Is Chicken Green-Sensitive Cone Visual Pigment a Rhodopsin-like Pigment? A Comparative Study of the Molecular Properties between Chicken Green and Rhodopsin†

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Received May 16, 1994; Revised Manuscript Received June 16, 1994®

ABSTRACT: Chicken green is a visual pigment present in chicken green-sensitive cones and has an amino acid sequence more similar than any other cone visual pigments to the rod visual pigments, rhodopsins. Here we have investigated the molecular properties of chicken green and compared them with those of rhodopsin to elucidate whether or not chicken green is a rhodopsin-like pigment. While chicken green has a molecular extinction coefficient and a photosensitivity very similar to those of rhodopsin, it displays faster regeneration from 11-*cis*-retinal and opsin and faster formation and decay of the physiologically active meta II intermediate than rhodopsin. These differences correlate with the physiological difference between cones and rods. Thus in spite of the similarity in amino acid sequence, chicken green displays molecular properties required for a cone visual pigment that are clearly different from those of rhodopsin.

In the eyes of most vertebrates there are two types of photoreceptor cells, rods and cones, which mediate scotopic (twilight) and photopic (daylight) vision, respectively. Rods are more sensitive to light than cones, while cones display rapid photoresponse and rapid adaptation as compared with rods (Wald et al., 1955; Schnapf & Baylor, 1987). In contrast with extensive studies on the visual transduction process in rods, little is known about the process in cones, which has hampered the elucidation of the molecular mechanisms leading to the differences in physiological function between rods and cones. However, the recent development of molecular cloning techniques has led to the determination of the primary structures of several proteins in cones and revealed that these proteins have amino acid sequences similar to but considerably different from those in rods (Nathans et al., 1986; Lerea et al., 1986; Li et al., 1990; Kuwata et al., 1990; Okano et al., 1992a). Therefore, it has been speculated that the physiological properites of rods and cones would be determined by the molecular properties of their phototransduction proteins which differ in their amino acid sequences. In fact, biochemical and spectroscopic studies revealed that iodopsin, a visual pigment present in chicken red-sensitive cones which has an amino acid sequence with 40% identity to the rod visual pigment rhodopsin (Kuwata et al., 1990; Tokunaga et al., 1990), and cGMP-phosphodiesterase in cones, which has 60% identity to its rod counterpart (Li et al., 1990), display several interesting properties which might partially account for the difference in photoresponse between rods and cones (Yoshizawa et al., 1991; Yoshizawa, 1992; Shichida et al., 1993; Okada et al., 1994; Gillespie & Beavo, 1988).

While most vertebrates have only a single form of rod visual pigment, rhodopsin, they have multiple types of cone visual pigments. For example, humans have three types of cone visual pigments, human red, green, and blue (Brown et al., 1964; Mark et al., 1964), and the chicken has four types of cone visual pigments, chicken red (iodopsin), green, blue, and violet (Yen & Fager, 1984; Okano et al., 1989). Determination of the amino acid sequences of these pigments (Nathans et al., 1986; Kuwata et al., 1990; Tokunaga et al., 1990; Wang et al., 1992; Okano et al., 1992a) revealed the interesting result that, among these pigments, chicken green, a visual pigment present in chicken green-sensitive cones (Oishi et al., 1990; Raymond et al., 1993), shows high homology in amino acid sequence with vertebrate rhodopsins (higher than 71% identity) but less than 40% identity with the other cone visual pigments (Wang et al., 1992; Okano et al., 1992a). Furthermore, a phylogenetic tree constructed on the basis of amino acid identity clearly shows that chicken green clusters with vertebrate rhodopsins to form one of the four groups of visual pigments, while the other groups contain only cone-type visual pigments (Okano et al., 1992a). On the basis of these results, one might speculate that chicken green should be classified among rod pigments rather than among cone pigments. However, comparison of the amino acid sequences among chicken green, rhodopsin, and other cone visual pigments suggests that, like other cone visual pigments, chicken green has an isoelectric point more basic than rhodopsin (Okano et al., 1992a), which is consistent with the lack of affinity of chicken green and other cone visual pigments for DEAE-Sepharose (Yen & Fager, 1984; Okano et al., 1989). In addition, chicken green and other cone visual pigments are unstable in the presence of hydroxylamine even in the dark (Yen & Fager, 1984; Okano et al., 1989; Wang et al., 1992). Therefore, it is an important question whether chicken green shows physiological properties similar to those of rhodopsin or other cone pigments.

In relation to the faster dark adaptation and faster response of cones than rods, the expected properties of cone visual pigments would be faster regeneration from their opsin moieties and the chromophore, 11-*cis*-retinal (Wald et al., 1955), and

† This work was supported in part by Grants-in-Aid for Scientific and Cooperative Research from the Japanese Ministry of Education, Science and Culture, JSPS fellows, a grant from the Human Frontier Science Program, and the Ciba-Geigy Foundation (Japan) for the Promotion of Science.

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® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

0006-2960/94/0433-9040$04.50/0

faster formation of the physiologically active intermediate which can bind to and activate the retinal G-protein, transducin (Shichida et al., 1993; Okada et al., 1994). In addition, a shorter lifetime of the physiologically active intermediate could be one of the molecular bases for the lower photosensitivity of cones than rods (Okada et al., 1994), because photosensitivity of the cells is related to the extent of amplification of the light signal which depends on how many transducins are activated (Kühn et al., 1981; Stryer et al., 1981). Therefore, we have investigated the molecular properties of chicken green and compared them to those of rhodopsin with special attention to the physiological difference between rods and cones.

MATERIALS AND METHODS

Preparation of Chicken Green, Rhodopsin, and Iodopsin and Their Opsin Moieties. Chicken green (λ_{max} = 508 nm) was purified from chicken retinas by a method modified from that described previously (Okano et al., 1989). Visual pigments in photoreceptor outer segments isolated from about 4000 chicken retinas were extracted with buffer A (50 mM HEPES,[1] 140 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, 50 KIU/mL aprotinin, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 6.6) supplemented with 0.75% CHAPS and 1 mg/mL PC. After the CHAPS and PC concentration of the extract was lowered to 0.6% and 0.8 mg/mL, respectively, the extract was applied to a ConA–Sepharose (Pharmacia) column (16 mm × 300 mm) which had been equilibrated with buffer A supplemented with 0.6% CHAPS and 0.8 mg/mL PC (buffer B). After elution of a mixture of chicken violet, blue, and iodopsin with buffer B supplemented with 1.5 mM methyl α-mannoside, a mixture of chicken green and rhodopsin was eluted with buffer C (buffer B with a NaCl concentration of 10 mM) supplemented with 200 mM methyl α-mannoside. Glycerol was added to the mixture to make a final concentration of 15.8% (v/v) to stabilize chicken green and rhodopsin (Okano et al., 1989). The eluate was then applied to a DEAE-Sepharose (Pharmacia) column (16 mm × 300 mm) which had been equilibrated with buffer D [buffer C further supplemented with 15.8% (v/v) glycerol]. Under our experimental conditions, rhodopsin was adsorbed to the column, while chicken green passed through the column. Thus a fraction containing mostly chicken green was obtained as a passed-through fraction. This faction was further applied to a CM-Sepharose (Pharmacia) column (16 mm × 300 mm), from which chicken green was eluted by buffer B supplemented with 15.8% (v/v) glycerol. For low-temperature experiments at −8 °C or −185 °C, glycerol was further added to the chicken green sample to a final concentration of 56% (v/v) or 72% (v/v), respectively.

G-Photopsin (the apoprotein of chicken green) was prepared by irradiation of a chicken green sample with an orange light (>520 nm) in the presence of 10 mM hydroxylamine, followed by purification by means of ConA–Sepharose column chromatography, as described above.

Chicken rhodopsin, iodopsin, and their opsin moieties were prepared by the method previously described (Okano et al., 1992b).

Regeneration of Visual Pigments. 11-*cis*-Retinal was prepared by irradiation of *all-trans*-retinal (Sigma) dissolved in acetonitrile with light from a 2-kW xenon lamp (Ushio Co. Ltd.) for 30 min at 0 °C and purified by means of HPLC (Maeda et al., 1977). It was dissolved in ethanol to make a 7.5 μM solution. Then 5 μL of ethanol solution was added to 250 μL of each opsin solution (150 nM, pH 6.6) at 2 °C, followed by incubation at this temperature until the changes of absorbance due to the regeneration of the pigment were saturated. Wavelengths for monitoring were 530 nm for chicken green and rhodopsin and 570 nm for iodopsin using a spectrophotometer (Shimadzu UV-3000) operated in a time-scan mode. A sample cell holder connected with a thermostatic circulator (RTE-220, Neslab) was installed in the sample compartment of the spectrophotometer to keep the sample at a constant temperature (2 °C). The experimental data were analyzed by a computer (PC9801RA, NEC).

Low-Temperature Spectroscopy. For spectral measurements at low temperatures, a MPS-2000 recording spectrophotometer (Shimdazu) equipped with a special glass cryostat (Yoshizawa & Shichida, 1982) or a combination of a cryostat (CF-1204, Oxford) and a temperature controller (ITC-4, Oxford) was used. The spectral data were stored and analyzed by a personal computer (PC-9801RA, NEC). The light source was a 1-kW tungsten–halogen lamp (Rikagaku-Seiki) which had passed through an interference filter (Nihonshinku, half-bandwidth = 2 nm) or glass cutoff filter (Toshiba, VO 59). A 5-cm water layer was placed in front of the projector lamp to remove infrared radiation. For correction of the light scattering of the sample at −185 °C, opal glass was placed in both the sample and reference beams (Yoshizawa & Shichida, 1982).

RESULTS

Molecular Extinction Coefficient of Chicken Green. The molecular extinction coefficient of chicken green relative to that of iodopsin was estimated from the following experiments using a sample containing chicken green and iodopsin[2] (Figure 1A). The sample (curve 1 in Figure 1A) was first irradiated with a red light (>590 nm) to bleach chicken green and iodopsin (curve 2 in Figure 1A). Second, successive portions of 11-*cis*-retinal dissolved in ethanol (150 μM 11-*cis*-retinal in 1 μL of solution) were added, and the spectra were recorded 10 min after each addition (curves 3–8 in Figure 1A). The difference spectra were calculated from the spectra before and after respective additions of 11-*cis*-retinal (Figure 1B). Since the difference spectrum calculated from the spectra before and after each addition is a composite of the spectra of chicken green and iodopsin, percent compositions of chicken green and iodopsin were estimated by simulating the difference spectrum with the spectra of chicken green and iodopsin (Figure 1C). Under the experimental conditions, the sum of the molar amounts of regenerated chicken green and iodopsin would be identical in each addition of 11-*cis*-retinal, and the following equations are derived:

$$\frac{Abs_{green}}{\epsilon_{green}} + \frac{Abs_{iod}}{\epsilon_{iod}} = [11\text{-}cis\text{-retinal}] = \text{const}$$

$$Abs_{green} = -\left(\frac{\epsilon_{green}}{\epsilon_{iod}}\right) Abs_{iod} + \text{const}$$

where Abs_{green} and Abs_{iod} are the absorbances at the maxima of regenerated chicken green and iodopsin in each addition of 11-*cis*-retinal, respectively, and ϵ_{green} and ϵ_{iod} are the molecular extinction coefficients of chicken green and iodopsin, respectively. Therefore, when the Abs_{green} estimated in each

[1] Abbreviations: 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.

[2] Because of the availability of an extraordinarily low concentration of apoprotein of chicken green, we estimated an extinction coefficient of chicken green by using iodopsin as an inner control.

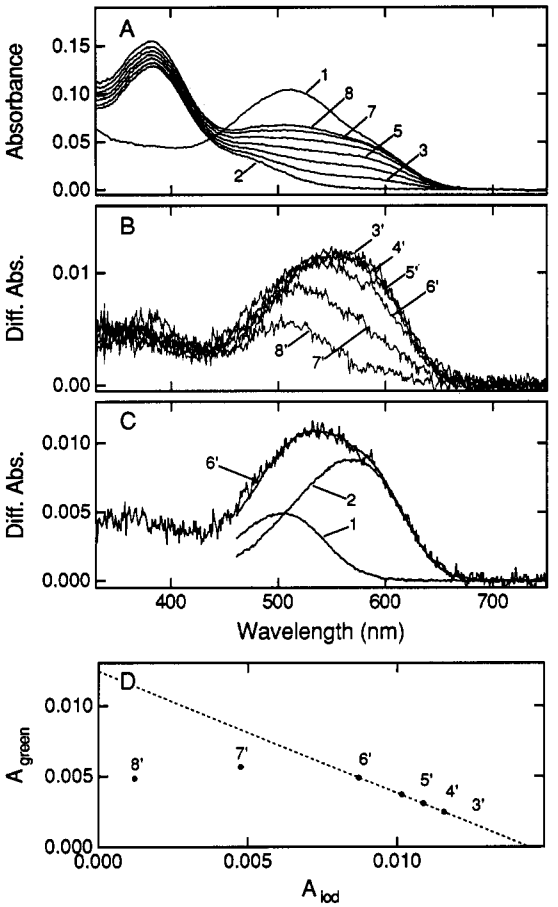

FIGURE 1: Estimation of the molecular extinction coefficient of chicken green. (A) A mixture of chicken green and iodopsin (curve 1) was irradiated with >590 nm of light for 40 min at 2 °C (curve 2); then successive portions of 11-*cis*-retinal dissolved in ethanol (150 μM 11-*cis*-retinal in 1 μL of solution) were added, and the spectra were recorded 10 min after each addition (curves 3–8). (B) Difference spectra calculated from the spectra before and after each addition of 11-*cis*-retinal. (C) Difference spectrum calculated before and after the fifth addition of 11-*cis*-retinal to the sample (curve 6′) simulated with the sum of the spectra of chicken green (curve 1) and iodopsin (curve 2). The smooth curve which is overlapped with curve 6′ is the fitted spectrum composed of 38% chicken green and 62% iodopsin. (D) Relationship between the amounts of chicken green and iodopsin regenerated by addition of 11-*cis*-retinal to the sample. The amounts of chicken green were plotted against those of iodopsin.

addition of 11-*cis*-retinal are plotted against Abs_{iod}, the slope should exhibit the ratio of the molecular extinction coefficient of chicken green and iodopsin. In Figure 1D, the first four points are on a linear line, while the others were below the line. The deviation from the linear line indicated the incomplete binding of 11-*cis*-retinal to the opsins due to the absence of enough amounts of opsins remaining in the sample. Therefore, these points were neglected, and the slope was estimated to be 0.86. Since the extinction coeffiicent of iodopsin was reported to be 47 200 (Okano et al., 1992b), that of chicken green was calculated to be 40 800, which is very close to that of rhodopsin (40 700; Okano et al., 1992b).

Rate of Regeneration of Chicken Green. In relation to the rapid dark adaptation of cones as compared with rods, it is of interest to investigate whether chicken green exhibits a regeneration rate similar to that of iodopsin or rhodopsin. Figure 2 shows the regeneration processes of chicken green, iodopsin, and rhodopsin after addition of 11-*cis*-retinal to the respective opsin solutions at 2 °C. Since an equimolar amount of 11-*cis*-retinal was added to each opsin solution (see Materials and Methods), the course of regeneration, a bimolecular, should be simulated by a hyperbolic curve. Solid curves in

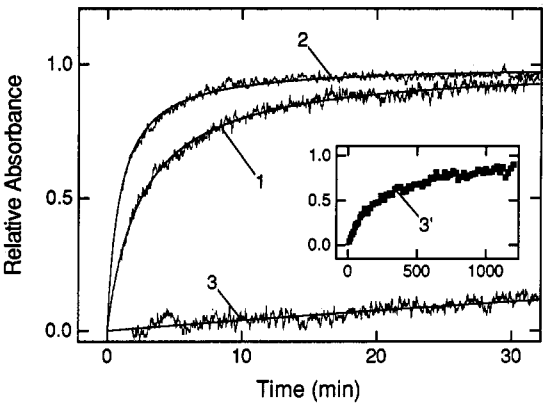

FIGURE 2: Time courses of regeneration of chicken green, iodopsin, and rhodopsin. 11-*cis*-Retinal dissolved in 5 μL of ethanol (7.5 μM) was added to 250 μL of each opsin solution (150 nM, pH 6.6) at 0 °C, followed by incubation at this temperature until the changes of absorbance due to the regeneration of the pigments were saturated. Wavelengths for monitoring are 530 nm in chicken green (curve 1) and rhodopsin (curve 3) and 570 nm in iodopsin (curve 2). Solid curves are the fitted hyperbolic curves with time constants of 2.5 min, 1 min, and 4 h for chicken green, iodopsin, and rhodopsin, respectively. Inset: Regeneration process of chicken rhodopsin monitored on a longer time scale.

the figure are the fitted hyperbolic curves with time constants of 2.5 min, 1 min, and 4 h for chicken green, iodopsin, and rhodopsin, respectively. Therefore, chicken green regenerated about 100 times faster than rhodopsin with a rate constant comparable to that of iodopsin. These results strongly suggest that the rapid regeneration is one of the common properties of cone visual pigments independent on the extent of amino acid identity.

Formation of the Batho Intermediate and Photosensitivity of Chicken Green. Irradiation of chicken green with 501-nm light at −185 °C resulted in a photo-steady-state mixture of chicken green, its 9-*cis* pigment, and batho intermediate. Typical experimental results are shown in Figure 3A. Under similar experimental conditions, chicken rhodopsin also converted to a photo-steady-state mixture composed of rhodopsin, isorhodopsin, and bathorhodopsin (Figure 3B). In Figure 3C, the changes of absorbance at 550 nm due to the formation of batho intermediates of chicken green and rhodopsin are plotted as a function of time for irradiation. That the time plot for chicken green is identical with that for rhodopsin clearly showed that chicken green has photosensitivity identical with that of rhodopsin. In the previous section, we showed that the molecular extinction coefficient of chicken green is almost identical with that of rhodopsin, indicating that the quantum yield of chicken green is also identical with that of rhodopsin.

Formation and Decay of the Meta II Intermediate of Chicken Green. To elucidate whether or not chicken green has a meta II intermediate whose thermal behavior is similar to that of rhodopsin (metarhodopsin II), we have investigated thermal reactions of a photoactivated chicken green and compared them to those of a photoactivated rhodopsin. Figure 4 shows the time courses of absorbance changes at 450 nm (chicken green), 500 nm (rhodopsin), and 380 nm (both pigments) after irradiation of chicken green and rhodopsin with an orange light (>570 nm) at −8 °C. In both pigments, triphasic absorbance changes were observed: The first phase, a decrease of absorbance at 450 or 500 nm and increases at 380 nm, is due to the decay of the meta I intermediate with the concurrent formation of the meta II intermediate of chicken green or rhodopsin. The second phase, the reversal changes of the absorbances, reflects the decay of the meta II intermediate with the concurrent formation of the meta III intermediate.

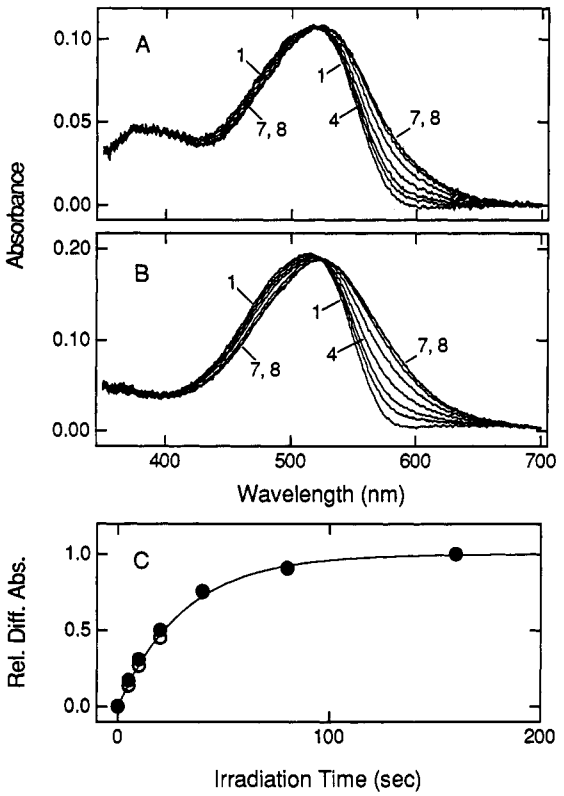

FIGURE 3: Spectral changes in the course of the conversion of chicken green and rhodopsin to the respective batho intermediates. (A or B) Chicken green–72% glycerol mixture (pH 6.6, curve 1 in panel A) or rhodopsin–72% glycerol mixture (curve 1 in panel B) was successively irradiated with 501 nm of light at −185 °C for a total of 5, 10, 20, 40, 80, 160, and 320 s (curves 2–8 in panel A or B). (C) Changes of absorbance at 550 nm due to the formation of batho intermediates of chicken green (open circles) and chicken rhodopsin (closed circles) were plotted as a function of time for irradiation. The solid curve is the simulated exponential curve.

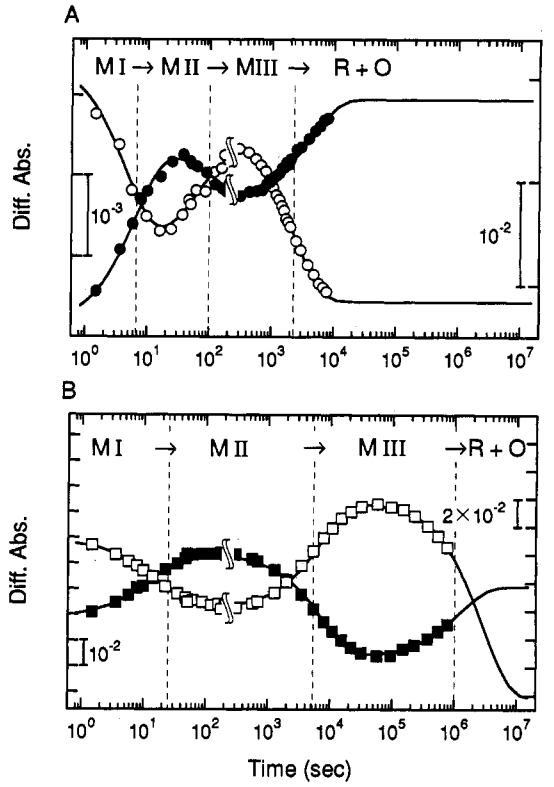

FIGURE 4: Time plots showing changes in absorbance due to formation and decay of three meta intermediates in chicken green (A) and rhodopsin (B). Each pigment was cooled to −8 °C and irradiated with >570 nm of light, followed by monitoring the absorbance changes at 450 nm (chicken green, open circles) or 500 nm (rhodopsin, open squares) and 380 nm (chicken green, closed circles; chicken rhodopsin, closed squares) at this temperature. Note that absorbance changes are plotted against a logarithmic time scale. The processes proceeding up to 3×10^2 s were monitored by the time scan mode of the spectrophotometer after irradiation of the sample of 2 s, while the later processes were monitored by the repeat scan mode of spectra after irradiation of the sample for 30 s. The changes of absorbance in early and later processes were combined at 3×10^2 s after correction for the changes of absorbance at the later stage to that at the early stage. Solid and dashed curves are the fitted three single-exponential curves for the changes of absorbance at 450 (or 500) and 380 nm, respectively. Vertical bars on each side of the panels represent the scales of absorbance in early and later processes, respectively. Vertical broken lines in the panels represent the time constants of the respective processes.

The subsequent decrease of absorbance at 450 or 500 nm and increase at 380 nm shows the dissociation process of the meta III intermediate of chicken green or rhodopsin to *all-trans*-retinal and opsin. The experimental data can be expressed by three successive single-exponential curves with times constants of 6.7, 100, and 2300 s in chicken green and 25, 5300, and 1.0×10^6 s in rhodopsin, respectively. From these results, it is clear that the meta II intermediate of chicken green is present, having formation and decay time constants about 4 and 50 times smaller than those of metarhodopsin II. It should be noted that the thermal behavior of the meta II intermediate of chicken green is very similar to that of iodopsin (Shichida et al., 1993; Okada et al., 1994).

DISCUSSION

The results described above clearly show that chicken green has a molecular extinction coefficient and a photosensitivity very similar to those of rhodopsin and iodopsin, while chicken green exhibits a rate of regeneration from 11-*cis*-retinal and opsin, and those of formation and decay of the physiologically active intermediate (meta II intermediate) more similar to that of iodopsin than rhodopsin. Therefore, we suggest that chicken green is functionally not a rhodopsin-like pigment but really a cone visual pigment.

The photosensitivity of a visual pigment is one of the important physiological properties that determines the photosensitivity of a photoreceptor cell. Since our previous study showed that iodopsin displays a photosensitivity similar to that of rhodopsin's (Okano et al., 1992b), we expected that chicken green has a photosensitivity similar to those of rhodopsin and iodopsin. In fact, the present results clearly showed that this is the case and that chicken green also converts to a batho intermediate. Since the phylogenetic tree constructed on the basis of amino acid identity (Okano et al., 1992a) indicates that rhodopsin of the lowest vertebrate (lampley) and that of higher vertebrates diverged much later than the divergence of the cone visual pigments into four groups, it is reasonable to speculate that the high photosensitivity of visual pigments which originates from the cis–trans photoisomerization of the chromophore had been acquired in the early period of the evolution of visual pigments.

In the rhodopsin system, the later intermediate, metarhodopsin II, is a physiologically active intermediate in the sense that it can activate transducin, which triggers the enzymatic cascade system in rods (Fukada & Yoshizawa, 1981; Kühn et al., 1981; Stryer et al., 1981). In relation to the faster response of cones than rods (Nakatani & Yau, 1988), one might expect that cone pigments would be faster in the formation of physiologically active intermediates than rhodopsins. Furthermore, the lifetime of the active intermediate should be closely related to the extent of amplification

of the light signal, because activation of transducin by metarhodopsin II is the first step of amplification in the signal transduction system. The present results showed that chicken green has a meta II intermediate whose formation and decay time constants are 4 and 50 times smaller than those of metarhodopsin II. Since phosphorylation of the active intermediate is thought to be one of the essential steps of the signal amplification and shut-off mechanisms (Kühn, 1984; Palczewski et al., 1988; Fukada et al., 1990), a direct relationship between the thermal behavior of meta II intermediates and the photosensitivity of photoreceptor cells is not clear yet. However, it is interesting to note that the differences in time constant between the meta II intermediate of chicken green and rhodopsin are well correlated to the differences in photoresponse and photosensitivity between cones and rods. In fact, it was reported that cones can respond about 4 times faster than rods and have a photosensitivity about 100 times less than rods (Schnapf & Baylor, 1987).

The faster regeneration rates of cone visual pigments and the less stable properties of their meta II intermediates suggest that the protein moieties of cone visual pigments tend to change their conformation more easily than those of rhodopsins. In relation to the physiological functions of photoreceptor cells, rapid regeneraiton of visual pigment would be advantageous for rapid dark adaptation, while instability of the meta II intermediate would be disadvantageous for signal amplification. We therefore speculate that animals have acquired rhodopsins to construct a high signal amplification system at the expense of rapid response and dark adaptation in the course of evolution.

Our results clearly show that overall homology in amino acid sequence is not a clear measure of the molecular properties of visual pigments as related to the difference in physiological function between rods and cones. Rather, the close similarity in molecular property between chicken green and iodopsin suggests the presence of distinctive amino acid residues which determine these properties. Like rhodopsin, chicken green has two aspargines at positions 2 and 15 and two cysteines at positions 322 and 323 (Okano et al., 1992a; Wang et al., 1992) which are the putative glycosylation (Hargrave et al., 1983) and palmitoylation (Ovchinnikov et al., 1988) sites, respectively, while iodopsin has only asparagine at position 15 (Kuwata et al., 1990; Tokunaga et al., 1990). Therefore, posttranslational modifications at these positions may not determine the difference in molecular properties between cone visual pigments, chicken green and iodopsin, and rhodopsin. The most prominent difference between chicken green and rhodopsin is the isoelectric points (p*I*) calculated from their deduced amino acid sequences (Okano et al., 1992a). Chicken green as well as the other cone pigments is basic, while rhodopsins are acidic. This fact implies that the decrease in basic properties of the protein moiety may reduce the rate of thermal reaction but not the photochemical reaction of the visual pigment. In fact, the change of pH of a chicken green sample from 6.6 to 10.0 resulted in slow formation and decay of the meta II intermediate (Imai et al., manuscript in preparation). Therefore, the difference in rate constant could be explained by the difference in electric property between these pigments. This hypothesis could be tested by reaction kinetic experiments of mutant cone pigments whose basic amino acid residue(s) is (are) replaced by acidic amino acid residue(s).

ACKNOWLEDGMENT

We thank Drs. T. Okano and T. Okada and Prof. Maeda for their helpful discussions and suggestions.

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