Chromophore Configuration of Iodopsin and Its Photoproducts Formed at Low Temperatures†

Yasushi Imamoto,‡,§ Tôru Yoshizawa,‖ and Yoshinori Shichida*,‡

Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606-01, Japan, and Department of Information Systems Engineering, Faculty of Engineering, Osaka Sangyo University, Osaka 574, Japan

Received June 21, 1996; Revised Manuscript Received September 9, 1996⊗

ABSTRACT: The photochemical reactions of iodopsin at low temperatures were investigated by a combination of absorption spectroscopy and chromophore extraction to show the formation of isomeric photoproducts other than the all-trans intermediates. We first confirmed that the chromophore in iodopsin is an 11-*cis*-retinal. Next, iodopsin samples were irradiated with light of different wavelengths at selected temperatures ranging from −190 to 0 °C, and their retinylidene chromophores were extracted as oximes after warming the sample to 0 °C. The isomeric composition of the extracted chromophores was analyzed by high-performance liquid chromatography. It was confirmed that bathoiodopsin produced at −190 °C has an all-trans chromophore, but a considerable amount of its chromophore thermally reisomerizes to the 11-cis form upon warming. Photoproducts formerly assigned as lumi- and metaiodopsins [Yoshizawa, T., & Wald, G. (1967) *Nature 214*, 566−571; Hubbard, R., & Kropf, A. (1959) *Nature 183*, 448−450] were produced by extensive irradiation of iodopsin at −80 and −40 °C, respectively, with red light, but their chromophores were identified to be 7-*cis*-retinals instead of *all-trans*-retinals. Thus these photoproducts are artificial byproducts formed as a result of photon absorption by all-trans intermediates. The absorption spectrum of the 7-cis product formed from bovine rhodopsin shows no spectral shift when it is warmed from −80 to 0 °C, but the spectrum of 7-cis species formed from iodopsin shifted about 40 nm to the blue at a transition temperature of −60 °C. This result indicates a unique chromophore−opsin interaction in iodopsin. Four all-trans intermediates of iodopsin were identified above −80 °C under irradiation conditions in which no 7-cis products accumulated. Their absorption maxima were estimated to be ∼570, ∼530, ∼470, and ∼380 nm. These species should correspond to BL-iodopsin, lumiiodopsin, metaiodopsin I, and metaiodopsin II, respectively, assigned by room temperature laser photolysis [Shichida, Y., Okada, T., Kandori, H., Fukada, Y., & Yoshizawa, T. (1993) *Biochemistry 32*, 10832−10838].

Color vision is mediated by several types of cone photoreceptor cells. Each type of cone cell has a visual pigment with a characteristic absorption maximum. The chicken has four classes of cone pigments, maximally absorbing either orange (571 nm), green (508 nm), blue (455 nm), or violet (415 nm) light (Yen & Fager, 1984; Okano et al., 1989). The pigment absorbing at 571 nm is called iodopsin and is the most characterized cone visual pigment (Wald et al., 1955; Yoshizawa & Imamoto, 1995).

Room temperature laser photolysis revealed that, like the rod visual pigment rhodopsin, iodopsin, after it absorbs a photon, bleaches through several thermolabile intermediates (Kandori et al., 1990; Shichida et al., 1993) which are analogous to those observed in the bleaching of rhodopsin. The reaction of iodopsin additionally has a unique intermediate called BL-iodopsin between batho- and lumiiodopsins (Shichida et al., 1993). The BL-iodopsin might correspond to the BSI(BL) intermediate of rhodopsin (Shichida et al., 1981; Albeck et al., 1989; Randall et al., 1991; Hug et al., 1990; Okada et al., 1991), but the absorption characteristics of the two species are quite different, suggesting that the change in chromophore−opsin interaction during the bleaching process of iodopsin does not strictly parallel that of rhodopsin. However, the limited amount of iodopsin sample available for spectroscopic measurements hampered the detailed analysis of the bleaching process of iodopsin at room temperature.

Low-temperature UV−visible spectroscopy is a useful technique for measuring the precise spectrum of a limited amount of photolabile visual pigment (Yoshizawa & Shichida, 1982; Imai et al., 1994). Vibrational spectroscopy with the aid of low-temperature techniques has also revealed detailed structure of chromophore and/or protein moieties in photointermediates (Mathies et al., 1987; Kitagawa & Maeda, 1990). In fact, the chromophore structure of bathoiodopsin and the red-shift mechanism in iodopsin have been investigated by means of low-temperature resonance Raman spectroscopy (Lin et al., 1994). However, bathoiodopsin produced at liquid nitrogen temperature mainly converts back to the original iodopsin when it is warmed above liquid nitrogen temperature (Yoshizawa & Wald, 1967; Imamoto et al., 1989). This property makes it very difficult to apply low-temperature spectroscopy to study changes in chromophore−protein interactions that eventually cause the

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

* Correspondence should be addressed to this author.

‡ Kyoto University.

§ Present address: Department of Earth and Space Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560, Japan.

‖ Osaka Sangyo University.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

S0006-2960(96)01485-7 CCC: $12.00

formation of the physiologically active intermediate which binds to and activates transducin (Okada et al., 1994).

In an attempt to identify intermediates subsequent to bathoiodopsin at low temperature, Yoshizawa and Wald (1967) extensively irradiated iodopsin with >595-nm light at −80 °C and clearly observed the formation of a photoproduct displaying an absorption maximum at 515 nm. They concluded that this product is lumiiodopsin and that extensive irradiation is indispensable for its accumulation because a considerable amount of bathoiodopsin reverts to the original iodopsin at this temperature. These results supported earlier observations that the photosensitivity of iodopsin was greatly reduced as the temperature of the sample was lowered (Hubbard & Kropf, 1959). However, the absorption maximum of lumiiodopsin trapped at −80 °C was considerably blue-shifted from those of BL-iodopsin and lumiiodopsin (571 and 535 nm, respectively) detected at room temperature (Shichida et al., 1993), and this shift is inconsistent with a general property of retinal proteins that their absorption maxima shift to longer wavelengths as the temperature is lowered.

Assuming that the species trapped at −80 °C is different from lumiiodopsin at room temperature, the most plausible explanation would be a different chromophore configuration between these products. Because the photobleaching intermediates are stably trapped at low temperatures, they can absorb successive photons and their chromophores may possibly photoisomerize. Indeed, it is well-known that the all-trans chromophore of bathorhodopsin isomerizes to the 11-cis and 9-cis forms (rhodopsin and isorhodopsin) at liquid nitrogen temperature (Yoshizawa & Wald, 1963) and that the chromophore of lumirhodopsin isomerizes to the 7-cis and 13-cis forms in addition to the 11-cis and 9-cis forms at −80 °C (Maeda et al., 1978a, 1979). Therefore, we determined the chromophore configuration of the photoproducts of iodopsin formed at temperatures ranging from −190 to 0 °C by means of chromophore extraction followed by HPLC[1] analysis. The present findings clearly show that the photoproduct produced by extensive irradiation of iodopsin with red light at −80 or −40 °C has a 7-cis chromophore, whereas native intermediates containing all-trans chromophores are identified under irradiation conditions in which no 7-cis products accumulate. Furthermore, the 7-cis product formed at −80 °C displays a spectral shift upon warming unlike that of rhodopsin. On the basis of the present experimental results, the difference in chromophore−opsin interaction between rhodopsin and iodopsin is discussed.

MATERIALS AND METHODS

Preparation of 11-cis-Retinal and Retinal Oxime Standard Mixture. The mixture of retinal isomers was prepared by irradiating *all-trans*-retinal dissolved in acetonitrile overnight with a fluorescent lamp. 11-*cis*-Retinal was isolated from this mixture by HPLC (Maeda et al., 1978b) and used for the regeneration of pigments. The other retinal isomers (mono-cis and all-trans forms) were also isolated. Comparable amounts of the retinal isomers were mixed together in methanol, followed by the addition of 1/10 volume of an aqueous solution of 1 M hydroxylamine (pH 7.0). After the solvent was evaporated, the retinal oximes were extracted with hexane and used as the retinal oxime standard mixture.

Preparation of Iodopsin. Iodopsin was isolated from fresh chicken retinas as previously reported (Okano et al., 1989). The rod and cone outer segments were isolated by means of 40% (w/v) sucrose flotation in buffer A [50 mM HEPES, 140 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 4 μg/mL leupeptin, 50 kallikrein inhibitor units/mL aprotinin, pH 6.6]. They were washed eight times with buffer A by centrifugation and resuspended in buffer B (buffer A supplemented with 0.75% CHAPS, 1.0 mg/mL PC, 1 mM $MnCl_2$, and 1 mM $CaCl_2$). After the bleached pigments were regenerated by addition of 11-*cis*-retinal, the suspension was centrifuged (60000*g* × 60 min) to obtain a clear supernatant containing visual pigments. The extract was diluted 1.25 times with buffer A to adjust the concentration of CHAPS and PC to 0.6% and 0.8 mg/mL, respectively, and applied to a concanavalin A Sepharose (Pharmacia) column (16 mm × 270 mm) which had been equilibrated with buffer C (buffer A supplemented with 0.6% CHAPS, 0.8 mg/mL PC, 1 mM $MnCl_2$, and 1 mM $CaCl_2$). Iodopsin was eluted from the column with buffer C supplemented with 1.0−1.5 mM methyl α-mannoside. The eluate was supplemented with $^1/_5$ to $^1/_7$ volume of glycerol to stabilize iodopsin.

The iodopsin sample was concentrated 5 times by ultrafiltration membrane (Amicon, YM30) and dialyzed against buffer D [buffer C containing 20% (w/v) glycerol] to remove methyl α-mannoside. Then it was applied to a second concanavalin A column (16 mm × 50 mm). The column was washed with buffer D supplemented with 10 mM hydroxylamine to remove free retinal. Then it was washed with buffer D, followed by elution of iodopsin with buffer D supplemented with 100 mM methyl α-mannoside. An equivalent volume of glycerol was added to the eluate [final concentration, 57% (v/v)]. The sample composition was determined by partial-bleaching method (Okano et al., 1989) to be as follows: 90% iodopsin, 6% chicken blue, 2% chicken violet, and 2% mixture of rhodopsin and chicken green. In this calculation, we used reported values for the extinction coefficients of chicken red (47 200, Okano et al., 1992) and green (40 800, Shichida et al., 1993) and rhodopsin (40 700, Okano et al., 1992) and assumed that those of chicken blue and violet are identical to that of rhodopsin.

Low-Temperature Spectrophotometry. Absorption spectra were recorded on a Shimadzu MPS-2000 recording spectrophotometer equipped with a glass optical cryostat containing a specially designed optical cell (path length, 5 mm) (Yoshizawa & Shichida, 1982; Imamoto et al., 1989). To compensate for light scattering by cracks in the sample formed at liquid nitrogen temperature, opal glasses were placed in front of the photomultiplier. A 1-kW tungsten−halogen lamp (Master HILUX, Rikagaku-Seiki) was used as the light source for irradiation of the sample. The irradiation wavelength was selected with a glass cutoff filter (R-68, R-67, R-65, or R-61, Toshiba) or an interference filter (547 or 501 nm, Nihonshinku, or KL62, Toshiba).

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PC, L-α-phosphatidylcholine from fresh egg yolk; HPLC, high-performance liquid chromatography; 11s, 11-*cis*-15-*syn*-retinal oxime; 11a, 11-*cis*-15-*anti*-retinal oxime; 9s, 9-*cis*-15-*syn*-retinal oxime; 9a, 9-*cis*-15-*anti*-retinal oxime; 7s, 7-*cis*-15-*syn*-retinal oxime; 7a, 7-*cis*-15-*anti*-retinal oxime; Ts, *all-trans*-15-*syn*-retinal oxime; Ta, *all-trans*-15-*anti*-retinal oxime; 15E, (15*E*)-retinal oxime; 15Z, (15*Z*)-retinal oxime; ppR, *pharaonis* phoborhodopsin.

Chromophore Extraction and HPLC Analysis. The retinylidene chromophore was extracted from the sample as an oxime according to the method previously described (Shichida et al., 1988; Imamoto et al., 1992). Briefly, the sample containing iodopsin and/or photoproduct(s) was collected from the optical cell after low-temperature spectroscopy and placed on ice. Its volume was adjusted to 0.9 mL with buffer D. The solution was supplemented with 1 M hydroxylamine solution at pH 7 (0.1 mL), methanol (1 mL), and dichloromethane (1 mL) and mixed vigorously with an ULTRA-TURRAX homogenizer (Janke & Kunkel) for 30 s on ice to denature photopsin (protein moiety of iodopsin). Hexane (5 mL) was added to the sample, followed by vigorous mixing for 30 s to transfer the retinal oximes to the hexane layer. After centrifugation, the hexane layer was collected, and the hexane extraction was repeated. The collected hexane layer was dried over anhydrous Na_2SO_4 and evaporated under a N_2 stream. The residue was dissolved in 50 μL of hexane. The extraction yield of the chromophore was more than 95%.

The configuration of the extracted retinal oxime was analyzed by HPLC (LC-7A system, Shimadzu) equipped with a silica column (6 × 150 mm, A0123, YMC) (Imamoto et al., 1992). The solvent was composed of 98.8% (v/v) benzene, 1.0% (v/v) diethyl ether, and 0.2% (v/v) 2-propanol, and the flow rate was 1.0 mL/min. The HPLC patterns were obtained by monitoring the absorbance at 360 nm. Each peak in the HPLC pattern was assigned by comparison of its retention time with those of the retinal oxime standards. The isomeric composition of retinal oximes was calculated from the area of the peaks and the molar extinction coefficients at 360 nm (Trehan et al., 1990): 11s, 35 000; 11a, 29 600; 9s, 39 300; 9a, 30 600; 7s, 47 000; 7a, 46 200; Ts, 54 900; Ta, 51 600. Throughout the present experiments, the amount of 13-*cis*-retinal oxime (13s and 13a) was negligible (less than 2%).

RESULTS

Chromophore Configuration of Iodopsin and Its Bleaching Products at 0 °C. To confirm the generally accepted idea that iodopsin has 11-*cis*-retinal as its chromophore and that light isomerizes the chromophore into the all-trans form, we first extracted the retinal chromophores of iodopsin and its bleaching products at 0 °C and identified their configuration. In these experiments, we also examined the ratio of 15-syn and 15-anti forms of the extracted retinal oxime which might depend on the extraction conditions (Shichida et al., 1988; Imamoto et al., 1992).

Three aliquots of iodopsin/57%-glycerol sample were subjected to the following experiments. Iodopsin in the first aliquot was denatured by adding equal volumes of methanol and dichloromethane at 0 °C in the dark. Hydroxylamine (1 M neutralized solution) was also simultaneously added to the sample at a final concentration of 100 mM. Iodopsin in the second aliquot was first bleached by adding 100 mM hydroxylamine in the dark at 20 °C, followed by denaturing the photopsin by adding equal volumes of methanol and dichloromethane. In the former experiment, hydroxylamine attacks the chromophore of denatured iodopsin, while in the latter, it attacks the chromophore in intact iodopsin. The third aliquot was irradiated with >660-nm light in the presence of 50 mM hydroxylamine at 0 °C (Figure 1a), and then photopsin was denatured by methanol and dichloromethane. Under this condition, the chromophore of photobleaching product(s) is expected to immediately react with hydroxylamine before denaturation of photopsin.

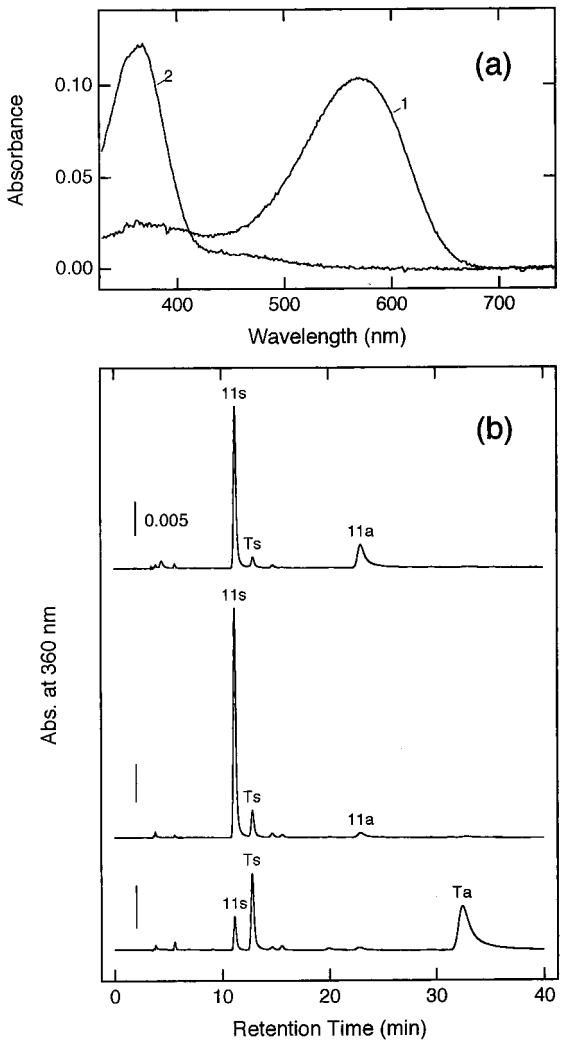

FIGURE 1: Chromophore configuration of iodopsin and its bleaching products. (a) An iodopsin/57%-glycerol sample was supplemented with 50 mM hydroxylamine (curve 1) and irradiated with >660-nm light for 40 min at 0 °C (curve 2). Absorption spectra were recorded at 0 °C. (b) The HPLC patterns of retinal oximes extracted from the following samples after denaturation of the protein moiety of iodopsin. Upper trace: the sample shown by curve 1 in panel a. Middle trace: iodopsin/57%-glycerol sample which was bleached by incubation in the presence of 100 mM hydroxylamine in the dark for 8 h at 20 °C. Lower trace: the sample shown by curve 2 in panel a. These traces were normalized to make the total amounts of retinal oxime in the sample equal.

The retinal oximes extracted from the three aliquots were analyzed by HPLC (Figure 1b, Table 1). From the first (Figure 1b, top; Table 1, line 1) and second (Figure 1b, middle; Table 1, line 2) aliquots, 11-*cis*-retinal oxime was mainly extracted. A small amount of *all-trans*-retinal oxime was also detected, probably because some 11-*cis*-retinal oxime isomerized during the extraction manipulations and/or a small amount of *all-trans*-retinal was still present in the sample even after the pigment purification procedures. From the third aliquot (Figure 1b, bottom; Table 1, line 3), *all-trans*-retinal oxime was mainly extracted. The percentage of extracted 11-*cis*-retinal oxime in this aliquot was 15%, which is larger than the amount (10%) of pigments other than iodopsin estimated to be present in the sample by the

Table 1: Isomeric Composition of Retinal Oximes Extracted from the Samples

				11-cis (%)			9-cis (%)			7-cis (%)			all-trans (%)		
	temp (°C)	light (nm)	time (min)	s[a]	a	s + a	s	a	s + a	s	a	s + a	s	a	s + a
1	0	dark[b]		60	34	94	1	0	1	0	0	0	4	1	5
2	20	dark[c]		84	5	89	2	0	2	0	0	0	8	1	9
3	0	>660	40	12	3	15	2	0	2	0	0	0	22	61	83
4	−190	547	5	50	26	76	6	2	8	0	0	0	12	4	16
5	−190	>630	32	14	6	20	52	23	75	0	0	0	3	2	5
6	−80	>610	512	10	4	14	1	0	1	43	39	82	3	0	3
7	−40	620	120	12	5	17	5	0	5	28	14	42	23	13	36
8	−20	620	40	12	5	17	1	0	1	0	0	0	49	33	82
9	−80	501	10	53	28	81	2	0	2	0	0	0	14	3	17
10	−40	>650	16	38	18	56	3	1	4	0	0	0	32	8	40

[a] s, 15-syn form; a, 15-anti form, s + a, sum of s and a. [b] Bleached with methanol/dichloromethane. [c] Bleached with 100 mM hydroxylamine.

partial-bleaching method. The discrepancy might originate from errors in the assumed extinction coefficients of chicken blue and violet and/or the presence of free 11-*cis*-retinal in the sample. The retinal oximes extracted from the second and third aliquots were predominantly in 15-syn and 15-anti forms, respectively. The stereoselective formation of retinal oxime extracted under nondenaturation conditions of photopsin will be discussed in the Discussion.

Chromophore Configuration of Bathoiodopsin and Isoiodopsin. The above experiments confirmed that iodopsin has 11-*cis*-retinal as its chromophore and bleaches to *all-trans*-retinal and photopsin. We next examined the chromophore configuration of bathoiodopsin and isoiodopsin produced by irradiation of iodopsin at −190 °C (Figure 2).

The iodopsin/57%-glycerol sample was cooled to −190 °C (Figure 2b, curve 2) and irradiated with 547-nm light for 320 s, which induced a red shift of the absorption spectrum due to the formation of a photo-steady-state mixture containing mostly bathoiodopsin (Figure 2b, curve 3). As reported previously (Yoshizawa & Wald, 1967; Imamoto et al., 1989), about 7% of iodopsin bleach was observed after warming the mixture to 0 °C (Figure 2a, curve 4), indicating that most of the bathoiodopsin thermally reverted to iodopsin, while a small but detectable amount bleached (Yoshizawa & Wald, 1967; Imamoto et al., 1989). The HPLC pattern of the extracted chromophore (Figure 2c, upper) shows peaks corresponding to 11-*cis*-, *all-trans*-, and 9-*cis*-retinal oximes. In comparison with the isomeric forms extracted from the iodopsin sample (Table 1, line 1), about 10% of the all-trans form was detected (Table 1, line 4). Although some of the all-trans form extracted from the irradiated sample might originate from the bleaching products of visual pigments other than iodopsin present in the sample, these results also strongly suggest that the chromophore of bathoiodopsin is in an all-trans form. The 9-cis form might originate from isoiodopsin in the photo-steady-state mixture (see below).

The irradiation of iodopsin with red light at −190 °C causes the blue shift of the absorption spectrum, owing to the formation of a photo-steady-state mixture mainly containing isoiodopsin (Figure 2b, curve 5). The HPLC pattern of the chromophores extracted after warming of the mixture to 0 °C (Figure 2c, lower; Table 1, line 5) clearly shows that isoiodopsin has 9-*cis*-retinal as its chromophore.

Chromophore Configuration of Photoproducts Produced by Extensive Irradiation at −80 and −40 °C. It has been reported that lumi- and metaiodopsins could be produced at −80 and −40 °C by extensive irradiation of iodopsin (Yoshizawa & Wald, 1967; Hubbard & Kropf, 1957). However, the spectral properties of these intermediates are different from those observed at room temperature (Shichida et al., 1993). Therefore, the photoreactions observed in the previous low-temperature spectroscopy were first reproduced using the purified iodopsin sample, and chromophore configurations of these products were determined.

The iodopsin/57%-glycerol sample was cooled to −80 °C and irradiated with >610-nm light as in Yoshizawa & Wald (1967) (Figure 3). The spectrum of the sample shifted further to the blue as the irradiation time increased, and a product with an absorption maximum at 500 nm was finally produced (Figure 3b). To observe the thermal reaction of this product, the sample was warmed in a stepwise manner to 0 °C and then cooled back down to −80 °C, and its spectrum was recorded (Figure 3c). The data show that the 500-nm product converts to a 460-nm product at a transition temperature of about −60 °C. This 460-nm product did not bleach even at 0 °C (Figure 3a), and a considerable amount of 7-*cis*-retinal oxime was extracted from this warmed sample (Figure 3d; Table 1, line 6). Therefore, the photoproduct formed at −80 °C is not a normal bleaching intermediate of iodopsin, but a byproduct containing a 7-*cis*-retinal chromophore.

We also investigated the photochemical reaction of iodopsin at −40 °C using irradiation conditions similar to those described by Hubbard and Kropf (1957) (Figure 4). The irradiation resulted in a blue shift of the absorption spectrum (Figure 4b), and a photoproduct with an absorption maximum at 460 nm was finally formed. The intersection of the spectra shifted from 545 to 470 nm during the irradiations, indicating the presence of at least two conversion processes. The sample was then warmed in a stepwise manner to 0 °C to characterize the thermal reactions of the photoproducts (Figure 4c). The absorbance at about 470 nm decreased with concurrent increases at 380 and 580 nm. At 0 °C, about two-thirds of the absorbance at 470 nm still remained (Figure 4a). These results suggest the presence of two products, one that is thermally unstable and another that is stable. HPLC analysis of the extracted chromophores showed that the sample contained comparable amounts of all-trans and 7-cis isomers (Figure 4d; Table 1, line 7). The thermally stable component is assigned as the 7-cis product, and the observed spectral changes are probably due to thermal decay of an all-trans intermediate. Since it is reasonable to assume that the 380-nm product formed from the all-trans intermediate is metaiodopsin II, the all-trans intermediate should be metaiodopsin I. Then the absorbance increase at 590 nm would be due to the thermal recovery of iodopsin from

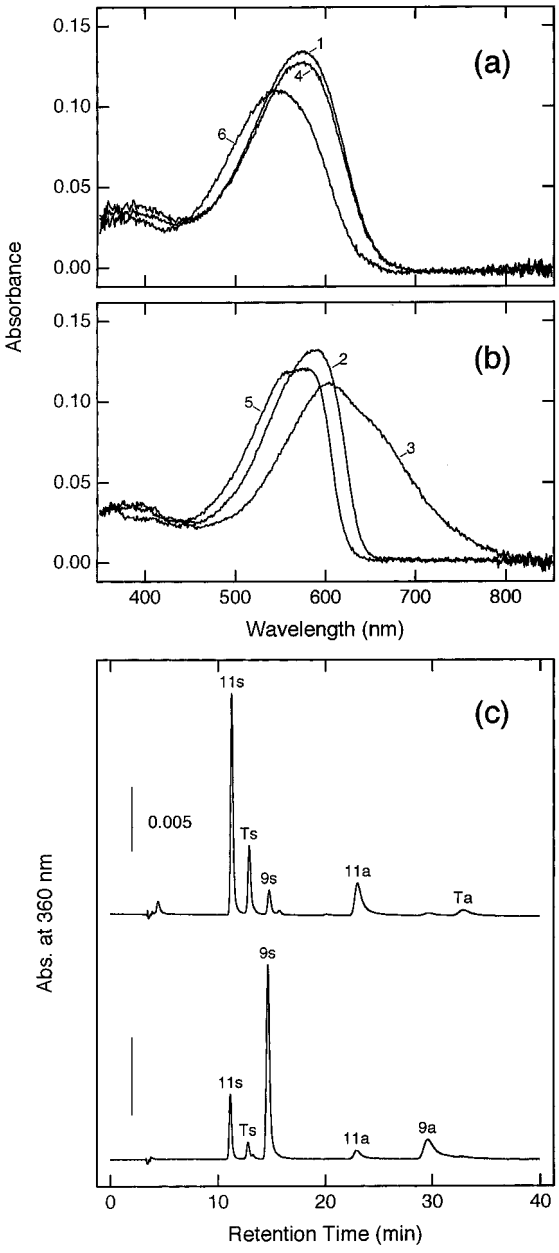

FIGURE 2: Chromophore configuration of bathoiodopsin and isoiodopsin. (a and b) An aliquot of a iodopsin/57%-glycerol sample (0 °C, curve 1 in panel a) was cooled to −190 °C (curve 2 in panel b) and irradiated with 547-nm light for 320 s to form bathoiodopsin (curve 3 in panel b). The sample was then warmed to 0 °C (curve 4 in panel a), and the chromophore was extracted. Another aliquot was cooled to −190 °C and irradiated with >630-nm light for 32 min to form isoiodopsin (curve 5 in panel b), followed by warming of the sample to 0 °C (curve 6 in panel a), and the chromophore was extracted. (c) The HPLC patterns of extracted retinal oxime from the thermal products of bathoiodopsin (upper) and isoiodopsin (lower). These traces were normalized to make the total amounts of retinal oxime in the sample equal.

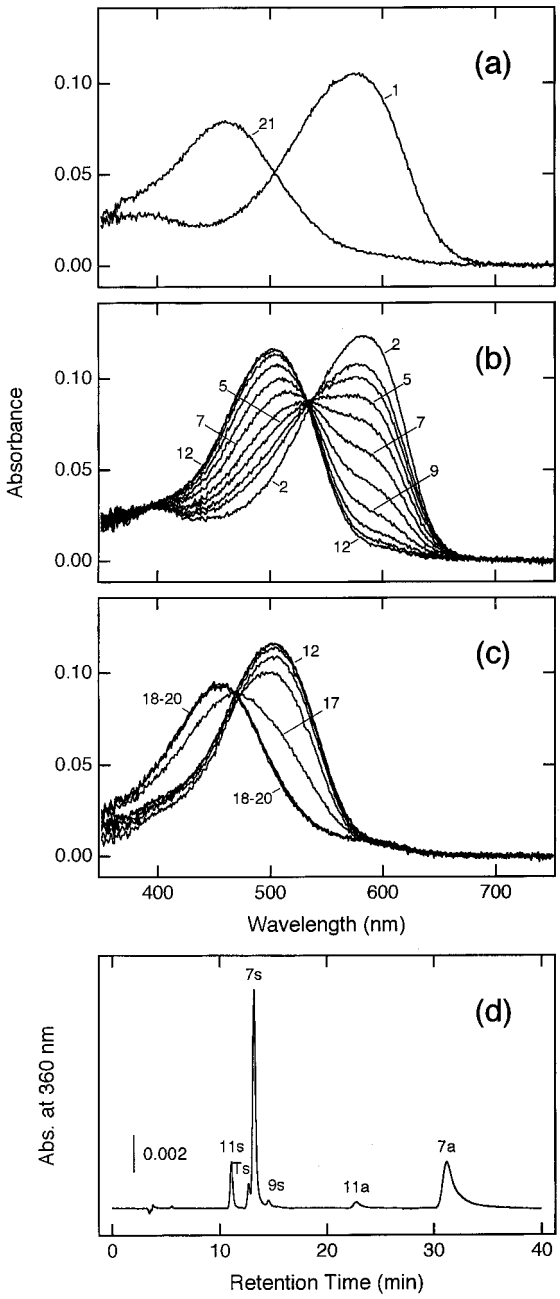

FIGURE 3: Chromophore configuration of the photoproduct produced by extensive irradiation of iodopsin at −80 °C. An iodopsin/57%-glycerol sample (curve 1 in panel a, 0 °C) was cooled to −80 °C (curve 2 in panel b) and irradiated with >610-nm light for a total of 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 min (curves 3−12 in panel b, respectively). Then the sample was warmed in a stepwise manner to −70, −60, −50, −40, −30, −20, −10, and 0 °C, and the spectra were recorded at −80 °C (curves 13−20 in panel c, respectively). The sample was then rewarmed to 0 °C (curve 21 in panel a), followed by chromophore extraction. The extracted retinal oxime was analyzed by HPLC (d).

metaiodopsin I (Imamoto et al., 1994; Tachibanaki et al., 1995).

We further investigated the photochemical reaction of iodopsin at −20 °C to test for the formation of the 7-cis product (Figure 5). The irradiation caused a rapid decrease in absorbance at 570 nm and increases at 470 and 380 nm (Figure 5b, curves 3−6). Further irradiation resulted in a gradual decrease of the absorbance at about 530 nm with a concurrent increase at 380 nm (Figure 5b, curves 6−11). The thermal reaction of the irradiated sample was also investigated. The decrease in absorbance at about 470 nm is accompanied by increases at 380 and 580 nm (Figure 5c). In addition, the difference spectrum between curves 11 and 13 in Figure 5c is almost identical to that between curves 14 and 18 in Figure 4c, indicating that the intermediate produced at −20 °C is the same as the thermally unstable intermediate produced at −40 °C. The chromophore extracted from this sample was mainly in the all-trans form (Figure 5d; Table 1, line 8). This indicates that the product is a mixture of only metaiodopsin I and II and that no

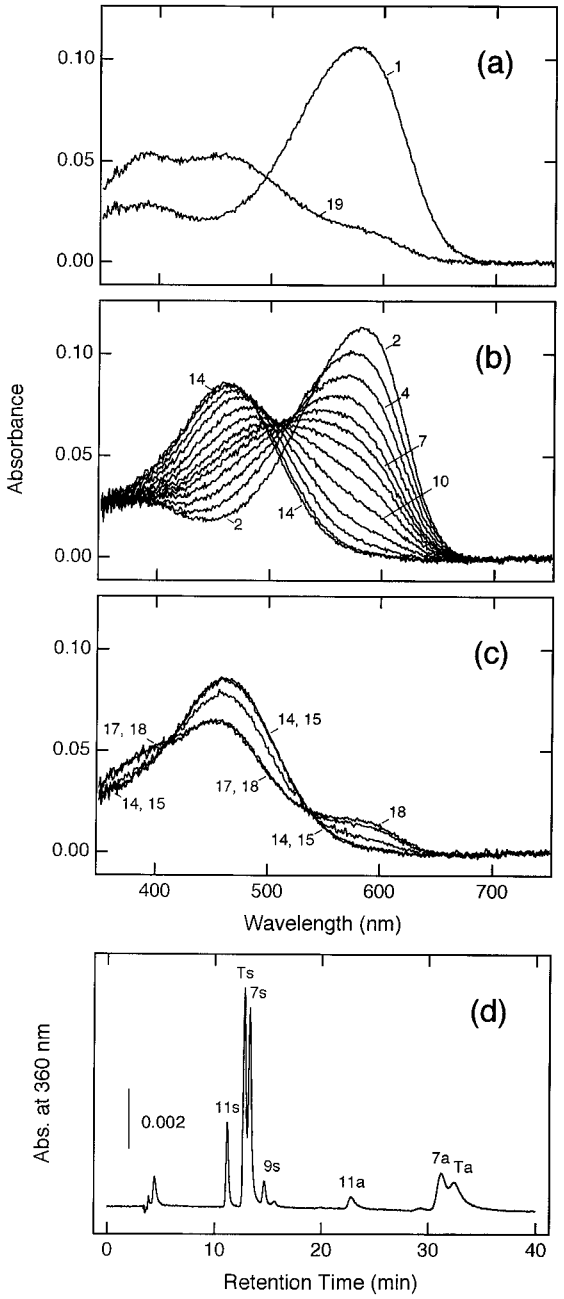

FIGURE 4: Chromophore configuration of the photoproduct produced by extensive irradiation of iodopsin at −40 °C. An iodopsin/57%-glycerol sample (0 °C, curve 1 in panel a) was cooled to −40 °C (curve 2 in panel b) and irradiated with 620-nm light for a total of 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120, and 7200 s (curves 3−14 in panel b, respectively). Then the sample was warmed in a stepwise manner to −30, −20, −10, and 0 °C, and the spectra were recorded at −40 °C (curves 15−18 in panel c, respectively). The sample was then rewarmed to 0 °C (curve 18 in panel a), followed by chromophore extraction. The extracted retinal oxime was analyzed by HPLC (d).

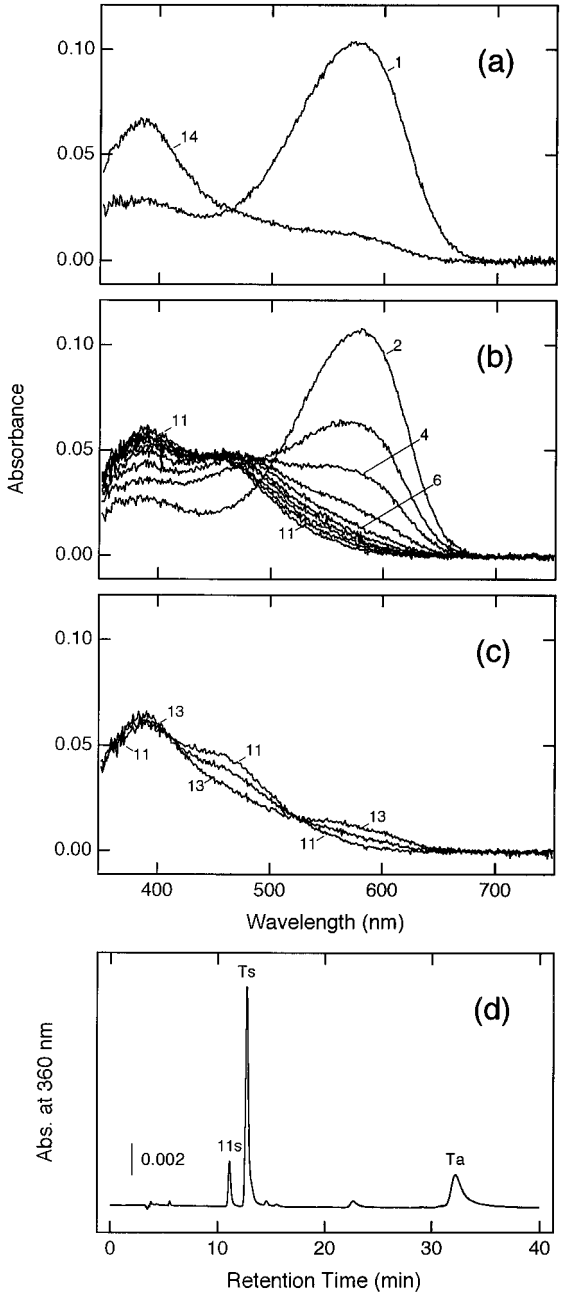

FIGURE 5: Chromophore configuration of the photoproduct produced by irradiation of iodopsin at −20 °C. An iodopsin/57%-glycerol sample (0 °C, curve 1 in panel a) was cooled to −20 °C (curve 2 in panel b) and irradiated with 620-nm light for a total of 5, 10, 20, 40, 80, 160, 320, 640, and 1280 s (curves 3−11 in panel b, respectively). Then the sample was warmed to −10 and 0 °C, and the spectra were measured at −20 °C (curves 12 and 13 in panel c, respectively). The sample was then warmed to 0 °C (curve 14 in panel a), followed by extraction of the chromophore. The extracted retinal oxime was analyzed by HPLC (d).

byproduct was produced from the irradiation of the iodopsin sample at −20 °C.

Identification of All-Trans Intermediates Formed above −80 °C. Since the irradiation of iodopsin with red light at −80 and −40 °C causes the formation of the 7-cis product, two irradiation conditions were tested to determine if all-trans products could be produced without an accumulation of the 7-cis product. We selected an irradiation wavelength which would be preferentially absorbed by the 7-cis product (Figure 6) and another wavelength that is absorbed only by iodopsin (Figure 7).

Since the 7-cis product formed at −80 °C has an absorption maximum at 500 nm, we expected that irradiation of iodopsin with 500-nm light would cause preferential conversion of the 7-cis product into an all-trans product. Consistent with this idea, no 7-cis product accumulated under 500-nm irradiation (Figure 6c; Table 1, line 9). The spectral red shift induced by this irradiation was small (Figure 6b, curve 3). The formation of metaiodopsin II is evidenced by the increase in absorbance at 380 nm (Figure 6a, curve 4)

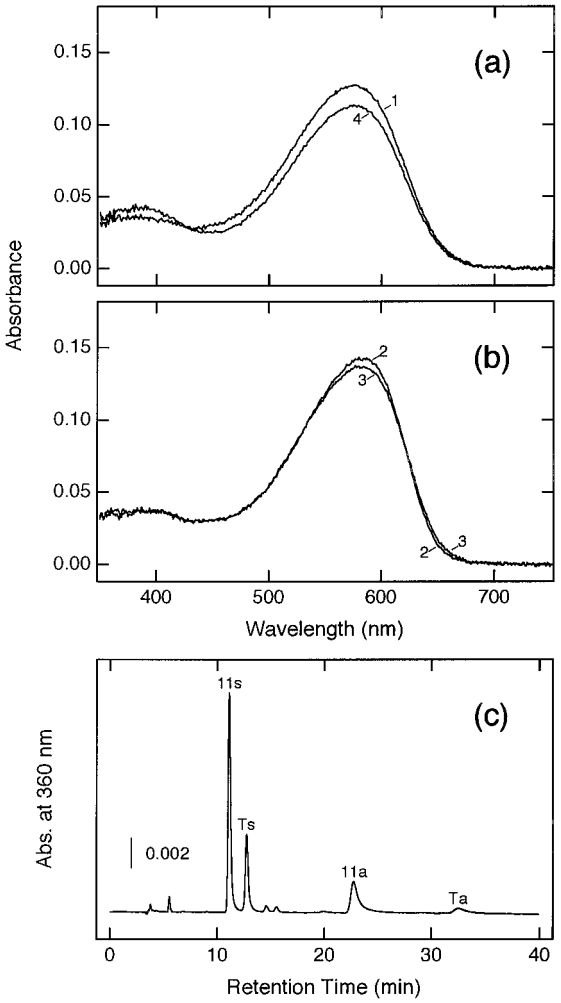

FIGURE 6: Formation of all-trans intermediate of iodopsin at −80 °C. An iodopsin/57%-glycerol sample (0 °C, curve 1 in panel a) was cooled to −80 °C (curve 2 in panel b) and irradiated with 501-nm light for 640 s (curves 3 in panel b). Then the sample was warmed to 0 °C (curve 4 in panel a), followed by extraction of the chromophore. The extracted retinal oxime was analyzed by HPLC (c).

and the extraction of more than 10% of the all-trans product (Figure 6c; Table 1, line 9). We conclude that all-trans product(s) is produced at this temperature. The product might have an absorption maximum slightly longer than that of iodopsin, because an increase in absorbance at longer wavelengths was observed after the irradiation (Figure 6b, curve 3).

Since the irradiation of iodopsin with red light at −40 °C induces a two-step conversion that results in the formation of a mixture of all-trans and 7-cis products (Figure 4), it is reasonable to speculate that the first conversion is due to the photochemical reaction of iodopsin to the all-trans product and the second is that of the all-trans product to the 7-cis product. Thus, in order to minimize the photochemical reaction of the all-trans product, we irradiated the iodopsin sample at −40 °C with a deep-red light (>650 nm) (Figure 7), assuming that the all-trans product(s) present at −40 °C has negligible absorbance at the longer wavelengths. The irradiation induced spectral changes similar to those observed at the early stage of irradiation with 620-nm light (Figure 4b), but no production of 7-cis product, if any, was observed (Figure 7d; Table 1, line 10). It should be noted that no isosbestic point was formed during the irradiation even

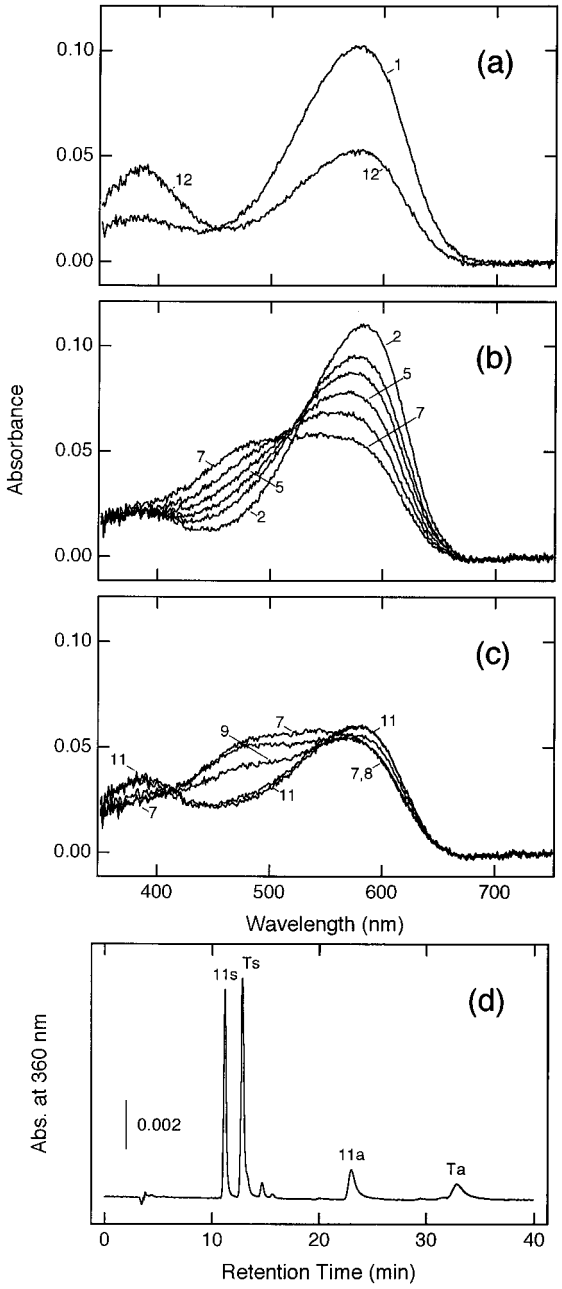

FIGURE 7: Formation of all-trans intermediates of iodopsin at −40 °C. An iodopsin/57%-glycerol sample (0 °C, curve 1 in panel a) was cooled to −40 °C (curve 2 in panel b) and irradiated with >650-nm light for a total of 5, 10, 20, 40, and 80 s (curves 3−7 in panel b, respectively). Then the sample was warmed in a stepwise manner to −30, −20, −10, and 0 °C, and the spectra were measured at −40 °C (curves 8−11 in panel c, respectively). The sample was then warmed to 0 °C (curve 12 in panel a), followed by extraction of the chromophore. The extracted retinal oxime was analyzed by HPLC (d).

though the accumulation of the 7-cis product could not be detected, suggesting that the all-trans product is a mixture of at least two intermediates.

DISCUSSION

In the present study, the all-trans intermediates of iodopsin formed at low temperatures were distinguished from 7-cis products by determination of their chromophore configurations. Because iodopsin has 11-*cis*-retinal as its chromophore and bleaches to *all-trans*-retinal and photopsin (Figure 1), the photoproduct with 7-*cis*-retinal should be a byproduct

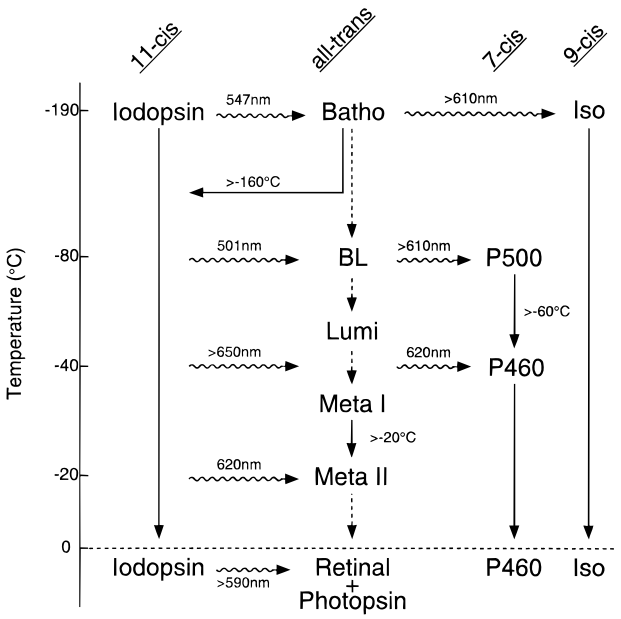

FIGURE 8: Proposed scheme for the photoreactions of iodopsin. Wavy and straight lines represent photochemical and thermal reactions, respectively. The broken lines represent thermal reactions whose transition temperatures were not determined. See text for details.

formed from an all-trans intermediate by photon absorption. We confirmed that at least four intermediates having all-trans chromophores are present at temperatures ranging from −80 to −20 °C. The photoreactions of iodopsin at low temperature are summarized in Figure 8.

Formation of All-Trans Intermediates. The irradiation of iodopsin with 501-nm light at −80 °C induced a small spectral red shift, suggesting that the intermediate produced at this temperature has an absorption maximum similar to that of iodopsin. This species might correspond to BL-iodopsin detected by flash photolysis at room temperature (Shichida et al., 1993). A small accumulation of the all-trans intermediate might originate from the apparent reduction of quantum yield of iodopsin due to the reversion of bathoiodopsin to the original iodopsin at this temperature (Yoshizawa & Wald, 1967). On the other hand, a large spectral blue shift was observed when iodopsin was irradiated with >650-nm light at −40 °C, although no 7-cis product was produced. The gradual blue shift of the absorption spectrum in the absence of an isosbestic point suggests the formation of at least two intermediates whose absorption maxima are shorter than that of iodopsin. Because the later intermediate formed at −40 °C shows a thermal reaction similar to the intermediate formed at −20 °C and it converts to the 380-nm intermediate (metaiodopsin II), this species should be assigned as metaiodopsin I. It follows that the earlier intermediate, which is produced and decays during irradiation at −40 °C, is lumiiodopsin. However, a small accumulation of BL-iodopsin and the thermal reversion of metaiodopsin I to the original iodopsin hampered efforts to estimate the precise absorption spectra of the intermediates. Preliminary calculations using methods previously reported (Imai et al., 1994; Tachibanaki et al., 1995) suggest that the absorption maximum is ∼570 nm for BL-iodopsin, ∼530 nm for lumiiodopsin, ∼470 nm for metaiodopsin I, and ∼380 nm for metaiodopsin II.

The 80-s irradiation of iodopsin at −40 °C with >650-nm light yielded a considerable amount of metaiodopsin I (more than 40%) even though some fraction of metaiodopsin I converted to the original iodopsin upon warming to 0 °C. The amount of metaiodopsin I produced by this irradiation is comparable to the amount of metarhodopsin I formed by the 80-s irradiation of rhodopsin with >580-nm light (data not shown). Since the absorption maximum of iodopsin is about 70-nm red-shifted from that of rhodopsin, these results suggest that bathoiodopsin produced at this temperature does not revert to the original iodopsin but preferentially converts to metaiodopsin I through BL-iodopsin and lumiiodopsin. Therefore, the critical temperature below which the reversion of bathoiodopsin occurs is located between −80 and −40 °C. Although the mechanism of the reverse reaction is not clear yet, it might be correlated with the presence of spectral change upon warming of the 7-cis product from −80 °C (see below).

Difference between the Thermal Behaviors of 7-Cis Products of Rhodopsin and Iodopsin. The identification of isomeric forms other than all-trans in some photoproducts gives some insight into structural changes of the protein moiety during the bleaching process. Maeda et al. (1978a, 1979) showed the formation of 7-cis chromophore in the binding site of lumirhodopsin, but not in that of bathorhodopsin. This suggests that a conformational change which allows the formation of a 7-cis chromophore occurs during the batho-to-lumi transition. The present results also show the temperature-dependent formation of a 7-cis chromophore in the iodopsin system. However, there is a critical difference between the thermal behaviors of the 7-cis product produced in rhodopsin and iodopsin at −80 °C. Namely, the 7-cis product formed from iodopsin at −80 °C displays a spectral shift from 500 nm to 460 nm upon warming to 0 °C, whereas the 7-cis species in bovine rhodopsin shows no spectral shift (Maeda et al., 1978a; Shichida et al., unpublished results). Since the thermal decay species of the 7-cis product of iodopsin formed at −80 °C has an absorption maximum (∼460 nm) similar to that of 7-*cis*-iodopsin which is reconstituted from photopsin and 7-*cis*-retinal (Fukada et al., 1990), we infer that the chromophore−opsin interaction in the 7-cis product relaxes to a stable configuration as the temperature is raised to 0 °C. On the other hand, the 7-cis product formed from rhodopsin at −80 °C has an absorption maximum similar to that of 7-*cis*-rhodopsin (450 nm, DeGrip et al., 1976; Maeda et al., 1978a), suggesting that a relaxed 7-*cis*-rhodopsin has already formed in the binding site of lumirhodopsin. These results clearly show that the chromophore binding site of the iodopsin intermediate produced at −80 °C, which is most likely BL-iodopsin, is different in shape from that of lumirhodopsin, although the binding site for the 11-cis chromophore in the ground-state iodopsin is similar to that of rhodopsin (Fukada et al., 1990). Since the irradiation of iodopsin at −40 °C causes the formation of lumiiodopsin as well as metaiodopsin I, and the transition temperature of the 7-cis product formed at −80 °C to its relaxed thermal product is about −60 °C, the chromophore binding site of lumiiodopsin might be able to accommodate a relaxed 7-cis chromophore like the binding site of lumirhodopsin. Thus, iodopsin has a unique intermediate, BL-iodopsin, in its bleaching process. Although the mechanism of the formation of this unique protein state in iodopsin is not yet clear, it is interesting to speculate that the binding of chloride (Knowles, 1976; Fager & Fager, 1979; Shichida et al., 1990; Tachibanaki et al., 1995) might influence the shape

of chromophore binding site. This possibility is now being investigated.

Stereoselective Formation of Retinal Oximes. When retinal oxime was formed by the addition of hydroxylamine after denaturation of iodopsin by methanol and dichloromethane, the ratio of 11s (15E) and 11a (15Z) is about 2:1 (Table 1, line 1). On the other hand, most of the retinal oxime extracted from iodopsin that was *dark*-bleached or irradiated in the presence of hydroxylamine under nondenaturation conditions is in the 11s (15E) or Ta (15Z) form (Table 1, lines 2 and 3). These results suggest that access to the chromophore binding site in iodopsin, as well as its bleaching intermediate(s), is restricted. As a result, the chromophore is partially protected by the protein moiety which effectively directs the approach of hydroxylamine to its imine group. The stereoselective formation of retinal oxime was also reported in partially denatured bacteriorhodopsin (Oesterhelt et al., 1973) and *pharaonis* phoborhodopsin (ppR) (Imamoto et al., 1992). In ppR, the retinal oxime extracted in the dark is mainly Ts (15E), whereas on irradiation under nondenaturation conditions the stereoisomer is 13a (15Z). Although the isomeric forms of retinylidene species are different between iodopsin and ppR, the stereoselective formation of retinal oxime is a common feature of both pigments.

It is reasonable to assume that the chromophores in iodopsin and ppR have a C═N anti (trans, 15E) configuration of protonated Schiff base, because the chromophores in other related retinal proteins (rhodopsin, bacteriorhodopsin, halorhodopsin, and sensory rhodopsin) have this configuration. Therefore, the stereoselective formation of retinal oxime between these pigments exhibits similarity in that the hydroxyl group is directed opposite to the proton on the Schiff base (15E). This suggests that a Schiff base proton and/or counterion may interfere with the displacement of a hydroxyl group at this position. On the other hand, retinal oximes extracted from the photobleaching intermediates in iodopsin and ppR have the hydroxyl group in the 15Z configuration. The ppR intermediate which interacts with hydroxylamine is the M intermediate (Imamoto et al., 1992), and the corresponding intermediate of iodopsin is probably metaiodopsin II. The retinylidene Schiff base in each intermediate is deprotonated and oriented in the opposite direction from that in the original species as a result of isomerization. From these considerations, two explanations would be possible. One is that the retinal oxime in the 15-anti (15Z) configuration is preferentially formed if the interaction between the Schiff base and its counterion is absent. The other is that the hydroxyl group is positioned on the opposite side of the counterion due to the steric interactions between them. The latter explanation is likely because the present experiments suggest that the stereoselective formation is not an intrinsic property of the Schiff base but is induced by the native chromophore binding site.

ACKNOWLEDGMENT

We thank Dr. H. Imai for very valuable discussions during the course of preparation of the manuscript. We also thank Dr. Steven Lin at Rockefeller University for his critical reading of the manuscript.

REFERENCES

Albeck, A., Friedman, N., Ottolenghi, M., Sheves, M., Einterz, C. M., Hug, S. J., Lewis, J. W., & Kliger, D. S. (1989) *Biophys. J. 55*, 233−241.
DeGrip, W. J., Liu, R. S. H., Ramamurthy, V., & Asato, A. E. (1976) *Nature (London) 262*, 416−418.
Fager, L. Y., & Fager, R. S. (1979) *Exp. Eye Res. 29*, 401−408.
Fukada, Y., Okano, T., Shichida, Y., Yoshizawa, T., Trehan, A., Mead, D., Denny, M., Asato, A. E., & Liu, R. S. H. (1990) *Biochemistry 29*, 3133−3140.
Hubbard, R., & Kropf, A. (1959) *Nature 183*, 448−450.
Hug, S. J., Lewis, J. W., Einterz, C. M., Thorgeirsson, T. E., & Kliger, D. S. (1990) *Biochemistry 29*, 1475−1485.
Imai, H., Mizukami, T., Imamoto, Y., & Shichida, Y. (1994) *Biochemistry 33*, 14351−14358.
Imamoto, Y., Kandori, H., Okano, T., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1989) *Biochemistry 28, 9412*−9416.
Imamoto, Y., Shichida, Y., Hirayama, J., Tomioka, H., Kamo, N., & Yoshizawa, T. (1992) *Biochemistry 31*, 2523−2528.
Imamoto, Y., Imai, H., Yoshizawa, T., & Shichida, Y. (1994) *FEBS Lett. 354*, 165−168.
Kandori, H., Mizukami, T., Okada, T., Imamoto, Y., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8908−8912.
Kitagawa, T., & Maeda, A. (1989) *Photochem. Photobiol. 50*, 883−894.
Knowles, A. (1976) *Biochem. Biophys. Res. Commun. 73*, 56−62.
Lin, S. W., Imamoto, Y., Fukada, Y., Shichida, Y., Yoshizawa, T., & Mathies, R. A. (1994) *Biochemistry 33*, 2151−2160.
Maeda, A., Ogurusu, T., Shichida, Y., Tokunaga, F., & Yoshizawa, T. (1978a) *FEBS Lett. 92*, 77−80.
Maeda, A., Shichida, Y., & Yoshizawa, T. (1978b) *J. Biochem. 83*, 661−663.
Maeda, A., Shichida, Y., & Yoshizawa, T. (1979) *Biochemistry 18*, 1449−1453.
Mathies, R. A., Smith, S. O., & Palings, I. (1987) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 2, pp 59−108, John Wiley and Sons, Inc., New York.
Oesterhelt, D., Meentzen, M., & Schuhmann, L. (1973) *Eur. J. Biochem. 40*, 453−463.
Okada, T., Kandori, H., Shichida, Y., Yoshizawa, T., Denny, M., Zhang, B. W., Asato, A. E., & Liu, R. S. H. (1991) *Biochemistry 30*, 4796−4802.
Okada, T., Matsuda, T., Kandori, H., Fukada, Y., Yoshizawa, T., & Shichida, Y. (1994) *Biochemistry 33*, 4940−4946.
Okano, T., Fukada, Y., Artamonov, I. D., & Yoshizawa, T. (1989) *Biochemistry 28*, 8848−8856.
Okano, T., Fukada, Y., Shichida, Y., & Yoshizawa, T. (1992) *Photochem. Photobiol. 56*, 995−1001.
Randall, C. E., Lewis, J. W., Hug, S. J., Bjorling, S. C., Eisner-Shanas, I., Friedman, N., Ottolenghi, M., Sheves, M., & Kliger, D. S. (1991) *J. Am. Chem. Soc. 113*, 3473−3485.
Shichida, Y., Kropf, A., & Yoshizawa, T. (1981) *Biochemistry 20*, 1962−1968.
Shichida, Y., Nakamura, K., Yoshizawa, T., Trehan, A., Denny, M., & Liu, R. S. H. (1988) *Biochemistry 27*, 6495−6499.
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.
Tachibanaki, S., Imamoto, Y., Imai, H., & Shichida, Y. (1995) *Biochemistry 34*, 13170−13175.
Trehan, A., Liu, R. S. H., Shichida, Y., Imamoto, Y., Nakamura, K., & Yoshizawa, T. (1990) *Bioorg. Chem. 18*, 30−40.
Wald, G., Brown, P. K., & Smith, P. H. (1955) *J. Gen. Physiol. 38*, 623−681.
Yen, L., & Fager, R. S. (1994) *Vision Res. 24*, 1555−1562.
Yoshizawa, T., & Wald, G. (1963) *Nature 197*, 1279−1286.
Yoshizawa, T., & Wald, G. (1967) *Nature 214*, 566−571.
Yoshizawa, T., & Shichida, Y. (1982) *Methods Enzymol. 81*, 333−354.
Yoshizawa, T., & Imamoto, Y. (1995) *Biophys. Chem. 56*, 57−62.

BI9614850