Difference in Molecular Properties between Chicken Green and Rhodopsin as Related to the Functional Difference between Cone and Rod Photoreceptor Cells†

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ABSTRACT: Using low-temperature spectroscopy, we have investigated the photobleaching process of chicken green, a green-sensitive cone visual pigment present in chicken retina, and compared it to that of rhodopsin, a rod visual pigment. Like rhodopsin, chicken green converts to *all-trans*-retinal and opsin through batho, lumi, and meta I, II, and III intermediates. However, all of the intermediates of chicken green except lumi, are less stable than the corresponding intermediates of rhodopsin. While early intermediates, batho and lumi are similar in absorption maxima between chicken green and rhodopsin, the meta intermediates of chicken green are about 20 nm blue shifted from those of rhodopsin. Low-temperature time-resolved spectroscopy was applied to estimate the thermodynamic properties of meta intermediates, and it indicated that the less stable properties of meta II and III intermediates of chicken green originate from the smaller activation enthalpies. The decay of the meta II intermediate of chicken green is greatly suppressed when a chicken green sample is irradiated at alkaline conditions while the net charge becomes similar to that of rhodopsin at neutral conditions. These results strongly suggest that the functional properties of chicken green that are different from those of rhodopsin are regulated by the dissociative amino acid residue(s).

Chicken green, a green-sensitive cone visual pigment present in chicken retina, is one of the key visual pigments used to elucidate the functional difference between rod and cone photoreceptor cells at a visual pigment level. In spite of the high similarity in amino acid sequence and absorption spectrum between chicken green and rhodopsin, a rod visual pigment (Okano et al., 1992; Wang et al., 1992), chicken green displays faster regeneration from 11-*cis*-retinal and opsin and faster formation and decay of the physiologically active meta[1] II intermediate than rhodopsin (Shichida et al., 1994), in addition to instability against hydroxylamine (Yen-Fager & Fager, 1984). These results correlate well with the functional difference between cone and rod photoreceptor cells (Schnapf & Baylor, 1986). Since the other cone visual pigments, chicken red (iodopsin) and chicken blue, display thermal behavior of meta II intermediates similar to that of chicken green (Shichida et al., 1993; Okada et al., 1994; Imai et al., manuscript in preparation), less stable properties of meta II intermediates would be common in all of the cone visual pigments and therein lies a key difference in molecular structure between rod and cone visual pigments. In the present study, we have further investigated the spectral and thermal properties of intermediates of chicken green and compared them with those of rhodopsin in order to elucidate the molecular difference between cone and rod visual pigments.

Since the difference in thermal behavior of meta II intermediate between chicken green and rhodopsin originates from the difference in activation free energy, it is of interest to compare the thermodynamic properties of these intermediates. Furthermore, estimation of thermodynamic parameters would provide a clue to elucidate the difference in molecular properties between chicken green and rhodopsin. However, the availability of an extraordinarily small amount of chicken green has hampered kinetic measurements using the conventional flash photolysis technique. Recently, we showed that low-temperature time-resolved spectroscopy has a great advantage for the measurement of precise spectral changes using only a small amount of visual pigment (Imai et al., 1994). Furthermore, we showed that one of the explanations for the apparent difference in the bleaching process of rhodopsin between room and low temperatures is the temperature dependence of the free energies of the intermediates (Imai et al., 1994). Thus, we applied this technique to the measurement of the bleaching process of chicken green, paying special attention to the temperature dependence of formation and decay rate constants of meta intermediates.

In spite of the great variety in amino acid sequence among cone visual pigments, all of the cone visual pigments whose amino acid sequences were determined have more positive calculated net charges than those of rhodopsin. Namely, when the net charges of the cone visual pigments and

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[1] Abbreviations: cRh, chicken rhodopsin; cG, chicken green-sensitive cone visual pigment; batho, batho intermediate; lumi, lumi intermediate; meta, meta intermediate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; ConA, concanavalin A; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; KIU, kallikrein inhibitor units; SVD, singular value decomposition.

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rhodopsins are plotted against environmental pH, the cone visual pigments clearly split from the rhodopsins (Okano et al., 1992). Thus, the difference in net charge between cone visual pigments and rhodopsins would be one of the important factors regulating the thermal behavior of meta II intermediate. Therefore, we changed the pH of chicken green sample to an alkaline condition, where the calculated net charge of chicken green is almost identical with that of rhodopsin at pH 7, and investigated the thermal behavior of meta II intermediate.

MATERIALS AND METHODS

Purification of Chicken Green and Rhodopsin. Chicken green and rhodopsin were extracted from chicken retinas by a mixture of CHAPS and PC and purified by means of column chromatography (Okano et al., 1989; Shichida et al., 1994). Briefly, a mixture of cone and rod outer segments was isolated from 2000 flesh chicken retinas by means of a 40% (w/v) sucrose flotation method in buffer P [50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, 50 KIU/mL aprotinin, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7] under red light (>620 nm). Since a considerable amount of the visual pigments was bleached during the course of isolation of retinas from eyes, it was regenerated by adding 2 times molar excess 11-*cis*-retinal. The following manipulations were carried out in the dark or dim red light (>660 nm). To extract the visual pigments, the outer segments were suspended in buffer P supplemented with 0.75% (w/v) CHAPS and 1 mg/mL PC and incubated at 4 °C for 1 h. The extract obtained by homogenization and centrifugation (60000*g*, 1 h) was diluted with buffer P to lower the final concentrations of CHAPS and PC to 0.6% and 0.8 mg/mL, respectively. The extraction procedure was repeated three times.

Pigments in the extract were then adsorbed to a concanavalin A−Sepharose affinity column (Pharmacia). To remove bound materials containing oil droplets, the column was washed with 20 bed vol of buffer A (buffer P with 0.6% CHAPS and 0.8 mg/mL PC). Then to reduce the NaCl concentration for the next ion exchange column chromatography, the column was washed with buffer A whose NaCl concentration was 10 mM (buffer B). After chicken violet, chicken blue, and iodopsin were eluted with buffer B supplemented with a low concentration (1.5−5 mM) of methyl α-D-mannoside, the mixture of chicken green and rhodopsin was eluted with buffer B supplemented with 200 mM methyl α-D-mannoside. To stabilize chicken green, glycerol was added to the eluate to give a final concentration of 20% (w/v), and the eluate was then applied to a DEAE−Sepharose column (Pharmacia). Chicken green was obtained as a passed-through fraction. To obtain a concentrated chicken green sample, the fraction was applied to the CM-Sepharose column (Pharmacia), followed by elution with buffer D-20 [20% glycerol (w/v), 0.6% CHAPS, 0.8 mg/mL PC, 50 mM HEPES, 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 7]. The eluate was dialyzed against buffer D-20 overnight in order to adjust the buffer conditions. Chicken rhodopsin was eluted from the DEAE−Sepharose column with buffer D-20 after chicken green was completely removed from the column. If necessary, the samples were concentrated with an ultrafiltration membrane (Amicon YM-30) followed by dialysis against buffer D-20 overnight. The pH of the sample was adjusted by adding 1 N NaOH or HCl at 4 °C. Finally, twice the volume of glycerol was added to the green and the rhodopsin samples for conventional low-temperature spectroscopy, or an equal volume was added for low-temperature time-resolved spectroscopy. Before each experiment, iodopsin contaminated in the sample was bleached by irradiation with >660 nm light for 40 min at 0 °C.

Spectrophotometry. Absorption spectra were recorded with a Shimadzu Model MPS-2000 spectrophotometer interfaced with an NEC PC-9801 computer. A glass optical cryostat (Yoshizawa & Shichida, 1982) was used to keep the sample at low temperatures for conventional low-temperature spectroscopy. The system for the measurement of absorption spectra was reported previously (Yoshizawa & Shichida, 1982; Imamoto et al., 1989). An Oxford Model CF-1204 cryostat was used for low-temperature time-resolved spectroscopy. The temperature of the sample was maintained within 0.1 °C with a temperature controller (ITC-4, Oxford). The sample was irradiated with light from a 1 kW tungsten−halogen lamp (Rikagaku Seiki) that had been passed through a glass cutoff filter (VR68, VR65, VR59, VO56, VY50, Toshiba) or an interference filter (501 nm, Nihonshinku). To regulate the intensity of light, a neutral density filter (TND25, Toshiba) or convex lens was used. A 5 cm water layer was placed in front of the light source in order to remove heat radiation from the irradiation light. To confirm that low-temperature irradiation did not form retinal isomers other than the all-trans, 11-cis, and 9-cis forms, the chromophores were extracted from the irradiated sample after the experiments, and their isomeric composition was analyzed by means of the HPLC (YMC-A0123, Yamamura) method (Imamoto et al., 1992). The absorption spectrum of each intermediate was calculated by the methods described previously (Yoshizawa & Shichida, 1982; Imai et al., 1994).

RESULTS

Batho and Lumi Intermediates of Chicken Green. The absorption maximum of chicken green was 508 nm at 0 °C (data not shown), which was identical with that previously reported (Okano et al., 1989). When cooled to −185 °C, the spectrum was sharpened and the maximum was shifted to 514 nm (curve 1 in Figure 1A). Irradiation with green light (501 nm) at −185 °C caused the formation of batho intermediate (cG-batho) as reported previously (Shichida et al., 1994), and the photoreversibility among chicken green, cG-batho, and isopigment (cG-iso) was also demonstrated (Figure 1A). Spectral properties and photosensitivities were almost identical with those observed in chicken (inset of Figure 1A) and bovine rhodopsins (Yoshizawa & Wald, 1963).

Next, the thermal stability of cG-batho was estimated by warming the photo-steady-state mixture containing mainly cG-batho in a stepwise manner (Figure 1B). The shift in absorption spectrum was observed when the sample was warmed above −140 °C (curve 9 in Figure 1B), indicating the transition temperature to be −140 °C. After the photo-steady-state mixture was warmed to 20 °C, the loss of chicken green was estimated to be 58% (data not shown), which was almost identical with that in rhodopsin (52%). Therefore, cG-batho would not revert to original chicken green, which is contrast to the case of iodopsin, whose batho

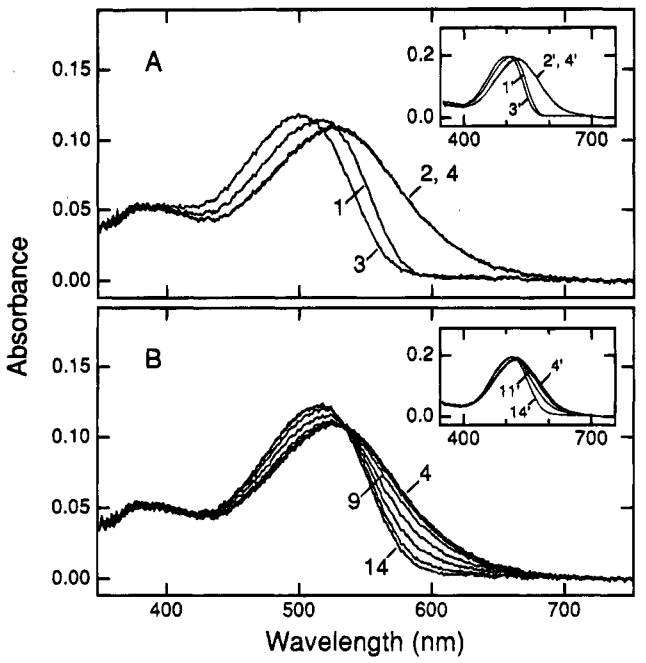

FIGURE 1: Formation and decay of batho intermediates of chicken green and rhodopsin (inset) measured by conventional low-temperature spectroscopy. (A) A chicken green or rhodopsin (inset) sample was cooled to −185 °C (curve 1), followed by irradiation with green light (501 nm) for 120 s until the photo-steady-state mixture containing mainly batho (curve 2) was formed. Then it was irradiated with orange light (>540 nm) for 480 s to form a mixture containing mainly isopigment (curve 3). The sample was irradiated again with green light (501 nm) for 120 s (curve 4) to form the photo-steady-state mixture which was identical in shape with that formed by the first irradiation. (B) The photo-steady-state mixture, containing mainly batho (curve 4) was warmed in a stepwise manner to −180, −170, −160, −150, −140, −130, −120, −110, −100, and −90 °C, and the spectra were recorded at −185 °C (curves 5−14, respectively).

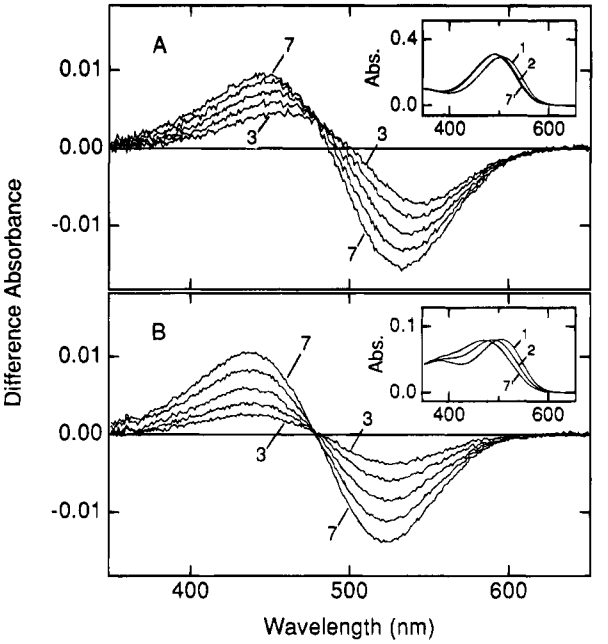

FIGURE 2: Course of the conversion from lumi to meta I of rhodopsin (A) and chicken green (B) recorded at −40 °C. Each pigment/56% glycerol mixture was cooled to −40 °C (curve 1, inset), followed by irradiation with orange light (>570 nm) for 30 s (curve 2, inset). Absorption spectra were then recorded at 2, 4, 8, 16, and 32 min after irradiation, and the difference spectra obtained by subtracting curve 2 from these spectra were calculated (curves 3−7).

intermediate thermally reverts to original iodopsin (Yoshizawa & Wald, 1967; Imamoto et al., 1989). Under similar experimental conditions, cRh-batho produced by the irradiation of chicken rhodopsin at −185 °C converted to cRh-lumi at −120 °C (curve 11 in inset of Figure 1B). Thus, the transition temperature of cRh-batho is 20 °C higher than that of cG-batho. The absorption spectra of batho and lumi intermediates were calculated by a method previously reported (Yoshizawa & Shichida, 1982; Imai et al., 1994). The maxima of cG-batho and cRh-batho were identical (549 nm), and those of cG-lumi and cRh-lumi were 511 and 503 nm, respectively. The difference in absorption maximum between cG-lumi and cRh-lumi is similar to that between original chicken green (511 nm) and rhodopsin (509 nm) at −80 °C.

Thermal Behavior of Three Meta Intermediates in Chicken Green and Rhodopsin. By using low-temperature time-resolved spectroscopy, we observed the thermal behavior of intermediates of chicken green and rhodopsin after irradiation of these pigments at temperatures ranging from −40 to −10 °C (Figures 2−4). In rhodopsin, an increase in absorbance at about 460 nm and a decrease at about 530 nm were observed at −40 °C (Figure 2A), indicating the conversion from lumi to meta I (Imai et al., 1994). However, the gradual shift of intersection points to shorter wavelengths was observed. This is due to the formation of a meta I-like intermediate, in addition to lumi and meta I, which is now confirmed by means of singular value decomposition (SVD) analysis (Mizukami et al., manuscript in preparation). On the other hand, a clear isosbestic point was observed during the lumi−meta I transition in chicken green (Figure 2B). This process was confirmed to be single-order reaction by SVD analysis (data not shown). It should be noted that the positive (440 nm) and negative (525 nm) peaks in curve 7 of Figure 2B are 20 nm blue shifted from those (460 and 545 nm) in curve 3 of Figure 2A. Since the absorption maximum of cG-lumi is almost the same as that of cRh-lumi, this result suggests that cG-meta I has an absorption maximum 20 nm blue shifted from that of cRh-meta I.

At −30 °C, the conversion from lumi to meta II via meta I was observed in rhodopsin, but they were not well separated as shown by the gradual shift of the intersection points of the spectra (Figure 3). However, at −20 and −10 °C, the stepwise conversion was clearly observed. Namely, the conversions from meta I to meta II (Figure 3B, top), from meta II to meta III (Figure 3B, bottom, and 3C, top), and from meta III to *all-trans*-retinal plus opsin (Figure 3C, bottom) were observed with clear isosbestic points, indicating that each intermediate converts to the next intermediate without branching.

The experimental results for chicken green (Figure 4) are somewhat different from those of rhodopsin. First, at −30 °C, the early spectral change (Figure 4A, top) was similar to that in rhodopsin (Figure 3A), while it was followed by a broad absorbance decrease (Figure 4A, bottom). Since the absorbance at about 380 nm increased in this phase and no spectral changes were observed when the sample was further warmed to +20 °C, this phase could be due to the dissociation process of intermediate into *all-trans*-retinal and opsin. A similar spectral change was observed at −20 °C (Figure 4B, bottom) after the formation of meta II. These spectral changes were similar in shape to that in the decay

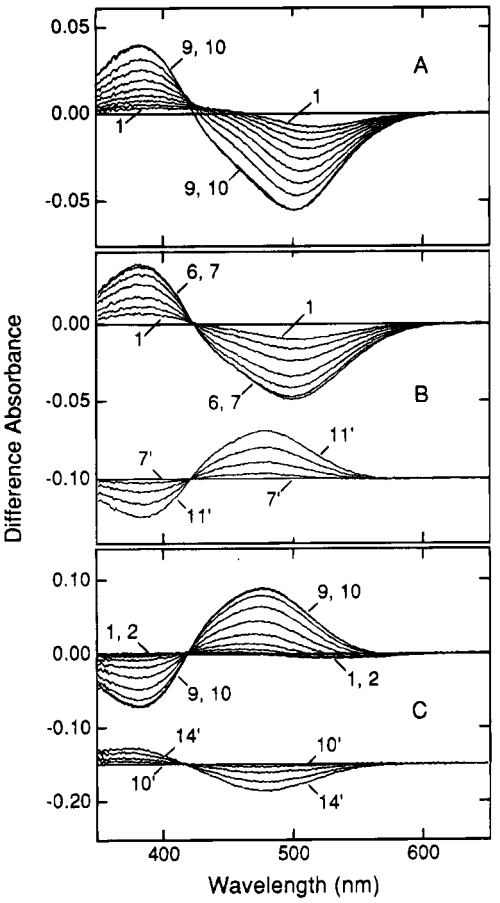

FIGURE 3: Thermal reactions of meta intermediates of chicken rhodopsin measured at −30 (A), −20 (B), and −10 °C (C) by low-temperature time-resolved spectroscopy. A rhodopsin/56% glycerol mixture was cooled to each temperature and irradiated with orange light (>570 nm) for 30 s. Absorption spectra were recorded immediately (0 min) and 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 15 424 min after irradiation. (A) Difference spectra calculated by subtracting the spectrum recorded immediately after irradiation from those recorded at 2−1024 min after irradiation (curves 1−10). (B) Top: Difference spectra calculated by subtracting the spectrum recorded immediately after irradiation from those recorded at 2−128 min after irradiation (curves 1−7). Bottom: Difference spectra calculated by subtracting the spectrum recorded at 128 min after irradiation from those recorded at 128−2048 min after irradiation (curves 7′−11′). (C) Top: Difference spectra calculated by subtracting the spectrum recorded immediately after irradiation from those recorded at 2−1024 min after irradiation (curves 1−10). Bottom: Difference spectra calculated by subtracting the spectrum recorded at 1024 min after irradiation from those recorded at 1024−15424 min after irradiation (curves 10′−14′).

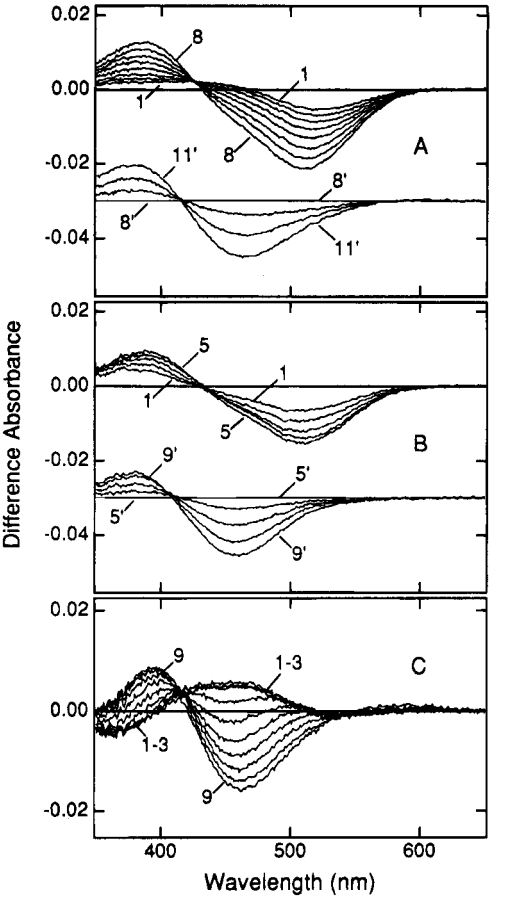

FIGURE 4: Thermal reactions of meta intermediates of chicken green measured at −30 (A), −20 (B), and −10 °C (C) by low-temperature time-resolved spectroscopy. A chicken green/56% glycerol mixture was cooled to each temperature and irradiated with orange light (>570 nm) for 30 s. Absorption spectra were then recorded immediately (0 min) and 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, and 2048 min after irradiation. (A) Top: Difference spectra calculated by subtracting the spectrum recorded immediately after irradiation from those recorded at 2−256 min after irradiation (curves 1−8). Bottom: Difference spectra calculated by subtracting the spectrum recorded at 256 min after irradiation from those recorded at 256−2048 min after irradiation (curves 8′−11′). (B) Top: Difference spectra calculated by subtracting the spectrum recorded immediately after irradiation from those recorded at 2−32 min after irradiation (curves 1−5). Bottom: Difference spectra calculated by subtracting the spectrum recorded at 32 min after irradiation from those recorded at 32−512 min after irradiation (curves 5′−9′). (C) Difference spectra calculated by subtracting the spectrum recorded immediately after irradiation from those recorded at 2−512 min after irradiation (curves 1−9).

process of cRh-meta III (Figure 3C, bottom). Therefore, we concluded that the phase demonstrates the decay of cG-meta III. Although the formation process of meta III was not observed at −30 and −20 °C, it was clearly observed at −10 (curves 1−3 in Figure 4C) and −15 °C (data not shown). Therefore, the formation time constant of cG-meta III became similar to that of meta II when the temperature was lowered. It should be noted that cG-meta II and cG-meta III displayed absorption maxima about 20 nm blue shifted (Figure 4C) from those of cRh-meta II and cRh-meta III (Figure 3C), respectively.

From the experiments performed at various temperatures ranging from −30 to +20 °C, absorbance changes at several wavelengths after irradiation were fitted with exponential time functions in order to estimate the thermodynamic parameters. A single-wavelength measurement was applied to monitor the change in absorbance in the time range 0−200 s after a 2 s irradiation of the sample with focused orange light (>570 nm). Figure 5 shows Arrhenius plots of the observed rate constants. Activation parameters in each reaction were calculated from the following equations:

$$\ln k = -(\Delta G^{\ddagger}/RT) + \ln(k_B T/h) \quad (1)$$

$$\ln k = -(\Delta H^{\ddagger}/R)(1/T) + (\Delta S^{\ddagger}/R) + \ln(k_B T/h) \quad (2)$$

$$\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T \quad (3)$$

where $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, and $\Delta S^{\ddagger}$ are the difference activation enthalpy, difference activation free energy, and difference activation entropy, respectively, and R, k_B, h, and T are the

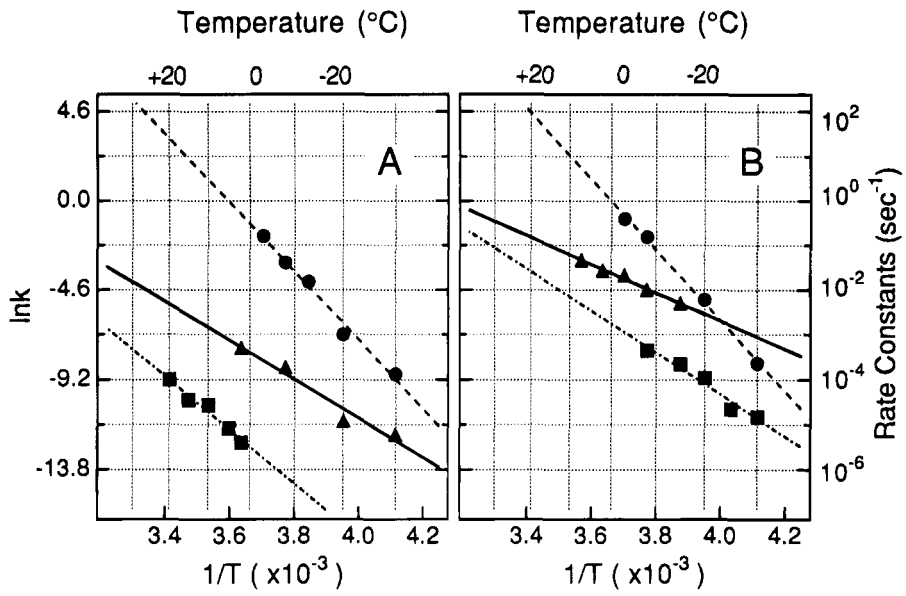

FIGURE 5: Temperature dependence on the formation and decay of the meta intermediates of rhodopsin (A) and chicken green (B). Apparent rate constants of meta II formation, meta II decay, and meta III decay (circles, triangles, and squares, respectively) are plotted in an Arrhenius manner, and the experimental points were fitted with straight lines (dashed, solid, and dotted lines, respectively) by a least-squares method.

Table 1: Thermodynamic Parameters of Formation and Decay of Meta Intermediates[a]

	chicken rhodopsin		chicken green	
	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/mol/deg)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (cal/mol/deg)
meta II form	34.7 (2.4)	66.3 (9.5)	35.7 (1.4)	72.5 (5.3)
meta II decay	19.3 (3.0)	−3.2 (11.8)	14.0 (0.9)	−14.6 (3.5)
meta III decay	26.8 (2.8)	14.7 (10.0)	20.8 (2.7)	5.1 (10.5)

[a] Numbers in parentheses are standard deviations.

gas constant, Boltzmann's constant, Planck's constant and temperature, respectively. The values obtained are listed in Table 1.

Photobleaching Process of Chicken Green and Rhodopsin at pH 10. According to previous calculation (Okano et al., 1992), chicken green has a positive net charge at neutral pH, while rhodopsin has negative charge. Thus, we changed the pH of the chicken green sample to 10 to make the net charge of chicken green similar to that of rhodopsin at pH 7. Like rhodopsin, but unlike iodopsin (Wald et al., 1955), chicken green was stable and the absorption spectrum was rarely changed in the course of alkalization of the sample from pH 7 (data not shown). Figure 6A,B shows the spectral changes due to the conversion from meta II to meta III in both chicken green and rhodopsin at pH 10, respectively. Decay of cG-meta II (Figure 6A) was 7 times slower than that of cG-meta II at pH 7 (Shichida et al., 1994; Figure 4C), while the decay of cRh-meta II (Figure 6B) was 3 times faster than that at pH 7 (Figure 3C). To demonstrate the change in the decay time constant of meta II more clearly, the absorbance change at 380 nm was plotted against the incubation time (Figure 6C,D). This figure clearly shows that the decay time constant of cG-meta II at pH 10 (Figure 6C) was close to that of cRh-meta II at both pH 7 and 10 (Figure 6D), as compared to that of cG-meta II in neutral conditions (Figure 6C). To make it clear that no irreversible reaction took place during pH change manipulation, we observed the decay processes of meta II (Figure 6C,D) in the samples once changed to pH 10 and reverted to pH 7 and confirmed that the decay time constants were identical with those without alkalization (Figure 6C,D).

DISCUSSION

In the present study, we showed that chicken green exhibits a bleaching process similar to that of rhodopsin. However, almost all of the intermediates are less stable than the respective intermediates of rhodopsin, and the absorption maxima of meta intermediates were located at wavelengths about 20 nm shorter than those of meta intermediates of rhodopsin. Furthermore, it was suggested from the pH change experiments that the difference in total net charge between chicken green and rhodopsin is one of the important factors regulating the thermal stability of the meta II intermediate. Now, we will discuss the difference in molecular properties between chicken green and rhodopsin on the basis of the acquired results.

Spectroscopic Properties. One of the interesting differences between chicken green and rhodopsin is that meta intermediates of chicken green (cG-meta I, II, and III) exhibit absorption maxima at about 20 nm shorter wavelengths than those of rhodopsin (cRh-meta I, II, and III), while the original pigment, cG-batho, and cG-lumi exhibit absorption maxima close to those of their rhodopsin counterparts. The fact that the difference appears only in the meta intermediates suggests a notable change in the chromophore−opsin interaction taking place in the lumi−meta I transition. Because our previous study (Imai et al., 1994) revealed that the protein reaches an adapted conformation for the all-trans chromophore in the meta I stage, it is reasonable that the early intermediates, batho and lumi, retain a protein conformation similar to that of the original state, while meta intermediates are in conformations different from that of the original state. In this context, it is of interest to speculate about the molecular differences between chicken green and rhodopsin, which might explain the differences in absorption maxima of meta intermediates.

Recent accumulated evidence has suggested that the absorption spectrum of the visual pigment is regulated by a

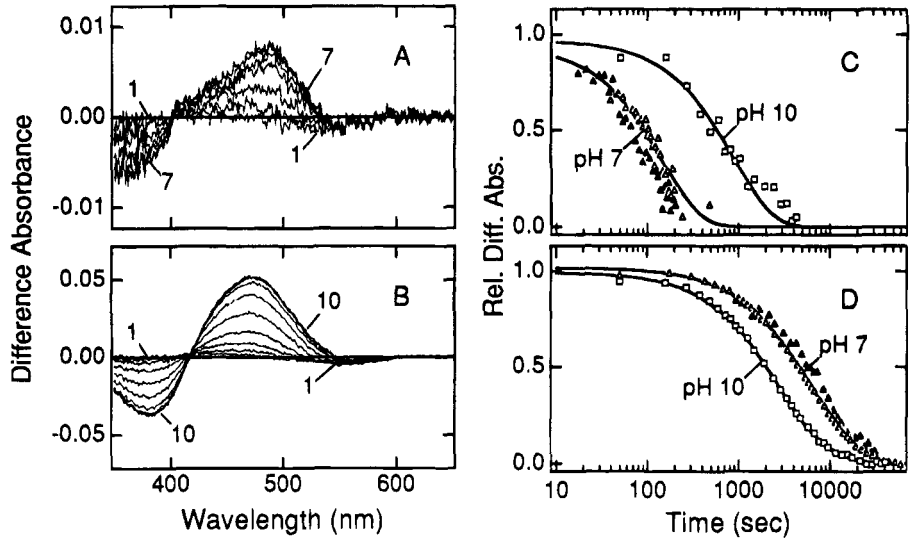

FIGURE 6: Thermal reactions of meta II intermediates of chicken green (A) and rhodopsin (B) at pH 10 measured by low-temperature time-resolved spectroscopy. The pigment/56% glycerol mixture was cooled to −10 °C, followed by irradiation with orange light (>570 nm) for 30 s. Absorption spectra were then recorded immediately (0 min) and 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 min after irradiation. In panels A and B, difference spectra calculated by subtracting the spectrum recorded immediately after irradiation from those recorded at 2−1024 min after irradiation are shown (curves 1−10). Absorbance at 380 nm of the sample is plotted against incubation time after irradiation of chicken green (C) and rhodopsin (D). Open triangles and squares are the data obtained from the sample at pH 7 and 10, respectively. Closed triangles are those obtained from the sample once changed to pH 10 and reverted to pH 7.

dissociative or polarized residue(s) present near the chromophore transmembrane region. Since chicken green has an amino acid sequence highly similar to that of rhodopsin (Wang et al., 1992; Okano et al., 1992), only a limited number of amino acid residues are present that differ from in each other. Among the residues, glutamic acid 122 (E122) of chicken rhodopsin, which is replaced by glutamine (Q122) in chicken green, is interesting because site-directed mutation of E122 to Q in rhodopsin caused an 18−20 nm blue shift of the absorption maximum from the original bovine rhodopsin (Sakmar et al., 1989; Zhukovsky & Oprian, 1989; Nathans, 1990) and chicken rhodopsin (Imai et al., unpublished data). In spite of the replacement of the residue, chicken green exhibits an absorption maximum similar to that of rhodopsin, while one of the visual pigments present in gecko retina, called gecko P467, which has an amino acid sequence highly homologous to chicken green and contains Q122 (Kojima et al., 1992), exhibits a maximum (467 nm) considerably blue shifted from that of rhodopsin. Similar absorption maxima between chicken green and rhodopsin were explained previously by the presence of the other polarizable amino acid residue(s), which might compensate for the effect of the blue shift due to the replacement of E122 (Kojima et al., 1992). If one assumes that the regulation mechanism of the absorption maximum of the intermediate is similar to that of the original pigment, the absorption maxima of cG-batho and cG-lumi should resemble those of cRh-batho and cRh-lumi, respectively, because they have a protein conformation similar to that of the original pigment. On the other hand, the shift in absorption maxima observed in meta intermediates is similar to that due to the replacement of E122 by Q122. Therefore, we speculate that the replacement of E122 by Q122 is one of the main factors of the difference in absorption maxima between meta intermediates of chicken green and of rhodopsin, and large protein motion occurring in the lumi−meta I transition would remove the effect of the compensative residue(s). This speculation is further supported by the fact that meta intermediates of P467 (Kojima et al., 1995) and meta II and III of the E122Q mutant of chicken rhodopsin (Imai et al., manuscript in preparation) exhibit absorption maxima considerably blue shifted from those of cRh-meta intermediates.

Thermodynamic Properties. As shown in Table 1, the magnitudes of thermodynamic parameters for the formation of cG-meta II are qualitatively similar to those of cRh-meta II, while those for the decay of cG-meta II and III are considerably different from those of cRh-meta II and III. Since a reaction becomes faster as activation enthalpy becomes smaller, the faster decay of cG-meta II and III is due to smaller activation enthalpies for these intermediates. If one assumes that the activation enthalpy reflects a number of breakages in the hydrogen-bonding network system of the protein, a greater number of breakages would take place during the decay of meta II and III in rhodopsin than in chicken green. Therefore, the intermediates of rhodopsin would be in a more tightly fixed conformation than those of chicken green.

The formation and decay time constants of meta intermediates at room temperature (+20 °C) in both chicken green and rhodopsin were estimated by extrapolation of the temperature dependence of the time constants (Figure 5) and activation energies (Table 1) obtained at low temperatures. The results showed that cG-meta II forms and decays with 10 ms and 7 s at +20 °C, respectively, which are 3 and 30 times faster than cRh-meta II (Figure 7). In addition, the decay time constant of cG-meta III is 40 s, which is 270 times faster than that of cRh-meta III. It should be noted that the estimated time constants of rhodopsin are in good agreement with those measured at room temperature by laser photolysis (Okada et al., unpublished data), indicating the appropriateness of this approach. Previously, we reported that formation and decay of cG-meta II and III at −10 °C are considerably faster than those of cRh-meta II and III (Shichida et al., 1994). We concluded that this trend is conserved at a wide temperature range, including physiological temperature.

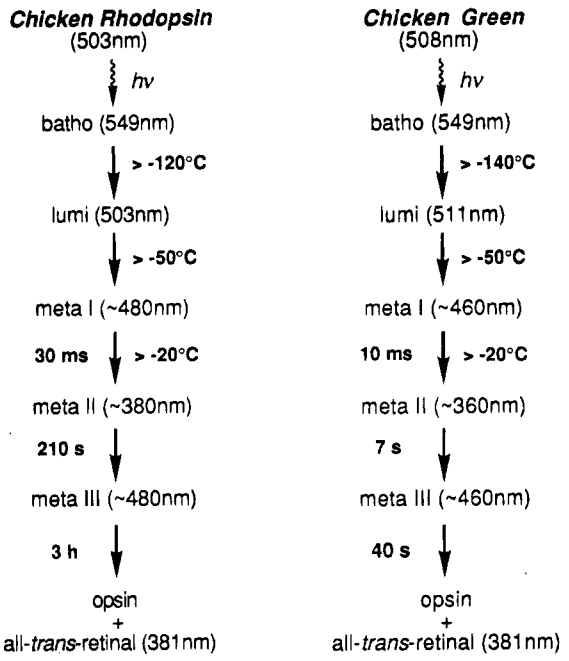

FIGURE 7: Photobleaching process of chicken green and rhodopsin. Transition temperatures and estimated time constants at room temperature are shown on the right- and left-hand sides of the arrows, respectively.

Effect of pH and Evolution of Visual Pigments. The decay of cG-meta II was greatly suppressed at pH 10, and the decay time constant became close to that of cRh-meta II. These results clearly show the relationship between the net charge of the visual pigment, which reflects the number of dissociative amino acid residues, and the thermal behavior of meta II. Since we observed the decay of meta II only at pH 7 and 10, it is difficult to determine the molecular event(s) that interprets these changes. One is that a dissociative amino acid residue(s) present only in chicken green is(are) titrated when the sample is alkalized, and the other is that the decay is suppressed by low concentrations of proton at alkaline conditions. The interesting phenomenon is that the decay of cG-meta II was suppressed, while that of cRh-meta II was enhanced at alkaline conditions. Since the enhancement in the decay of cRh-meta II was explained by the release of a proton from the protein to the outer environment (Ostroy, 1974), a proton could be taken into the protein during the decay of cG-meta II. If so, a molecular event occurring in this process is in contrast between chicken green and rhodopsin.

Since the dissociative amino acid residues present only in chicken green but not in chicken rhodopsin are well conserved in the other cone visual pigments (Okano et al., 1992), the relationship between the net charge and the thermal behavior of meta II obtained in the present study could be expanded to the difference between rod and cone visual pigments. In fact, all of the cone visual pigments investigated so far display rapid decay of meta II compared with rhodopsin, and total net charges are positive at neutral conditions. It has been shown that rhodopsin has evolved out of cone visual pigments, accompanied by an increase in net negative charge of the pigment (Okano et al., 1992). Taken together, animals have acquired rhodopsin by changing the electric properties of cone visual pigments to obtain a capacity for high signal amplification by stabilizing meta II.

It should be noted that, in the present study, we showed the difference between the intrinsic lifetimes of meta intermediates of chicken green and rhodopsin. Recent biochemical studies have suggested that the lifetime of "activated rhodopsin" in photoreceptor cells is controlled by the regulatory proteins (rhodopsin kinase and arrestin), although only a few reported on the regulation mechanism of "activated cone pigment" (Fukada et al., 1990). Thus, it is difficult at present to speculate about the relationship between the lifetime of "activated visual pigment" and the intrinsic lifetime of meta II, and more extensive studies on the regulation mechanism in cone visual pigments would be necessary. Low-temperature time-resolved spectroscopy will be one of the powerful techniques used to investigate such a complicated dynamic system.

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