicken green at positions 122 and 189. The meta-III decay rates were slowed by 6.8, 320, or 280 times for Q122E, P189I, or Q122E/P189I mutation, respectively. These results clearly indicate that the residues at positions 122 and 189 are responsible for the fast meta-III decay in chicken green. As is the case of the meta-II decay rate, the replacement of the residue at position 189 is more effective than at position 122.

We performed similar analysis on rhodopsin. Interestingly, the rhodopsin mutants at positions 122 and 189 showed no changes in meta-III decay (Figure 4C,D), which is in constrast to the case of chicken green. This is also very different from the case of meta-II decay, where the mutations at these positions remarkably accelerated the meta-II decay process (*13*). Therefore, we can infer that there are amino acid residues other than the residues at positions 122 and 189 that regulate the meta-III decay in rhodopsin.

To obtain more information about the amino acid residues that regulate the meta-III decay in rhodopsin, we constructed the chimerical mutants derived from chicken green and rhodopsin. Results from a previous study using the chimerical mutants between bovine rhodopsin and gecko blue pigments suggested that the residues responsible for the slow decay of meta-III in rhodopsin are situated in the region from the second-extracellular loop to the C-terminus (*32*). On the basis of these results, we constructed the chimerical mutants in the region from the N-terminus to helices 4, 5, 6, and 7 where rhodopsin was replaced in a stepwise manner by the corresponding regions in chicken green (Figure 5 and Table 3). The chimerical mutant, N-H4(1−173)cG/cRh, which has the chicken green sequence in the region from N-terminus to helix 4 and the rhodopsin sequence in the remaining regions, showed that the meta-III decay rate was almost identical to that of rhodopsin. This is consistent with a previous study (*32*). On the other hand, meta-III decay rate was gradually accelerated as the mutant contained more chicken green sequences. The chimerical mutants N-H5(1−231)cG/cRh, N-H6(1−276)cG/cRh, and N-H7(1−306)cG/cRh showed about 5, 50, and 400 times faster meta-III decays, respectively. These results demonstrated that several residues situated in the region from the second extracellular loop to helix 7 are responsible for the slow decay rate of meta-III in rhodopsin. It should be noted that the replacement of each rhodopsin helix with the corresponding helix of chicken green did not accelerate the meta-III decay (Table 3). Therefore, several residues could cooperatively regulate the meta-III decay rate of rhodopsin.

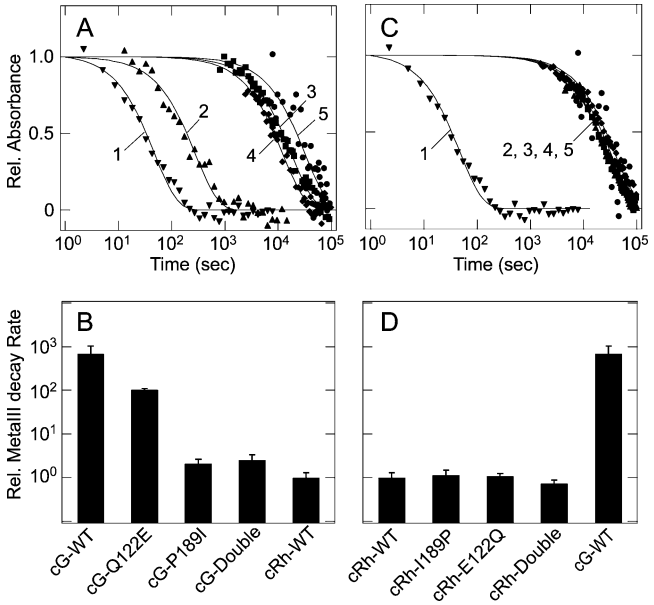

FIGURE 4: Meta-III decay rates of chicken green, rhodopsin, and their site-directed mutants (A) The meta-III decay time course of wild-type chicken green and its mutants. The samples in buffer B were cooled to 2 °C. The absorption spectra of the wild-type chicken green and Q122E mutant were measured using a CCD spectrophotometer before and after irradiation with a yellow light (>500 nm) from flash lamp (~10 μs). The absorption spectra of other samples were measured by MPS-2000 spectrophotometer before and after irradiation with an orange light (>520 nm) for 6 s. The absorption spectra were then continuously recorded at the same temperature until the change was obviously finished. The absorbance changes at 530 nm (cRh-WT, circles) and 480 nm (cG-WT, reverse triangles; cG-Q122E, triangles; cG-P189I, squares; and cG-double, diamonds) are plotted versus the incubation time after irradiation. The decrease in absorbance could be fitted by single-exponential curves whose time constants are 46.8 s (cG-WT, curve 1), 280 s (Q122E, curve 2), 12 900 s (P189I, curve 3), 11 400 s (double, curve 4), and 27 000 s (cRh-WT, curve 5), respectively. (B) The meta-III decay rate constants of chicken green and its mutants are represented relative to wild-type rhodopsin. Error bars represent the standard deviations estimated from three independent experiments. (C) The meta-III decay time course of wild-type chicken rhodopsin and its mutants. The absorption spectra of chicken rhodopsin and its mutants were measured by a MPS-2000 spectrophotometer before and after irradiation with an orange light (>520 nm) for 6 s. The absorbance changes at 530 nm (cRh-WT, circles) and 480 nm (cG-WT, reverse triangles; cRh-E122Q, triangles; cRh-I189P, squares; and cG-double, diamonds) are plotted versus the incubation time after irradiation. The decrease in absorbance could be fitted by the single-exponential curves whose time constants are 27 000 s (cRh-WT, curve 5), 26 000 s (I189P, curve 2), 26 800 s (E122Q, curve 3), 36 700 s (double, curve 4), and 46.8 s (cG-WT, curve 1), respectively. (D) The meta-III decay rate constants of chicken rhodopsin and its mutants are represented relative to wild-type rhodopsin. Error bars represent the standard deviations estimated from three independent experiments.

DISCUSSION

In the present study, we compared the meta-III decay rate of rhodopsin with that of chicken green and found that they exhibit about 700 times difference. Faster decay of meta-III is a common character among cone visual pigments (*5*, *8*). Thus, it might be related to the faster recovery of cone photoreceptor cells from the bleached state (*33*). In the following section, we shall discuss the amino acid residues responsible for the differences in meta-III decay rates between rod and cone pigments and their functional diversity in the course of molecular evolution.

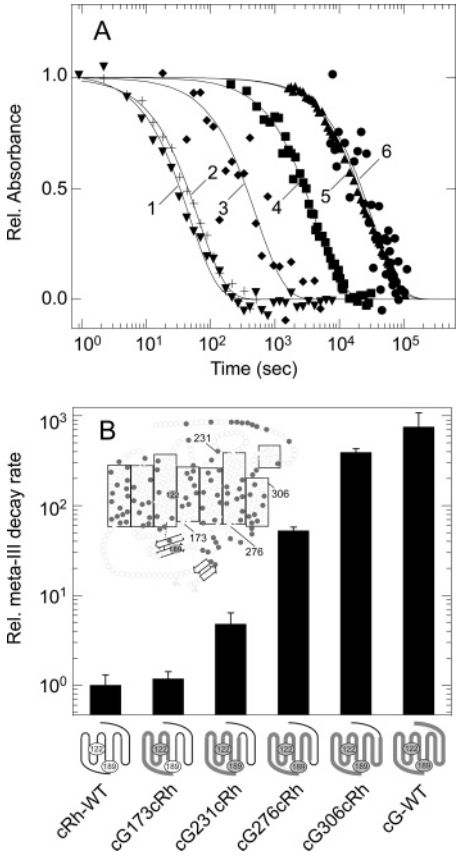

FIGURE 5: Meta-III decay rates of the chimerical mutants derived from chicken green and rhodopsin. (A) The meta-III decay time-course of wild-type chicken rhodopsin, chicken green, and chimerical mutants. Samples in buffer B were cooled to 2 °C. The absorption spectra of wild-type chicken green, N-H6(1−276)cG/cRh, and N-H7(1−306)cG/cRh chimeras were measured by CCD spectrophotometer before and after irradiation with a yellow light (>500 nm) from flash lamp (~10 μs). The absorption spectra of other samples were measured using a MPS-2000 spectrophotometer before and after irradiation with an orange light (>520 nm) for 6 s. Absorption spectra were then continuously recorded at the same temperature. The absorbance changes at 530 nm (cRh-WT, circles) and 480 nm (cG-WT, reverse triangles; N-H7(1−306)cG/cRh, crosses; N-H6(1−276)cG/cRh, diamonds, N-H5(1−231)cG/cRh, squares; and N-H4(1−173)cG/cRh, triangles) are plotted versus the incubation time after irradiation. The decrease in absorbance could be fitted by the single-exponential curves whose time constants are 27 000 s (cRh-WT, curve 6), 20 800 s (N-H4(1−173)cG/cRh, curve 5), 6130 s (N-H5(1−231)cG/cRh, curve 4), 490 s (N-H6(1−276)-cG/cRh, curve 3), 68.2 s (N-H7(1−306)cG/cRh, curve 2), and 46.8 s (cG-WT, curve 1), respectively. (B) The meta-III decay rate constants of wild-type chicken rhodopsin, chicken green, and chimerical mutants. The wild type and chimerical pigment structures are denoted in the figure. The region from chicken rhodopsin is shown as a thin black line, and the region from chicken green is shown as a bold gray line. The residues at the sites of 122 and 189 are also denoted in the pigment structures. The white and gray background represents the residues of chicken rhodopsin and chicken green, respectively. Error bars represent the standard deviations estimated from three indepen dent experiments. (Inset) The secondary structures of chicken green and rhodopsin. Residues common to chicken green and rhodopsin and the difference between them are denoted by the white and gray circles, respectively. The residues at the sites 173, 231, 276, and 306 are the boundary residues of the chimerical mutants.

Opsin Formation of Chicken Rhodopsin and Green. We examined the reaction kinetics using the DM-solubilized rhodopsin and chicken green samples. These results showed that all the photoactivated rhodopsins converted to opsin

Table 3: Relative Meta-III Decay Rate of Chicken Rhodopsin Chimerical Mutants[a]

N-chimera	decay rate	E2/H5/H6/H7-chimera	decay rate
N-H4(1−173)cG/cRh	1.2 (0.22)	E2(173−202)cG/cRh	3.2 (1.1)
N-H5(1−231)cG/cRh	4.8 (1.6)	H5(203−231)cG/cRh	0.78 (0.06)
N-H6(1−276)cG/cRh	53 (5.0)	H6(245−276)cG/cRh	0.26 (0.01)
N-H7(1−306)cG/cRh	390 (36)	H7(277−306)cG/cRh	1.1 (0.67)

[a] The decay rate for wild-type rhodopsin under the same conditions was normalized to 1. The values in parentheses are standard deviations calculated from at least three independent experiments.

through meta-III, while 30% of the opsin was formed without going through meta-III in chicken green. This 30% of the opsin would be formed directly from meta-II in chicken green. The results we obtained on chicken rhodopsin were different from those obtained using ROS membranes at physiological temperature, where about 70% of opsin was formed directly from meta-II (*18*, *31*). In addition, about 50% of opsin was directly formed from meta-II in chicken rhodopsin solubilized by a detergent, CHAPS, in the presence of PC at 2 °C (data not shown). This difference could have been caused by different experimental conditions. On the other hand, the interesting observation is that the formation pathway of opsin was different between rhodopsin and chicken green, even under the same experimental conditions. Unlike the case of chicken rhodopsin, about 30% of opsin was probably directly formed from meta-II in chicken green. This result suggests that unprotonated Schiff base in chicken green would be more unstable than in rhodopsin.

It should be noted that, in chicken green, only about 70% of the opsin was regenerated when chicken green was irradiated in the presence of 11-*cis*-retinal. The percentage of the regenerated opsin in the totally formed opsin is similar to that of opsin formed from meta-III. This similarity was observed when chicken green was solubilized by a detergent, CHAPS, in the presence of PC, where pigments were also regenerated only from opsin that was formed from meta-III (data not shown). These results suggest that opsin directly formed from meta-II could not be regenerated even in the presence of 11-*cis*-retinal. It was reported that the conformation of meta-II is more distorted from the original state than that of meta-III in bovine rhodopsin (*17*, *34*). Thus, it is likely that chicken green opsin formed from meta-II might have a structure that is more distorted than that of a regenerable opsin. Due to its specific interaction with detergent, this structure could probably not recover to regenerable opsin.

Amino Acid Residues Responsible for the Meta-III Decay Rate. Since the difference in the meta-III decay rates between rhodopsin and chicken green is about 700 times, the identification of amino acid residues responsible for the difference is important. We identified the amino acid residues responsible for the meta-II decay rate, and therefore, we could examine whether these residues were also responsible for the meta-III decay rate. The replacements of the residues at positions 122 and 189 of chicken green with corresponding rhodopsin residues resulted in the conversion of the meta-III decay rate into that observed in rhodopsin, indicating that these residues are also responsible for the meta-III decay rate.

In contrast, several amino acid residues other than the residues at 122 and 189 would be responsible for the slow decay of meta-III of rhodopsin. These residues are situated in the region from the second extracellular loop to helix 7, as detected by the experiments using chimerical mutants between rhodopsin and chicken green. It should be noted that rhodopsin mutants with a replaced helix 5, 6, or 7 of chicken green did not accelerate the meta-III decay rate (Table 3). Therefore, amino acid residues at different helices synergistically regulate the meta-III decay rate in rhodopsin.

In previous studies, phylogenetic analysis of vertebrate visual pigments showed that rhodopsins have evolved out of cone visual pigments and that an ancestral visual pigment would have a cone-pigment-like nature in regard to the molecular properties (*3*, *13*). The fact that the several residues regulate the meta-III decay rate of rhodopsin suggests that the mutations were accumulated to reinforce the molecular properties of rhodopsin in the course of evolution. Because chicken green has a high sequence similarity to rhodopsin, but shows a typical cone pigment nature, further mutational analysis between chicken green and rhodopsin is necessary to make the regulation mechanism of the decay rate of meta-intermediates and the stability of dark state of pigments even clearer.

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