Nanosecond Laser Photolysis of Iodopsin, a Chicken Red-Sensitive Cone Visual Pigment†

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ABSTRACT: The photobleaching process of iodopsin (a chicken red-sensitive cone visual pigment) purified in a detergent system containing CHAPS and phosphatidylcholine was investigated by means of nanosecond laser photolysis at room temperature. Excitation of iodopsin with a nanosecond laser pulse (wavelength, 560 nm; pulse width, 17 ns) resulted in the formation of at least four intermediates on the nanosecond to millisecond time scale. The earliest intermediate detected had an absorption maximum at 571 nm, which was very close to that of original iodopsin (λ_{max} = 567 nm), and remarkably blue-shifted as compared with that of bathoiodopsin [λ_{max} = 625 nm; Kandori et al. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 8908–8912]. The intermediate, named BL-iodopsin, converted to the next intermediate, lumiiodopsin (λ_{max} = 535 nm), with a time constant of 130 ns. The BL intermediate had an absorption maximum just between batho- and lumiiodopsins, and an extinction coefficient comparable with these intermediates. These properties are different from those of the corresponding intermediate of rhodopsin [BL(BSI)-rhodopsin], suggesting that the binding of chloride to iodopsin, but not to rhodopsin, has an influence upon changes of the chromophore–opsin interaction in the early stage of photobleaching of iodopsin. Lumiiodopsin converted to metaiodopsin I (λ_{max} < 500 nm) with a time constant of 230 μs, and then to metaiodopsin II (λ_{max} = 390 nm) with a time constant of 6 ms. A thermal equilibrium between metaiodopsin I and II was established, but unlike meta intermediates of rhodopsin, they showed little temperature dependence.

Many vertebrate retinas including human retina contain two types of photoreceptor cells, rods and cones, to receive photon signals from the outer environment. Rods work under twilight conditions and are responsible for scotopic vision, while cones work under daylight conditions and are responsible for phototopic vision. There are several types of cones, each of which has a specific visual pigment having a respective absorption maximum (Brown & Wald, 1964; Marks et al., 1964; Yoshizawa et al., 1991). Integration of the photon signals from two or more types of cones enables animals to discriminate colors of objects. Since the photosensitivities of rods are extraordinarily high and their responses are easily saturated under normal room light (Schnapf & Baylor, 1987), extensive investigations of the molecular mechanism of visual transduction in cones are essential for understanding "vision" in daily life.

Historically, a cone visual pigment present in chicken retina was extracted by Wald (1937), who named it iodopsin. Subsequently, his group and others revealed several important properties of iodopsin (Wald et al., 1955). Like rhodopsin, iodopsin has an 11-*cis*-retinal as its chromophore, while its absorption maximum [562 nm in 2% digitonin (Wald et al., 1955); 571 nm in 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)[1] and 0.8 mg/mL PC (Okano et al., 1989)] is located at a longer wavelength than that of rhodopsin (λ_{max} = 501 nm; Okano et al., 1989). The rate of regeneration of iodopsin is about 500 times faster than that of rhodopsin (Wald et al., 1955), which may explain the rapid dark adaptation of cones as compared with rods. Although the shape of the chromophore binding site of iodopsin would be similar to that of rhodopsin (Fukada et al., 1990), the chromophore of iodopsin is easily attacked by a reagent of low molecular weight such as hydroxylamine, alum (Wald et al., 1955), or sodium borohydride (Matsumoto et al., 1975). Furthermore, the absorption spectrum of iodopsin is blue-shifted by depleting the chloride bound to the opsin moiety (Knowles, 1976; Fager & Fager, 1979). Later Shichida et al. (1990) revealed that the binding of chloride to iodopsin is essential for stabilizing the Schiff base linkage of the chromophore, resulting in inhibition of transfer of the retinylidene chromophore (Matsumoto et al., 1975) from iodopsin to scotopsin, the protein moiety of rhodopsin. A recent determination of the amino acid sequence of iodopsin (Kuwata et al., 1990; Tokunaga et al., 1990) enabled us to speculate about the chloride binding site of iodopsin.

The photobleaching process of iodopsin has been investigated by low-temperature spectrophotometry (Hubbard & Kropf, 1959; Yoshizawa & Wald, 1967), by which several intermediates have been identified. One of the interesting results is that a bathochromic photoproduct (bathoiodopsin) produced by irradiation of iodopsin at liquid nitrogen temperature does not thermally bleach to *all-trans*-retinal and R-photopsin (the protein moiety of iodopsin) but reverts to the original iodopsin

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from egg yolk; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Con A, concanavalin A.

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(Yoshizawa & Wald, 1967). This result is in contrast with that of rhodopsin in which bathorhodopsin produced at liquid nitrogen temperature thermally bleaches to *all-trans*-retinal and the protein moiety (scotopsin) through several thermolabile intermediates. Recently, the reverse reaction of bathoiodopsin to iodopsin was investigated in relation to the binding of chloride to iodopsin: When the chloride bound to iodopsin was replaced by nitrate, bathoiodopsin converted to lumiiodopsin (Imamoto et al., 1989). From these results, a possible influence of chloride during the batho–lumi transition of iodopsin was reported (Imamoto et al., 1989).

Contrary to the low-temperature experiments, our picosecond laser photolysis experiments clearly showed that bathoiodopsin which was produced at room temperature from iodopsin in chloride-bound form (native iodopsin) bleached at *all-trans*-retinal and R-photopsin (Kandori et al., 1990). Therefore, it is of interest to investigate whether the intermediate formed from bathoiodopsin at room temperature is identical to lumiiodopsin detected by low-temperature spectrophotometry (Hubbard & Kropf, 1959; Yoshizawa & Wald, 1967). Furthermore, identification of intermediates appearing in the bleaching process of iodopsin may provide new insight into differences in the photobleaching behavior between iodopsin and rhodopsin.

The present paper is devoted to the study of nanosecond laser photolysis of iodopsin. The experimental results showed the presence of a distinct intermediate between batho- and lumiiodopsins. The intermediate, named BL-iodopsin, has an absorption maximum just between batho- and lumiiodopsins and an extinction coefficient comparable with them. Other intermediates corresponding to lumi, meta I, and meta II intermediates of rhodopsin were also identified. Furthermore, it was demonstrated that unlike rhodopsin, iodopsin showed little temperature dependence in the equilibrium state between meta I and meta II intermediates.

MATERIALS AND METHODS

Preparation of Iodopsin. Iodopsin was extracted and purified from 2000 chicken retinas according to the method of Okano et al. (1989) modified from that described previously (Shichida et al., 1989). All procedures were performed at 4 °C. Briefly, chicken photoreceptor outer segments were isolated by a sucrose flotation method [40% (w/v) sucrose in buffer A (50 mM HEPES, 140 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol, 0.1 mM $PhMeSO_2F$, 50 kalikrein inhibitor units/mL aprotinin, and 4 μg/mL leupeptin), pH 6.6]. The outer segments were then washed with buffer A and centrifuged (20000g × 60 min) 6 times to remove the oil droplets. Cone pigments as well as rhodopsin were extracted from the outer segments by buffer A supplemented with 0.75% CHAPS and 1 mg/mL PC (extraction buffer). Though all the procedures described above were performed under red light (>630 nm), visual pigments in the sample partially bleached. Then, an ethanol solution of 11-*cis*-retinal, whose molar amount was about twice that of the bleached visual pigments, was added to the extraction buffer for regeneration of the visual pigments. Subsequent operations were carried out in complete darkness or under dim red light (>680 nm). A clear supernatant containing visual pigments was obtained by centrifugation (20000g × 60 min). The supernatant (4 volumes) was diluted with buffer A (1 volume) for decreasing the concentration of CHAPS and PC to 0.6% and 0.8 mg/mL, respectively, and then loaded on a concanavalin A–Sepharose (Pharmacia) affinity column. Iodopsin was eluted from the column by buffer A supplemented with 1.5 mM methyl α-mannoside, 0.6% CHAPS, and 0.8 mg/mL PC. The fractions containing mainly iodopsin were collected and then mixed together, followed by addition of glycerol to a final concentration of 20% (w/v). After removal of the methyl α-mannoside by dialysis against buffer A supplemented with 0.6% CHAPS, 0.8 mg/mL PC, and 20% glycerol, the fractions were loaded again on a Con A column. The iodopsin-rich fractions were obtained from the column by elution with buffer A supplemented with 100 mM methyl α-mannoside, 0.6% CHAPS, 0.8 mg/mL PC, and 20% glycerol. These fractions were mixed together and concentrated by means of a ultrafiltration membrane (Amicon, YM100).

Preparation of Chicken and Bovine Rhodopsins. All procedures were performed in complete darkness or under dim red light (>680 nm) at 4 °C. Chicken rhodopsin was purified from the extract containing rhodopsin and cone visual pigments by means of two steps of column chromatography described below (Okano et al., 1992). The extract was loaded on a Con A column, and a mixture containing rhodopsin and chicken green was eluted by buffer B (buffer A whose NaCl concentration was 10 mM) supplemented with 100 mM methyl α-mannoside, 0.6% CHAPS, and 0.8 mg/mL PC. After addition of glycerol to the final concentration of 20%, the mixture was loaded on a DEAE-Sepharose (Pharmacia) column from which rhodopsin was eluted by buffer A supplemented with 0.6% CHAPS, 0.8 mg/mL PC, and 20% glycerol, followed by concentration by means of a ultrafiltration membrane (Amicon, YM100).

Bovine rod outer segments were prepared according to the method described previously (Shichida et al., 1987). Rhodopsin was extracted from the rod outer segments with buffer B supplemented with 0.75% CHAPS and 1 mg/mL PC, followed by centrifugation (20000g × 60 min) to get a clear supernatant. It (4 volumes) was diluted with buffer B (1 volume) to decrease the concentrations of CHAPS and PC to 0.6% and 0.8 mg/mL, respectively, and then loaded on a DEAE-Sepharose column, from which rhodopsin was eluted by buffer A supplemented with 0.6% CHAPS, 0.8 mg/mL PC, and 20% glycerol. The rhodopsin solution thus obtained was concentrated by means of a ultrafiltration membrane (Amicon, YM100).

Nanosecond Laser Photolysis. Absorbance changes (kinetics) at selected wavelengths and transient absorption spectra at selected times after the excitation of iodopsin were measured by using a nanosecond laser photolysis system described previously (Okada et al., 1991; Shichida et al., 1991). The sample was excited with a 17-ns laser pulse (wavelength, 560 nm for iodopsin and 460 nm for rhodopsins) from an excimer-pumped dye laser (Lambda Physik EMG101 MSC, FL3002). The excitation energy was about 40 μJ/mm^2. A photographic flash lamp (622, Sanpak) or a xenon continuous lamp (L2274, Hamamatsu) coupled with a shutter system was used as a light source of a monitoring light for kinetic measurements, while only the flash lamp was used for spectral measurements. The excitation and monitoring lights entered the sample at 90° to one another. A magic-angle excitation was applied for neglecting absorbance changes due to tumbling of the molecule or reorientation of the chromophore after photoexcitation of iodopsin.

For the kinetic measurements, the monitoring light was focused onto an aperture of 1.8-mm diameter in front of the sample cell (2 × 2 mm) through an interference filter, and then focused onto a slit of a monochromator (H25 Jobin Yvon). The light intensity was measured by a photomultiplier (R666s,

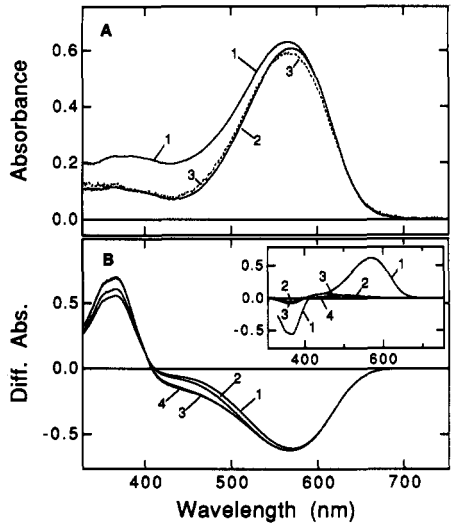

FIGURE 1: (A) Absorption spectrum of the sample used for nanosecond laser photolysis and calculated absorption spectra of iodopsin at 0 (curve 2) and 20 °C (curve 3). Details of the calculation of the absorption spectra of iodopsin at 0 and 20 °C are described in the text. (B) Absorption spectra obtained by stepwise bleaching of the sample with >660-, >590-, >520-, and >480-nm light (partial bleaching method). After addition of a neutralized hydroxylamine solution (1 M) to the sample to the final concentration of 10 mM (base line), it was successively irradiated with >660-nm light for 80 min (curve 1), >590-nm light for 40 min (curve 2), >520-nm light for 20 min (curve 3), and >480-nm light for 10 min (curve 4). Inset: Difference absorption spectrum calculated before and after irradiation with >660-nm (curve 1), >590-nm (curve 2), >520-nm (curve 3), and >480-nm (curve 4) light.

Hamamatsu) whose signals were monitored by a storage scope (TS8123, Iwatsu). The digitized light signals were then processed by a computer (PC9801, NEC). The time resolution of the storage scope was 0.39 ns per channel.

For the spectral measurements, a monitoring pulse was divided into two pulses: One was focused on an aperture of 1.8-mm diameter in front of the sample cell (2 × 2 mm) (sample side) and the other on another aperture of the same diameter (reference side). Each pulse was focused onto the slit of each polychromator (82-499, Jarrell Ash), and its intensity was detected by an SMA (spectrometric multichannel analyzer) detector (IRY-700G, Princeton Instruments). Signals from the detectors were digitized by SMA controllers (ST-100, Princeton Instruments) and transferred to a personal computer (PC-286, EPSON), followed by calculation of difference spectra. The gating widths of the SMA detectors were set at 17 ns by a high-voltage pulse generator (FG-100, Princeton Instruments). The gating jitter was negligible in the present experiments. The triggering time of each device was controlled by a four-channel digital delay/pulse generator (DG535, Stanford).

For measurements of the kinetics and transient spectra, 10 μL of the sample was used for each experiment. The sample in the optical cell was replaced after every single excitation in order to avoid disturbance due to absorbance changes by photoproducts and/or intermediates formed by the previous excitation.

RESULTS

Absorption Spectra of Iodopsin at 0 and 20 °C. Curve 1 in Figure 1A shows a absorption spectrum of the iodopsin sample used for the nanosecond laser photolysis. The spectrum was measured at 0 °C. To estimate the percentage of each visual pigment in the sample, 1 M neutralized hydroxylamine was added to the sample to a final concentration of 10 mM, followed by successive irradiation with >660-, >590-, >520-, and >480-nm light. These four irradiations resulted in bleaching of iodopsin, a mixture of rhodopsin and chicken green, chicken blue, and chicken violet, respectively (Okano et al., 1989; Kandori et al., 1990). The difference spectra calculated before and after respective irradiations are shown in the inset of Figure 1B. From these spectra, the sample was estimated to be composed of 87% iodopsin, 4% mixture of rhodopsin and chicken green, 8% chicken blue, and 1% chicken violet.

Now it is essential to estimate the absorption spectrum of pure iodopsin at 20 °C in order to calculate the absorption spectra of intermediates appearing in the bleaching process of iodopsin (see below). We have estimated an absorption spectrum of pure iodopsin at 0 °C, which was calculated by adding an absorption spectrum on an equimolar amount of retinal oxime to the difference spectrum between iodopsin and retinal oxime (curve 1 in the inset of Figure 1B). Since we were able to prepare a highly purified bovine rhodopsin, the absorption spectrum of retinal oxime was obtained by irradiation of bovine rhodopsin in the presence of 10 mM hydroxylamine. The molar extinction coefficient of retinal oxime was also estimated by comparing the absorbance at the maximum of retinal oxime to that of bovine rhodopsin (ϵ_{max} = 41 200 in the CHAPS/PC system; Okano et al., 1992). Using the ratio of the molar extinction coefficient between retinal oxime and iodopsin (ϵ_{max} = 47 200; Okano et al., 1992), we estimated the absorption spectrum of an equimolar amount of retinal oxime. The calculated spectrum (curve 2 in Figure 1A) displays an absorption maximum at 571 nm, which is consistent with that reported previously (Okano et al., 1989).

The temperature dependence of the absorption spectrum of iodopsin was then examined as follows: Since the sample is mainly composed of iodopsin (87%), which is different in absorption maximum from the other visual pigments, the temperature dependence of the absorption spectrum of the sample can be mainly attributed to that of iodopsin. Therefore, the absorption spectrum of iodopsin at 20 °C was calculated by adding the difference spectrum between the spectra of samples at 0 and 20 °C to the spectrum of iodopsin at 0 °C. The spectrum obtained (curve 3 in Figure 1A) displayed an absorption maximum at 567 nm, which is 4 nm shorter than that at 0 °C, and a maximal absorbance 0.97 times smaller than that at 0 °C.

Spectral Changes Observed after Excitation of Iodopsin. Figure 2 shows transient absorption spectra (difference spectra between original iodopsin and its transients) and absorbance changes (kinetics) at selected wavelengths recorded on the nanosecond to millisecond time scale after excitation of iodopsin with a nanosecond laser pulse. The spectrum recorded at 50 ns after excitation (curve 1 in Figure 2A) displayed a triphasic shape composed of two maxima at about 660 and and 500 nm and a minimum at about 580 nm. As time elapsed, the triphasic spectral shape changed to a biphasic shape with decrease and increase of absorbances at about 660 nm and about 500 nm, respectively. An isosbestic point was observed at 550 nm during these spectral changes. The spectrum recorded 1 μs after the excitation (curve 3 in Figure 2A) was almost identical with that recorded 10 μs after the excitation (curve 4 in Figure 2A), indicating that the intermediate formed by the first conversion process is stable within this time scale. The kinetic measurements at 663 and 450 nm showed that this process was expressed by a single-exponential curve with a time constant of 130 ns (Figure 2D).

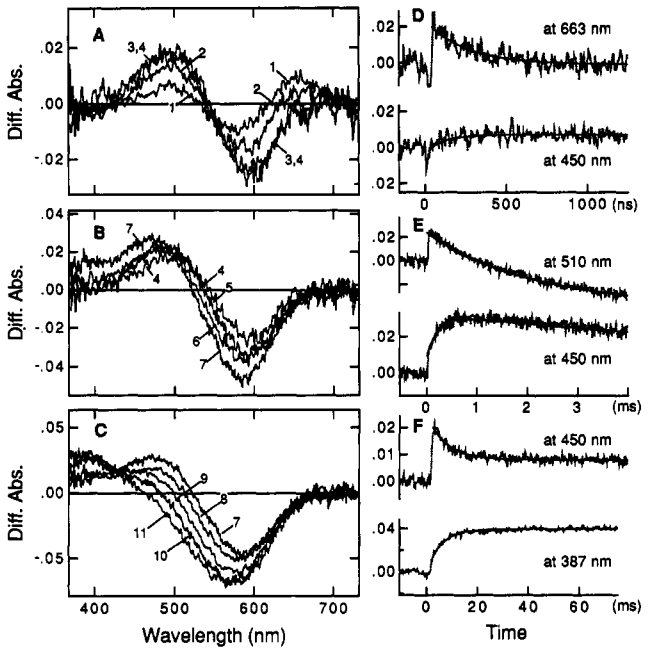

FIGURE 2: Transient absorption spectra at selected times (A–C) and kinetics at selected wavelengths (D–F) recorded after excitation of the iodopsin sample with a nanosecond laser pulse at 20 °C. (A–C) Times after excitation are 50 and 135 ns, 1, 10, 50, 200, and 300 μs, and 1, 2, 5, and 100 ms (curves 1–11). (D–F) Wavelengths for the kinetic measurements are shown in the figures. The smooth curves in panel D or panel F are the fitting curves having a time constant of 130 ns or 6 ms, respectively, and those in panel E are the two sequential single-exponential curves with time constants of 230 μs and 6 ms.

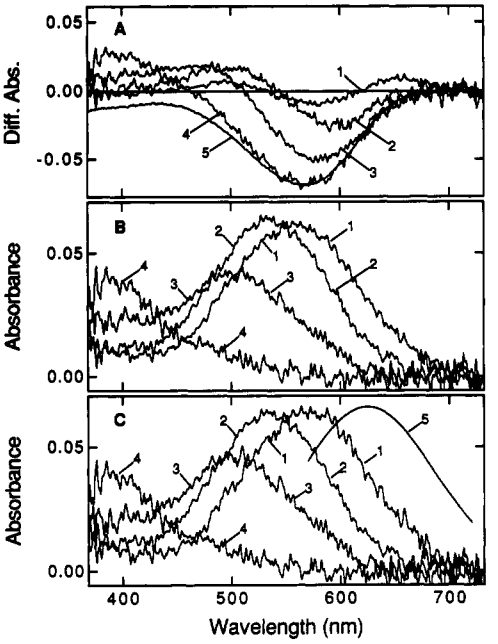

FIGURE 3: Transient absorption spectra at selected times after excitation of the iodopsin sample with a nanosecond laser pulse and calculated absorption spectra of the intermediates of iodopsin. (A) Transient absorption spectra recorded at 50 ns, 1 μs, 1 ms, and 100 ms (curves 1–4) after excitation of the iodopsin sample with a nanosecond laser pulse (560 nm, 17 ns). Curve 5 is an absorption spectrum of iodopsin at 20 °C whose maximal absorbance was adjusted to that of curve 4. (B) Absorption spectra calculated by subtracting curve 5 from curves 1–4 in panel A. (C) Absorption spectra of the intermediates of iodopsin calculated from curves 1–4 in panel B after estimation of the percentages of intermediates present at the time when each curve in panel A was recorded. Curve 5 is an absorption spectrum of an equimolar amount of bathoiodopsin obtained from a picosecond laser photolysis experiment (Kandori et al., 1990).

Further recording on the microsecond time scale displayed the conversion of this intermediate to the next intermediate (Figure 2B). There was an isosbestic point at about 500 nm. The latter intermediate was then converted to the next intermediate with an isosbestic point at 430 nm (Figure 2C). The kinetic profiles at 510 and 450 nm in Figure 2E were not fit by a single-exponential curve but by two sequential single-exponential curves whose time constants were 230 μs and 6 ms, respectively. On the other hand, the kinetic profiles at 450 and 387 nm in Figure 2F were fit by a single-exponential curve with the time constant of 6 ms. Therefore, the decay processes on the microsecond to millisecond time scale were approximated by two sequential single-exponential curves having time constants of 230 μs and 6 ms.

The results obtained clearly indicated that at least three intermediates and the product having an absorption maximum at about 390 nm are present in the bleaching process of iodopsin within the time scales recorded. The product has an absorption maximum very close to the meta II intermediate of rhodopsin (metarhodopsin II, λ_{max} = 380 nm). Furthermore, it displayed positive and negative CD signals in the wavelength regions around 390 and 280 nm, respectively, which are also very close to those of metarhodopsin II (Okada et al., manuscript in preparation). This fact clearly indicated that the product is not an *all-trans*-retinal dissociated from the opsin moiety of iodopsin but an intermediate corresponding to metarhodopsin II. Therefore, the product should be named the meta II intermediate of iodopsin (metaiodopsin II). The intermediate which converted to metaiodopsin II is then called metaiodopsin I, and the precursor of metaiodopsin I is lumiiodopsin. The first intermediate observed in the present experiments is not bathoiodopsin, because the calculated absorption spectrum of this intermediate (curve 1 in Figure 3C, see below) displays an absorption maximum (571 nm) largely different from that of bathoiodopsin (625 nm) previously reported (curve 5 in Figure 3C; Kandori et al., 1990). Therefore, we called this intermediate BL-iodopsin, which means the intermediate situated between batho- and lumiiodopsins. Because of our experimental limitation, we could not observe the conversion process from batho- to BL-iodopsin. Thus, the decay time constant of bathoiodopsin should be less than 50 ns.

Absorption Spectra of Intermediates of Iodopsin. Using the transient absorption spectra recorded at 50 ns, 1 μs, 1 ms, and 100 ms after the excitation (curves 1–4 in Figure 3A), we calculated the absolute absorption spectra of the intermediates (Figure 3C). The percentage of iodopsin in the sample bleached by the laser excitation was estimated as follows: The spectrum recorded at 100 ms after the excitation (curve 4 in Figure 3A) agreed well with that of iodopsin (curve 5 in Figure 3A) in the wavelength region longer than 560 nm, so that we assumed that the spectrum in this region reflects the bleaching of iodopsin in the sample. Thus, the percentage of the bleached iodopsin was estimated to be 11.5%. Using this value, the absorption spectra were calculated by adding the spectrum of 11.5% iodopsin in the sample (curve 5 in Figure 3A) to the respective transient spectra (Figure 3B). Then the percentage of intermediates present at the time when each transient spectrum was recorded were estimated by a kinetic simulation of the assumption that the four intermediates are sequentially produced and there are no reverse reactions in these processes (BL → lumi → meta I → meta II). Thus, the spectrum calculated from the transient spectrum recorded at 1 μs or 100 ms after the excitation (curve 2 or 4 in Figure 3B) was ascribed to that of lumiiodopsin or metaiodopsin II,

respectively, because the simulation indicated that almost only lumiiodopsin (99.6%) or metaiodopsin II (100%) was present at 1 μs and 100 ms, respectively. On the other hand, the spectrum calculated from the spectra recorded at 50 ns after the excitation (curve 1 in Figure 1B) was a composite of those of 68.1% BL-iodopsin and 31.9% lumiiodopsin. Therefore, the spectrum of BL-iodopsin (curve 1 in Figure 3C) was calculated by subtracting 31.9% lumiiodopsin (curve 2 in Figure 3B) from that (curve 1 in Figure 3B) calculated from the transient spectrum recorded at 50 ns after the excitation. The spectrum calculated from that recorded at 1 ms after the excitation (curve 3 in Figure 3B) was a composite of the spectra of 1.3% lumiiodopsin, 86.7% metaiodopsin I, and 12% metaiodopsin II. Consequently, the spectrum of metaiodopsin I (curve 3 in Figure 3C) was calculated by subtracting the contribution of the spectra of lumiiodopsin and metaiodopsin II from curve 3 in Figure 3B.

It should be noted that the spectrum of BL-iodopsin displays an absorption maximum (571 nm) at a wavelength 54 nm shorter and 36 nm longer than those of bathoiodopsin (625 nm) and lumiiodopsin (535 nm) and shows a half-band-width (137 nm, 4300 cm^{-1}) smaller and larger than bathoiodopsin (146 nm, 3800 cm^{-1}) and lumiiodopsin (128 nm, 4500 cm^{-1}), respectively. Since the spectrum of BL-iodopsin is not simulated by any combination of the spectra of batho- and lumiiodopsins, BL-iodopsin is not a mixture of batho- and lumiiodopsins but a distinct intermediate.

Temperature Dependence of the Equilibrium between Meta I and Meta II Intermediates. On the assumption that there are no reverse reactions in the bleaching process of iodopsin, the spectrum calculated from the spectrum recorded at 100 ms after the excitation was expected to be that of metaiodopsin II. However, the spectrum displays a longer wavelength tail (curve 4 in Figure 3C), suggesting that the spectrum reflects not metaiodopsin II alone but a composite of metaiodopsins I and II. Since the time constant of the apparent conversion from metaiodopsin I to metaiodopsin II was 6 ms, the existence of metaiodopsin I in the sample even at 100 ms after the excitation strongly suggests the presence of a back-reaction from metaiodopsin II to I, resulting in formation of an equilibrium state between metaiodopsins I and II. This is consistent with the fact that metarhodopsin II also forms an equilibrium state with metarhodopsin I (Matthews et al., 1963). Since the equilibrium between metarhodopsins I and II is reported to be temperature-dependent (Matthews et al., 1963), we have investigated the temperature dependence on the shape of the difference spectrum recorded after completion of the apparent conversion from metaiodopsin I to II.

We first investigated whether or not the established scheme of meta I → meta II equilibrium in rhodopsin was observed in our detergent system (CHAPS/PC system). When chicken or bovine rhodopsin was excited with a nanosecond laser pulse (wavelength, 460 nm; pulse width, 17 ns) at 3, 20, and 37 °C, the spectra which did not change their shapes on the time scale from 100 ms to 60 s were obtained (Figure 4B,C). The spectral shape dependend on the temperature at which each rhodopsin was excited. The difference spectra calculated from the spectra at 3 °C and those at 37 °C (insets of Figure 4B,C) displayed the typical difference spectra between metarhodopsins I and II. These results indicated the presence of an equilibrium state between metarhodopsins I and II in our detergent system. The time for reaching the equilibrium state depended on the temperature, but the decay of a mixture of metarhodopsins I and II to metarhodopsin III was at least 2 orders slower than the establishment of the equilibrium.

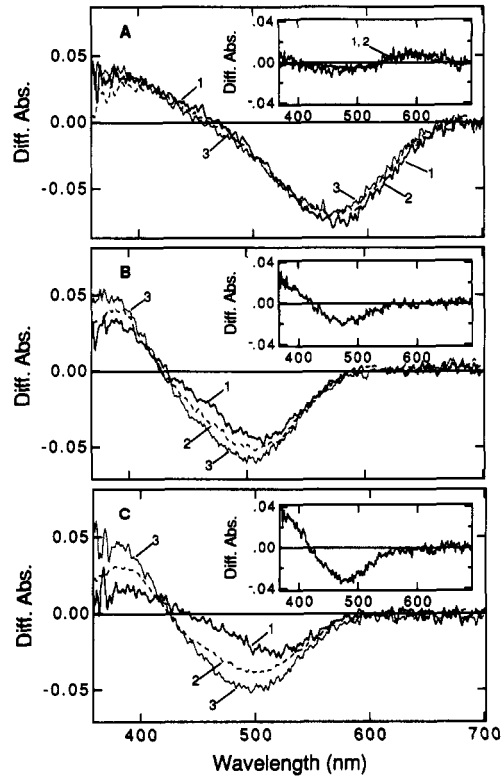

FIGURE 4: Temperature dependence of the thermal equilibrium between meta I and meta II intermediates of iodopsin and rhodopsins. (A) An iodopsin sample was excited with a nanosecond laser pulse (560 nm, 17 ns), and the transient absorption spectrum was recorded 10 s, 100 ms, or 10 ms after excitation at 3, 20, or 37 °C (curve 1, 2, or 3), respectively. Inset: curve 1, difference spectrum between curves 1 and 3 in panel A; curve 2, difference spectrum between the spectra of iodopsin at 3 and 20 °C. (B) A chicken rhodopsin sample was excited with a nanosecond laser pulse (460 nm, 17 ns), and the transient absorption spectrum was recorded after 30 s, 100 ms, or 10 ms excitation at 3, 20, or 37 °C (curve 1, 2, or 3), respectively. Inset: Difference spectrum between curves 1 and 3 in panel B. (C) A bovine rhodopsin sample was excited with a nanosecond laser pulse (460 nm, 17 ns), and the transient absorption spectrum was recorded 180 s, 100 ms, or 10 ms after excitation at 3, 20, or 37 °C (curve 1, 2, or 3), respectively. Inset: Difference spectrum between curves 1 and 3 in panel C.

Similar experiments were performed by using the iodopsin sample (Figure 4A). Contrary to rhodopsins, iodopsin showed almost no temperature dependence on the spectral shapes. The small differences among the spectra shown in Figure 4A may originate from the temperature dependence of the spectrum of iodopsin. In fact, the difference spectrum (cure 1 in the inset of Figure 4A) calculated from curves 1 and 3 in Figure 4A is very similar in shape to that calculated from the spectra of the original sample measured at 3 and 20 °C (curve 2 in the inset of Figure 4A).

DISCUSSION

The present results newly identified two intermediates, BL and meta II, in addition to the three intermediates, batho, lumi, and meta I, which were previously identified by low-temperature spectrophotometry (Hubbard & Kropf, 1959; Yoshizawa & Wald, 1967) and picosecond laser photolysis (Kandori et al., 1990). The photobleaching process of iodopsin is thus summarized as shown in Figure 5. The presence of BL-iodopsin may provide insight into the pecularity of the photobleaching process of iodopsin, i.e., the difference in thermal behavior between bathoiodopsins produced at room and low temperatures (Yoshizawa & Wald, 1967; Kandori et

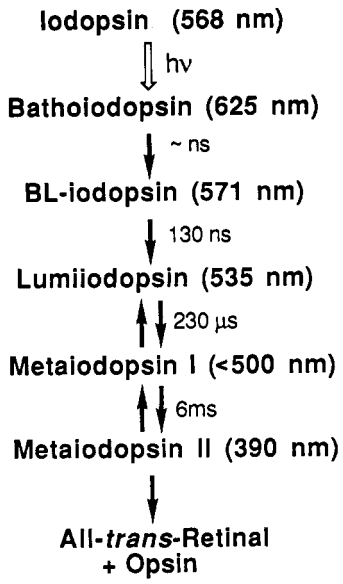

FIGURE 5: Photobleaching process of iodopsin. The values in parentheses show absorption maxima. The decay time constants are shown on the right-hand side of the arrows. The absorption maximum of bathoiodopsin was reported by Kandori et al. (1990).

al., 1990). Furthermore, the presence of metaiodopsin II which corresponds to the physiologically active intermediate of rhodopsin (metarhodopsin II) suggests that iodopsin can activate transducin in a manner similar to rhodopsin.

In relation to the difference in thermal behavior at low temperature between batho intermediates of iodopsin and rhodopsins, it is of interest to compare the absorption characteristics of BL-iodopsin with the corresponding intermediate of rhodopsin. BL-rhodopsin formed between batho- and lumirhodopsins was first reported in the photobleaching process of the rhodopsin analog having 13-demethylretinal as its chromophore (Shichida et al., 1981). Later a similar intermediate [named BSI (blue-shifted intermediate)] was detected at room temperature in the photobleaching processes of various artificial rhodopsins (Albeck et al., 1989; Randall et al., 1991) and native rhodopsin (Hug et al., 1990). BL-(BSI)-rhodopsin displays an absorption maximum shorter than those of batho- and lumirhodopsins, and an extinction coefficient much smaller than those of batho- and lumirhodopsins (Hug et al., 1990). In addition, formation of BL-rhodopsin was facilitated if the C_5=C_6 bond of the chromophore was saturated, the cyclohexenyl ring of the chromophore was truncated, or the methyl groups attached to the chromophore were removed (Schichida et al., 1981; Yoshizawa et al., 1984; Albeck et al., 1989; Einterz et al., 1990; Okada et al., 1991). These results suggested that the chromophore of BL-rhodopsin is in a more relaxed conformation than that of bathorhodopsin, while the chromophore–opsin interaction in BL-rhodopsin is less favorable than that in lumirhodopsin. Recent FTIR studies also supported this idea (Ganter et al., 1990, 1991).

Unlike BL-rhodopsin, BL-iodopsin not only has an absorption maximum just between batho- and lumiiodopsins but also is almost identical in extinction coefficient with batho- and lumiiodopsins. These facts strongly suggest that BL-iodopsin has a chromophore and a protein whose conformation is in an intermediate state between batho- and lumiiodopsins. Namely, in iodopsin, the concurrent relaxation of both the chromophore and the nearby protein proceeds during the batho- to lumiiodopsin transition via BL-iodopsin. As already described in the introduction, chloride bound to iodopsin affects the thermal behavior of bathoiodopsin (Imamoto et al., 1989). Therefore, we can speculate that the difference in the batho–lumi transition via the BL intermediate between rhodopsin and iodopsin may be due to the presence or absence of a chloride binding site in the protein moiety.

Compared to batho-, BL-, and lumiiodopsins, metaiodopsin I is small in maximal absorbance, long in wavelength tail, and large in absorbance around 400 nm (curve 3 in Figure 3C). The latter may originate from inadequate subtraction of the spectrum of metaiodopsin II in the calculation of this spectrum, because the spectrum of metaiodopsin II (curve 4 in Figure 3B) used for the calculation was not a pure spectrum of metaiodopsin II but a mixture of metaiodopsins I and II, whose contents could not be estimated by the simple bleaching scheme (see Results). Since we can estimate the spectrum of lumiiodopsin which is stable on the time scale from 1 to 10 μs, the shape of the spectrum of metaiodopsin I at wavelengths longer than λ_{max} was not due to inadequate subtraction of the spectrum of lumiiodopsin. There are at least two possible explanations for the presence of a longer wavelength tail: One is the presence of a thermal back-reaction to lumiiodopsin from metaiodopsin I. This may cause the presence of a considerable amount of lumiiodopsin even 1 ms after the excitation. The other is that the simple scheme (Figure 5) may not be enough to express the bleaching process of iodopsin. Further kinetic measurements of the bleaching process of iodopsin at various temperatures with detailed simulation analyses would be necessary for evaluation of these possibilities. This point is being investigated.

The temperature dependence on the thermal equilibrium between metarhodopsins I and II was reported by Matthews et al. (1963). Not only the present results confirmed it, but also the different temperature dependence between bovine and chicken rhodopsins was demonstrated (Figure 4B,C). On the other hand, meta intermediates of iodopsin show little temperature dependence, indicating that the thermodynamic parameters for meta intermediates of iodopsin are different from those of rhodopsins. The pH dependence on the equilibrium between metarhodopsins I and II was recently investigated by using mutant rhodopsin, in which each of the six histidine residues was replaced by phenylananine or cysteine (Weitz & Nathans, 1992). Then it is indicated that His-211 and possibly His-65 and His-152 are the residues which regulate the pH dependence of the equilibrium between metarhodopsins I and II. Since the residues found in the homologous positions in iodopsin are not histidine (Kuwata et al., 1990; Tokunaga et al., 1990), it is reasonable to speculate that meta intermediates of iodopsin could not show a similar pH dependence. All these results reflect the difference in properties between meta intermediates of iodopsin and rhodopsins.

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