Circular Dichroism of Metaiodopsin II and Its Binding to Transducin: A Comparative Study between Meta II Intermediates of Iodopsin and Rhodopsin†

Tetsuji Okada,‡ Takahiko Matsuda,‡,§ Hideki Kandori,‡ Yoshitaka Fukada,‡,§ Tôru Yoshizawa,∥ and Yoshinori Shichida*,‡

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan, and Department of Information Systems Engineering, Faculty of Engineering, Osaka Sangyo University, Daito-shi, Osaka 574, Japan

Received December 20, 1993; Revised Manuscript Received February 14, 1994®

ABSTRACT: Through low-temperature absorption and circular dichroism (CD) spectroscopies, and G-protein (transducin) binding experiments, we have investigated molecular properties of the meta II intermediate of iodopsin, a cone visual pigment present in chicken red-sensitive cones. The meta II intermediate of iodopsin (metaiodopsin II, λ_{max} = 390 nm) displayed a positive CD band at about 390 nm and a large negative CD band below 300 nm. It dissociated into *all-trans*-retinal and the protein moiety. A long-lived intermediate corresponding to the meta III intermediate of rhodopsin was not observed in iodopsin, under our experimental conditions. Decay of metaiodopsin II was significantly suppressed in the presence of transducin, but not in the presence of both transducin and GTP, indicating that metaiodopsin II can interact with transducin and activate it. Both metaiodopsin II and metarhodopsin II displayed a large negative CD band below 300 nm. This fact suggested that during the formation of both meta II intermediates, some aromatic amino acid residues and/or a disulfide bond are rearranged, which may be important for expression of catalytic activity for exchange of GDP to GTP on transducin. On the other hand, metaiodopsin II decayed more than 10 times faster than metarhodopsin II. This fact may be one of the reasons why cones are less photosensitive than rods.

Most vertebrate retinas contain two types of photoreceptor cells, rods and cones, which are responsible for scotopic and photopic vision, respectively. Rods and cones are different in light response from each other; rods display higher photosensitivity than cones, while cones display a faster response than rods. In contrast with extensive studies on the visual transduction process in rods, only a little is known about the process in cones. Recent advances in both biochemical and molecular biological studies on cone cells, however, revealed that they have several functional proteins similar to those in rods, suggesting that the visual transduction process in cones would be basically similar to that in rods. Therefore, the difference in light response between rods and cones would originate from different properties of the functional proteins present in the visual cells. Since it has been widely accepted that one of the molecular mechanisms for the high photosensitivity of rods is based on activation of hundreds of transducins catalyzed by one molecule of metarhodopsin II (Fukada & Yoshizawa, 1981; Kühn et al., 1981; Stryer et al., 1981), it is crucial to investigate whether or not cone pigments have biochemically active intermediates corresponding to metarhodopsin II.

Recently, we have investigated the photobleaching process of iodopsin, a visual pigment present in chicken red-sensitive cones, by means of nanosecond laser photolysis at room temperature, and found several intermediates in the bleaching process (Shichida et al., 1993). Among the intermediates, we identified the photoproduct (λ_{max} = 390 nm) formed with a time constant of 6 ms as the meta II intermediate of iodopsin (metaiodopsin II), because its absorption maximum is very close to that of metarhodopsin II (λ_{max} = 380 nm). Therefore, it is of interest to investigate the molecular properties of metaiodopsin II and compare them with those of metarhodopsin II in order to get a clue to elucidate the difference in the signal transduction process between cones and rods.

Current studies clearly showed that metaiodopsin II displayed specific circular dichroism (CD)[1] bands at its absorption maximum and below 300 nm, which are very similar to those of metarhodopsin II. Furthermore, like metarhodopsin II, metaiodopsin II had a binding ability to transducin, suggesting that it has a physiological role similar to metarhodopsin II. Thermal stabilities were, however, significantly different between metaiodopsin II and metarhodopsin II. This fact could be one of the molecular mechanisms closely connected with the difference in photosensitivity between cones and rods.

MATERIALS AND METHODS

Preparation of Iodopsin. Iodopsin was purified as described by Okano et al. (1989). Briefly, visual pigments in photoreceptor outer segments isolated from about 2000 chicken retinas were extracted with a buffer A (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

† This work was supported in part by Grants-in-Aid for Scientific Research and Cooperative Research from the Japanese Ministry of Education, Culture and Science, and by a Human Frontier Science Program.

* To whom correspondence should be addressed.

‡ Kyoto University.

§ Present address: Department of Pure and Applied Sciences, College of Arts and Sciences, The University of Tokyo, Tokyo 153, Japan.

∥ Osaka Sangyo University.

® Abstract published in *Advance ACS Abstracts*, April 1, 1994.

[1] Abbreviations: CD, circular dichroism; 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 unit(s); MOPS, 3-(*N*-morpholino)propanesulfonic acid; T_α, α subunit of transducin; $T_{\beta\gamma}$, βγ subunit of transducin; R-photopsin, protein moiety of iodopsin; scotopsin, protein moiety of rhodopsin.

0006-2960/94/0433-4940$04.50/0

6.6) supplemented with 0.75% CHAPS and 1 mg/mL PC. After the CHAPS and PC concentrations of the extract were lowered to 0.6% and 0.8 mg/mL, respectively, the extract was applied to a ConA–Sepharose (Pharmacia) column, from which iodopsin was eluted with buffer A supplemented with 1.5 mM methyl α-mannoside, 0.6% CHAPS, and 0.8 mg/mL PC. Glycerol was added to the eluate to a final concentration of 20%. To concentrate iodopsin, the eluate was loaded on another ConA column, from which iodopsin was eluted with buffer A supplemented with 100 mM methyl α-mannoside, 0.6% CHAPS, 0.8 mg/mL PC, and 20% glycerol. The eluate contained 87% iodopsin, 4% rhodopsin plus chicken green, 8% chicken blue, and 1% chicken violet. These values were estimated by a partial bleaching method (Okano et al., 1989). In the current study, the sample was irradiated with orange light (>570 nm) for investigation of the photoreaction of iodopsin. The irradiation caused the bleaching of iodopsin as well as rhodopsin and chicken green, but the contribution of the bleaching of rhodopsin and chicken green to the spectral change was about 4%.

Preparation of Rhodopsins. Chicken rhodopsin was extracted from the photoreceptor outer segments as a mixture of visual pigments, from which rhodopsin was purified in two steps by column chromatography. First, the mixture was applied to the ConA column described above, from which a mixture of chicken rhodopsin and chicken green was eluted with buffer B (buffer A whose NaCl concentration is 10 mM) supplemented with 100 mM methyl α-mannoside, 0.6% CHAPS, and 0.8 mg/mL PC. It was then applied to a DEAE-Sepharose CL-6B (Pharmacia) column, to which only rhodopsin was adsorbed. Rhodopsin bound to the column was eluted with buffer A supplemented with 0.6% CHAPS, 0.8 mg/mL PC, and 20% glycerol.

Bovine rod outer segments (ROS) were isolated from the retinas by a sucrose floatation method described previously (Shichida et al., 1987). Bovine rhodopsin was extracted from the outer segments with buffer A supplemented with 0.75% CHAPS and 1 mg/mL PC. After the CHAPS and PC concentrations of the extract were reduced to 0.6% and 0.8 mg/mL, respectively, the extract was applied to a DEAE-Sepharose CL-6B (Pharmacia) column, from which bovine rhodopsin was eluted with buffer A supplemented with 0.6% CHAPS, 0.8 mg/mL PC, and 20% glycerol.

Preparation of Transducin. Bovine rod transducin was purified by a procedure modified from that reported previously (Fukada et al., 1989). Briefly, the ROS membranes were isolated by a sucrose floatation method under a dim-red light and washed 5 times with hypotonic buffer (5 mM Tris-HCl, 0.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 7.2) under ordinary room light. To extract transducin, the membranes were suspended in hypotonic buffer supplemented with 0.1 mM GTP and centrifuged. The extract was applied to a Blue Sepharose CL-6B (Pharmacia) column which had been equilibrated with hyptonic buffer without GTP at 4 °C. Under our experimental conditions, the α subunit of transducin (T_α) was adsorbed to the column, while the βγ subunit ($T_{\beta\gamma}$) passed through the column. Thus, a purified T_α was obtained from the column after elution with buffer T (10 mM MOPS–NaOH, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 4 μg/mL leupeptin, and 50 KIU/mL aprotinin, pH 7.5) supplemented with 0.6 M NaCl. $T_{\beta\gamma}$, which passed through the Blue Sepharose column, was applied to a DEAE-Toyopearl 650S (Toyo Soda) column from which $T_{\beta\gamma}$ was eluted with the same buffer as that for the elution of T_α. To lower the salt concentration and to remove free guanine nucleotides, each fraction of T_α and $T_{\beta\gamma}$ was passed through a Superdex 75 (Pharmacia) column which had been equilibrated with buffer T supplemented with 0.1 M NaCl (buffer T′) at room temperature. It should be noted that $T_{\beta\gamma}$ was separated by this column into two fractions; one contained $T_{\beta\gamma}$ whose γ subunit had its C-terminal both farnesylated and carboxyl-methylated, while the other lacked the methyl group (Fukada et al., 1984). Only the methylated form of $T_{\beta\gamma}$ was used for the experiments because it has been recently reported that the methylation is important for the functional coupling with metarhodopsin II (Ohguro et al., 1991). Purified T_α and $T_{\beta\gamma}$ were concentrated by an Amicon ultrafiltration membrane (Centricon 30), and the concentrations were estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Measurements of Circular Dichroism. CD spectra were measured simultaneously with absorption spectra by a JASCO J-600 spectropolarimeter equipped with a thermostated cell holder. An optical cell of 1-cm path length was used for the measurements. A 75-W xenon lamp (LX75, ILC technology) was used as the light source. The sample was irradiated with colored light at wavelengths selected by a cutoff filter (Y47, Y52, or VO59, Toshiba). CD spectra were measured under the following settings of the spectropolarimeter: time constant, 0.5 s (for wavelength scan) or 4 s (for time scan); wavelength scan rate, 100 nm/min. Less than 5% of each pigment was bleached during the scan from 700 to 250 nm.

Measurements of Transducin Binding. The absorbance changes of both rhodopsin and iodopsin after irradiation in the presence of transducin were recorded by a Shimadzu MPS-2000 spectrophotometer equipped with an Oxford cryostat (CF-1204) and with a temperature controller (ITC-4, Oxford). The purified rhodopsin or iodopsin was mixed with buffer T′, or buffer T′ containing both T_α and $T_{\beta\gamma}$, or buffer T′ containing T_α, $T_{\beta\gamma}$, and excess (300 μM) GTP, to give final concentrations of 15 μM (T_α, $T_{\beta\gamma}$) and 5 μM (pigments). Each sample (120 μL) was put into an optical cell (path length = 5 mm) and cooled to the desired temperature (−10 °C), followed by measurement of the absorption spectrum. Then it was irradiated for 1 min with light from a tungsten–halogen lamp (1 kW, Sanko) which had passed through a glass cutoff filter (O58 for the rhodopsin sample or R63 for the iodopsin sample, Toshiba), and the spectra of the sample were repeatedly recorded at the fixed time interval (5 or 20 min).

RESULTS

CD and Absorption Spectra of Iodopsin and Its Photoproducts. In order to investigate whether or not metaiodopsin II observed by nanosecond laser photolysis (Shichida et al., 1993) displays intrinsic optical activity, we measured CD and absorption spectra before and after irradiation of iodopsin.

Our preliminary experiments demonstrated that irradiation of iodopsin with orange light (>570 nm) at 0 °C formed photoproducts having a broad positive CD band in the wavelength region from 500 to 300 nm. As time elapsed, however, the CD signal rapidly disappeared (data not shown). To stabilize the photoproducts enough for the spectral measurements, the iodopsin sample was cooled to −10 °C, where CD and absorption spectra were recorded (Figure 1).

Iodopsin displayed positive CD bands at about 560 and 360 nm (curve 1 in Figure 1), corresponding to the α- and β-bands of the absorption spectrum (573 and 380 nm, curve 1′ in Figure 1), respectively, and a negative CD band at about 280 nm. On irradiation of iodopsin with orange light (>570 nm), a broad positive CD band at about 400 nm was observed (curve 2 in

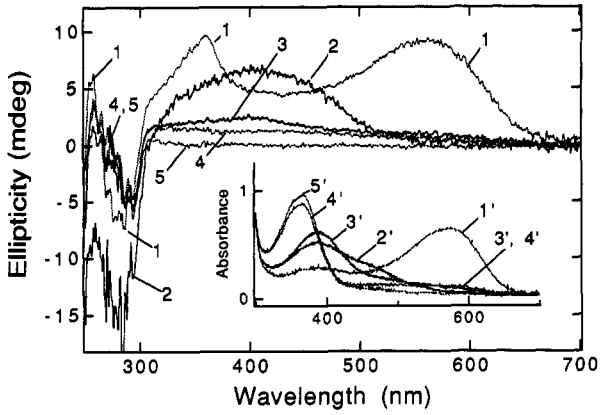

FIGURE 1: Circular dichroism and absorption spectra of chicken iodopsin and its photoproducts measured at −10 °C. Iodopsin purified in CHAPS/PC (curves 1, 1′) was irradiated with orange light at wavelengths longer than 570 nm for 30 s (curves 2, 2′) and then incubated for 60 min at −10 °C (curves 3, 3′). After addition of NH_2OH to a final concentration of 10 mM, it was incubated for 60 min (curves 4, 4′), followed by irradiation with light at wavelengths longer than 450 nm for 10 min (curves 5, 5′).

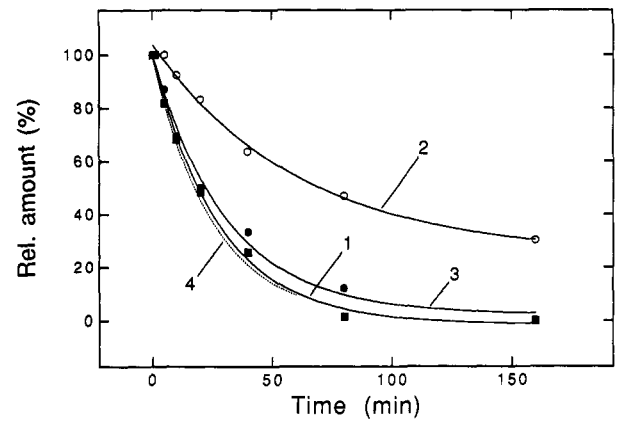

FIGURE 2: Effect of transducin on the stability of the meta II intermediate of iodopsin at −10 °C. The amount of metaiodopsin II was estimated by measuring absorption changes at 380 nm after irradiation of the iodopsin sample with red light (>610 nm) for 1 min. Each sample contained no transducin (filled squares, curve 1), transducin (open circles, curve 2), or both transducin and GTP (filled circles, curve 3). Each data set was fitted by an exponential curve (curves 1–3). The initial formation level of metaiodopsin II (100%) was the same for the three experiments (not normalized). The zero line represents the final absorbance of the experiments without transducin. The decay of CD at 380 nm measured without transducin at the same temperature (−10 °C) is also shown (curve 4).

Figure 1). Furthermore, the negative CD band observed in iodopsin was greatly enhanced. The corresponding absorption spectrum represented almost complete conversion of iodopsin to the photoproducts. The appearance of an absorption maximum at about 390 nm and a shoulder at about 470 nm (curve 2′ in Figure 1) indicated that two products having absorption maxima at about 390 nm and 470 nm were formed by the irradiation. Thus, the broad positive CD band at about 400 nm, which could not be separated into two bands, suggested that the product having an absorption maximum at about 470 nm displays an optical activity larger than the species having an absorption maximum at about 390 nm (see Figure 4).

Subsequent incubation for 60 min at this temperature (−10 °C) caused a decrease of the absorbance at about 470 nm and an increase at about 390 nm (curve 3′ in Figure 1). These absorbance changes corresponded to decreases in magnitude of both positive and negative CD bands (curve 3 in Figure 1). The disappearance of the positive CD band at about 400 nm clearly showed that the products formed by the irradiation decayed to *all-trans*-retinal and R-photopsin during the incubation. Then we added 1 M neutralized NH_2OH (pH 6.6) in a final concentration of 10 mM (curves 4 and 4′ in Figure 1) to the sample, followed by irradiation with yellow light (>450 nm) to bleach the residual pigments in the sample unreacted by the orange light (curves 5 and 5′ in Figure 1).

Since the product having an absorption maximum at about 390 nm displays CD (curves 2 and 2′ in Figure 1), it is not dissociated *all-trans*-retinal but an intermediate. Furthermore, the absorption maximum of this product is identical with metaiodopsin II detected by nanosecond laser photolysis (Shichida et al., 1993). Therefore, we concluded that metaiodopsin II displays optical activity.

The other intermediate having its absorption maximum at about 470 nm could be either the meta I or the meta III intermediate of iodopsin (metaiodopsin I or III). To get experimental evidence to distinguish between them, we measured the kinetics of the CD at 460 and 390 nm, respectively (data not shown). The results obtained showed that the CD at 460 nm disappeared simultaneously with that at 390 nm, suggesting that this intermediate has a decay time comparable to that of metaiodopsin II. In rhodopsins, the meta III intermediate is formed after prolonged incubation of a mixture of meta I and II intermediates and the meta I intermediate decays simultaneously with the meta II intermediate (see curves 2 and 3 in Figure 3B). Therefore, we tentatively conclude that this intermediate would be metaiodopsin I. However, because of our experimental limitations, the possibility that it might be metaiodopsin III cannot be fully excluded if the putative metaiodopsin III has absorption and CD spectra similar to metaiodopsin I and decays with a time constant similar to metaiodopsin II.

During incubation of the sample after irradiation with >570-nm light (curves 3 and 3′ in Figure 1), we observed small increases of the CD and absorbance signals at about 570 nm. The CD and absorption signals at about 570 nm did not change after the addition of hydroxylamine (curves 4 and 4′ in Figure 1), while the signals due to the residual metaiodopsins disappeared. Since the meta III intermediate of rhodopsin (metarhodopsin III) was reported to be highly unstable against hydroxylamine (Yoshizawa & Horiuchi, 1973), this product may not be a meta III intermediate of iodopsin but iodopsin regenerated during the incubation. In fact, irradiation of this sample with >660-nm light, by which only iodopsin is bleached, gave a difference spectrum similar to that between iodopsin and its retinal oxime.

Stabilization of Metaiodopsin II by Transducin. Next, we tested the ability of metaiodopsin II to bind transducin (Figure 2). Because we could not prepare enough transducin for the binding experiments using circular dichroism spectroscopy, we performed this experiment using absorption spectroscopy. Since the change of absorbance at 380 nm due to the conversion of metaiodopsin II to *all-trans*-retinal plus R-photopsin (curve 1 in Figure 2) correlated well with the decay of metaiodopsin II monitored by the decrease of CD at 380 nm (curve 4 in Figure 2), we can monitor the effect of transducin on the decay time constant of metaiodopsin II by means of absorption spectroscopy.

When iodopsin was irradiated after addition of transducin, the decay of metaiodopsin II was greatly suppressed (curve 2 in Figure 2). The time constant in the presence of transducin was about 2 times larger than that in the absence of transducin (curve 1 in Figure 2). This result clearly shows that transducin prolongs the decay of metaiodopsin II, suppressing the release

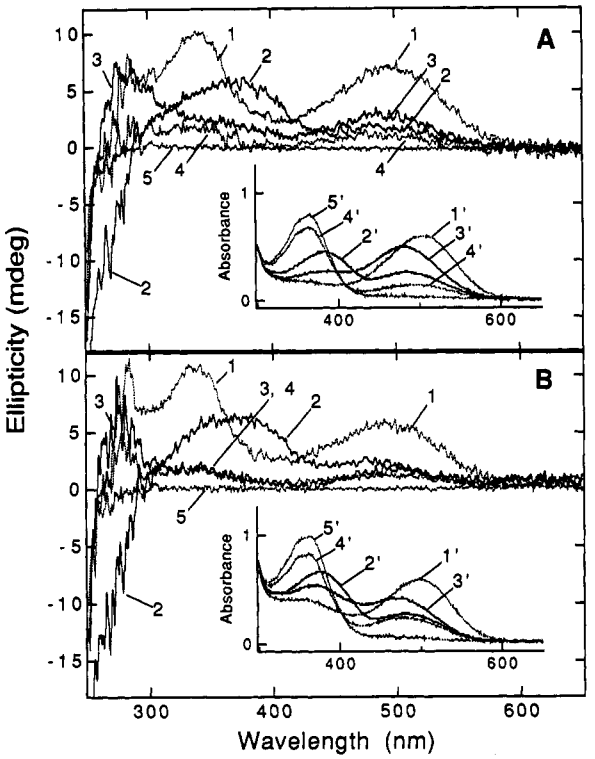

FIGURE 3: Circular dichroism and absorption spectra of chicken rhodopsin (A), bovine rhodopsin (B), and their photoproducts measured at 5 °C. The pigment purified in CHAPS/PC (curves 1, 1′) was irradiated with orange light at wavelengths longer than 500 nm for 30 s (curves 2, 2′) and then incubated for 60 min (A) or 120 min (B) at 5 °C (curves 3, 3′). After addition of NH_2OH to a final concentration of 10 mM, it was incubated for 60 min (curves 4, 4′), followed by irradiation with light at wavelengths longer than 450 nm for 10 min (curves 5, 5′).

of the retinal from R-photopsin. When the iodopsin sample contained both transducin and GTP, the decay of metaiodopsin II was almost identical with that in the absence of transducin (curve 3 in Figure 2). This suggests that the binding of transducin to metaiodopsin II can cause the GDP–GTP exchange reaction on transducin, resulting in release of transducin from metaiodopsin II. Therefore, we conclude that metaiodopsin II can interact with transducin and activate it.

CD and Absorption Spectra of Rhodopsins and Their Photoproducts. As already shown in Figure 1, the metaiodopsins display a negative CD signal at about 280 nm, which is much larger than that of iodopsin. This suggests that in the conversion from iodopsin to the metaiodopsins, the conformation of the chromophore as well as the interaction between the chromophore and nearby protein may significantly change. Since there is no report on the CD spectra of metarhodopsins in this wavelength region, we have measured CD spectra of chicken and bovine metarhodopsins to compare them with those of metaiodopsins. Figure 3 shows typical experiments on the CD and absorption spectra of chicken and bovine metarhodopsins. The experimental conditions were similar to those of iodopsin (Figure 1) except that all the experiments were carried out at 5 °C and the samples were irradiated with yellow light (>500 nm) for 30 s.

Chicken rhodopsin exhibited positive CD bands at 495 and 340 nm and a shoulder at about 280 nm (curve 1 in Figure 3A), corresponding to those of the α-, β-, and γ-bands of the absorption spectrum (503 nm and about 350 and 280 nm, curve 1′ in Figure 3A), respectively. When irradiated, the α-bands in both the CD and absorption spectra shifted to the

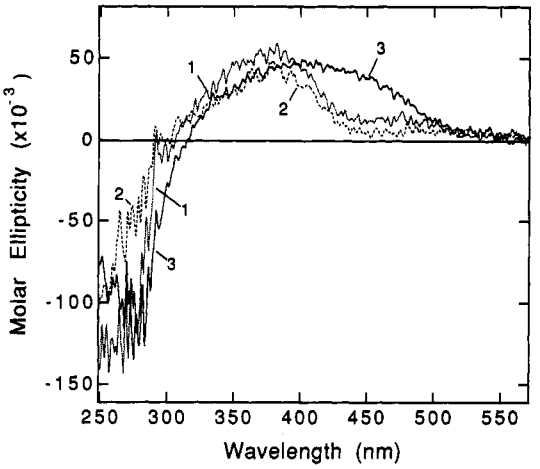

FIGURE 4: Difference CD spectra of meta intermediates. Each curve was calculated by subtracting curve 4 from curve 2 in Figures 1, 3A, or 3B. Curve 1, bovine metarhodopsins (at 5 °C); curve 2, chicken metarhodopsins (at 5 °C); curve 3, chicken metaiodopsins (at −10 °C).

blue with decreases of their intensities, and new CD and absorption bands at about 380 nm (curves 2 and 2′ in Figure 3A) were observed. These spectral changes should be ascribed to the formation of a mixture of metarhodopsins I and II, according to previous reports (Waggoner & Stryer, 1971; Yoshizawa & Horiuchi, 1973). It should be noted that a remarkable change in the CD spectrum was also observed in the wavelength region below 300 nm.

Subsequent incubation of the sample irradiated at 5 °C caused a decrease of the absorbance at about 380 nm and an increase at about 480 nm (curve 3′ in Figure 3A). These spectral changes are due to the conversion of a mixture of metarhodopsins I and II to metarhodopsin III. A noteworthy point is that the spectral change is opposite to that observed in the iodopsin system (curves 2′ and 3′ in Figure 1). Concurrently, the positive CD band at about 380 nm remarkably decreased, and the large negative CD band below 300 nm changed to a positive band at about 280 nm (curve 3 in Figure 3A). Furthermore, a slight increase of the CD at about 480 nm was also observed, indicating that chicken metarhodopsin III has a small positive CD band in its α-band. Upon addition of 1 M neutralized NH_2OH to a final concentration of 10 mM, the CD and absorbance at about 480 nm due to metarhodopsin III disappeared (curves 4 and 4′ in Figure 3A). The remaining CD and absorbance in the visible and near-UV (~350 nm) regions are attributed to the residual rhodopsin and 9-*cis*-rhodopsin (the latter was formed during the 30-s irradiation). They were completely bleached with a >450-nm light at 5 °C in the presence of 10 mM NH_2OH (curves 5 and 5′ in Figure 3A).

As shown in Figure 3B, bovine rhodopsin exhibited spectral changes similar to chicken rhodopsin. The only remarkable difference between the chicken and bovine rhodopsin systems is that chicken metarhodopsin III displays a small CD signal for the α-band, while bovine metarhodopsin III does not. The latter is consistent with that reported previously (Yoshizawa & Horiuchi, 1973).

CD Spectra of Meta Intermediates. In order to compare the CD spectra of the meta intermediates of iodopsin with those of rhodopsins, we calculated the difference CD spectra (Figure 4) from the spectra of the mixture of meta I and II intermediates and those measured after the addition of hydroxylamine (curves 2 and 4 in Figures 1 and 3A,B). Since a small amount of iodopsin is regenerated during incubation

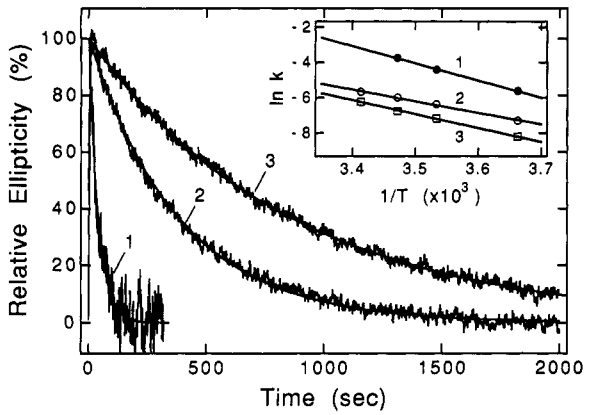

FIGURE 5: Kinetic recordings of circular dichroism at 380 nm after irradiation of iodopsin (curve 1), chicken rhodopsin (curve 2), and bovine rhodopsin (curve 3), with >570-nm light (for iodopsins) or >500-nm light (for rhodopsins) for 30 s at 15 °C. The kinetic curves obtained were expressed by single-exponential curves (smooth lines). Inset: temperature dependence of the decay rate constants of meta II intermediates. All the values plotted in the inset were determined by similar measurements as shown in the main panel. Line 1 (filled circles), iodopsin; line 2 (open circles), chicken rhodopsin; line 3 (open squares), bovine rhodopsin.

of a mixture of metaiodopsins I and II at −10 °C, we subtracted the contribution of regenerated iodopsin from the spectrum measured after addition of hydroxylamine.

In bovine rhodopsin (curve 1 in Figure 4), the difference CD spectrum displayed a positive band having a maximum at about 380 nm with a long-wavelength tail and a large negative band at about 275 nm. The positive CD band and the long-wavelength tail may be ascribed to metarhodopsins II and I, respectively. However, it is obscure whether or not the negative band can be ascribed to both metarhodopsins I and II, because the content of metarhodopsin I in the mixture is very small. Then we measured the CD spectrum of metarhodopsin I using a sample at pH 7.5 without any glycerol, conditions where a large amount of metarhodopsin I is formed after irradiation (Matthews et al., 1963). The results showed that metarhodopsin I displayed only a small negative CD signal at about 280 nm (Okada et al., unpublished results), indicating that the large negative CD band shown in curve 1 in Figure 4 is ascribed mainly to metarhodopsin II.

As shown in curve 2 in Figure 4, chicken metarhodopsins have a similar difference CD spectrum to bovine metarhodopsins, but the former was slightly smaller at about 280 nm than bovine metarhodopsins.

The difference CD spectrum of metaiodopsins (curve 3 in Figure 4) displayed a broad band in the visible region as compared with those of metarhodopsins, while the former displayed a negative CD band below 300 nm similar to the latter. The broad band in the visible region could be explained by the relative abundance of metaiodopsin I in the iodopsin sample. Since bovine metarhodopsin I (and also metarhodopsin III) did not display a large CD band below 300 nm, we assume that metaiodopsin I would display a small CD band at this region. Thus, metaiodopsin II would have a CD spectrum similar to metarhodopsin II.

Measurements of the Decay Process of Meta II Intermediates. We have shown that the intensity of CD at about 380 nm is a good measure of the meta II intermediates of iodopsin and rhodopsins. To compare the decay process of the meta II intermediates, the changes of CD at 380 nm were measured after irradiation of chicken iodopsin and rhodopsins at 15 °C (Figure 5). It is clear that the change of CD due to the decay of metaiodopsin II (curve 1 in Figure 5) is much faster than that due to chicken or bovine metarhodopsin II (curves 2 and 3 in Figure 5, respectively). The decay of the meta II intermediate of iodopsin, chicken rhodopsin, or bovine rhodopsin was expressed by a single-exponential curve whose time constant was 28, 265, or 596 s, respectively.

Similar experiments were performed at temperatures ranging from −10 to 15 °C to estimate the rate constants, which were plotted as a function of the reciprocal of the temperature (inset of Figure 5). From the slope of the fitted lines, the activation energies were estimated to be 19.5, 13.7, and 15.3 kcal/mol for meta II intermediates of iodopsin and chicken and bovine rhodopsins, respectively.

DISCUSSION

The present results revealed some important molecular properties of metaiodopsin II. Through CD spectroscopy and G-protein binding experiments, we have shown that metaiodopsin II is similar to metarhodopsin II in its chromophore–protein interaction and in the function to activate transducin. Because we have not yet been able to isolate a G-protein from cones, G-protein binding experiments of iodopsin were performed by use of G-protein from bovine rods. However, it is reasonable to speculate that metaiodopsin II would activate a cone G-protein in a manner similar to a rod G-protein, because the primary structure of the α subunit of G-protein in rods is 78% identical in amino acid sequence with that in cones (Lerea et al., 1986) and both have 40 amino acids at the carboxyl-terminal region, which may be an interaction site with the meta II intermediate (Navon & Fung, 1988; Hamm et al., 1988), completely identical with each other. Furthermore, similar spectroscopic characteristics of metaiodopsin II to metarhodopsin II enable us to speculate that metaiodopsin II would activate chicken cone G-protein in manner similar to metarhodopsin II.

The remarkable differences between metaiodopsin II and metarhodopsin II are found in their decay processes. In addition to the rapid decay of metaiodopsin II as compared with metarhodopsin II, metaiodopsin II does not decay to a long-lived intermediate corresponding to metarhodopsin III but to *all-trans*-retinal and R-photopsin, while it is evident that metarhodopsin II converts to an equilibrium mixture composed of metarhodopsins II and III (Chabre & Breton, 1979a). Although the physiological function of metarhodopsin III is not clear yet, it was demonstrated that the addition of transducin to an equilibrium mixture of metarhodopsins II and III converted metarhodopsin III to metarhodopsin II (Kibelbek et al., 1991). This suggests that metarhodopsin III can function to extend the survival of metarhodopsin II. Since the high photosensitivity of rods could be explained by the stability of metarhodopsin II enough to activate hundreds of transducins, it is likely that the less stable property of metaiodopsin II is one of the molecular mechanisms for the lower photosensitivity of cones compared to rods.

The decay time constant of metaiodopsin II at physiological temperature (40 °C in chicken) was estimated to be a few seconds by extrapolation of the Arrhenius line shown in the inset of Figure 5. Though none of the rates of electrophysiological responses of chicken cones have been reported, those of other animals are on the time scale of less than seconds (Baylor & Nunn, 1982; Nakatani & Yau, 1988). Therefore, it is likely that the physiological response of chicken red-sensitive cone is shorter than the decay time constant of metaiodopsin II estimated in this experiment. The contradiction may be explained by two factors: First, in the present study, the decay time of metaiodopsin II was estimated in the

Table 1: Molar Ellipticities of Three Visual Pigments

	$[\theta]_\alpha$[a]	(λ_{max})[c]	$[\theta]_\beta$[b]	(λ_{max})[c]
bovine rhodopsin	41400 ± 1700	(490)	77600 ± 2000	(335)
chicken rhodopsin	48300 ± 1700	(495)	71700 ± 2000	(340)
chicken iodopsin	70900 ± 2000	(560)	75600 ± 2200	(360)

[a] Molar ellipticity of the α-band (degrees centimeter squared per decimole). [b] Molar ellipticity of the β-band (degrees centimeter squared per decimole). [c] Wavelength (nanometers) of maximal ellipticity.

detergent system (CHAPS/PC), and it was different from that in the native membrane environment. Second, it is known that phosphorylation of metarhodopsin II and/or the binding of the 48-kDa protein (arrestin) to it quenches the signal transduction in rods (Sitaramayya & Liebman, 1983; Wilden et al., 1986). Our experiments on the phosphorylation of bleached iodopsin by rhodopsin kinase (Fukada et al., 1990) suggested a possible involvement of the phosphorylation in the shut-off mechanism of signal transduction in cones. Therefore, more detailed investigations are required for complete understanding of the rate of the physiological response in cones and the difference in photosensitivity between rods and cones.

The CD spectrum of iodopsin (curve 1 in Figure 1B) exhibits some interesting features different from those of rhodopsins. First, as shown in Table 1, iodopsin has a larger α-band than rhodopsins, while the β-bands are very similar to each other. Since the three visual pigments investigated here were solubilized with the same detergent system (CHAPS/PC), the difference cannot be due to different micellar environments (Waddell et al., 1976) but to different conformations of the retinylidene chromophore and/or chromophore–protein interactions. Since the ellipticity of the α-band of iodopsin is affected by chloride binding (Shichida et al., 1990), the difference may be related to the presence and absence of chloride binding site in these visual pigments.

Second, iodopsin displays a negative CD band in the wavelength region from 270 to 300 nm and a positive one from 260 to 270 nm, while rhodopsins have a positive CD band in these wavelength regions. R-Photopsin displayed CD bands similar to those of iodopsin in this wavelength region, although the magnitudes were slightly lower. On the other hand, scotopsins displayed almost no CD in these wavelength regions, except a negative band below 260 nm. The negative and positive CD bands in R-photopsin may originate from the aromatic residues which do not interact with the chromophore but with each other (coupled oscillators). Although it is difficult to assign the residues responsible for these signals, it is interesting that only iodopsin (R-photopsin) has three tryptophan residues (W161, W163, and W167) which are located in close contact with each other in helix IV (Kuwata et al., 1990; Tokunaga et al., 1990). Therefore, we tentatively conclude that these residues are the candidates for inducing the CD bands. Since the magnitudes of the CD bands increased when the chromophore was linked with R-photopsin, we can speculate that the binding of the chromophore may have some effect on interactions among these residues. Unlike iodopsin, rhodopsin displayed a relatively large change in CD at about 280 nm before and after bleaching. It is suggested that the chromophore of iodopsin may interact with nearby aromatic residue(s) in a manner different from those of rhodopsins.

In contrast with the different CD bands in the near-UV region between iodopsin and rhodopsin, metaiodopsins display CD bands very similar to metarhodopsins (Figure 4). This suggests that the physiologically active intermediates of both rhodopsin and iodopsin have the chromophore–opsin interaction and/or the conformation of the opsin moiety similar to each other. One of the plausible explanations for the appearance of a large negative CD band may be that some aromatic residues are rearranged during the formation of meta intermediates. This is consistent with earlier reports on rhodopsin using absorption spectra (Rafferty, 1979; Rafferty et al., 1980) or linear dichroism (Chabre & Breton, 1979b). Recent site-directed mutagenesis experiments on bovine rhodopsin identified four amino acid residues (E122, W126, W265, and Y268) which may be located in close contact with the chromophore of rhodopsin (Nakayama & Khorana, 1991). Since three of the aromatic amino acid residues (W126, W265, and Y 268) in bovine rhodopsin are conserved in chicken rhodopsin (Takao et al., 1988) and iodopsin (Kuwata et al., 1990), it is reasonable to speculate that these residues also interact with the chromophore in metaiodopsins. Site-directed mutagenesis experiments (Nakayama & Khorana, 1991) also suggested that rhodopsin mutants, in which each of these tryptophan residues was substituted by phenylalanine or alanine, displayed lower ability to activate transducin than the native rhodopsin. Therefore, these tryptophan residues may have an important role in producing the active intermediates of rhodopsin and iodopsin.

Another explanation may be due to the conformational change of the disulfide bond (Linderberg & Michl, 1970; Rauk, 1984) formed between two cysteines, C110 and C187, in bovine rhodopsin (Karnik et al., 1988; Karnik & Khorana, 1990) and cysteines at corresponding positions in iodopsin (Kuwata et al., 1990). Since all of the G-protein-coupled receptors whose sequence has been determined have the corresponding two cysteines, it would be interesting to speculate that some changes in the environment and/or the dihedral angle of the disulfide bond occur during the activation process of the receptor. This is consistent with a recent study on the cysteine mutants of bovine rhodopsin, in which the disulfide bond seems to stabilize the conformation of metarhodopsin II (Davidson et al., 1994). Further studies for testing the above hypothesis are now in progress.

ACKNOWLEDGMENT

We thank Prof. A. Maeda, Dr. Y. Imamoto, and H. Imai for their helpful discussions and suggestions.

REFERENCES

Baylor, D. A., & Nunn, B. (1982) *Methods Enzymol. 81*, 403–423.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Chabre, M., & Breton, J. (1979a) *Vision Res. 19*, 1005–1018.

Chabre, M., & Breton, J. (1979b) *Photochem. Photobiol. 30*, 295–299.

Davidson, F. F., Loewen, P. C., & Khorana, H. G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Fukada, Y., & Yoshizawa, T. (1981) *Biochim. Biophys. Acta 675*, 195–200.

Fukada, Y., Ohguro, H., Saito, T., Yoshizawa, T., & Akino, T. (1989) *J. Biol. Chem. 264*, 5937–5943.

Fukada, Y., Kokame, K., Okano, T., Shichida, Y., Yoshizawa, T., McDowell, J. H., Hargrave, P. A., & Palczewski, K. (1990) *Biochemistry 29*, 10102–10106.

Fukada, Y., Matsuda, T., Kokame, K., Takao, T., Shimonishi, Y., Akino, T., & Yoshizawa, T. (1994) *J. Biol. Chem.* (in press).

Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Hoenig, B., & Hofmann, K. P. (1988) *Science 241*, 832–835.

Karnik, S. S., & Khorana, H. G. (1990) *J. Biol. Chem. 265*, 17520–17524.
Karnik, S. S., Sakmar, T. P., Chen, H.-B., & Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8459–8463.
Kibelbek, J., Mitchell, D. C., Beach, J. M., & Litman, B. J. (1991) *Biochemistry 30*, 6761–6768.
Kühn, H., Bennett, N., Michel-Villaz, M., & Chabre, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6873–6877.
Kuwata, O., Imamoto, Y., Okano, T., Kokame, K., Kojima, D., Matsumoto, H., Morodome, A., Fukada, Y., Shichida, Y., Yasuda, K., Shimura, Y., & Yoshizawa, T. (1990) *FEBS Lett. 272*, 128–132.
Lerea, C. L., Somers, D. E., Hurley, J. B., Klock, I. B., & Bunt-Milam, A. H. (1986) *Science 234*, 77–80.
Linderberg, J., & Michl, J. (1970) *J. Am. Chem. Soc. 92*, 2619–2625.
Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–240.
Nakatani, K., & Yau, K.-W. (1988) *Nature 334*, 69–71.
Nakayama, T. A., & Khorana, H. G. (1991) *J. Biol. Chem. 266*, 4269–4275.
Navon, S. E., & Fung, B. K.-K. (1988) *J. Biol. Chem. 263*, 489–496.
Ohguro, H., Fukada, Y., Takao, T., Shimonishi, Y., Yoshizawa, T., & Akino, T. (1991) *EMBO J. 10*, 3669–3674.
Okano, T., Fukada, Y., Artamonov, I. D., & Yoshizawa, T. (1989) *Biochemistry 28*, 8848–8856.
Rafferty, C. N. (1979) *Photochem. Photobiol. 29*, 109–120.
Rafferty, C. N., Muellenberg, C. G., & Shichi, H. (1980) *Biochemistry 19*, 2145–2151.
Rauk, A. (1984) *J. Am. Chem. Soc. 106*, 6517–6524.
Shichida, Y., Ono, T., Yoshizawa, T., Matsumoto, H., Asato, A. E., Zingoni, J. P., & Liu, R. S. H. (1987) *Biochemistry 26*, 4422–4428.
Shichida, Y., Kato, T., Sasayama, S., Fukada, Y., & Yoshizawa, T. (1990) *Biochemistry 29*, 5843–5848.
Shichida, Y., Okada, T., Kandori, H., Fukada, Y., & Yoshizawa, T. (1993) *Biochemistry 32*, 10832–10838.
Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 1205–1209.
Stryer, L., Hurley, J. B., & Fung, B. K.-K. (1981) *Curr. Top. Membr. Transp. 15*, 93–108.
Takao, M., Yasui, A., & Tokunaga, F (1988) *Vision Res. 28*, 471–480.
Tokunaga, F., Iwasa, T., Miyagishi, M., & Kayada, S. (1990) *Biochem. Biophys. Res. Commun. 173*, 1212–1217.
Waddell, W. H., Yudd, A. P., & Nakanishi, K. (1976) *J. Am. Chem. Soc. 98*, 238–239.
Waggoner, A. S., & Stryer, L. (1971) *Biochemistry 10*, 3250–3254.
Wilden, U., Hall, S. W., & Kühn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174–1178.
Yoshizawa, T., & Horiuchi, S. (1973) in *Biochemistry and Physiology of Visual Pigments* (Langer, H., Ed.) pp 69–81, Springer-Verlag, Heidelberg.