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Bathoiodopsin, a primary intermediate of iodopsin at physiological temperature

(color vision/red-sensitive cone visual pigment/picosecond laser photolysis/photoisomerization)

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ABSTRACT **Measurement of the primary photochemical reaction of iodopsin, a chicken red-sensitive cone visual pigment, was carried out at room temperature by using picosecond (ps) laser photolysis. Excitation of iodopsin with a ps green pulse (pulse width, 21 ps) caused the instantaneous formation of a bathochromic product, which was stable on a ps time scale. This product may correspond to "bathoiodopsin," which was detected by low-temperature spectrophotometry. Although bathoiodopsin produced at the temperature of liquid nitrogen or helium reverted to the original pigment (iodopsin) on warming (above −170°C), the bathoiodopsin produced at physiological temperature decayed to all-*trans*-retinal and R-photopsin (the protein moiety of iodopsin) presumably through several intermediates. The absorption maximum of bathoiodopsin at room temperature was at 625 nm, a wavelength slightly shorter than that measured at low temperature (λ_{max}, 640 nm). The extinction coefficient of bathoiodopsin at room temperature was lower than that at low temperature and close to that of the original iodopsin at room temperature.**

The vertebrate retina contains two kinds of photoreceptor cells, rods and cones. A rod is a photoreceptor cell that has evolutionally acquired an extremely high photosensitivity so as to act under twilight conditions. The visual pigment in rods is called rhodopsin. Cones, however, act under daylight conditions and have a photoresponse with a wider dynamic range and a faster latency (1). Since there are at least three types of cones, each with its own distinct visual pigment possessing characteristic absorption maximum (2), integration of the photoresponses from cones generates the sensation of color in the visual cortex.

The primary photoreceptive mechanism in visual transduction has been extensively investigated in rods, which contain rhodopsin as the visual pigment. Rhodopsin has an 11-*cis*-retinylidene chromophore attached by a protonated Schiff base linkage to a specific lysine residue of the apoprotein opsin. The primary photochemical event in rhodopsin is an isomerization around the C_{11}=C_{12} bond of the 11-*cis*-retinylidene chromophore to form photorhodopsin, a highly twisted all-trans photoproduct (3, 4). The subsequent thermal reaction proceeds through several intermediates to form an enzymatically active intermediate, metarhodopsin II. This intermediate can bind to transducin, a guanine nucleotide-binding protein, to convert it into an active form by which cGMP phosphodiesterase can be activated (5). On the other hand, little is known about the cone visual pigments and the corresponding transduction mechanism. To elucidate the primary photoreceptive mechanism in cones, studies on the chemical nature and photochemical reaction of cone visual pigments are indispensable.

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In chicken retina, a red-sensitive cone visual pigment, iodopsin, is abundant in comparison with other cone visual pigments. Iodopsin is the only cone pigment that has been investigated biochemically. Like rhodopsin, iodopsin contains 11-*cis*-retinal as its chromophore. Investigations with retinal isomers and analogs suggested that iodopsin and rhodopsin have similarly shaped chromophore binding sites (6) but the point charges are in different locations (6, 7).

On absorption of light, iodopsin bleaches to all-*trans*-retinal and R-photopsin (the protein moiety of iodopsin) by way of lumiiodopsin and then metaiodopsin, according to low-temperature spectrophotometry (8). Yoshizawa and Wald (9) observed that irradiation of iodopsin at the temperature of liquid nitrogen produced bathoiodopsin. Prolonged irradiation of this sample formed a steady-state photo mixture composed of iodopsin, isoiodopsin, and bathoiodopsin. This photochemical behavior of iodopsin is very similar to that of rhodopsin (Fig. 1). On warming, however, an unexpected difference between iodopsin and rhodopsin was observed; unlike bathorhodopsin, bathoiodopsin was not thermally converted to lumiiodopsin but reverted back to iodopsin above −170°C. Thus the question arises whether bathoiodopsin is a physiological intermediate or an artifact produced only by irradiation at low temperature. To answer this question, a room temperature kinetic analysis after excitation of a purified iodopsin preparation with an ultrashort light pulse was required.

We have developed (11) a purification procedure for iodopsin that has permitted us to carry out the picosecond (ps) laser photolysis of iodopsin at room temperature. We report that bathoiodopsin was produced from iodopsin at room temperature and then decayed to all-*trans*-retinal and R-photopsin, presumably through several thermolabile intermediates.

MATERIALS AND METHODS

Preparation of Iodopsin. Iodopsin was prepared from 2000 chicken retinas according to the method of Okano *et al.* (11). All the procedures described below were performed under ice-chilled conditions. Photoreceptors from the outer segments of the retinas were isolated by the 40% (wt/vol) sucrose flotation method in a standard buffer [50 mM Hepes/140 mM NaCl/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/leupeptin (4 μg/ml)/aprotinin (50 kallikrein inhibitor units/ml)/1 mM $MnCl_2$/1 mM $CaCl_2$, pH 6.6] under red light (>630 nm). Since iodopsin absorbs light with wavelengths even longer than 630 nm, some iodopsin molecules in

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, phosphatidylcholine; mmϕ, mm in diameter.

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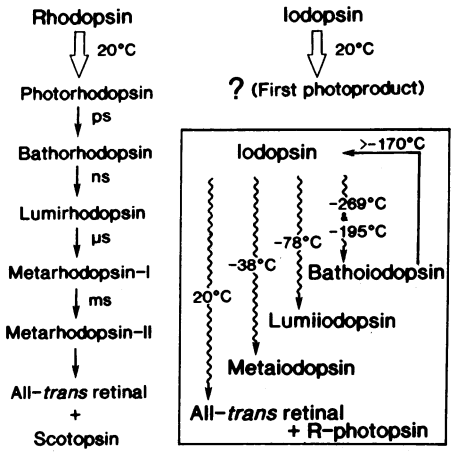

FIG. 1. Photobleaching of rhodopsin and iodopsin. Broad open and wavy arrows indicate photic reactions initiated by laser pulses and steady lights, respectively. Solid arrows indicate thermal reactions. (*Left*) Irradiation of cattle rhodopsin produces a primary intermediate, photorhodopsin. It decays to bathorhodopsin, lumirhodopsin, metarhodopsin-I, and metarhodopsin-II sequentially in the time scales of ps, ns, μs, and ms, respectively. (*Right*) Bleaching intermediates of chicken iodopsin at physiological temperature have not been investigated. Photoproducts (in the rectangle) of chicken iodopsin measured by low-temperature spectrophotometry (8–10) are shown.

the outer segments were bleached under red light, while the other visual pigments were not bleached. Then, about twice the amount of 11-*cis*-retinal relative to that of R-photopsin, estimated by a partial-bleaching method described below, was added to the outer segments to regenerate R-photopsin. Hereafter, all the procedures were performed under dim red light (>670 nm). All the visual pigments in the outer segments thus prepared were extracted with 0.75% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in the standard buffer containing phosphatidylcholine (PC; 1 mg/ml) from fresh egg yolk. The extract (4 vol) was then diluted with standard buffer (1 vol) to decrease the concentration of CHAPS and PC to 0.6% and 0.8 mg/ml, respectively.

Since a chicken retina contains five visual pigments (11) [red (iodopsin); green-, blue-, and violet-sensitive cone visual pigments; and rhodopsin], the amount of each visual pigment in the extract was estimated by the partial-bleaching method (11). Hydroxylamine (10 mM) was added to a portion of the extract at 4°C, where all the visual pigments were stable. The extract was first irradiated with light at wavelengths >660 nm for bleaching of only iodopsin. Then, the extract was irradiated with light of >590 nm for complete bleaching of rhodopsin and the green-sensitive pigment and then irradiated at >520 nm and >480 nm to bleach blue- and violet-sensitive pigments, respectively. The difference spectrum before and after each irradiation corresponded to that between the respective pigment and its photoproduct (all-*trans*-retinal oxime and opsin). Thus, the extract was estimated to contain 29.8% iodopsin (λ_{max}, 571 nm), 59.1% of a rhodopsin (λ_{max}, 503 nm) and green-sensitive pigment (λ_{max}, 508 nm) mixture, 9.2% blue-sensitive pigment (λ_{max}, 455 nm), and 1.9% violet-sensitive pigment (λ_{max}, 425 nm), on the assumption that molar extinction coefficients of all the pigments are identical.

Iodopsin was isolated from the other visual pigments, the oil droplets, and the added retinal by concanavalin A-Sepharose 4B affinity column chromatography; iodopsin was eluted from the column in equilibration buffer (standard buffer supplemented with 0.6% CHAPS and PC at 0.8 mg/ml) containing 1.5 mM α-methylmannoside. All the iodopsin-rich fractions were mixed and glycerol was added to a final concentration of 20% (wt/vol) to keep iodopsin stable. The sample thus prepared contained 80.6% iodopsin, 6.5% of a rhodopsin and green-sensitive pigment mixture, 10.0% blue-sensitive pigment, and 2.9% violet-sensitive pigment.

Since the maximum absorbance of the sample (≈0.04) was too low to do the laser photolytic experiment, it was concentrated by using a concanavalin A-Sepharose affinity column and then an ultrafiltration membrane (Amicon, YM100). The sample (600 ml) was dialyzed against the equilibration buffer to decrease α-methylmannoside to <0.06 mM and then adsorbed to a concanavalin A-Sepharose affinity column. The fraction, eluted with the equilibration buffer supplemented with 100 mM α-methylmannoside, had a maximum absorbance of 0.24. The eluate was concentrated about 20 times with the ultrafiltration membranes and then centrifuged.

Since iodopsin is unstable in the presence of hydroxylamine at room temperature, all the experiments herein were carried out in the absence of hydroxylamine.

An Optical System for ps Laser Photolysis. Transient absorption spectra and time-resolved absorbance changes were measured by a double-beam laser spectrophotometer linked to a ps laser apparatus (3). The sample was placed in an optical microcell (2 mm × 2 mm) and then excited with a frequency-doubled 532-nm light pulse (pulse width, 21 ps) from a mode-locked Nd^{3+}/yttrium/aluminum-garnet (YAG) laser (Matsui, Kawasaki, Japan). Transient absorption spectra at selected times after exciting the iodopsin sample were measured by a ps continuum (probe pulse), which was generated by focusing the fundamental pulse into a glass block. Both excitation and probe pulses were polarized by polarizers; the polarization angle between the two pulses was set at the "magic" angle, $\theta = 54.7°$. The ps probe pulse was split into two pulses by a half-mirror; one was passed through the sample and the other was not. Each probe pulse was focused on a slit of a polychrometer (Seki Technotron, Tokyo) and impinged onto a cryogenically cooled charge-coupled device (CCD) detector (CH-210, Photometrics, Tucson, AZ). Photosignals of the CCD detector were digitized in a CCD camera control system (series 200, Photometrics) and transferred to a personal computer (PC-9801, NEC, Tokyo), which was used to calculate the difference absorption spectrum. To reduce the large scattering of the excitation pulse (532 nm) that may disturb measurements of the ps absorption spectra, a cut-off filter (VO-55, Toshiba, Tokyo) was placed in front of the polychrometer. Thus, transient difference absorption spectra before and after excitation of a sample at wavelengths longer than 560 nm were recorded. The photon densities of the excitation pulses were monitored during the experiments by a biplanar photodiode (Hamamatsu Photonics, Hamamatsu, Japan), the outputs of which were calibrated by a joule meter (Gentec, Quebec) at the end of experiments.

For each experiment, 8 μl of the sample was held in a 2 mm × 2 mm optical cell. Experiments were carried out at 20°C. Each sample was excited three times with the excitation pulses for measuring the transient difference absorption spectra and then replaced by a new sample. No differences in spectral shape were observed among first, second, and third excitations. All the data thus obtained were corrected for the excitation photon density and analyzed by a computer (PC-9801, NEC).

RESULTS

An absorption spectrum of the iodopsin sample used is shown in Fig. 2*A*. The absorption maximum was at a shorter wavelength (560 nm) than that of a pure iodopsin (λ_{max}, 571 nm); the maximum absorbance was 0.885 for a 2-mm light path. To estimate the content of iodopsin and other pigments

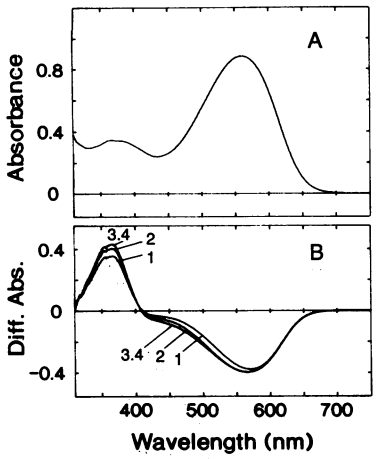

FIG. 2. (*A*) Absorption spectrum of the iodopsin sample (2-mm light path) in 0.6% CHAPS containing PC (0.8 mg/ml) and 20% glycerol in the standard buffer (pH 6.6) used for the ps laser photolysis (see text). (*B*) Difference absorption spectra (Diff. Abs.) between iodopsin and its photoproduct formed by a partial bleaching described below. The sample was diluted ≈1:11 with the equilibration buffer and the spectrum was measured through a 1-cm light path. Then the sample was irradiated with a dim red light (>660 nm) for 80 min (curve 1), a red light (>590 nm) for 40 min (curve 2), an orange light (>520 nm) for 20 min (curve 3), or a yellow light (>480 nm) for 10 min (curve 4). These four irradiations caused bleaching of iodopsin, a mixture of rhodopsin and green-sensitive pigment, blue-sensitive pigment, and a trace of violet-sensitive pigments, respectively.

in the sample, a portion of the sample was diluted ≈1:11 with equilibration buffer and subjected to the partial-bleaching method (Fig. 2*B*). The sample contained 83.9% iodopsin, 10.3% of a rhodopsin and green-sensitive pigment mixture, and 4.7% blue- and 1.1% violet-sensitive pigments. Relative absorbances at 532 nm (excitation wavelength) of the respective pigments in the sample were 0.87 (iodopsin), 0.12 (rhodopsin and green-sensitive pigment), 0.01 (blue-sensitive pigment), and 0.00 (violet-sensitive pigment).

As shown in Fig. 3, transient absorption spectra (difference spectra between iodopsin and its transient) were measured at 15 ps, 100 ps, and 1 ns after the excitation of the sample with

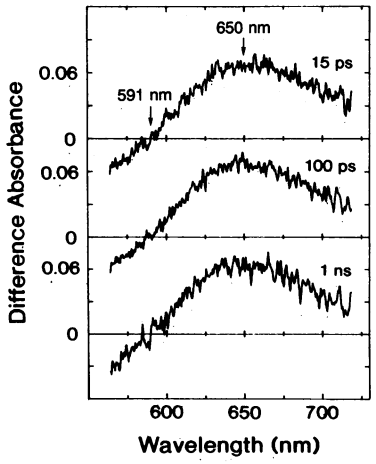

FIG. 3. Difference absorption spectra between the iodopsin sample and its bathochromic product 15 ps, 100 ps, or 1 ns (as indicated) after the excitation with a single green pulse (wavelength, 532 nm; pulse width, 21 ps) at room temperature (20°C). Each spectrum is an average of 31 (15 ps), 37 (100 ps), or 32 (1 ns) experiments. Averaged excitation photon density was 30.2 μJ/1.8 mmϕ. Each spectrum has an intersection point at 591 nm and a difference absorption maximum at 650 nm.

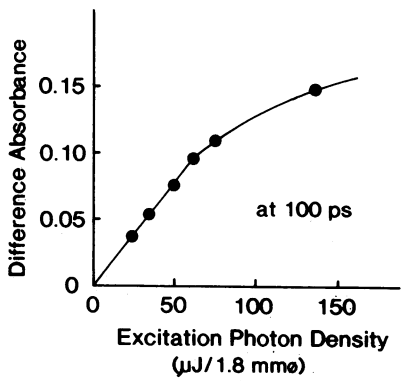

FIG. 4. Relationship between an increase in absorbance at 650 nm due to the excitation of iodopsin and the photon density of the excitation pulse (532 nm). The sample with a maximum absorbance of 0.62 (2-mm light path) was used. Absorbance changes were measured 100 ps after excitation. Each point is an average of five experiments.

the 532-nm laser pulse. Excitation photon densities of the pulses were in the range from 20 to 40 μJ/1.8 mm in diameter (mmϕ) in which no saturation effect due to the ps laser pulse was observed (see below). The three difference spectra in the figure were similar in shape to one another. In each spectrum, the absorbance at wavelengths <591 nm decreased, whereas absorbance at wavelengths >591 nm increased. The intersection point and the difference absorption maximum were at 591 and 650 nm, respectively. The generation process of this bathochromic photoproduct was too rapid to be detected within the time resolution of the laser apparatus (excitation and probe pulse widths, 21 ps) and the decay process was slower than the ps time scale. Fig. 4 shows a relationship between the excitation photon density and the increase in absorbance at 650 nm due to generation of the bathochromic product. A linear relationship was observed up to 60 μJ/1.8 mmϕ, indicating that the bathochromic product shown in Fig. 3 is produced directly from the original pigment iodopsin by a single-photon absorption process.

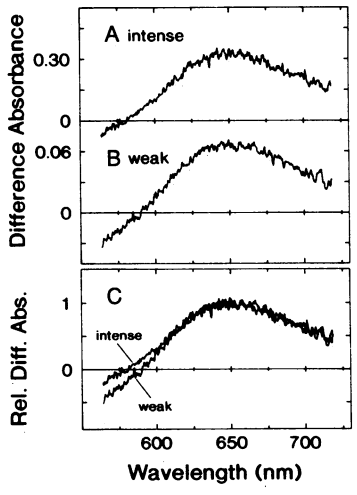

FIG. 5. (*A*) Difference absorption spectrum before and after excitation of the iodopsin sample with an intense green pulse (532 nm). The spectrum was measured 15 ps after the excitation with a pulse of 383 μJ/1.8 mmϕ. The spectrum is an average of six experiments. (*B*) Difference absorption spectrum between the bathochromic product (bathoiodopsin) and iodopsin. It is a sum of 100 spectra shown in Fig. 3. Excitation photon density was 30.2 μJ/1.8 mmϕ. (*C*) The spectra of *A* and *B* were superimposed. Both spectra were normalized at the 650-nm peaks. Rel. Diff. Abs., relative difference absorbance.

The sample was also excited with an intense pulse (383 μJ/1.8 mmϕ). Fig. 5*A* shows a transient absorption spectrum measured 15 ps after the excitation; the intersection point was at 580 nm and the difference absorption maximum was at 650 nm. The transient difference spectrum of the sample excited with a weak pulse (30.2 μJ/1.8 mmϕ), which is shown in Fig. 5*B*, was obtained from the sum of those presented in Fig. 3. This spectrum is similar in spectral shape at wavelengths >620 nm and in difference absorption maximum (650 nm) to that obtained by the intense pulse (Fig. 5*C*). The spectral shapes at wavelengths <620 nm, however, were clearly different from each other and the intersection point of the spectrum obtained by the intense excitation was at a wavelength 10 nm shorter than that seen with the weak excitation. The difference in spectral shape suggests that a photoreaction other than the back-reaction of the bathochromic product to iodopsin may take place if the excitation photon density exceeds 60 μJ/1.8 mmϕ.

DISCUSSION

The present study, which measures the primary photochemical reaction of a cone visual pigment at room temperature, has been carried out on the chicken red-sensitive cone pigment iodopsin by using a ps laser pulse. Since the sample contains mainly iodopsin (Fig. 2*B*), the results observed are predominantly attributed to the photochemical reaction of iodopsin.

According to the results shown in Fig. 3, a bathochromic product is generated immediately after the excitation. The product was stable on the ps time scale. It is likely to be identical to bathoiodopsin, which has been detected by low temperature spectrophotometry of iodopsin extracted with digitonin (9, 10) or CHAPS/PC (12), because the difference absorption maximum (≈650 nm in digitonin and CHAPS/PC) and the intersection point (595 nm in digitonin and 600 nm in CHAPS/PC) between bathoiodopsin and iodopsin at the temperature of liquid nitrogen were very close to those obtained here. Since bathoiodopsin produced at the temperatures of liquid nitrogen and helium reverted back to iodopsin on warming, it has been open to question whether or not bathoiodopsin is the primary product formed under a physiological temperature. The present results have clearly demonstrated the formation of bathoiodopsin at physiological temperature. The formation of bathoiodopsin suggests that the primary photochemical process in iodopsin is photoisomerization of the chromophore from 11-cis to all-trans, as inferred by Yoshizawa and Wald (9).

In rhodopsin systems, photorhodopsin was observed as the precursor of bathorhodopsin; the transient absorption spectrum 15 ps after excitation of cattle rhodopsin was located at a wavelength ≈10 nm longer than that 100 ps or 1 ns after the excitation (3). The time constant of the conversion from photorhodopsin to bathorhodopsin was determined to be 45 ps (13). The present experiment, however, did not demonstrate the presence of the bathoiodopsin precursor (photointermediate), because the transient spectrum measured 15 ps after the excitation did not change within 1 ns (Fig. 3). The failure to detect the precursor of bathoiodopsin implies at least two possibilities; one is that bathoiodopsin is the primary intermediate in the iodopsin system and is directly produced from an excited state of iodopsin, and the other is that the lifetime of a photointermediate of iodopsin is too short to be detected by the present apparatus. This may be resolved by fs laser photolysis of iodopsin.

As shown in Fig. 4, no photoreaction other than that of iodopsin to bathoiodopsin was observed using an excitation photon density up to 60 μJ/1.8 mmϕ. For cattle rhodopsin, the upper limit of the linear increase of bathorhodopsin as a function of the photon density of the excitation pulse was 20 μJ/1.8 mmϕ (14). This difference may be caused by a difference between iodopsin and cattle rhodopsin in the relative extinction coefficients at the excitation wavelength (532 nm), because the excitation wavelength is longer or shorter than the absorption maximum of rhodopsin (498 nm) or iodopsin (571 nm), respectively, so that bathoiodopsin absorbs the excitation pulse less than iodopsin, whereas photorhodopsin (570 nm) or bathorhodopsin (535 nm) absorbs more than rhodopsin.

The difference in shape between spectra measured with excitation of weak and intense laser pulses shown in Fig. 5 shows that intense excitation causes generation of a product other than bathoiodopsin. It is probably neither a 9-cis pigment (isoiodopsin) nor a hypsointermediate, because those hypsochromic products have lower absorbances at ≈591 nm than iodopsin and, therefore, it is not expected that the intersection point of the measured difference spectrum would shift to the blue when excited with the intense pulse. The product, which should have a larger absorbance at ≈591 nm than iodopsin, is presumably generated by photon absorption of bathoiodopsin or an excited state of iodopsin. It should be noted that the shift of intersection point of the spectrum caused by the intense excitation of iodopsin (Fig. 5) is in the opposite direction from that of rhodopsin (15, 16). These facts may indicate that the photoproducts of iodopsin and rhodopsin generated by excitation with a ps laser pulse have different photochemical behaviors.

Since bathoiodopsin produced at low temperature was reported to revert to iodopsin on warming (9), the question of whether or not bathoiodopsin produced at physiological temperature can revert to the original iodopsin was examined. We calculated the percentage of bleaching of iodopsin after the excitation with the laser pulse. The experimental procedure was similar to that described (14). Absorption spectra of the samples (8 μl) in an optical microcell were measured without hydroxylamine, and then samples were excited with the ps laser pulse and the absorption spectra were measured again ≈20 min after the excitation. Finally, the pigments in the sample were completely bleached with a continuous light (>480 nm) in the presence of hydroxylamine, and the absorption spectra were measured. The percentage of bleaching of the sample was estimated by calculating a ratio of difference absorbance at the maximum of the spectra before and after the excitation. The percentage of bleaching was calculated to be 8.52% per 30.2 μJ/1.8 mmϕ excitation (6.60% per 10.4 μJ/1.2 mmϕ). Absorbance of the iodopsin sample used for the experiment was 0.772 at its maximum and 0.692 at 532 nm. Under the same conditions, the percentage of bleaching of cattle rhodopsin extracted with 2% (wt/vol) digitonin was calculated to be 7.61% per 10.4 μJ/1.2 mmϕ, using the rhodopsin sample with an absorbance of 0.724 at 532 nm (14). Therefore, the photosensitivity ratio of iodopsin to cattle rhodopsin was estimated to be 0.87.‡ The fact that the photosensitivity of iodopsin is close to cattle rhodopsin suggests that bathoiodopsin at physiological temperature converts to its intermediate and bleaches like bathorhodopsin. The minor difference in photosensitivity between rhodopsin and iodopsin may be attributed to the difference in molecular extinction and/or quantum yield between them.

We have obtained a clue to the difference in thermal conversion between bathoiodopsins produced at room temperature and at a low temperature. Iodopsin has a chloride binding site and chloride binding affects the absorption maximum of iodopsin (18–20). Our low-temperature experiments (12) revealed that bathoiodopsin produced from chlo-

‡Our experiments (17) on the determination of the photosensitivity of iodopsin using a steady light source at room temperature showed that the photosensitivity ratio of iodopsin (CHAPS/PC) to cattle rhodopsin (2% digitonin) is 1.08.

ride-depleted iodopsin was thermally converted to the next intermediate, presumably lumiiodopsin, whereas bathoiodopsin produced from chloride-bound iodopsin (native iodopsin) reverted to iodopsin on warming. Therefore, the binding of chloride to the protein moiety may inhibit the conformational changes that induce the batho–lumi transition (12). Since we have found that bathoiodopsin produced at room temperature bleaches, the chloride bound to the protein moiety of iodopsin may be easily released at room temperature.

The absolute absorption spectrum of the intermediate gives us more information about the chromophore–protein interaction than the difference absorption spectrum between an original pigment and its intermediate. However, the precise measurement of the percentage of bleaching, which is indispensable for estimating the absolute absorption spectrum, is extraordinarily difficult in a ps laser experiment. In the ps laser apparatus, both excitation and probe pulses have such small diameters that all the molecules in the sample cell cannot be excited or monitored. Thus, diffusion of the excited molecule in the sample cell makes it difficult to accurately estimate the percentage of bleaching. We have developed a method for accurately estimating the percentage of bleaching of rhodopsin after laser excitation, in which a polyacrylamide gel was used to prevent diffusion of the excited molecules (21). Excitation of the rhodopsin sample with a pulse at 10.4 μJ/1.2 mmϕ caused bleaching of 7.61% of the rhodopsin in the sample, whereas excitation of the immobilized rhodopsin sample (2% digitonin extract) caused bleaching of 10.9% of the rhodopsin. This result indicates that the percentage of bleaching in a sample in solution would be underestimated by a factor of 1.43. Then, we may expect that the excitation of the immobilized iodopsin sample with a pulse at 30.2 μJ/1.8 mmϕ would result in bleaching 12.2% (8.52 $\times$ 1.43%) of the iodopsin in the sample. Since the polarization angle between excitation and probe pulses was 54.7° (magic angle), an artificial absorption change due to the rotation of the molecule after excitation should be negligible. Thus, an absolute absorption spectrum of bathoiodopsin can be calculated (Fig. 6) using the difference spectrum between bathoiodopsin and iodopsin (Fig. 5*B*) and the absolute absorption spectrum of iodopsin (Fig. 2*A*). The absorption maximum of the spectrum thus obtained was 620 nm (Fig. 6).

It should be noted that the spectrum of "bathoiodopsin" shown in Fig. 6 (solid line) must be composed of spectra of the photoproducts of iodopsin, rhodopsin, and the green-sensitive pigment, because the sample used for the experiment contained a small amount of visual pigments other than iodopsin. The rhodopsin and green-sensitive pigment in the sample contributed one-seventh of the absorbance at 532 nm. On the assumption that the absolute absorption spectra of the photoproducts produced from chicken rhodopsin and the green-sensitive pigment are identical to that of cattle bathorhodopsin (21), the absorption spectrum of bathoiodopsin was calculated (Fig. 6, solid line with calculated points). The absorption maximum of bathoiodopsin thus obtained was located at 625 nm, which is a slightly shorter wavelength than that (about 640 nm) obtained by low-temperature spectrophotometry at 78 K (9). The molar extinction coefficient of bathoiodopsin at room temperature is comparable to that of iodopsin at the same temperature but 1.5 times lower than the extinction coefficient at 78 K. Similar observations (ε_{max} of the bathointermediate at room temperature is lower than that at low temperature) have been reported in bacteriorhodopsin (22) and cattle rhodopsin (21).

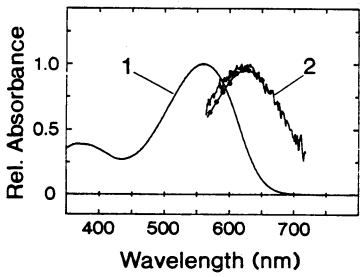

FIG. 6. Relative (Rel.) absorption spectra of iodopsin and bathoproduct at room temperature. Curve 1 shows the absorption spectrum of the iodopsin sample (same as that in Fig. 2*A*), and curve 2 shows the absorption spectrum of the bathoproduct that was calculated according to the method described in the text. Absorbance at the maximum of the iodopsin sample was normalized to 1. Absolute absorption spectrum of bathoiodopsin was drawn by a solid line with solid dots. Each dot was calculated by subtraction of the contribution of absorbance of the photoproduct from chicken rhodopsin and the green-sensitive pigment from the measured absorbance of the bathoproduct (curve 2) at each wavelength (see text). Its λ_{max} is at 625 nm.

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