Structure around C_6-C_7 Bond of the Chromophore in Bathorhodopsin: Low-Temperature Spectroscopy of 6s-*cis*-Locked Bicyclic Rhodopsin Analogs†

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ABSTRACT: To elucidate the structural changes near the β-ionone ring region of the chromophore during the photobleaching process of rhodopsin, the photochemical and subsequent thermal reactions of rhodopsin analogs, whose retinylidene chromophores were fixed in a 6s-cis form with a five-membered ring (6,5-rhodopsin) and a seven-membered ring (6,7-rhodopsin), respectively, were investigated by low-temperature spectroscopy. Like rhodopsin, both the rhodopsin analogs convert to the respective batho-intermediates upon absorption of light at −190 °C. The extinction coefficient of batho-intermediate of 6,5-rhodopsin is similar to that of bathorhodopsin, while that of 6,7-rhodopsin is considerably smaller than that of bathorhodopsin. Like bathorhodopsin, the batho-intermediate of 6,5-rhodopsin directly converts to lumi-intermediate, while that of 6,7-rhodopsin first converts to a blue-shifted intermediate and then to lumi-intermediate. These results strongly suggest that the structure around the β-ionone ring region of the bathorhodopsin chromophore resembles 6,5-retinal rather than 6,7-retinal. From the comparison of the structural features among retinal, 6,5-retinal, and 6,7-retinal, a possible conformation around C_6-C_7 bond of the bathorhodopsin chromophore is discussed.

The visual transduction process in rod photoreceptor cells is initiated by photon absorption by the visual pigment, rhodopsin. Rhodopsin contains an 11-*cis*-retinylidene chromophore bound to a specific lysine residue of the apoprotein opsin through a protonated Schiff base linkage. Light isomerizes the rhodopsin chromophore into highly twisted all-trans form in photorhodopsin (Shichida et al., 1984). Subsequent changes in chromophore/opsin interaction and/or conformational changes of the opsin moiety result in formation of a physiologically active intermediate, metarhodopsin II, which activates the enzymatic cascade system in the photoreceptor cells (Stryer et al., 1991). Accumulated evidences have now suggested that about 60% of the light energy absorbed by rhodopsin is stored in bathorhodopsin, the following intermediate of photorhodopsin (Cooper, 1979; Schick et al., 1987), due to the conformational distortions of the chromophore and/or charge separation between the positively charged Schiff base and its counterion (Yoshizawa & Wald, 1963; Honig et al., 1979; Birge et al., 1988). Thus it is the matter of interest to investigate how the light energy is stored in the bathorhodopsin state and how the energy stored in the chromophore transfers to the protein moiety.

There have been extensive studies to make clear the conformation of the bathorhodopsin chromophore using various spectroscopic techniques with the aids of synthetic retinal analogs and isomers [see Liu and Shichida (1991) and Yoshizawa and Kandori (1994) for recent reviews]. Since the rhodopsin chromophore is photoisomerized by change of the polyene part near the Schiff base (Shichida et al., 1987) and the red-shift in absorption spectrum from rhodopsin to bathorhodopsin is mainly due to the change in electrostatic interaction near the Schiff base (Lin et al., 1994), most investigations have been focused on the conformational changes near the Schiff base. However, analog studies as well as spectroscopic studies suggested that the interaction between β-ionone ring region of the chromophore and surrounding protein is somewhat different between rhodopsin and bathorhodopsin, because modification of the β-ionone ring of the chromophore alters the formation process of bathorhodopsin as well as the conversion process from bathorhodopsin to lumirhodopsin (Albeck et al., 1989; Ganter et al., 1989; Okada et al., 1991). Thus it is important to elucidate the structure near the β-ionone ring region of the chromophore in bathorhodopsin, which might be different from that of rhodopsin. From these consideration, we have synthesized two retinal analogs having fixed 6s-cis conformation by means of five-membered and seven-membered rings (Figure 1) and reported their spectroscopic properties (Ito et al., 1992; Wada et al., 1993).

6,7-Retinal has a $C_5{=}C_6-C_7{=}C_8$ structure close to that of the natural retinal, while that of 6,5-retinal is highly constrained to be completely planer due to the fixed five-membered ring. Thus absorption and CD[1] spectra of 6,7-rhodopsin are almost identical with those of rhodopsin, while 6,5-rhodopsin displays the spectra considerably different from

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

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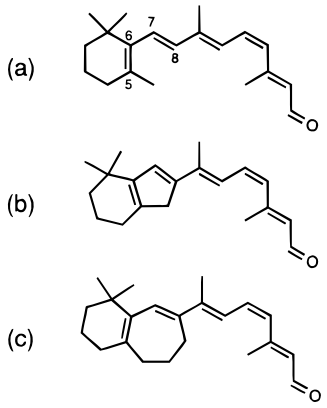

FIGURE 1: 11-*cis*-Retinal and its analogs used in the present experiments. (a) 11-*cis*-Retinal. (b) 6,5-Retinal. (c) 6,7-Retinal.

those of rhodopsin (Wada et al., 1993). However, current findings clearly showed that absorption characteristics of batho-intermediate relative to that of original pigment and thermal behavior of batho-intermediate in 6,5-rhodopsin are qualitatively similar to those in rhodopsin, while those in 6,7-rhodopsin are considerably different. From these results, a possible conformation around the C_6-C_7 bond of the bathorhodopsin chromophore is discussed.

MATERIALS AND METHODS

Preparations of 11-cis-Retinal and Its Analogs. 11-*cis*-Retinal was purified from isomeric mixture produced by irradiation of *all-trans*-retinal in acetonitrile using high-performance liquid chromatography (Maeda et al., 1978). Bicyclic retinals (6,5-retinal and 6,7-retinal, Figure 1) were prepared as reported previously (Ito et al., 1992; Wada et al., 1993).

Preparation of Bovine Opsin. Bovine opsin was purified in a CHAPS/PC mixture as described previously (Okano et al., 1989). Briefly, bovine rod outer segments were isolated by a conventional sucrose stepwise floatation method and suspended in buffer A (50 mM HEPES, 140 mM NaCl, 0.75% CHAPS, 1.0 mg/mL PC, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 6.6) to solubilize rhodopsin. After centrifugation (100000*g* $\times$ 60 min), the extract was supplemented with hydroxylamine at a final concentration of 50 mM and irradiated with orange light for 10 min to bleach rhodopsin into opsin and *all-trans*-retinal oxime. After addition of 1/5 volume of glycerol, the opsin solution was applied to concanavalin A-Sepharose affinity column (Pharmacia), which had been equilibrated with buffer A supplemented with 1/5 volume of glycerol. The retinal oxime was removed by washing the column with buffer B [50 mM HEPES, 0.6% CHAPS, 0.8 mg/mL PC, 20% (w/v) glycerol, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 6.6], and then opsin was eluted with buffer B supplemented with 200 mM α-methyl mannoside. The temperature of the column was kept at 4 °C.

Binding of Retinal Analogs and Bovine Opsin. 11-*cis*-Retinal or analog dissolved in a small amount of ethanol was added to the opsin preparation and incubated at 23 °C in the dark for 15−20 h. The amount of retinal or analog added to the opsin preparation was a 3−4-fold molar excess to opsin. In these experiments, the extinction coefficients of retinal analogs are assumed to be comparable to that of 11-*cis*-retinal (20 000 $M^{-1}cm^{-1}$; Gawinowicz et al., 1977).

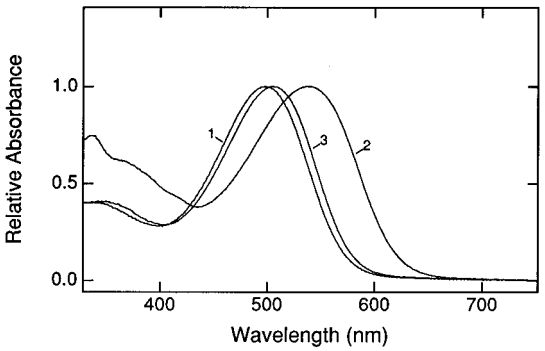

FIGURE 2: Absorption spectra of bovine rhodopsin (curve 1), 6,5-rhodopsin (curve 2), and 6,7-rhodopsin (curve 3) at 0 °C. Absorption maxima of rhodopsin, 6,5-rhodopsin, and 6,7-rhodopsin were located at 498, 535, and 503 nm, respectively.

Purification of Rhodopsin Analogs. Formed rhodopsin or analog preparation was applied to DEAE-Sepharose column (Pharmacia) which had been equilibrated with buffer C [50 mM HEPES, 0.6% CHAPS, 0.8 mg/mL PC, 20% (w/v) glycerol, pH 6.6]. The column was washed with buffer C supplemented with 10 mM hydroxylamine to remove the excess retinal or analog, and then hydroxylamine was removed by washing the column with buffer C. Rhodopsin or analog was eluted with buffer C supplemented with 140 mM NaCl. The temperature of the column was kept at 4 °C. For low temperature spectrophotometry, the eluate was 2−5-fold concentrated with ultrafiltration membrane (Amicon, YM-30), followed by addition of 2-fold volume of glycerol [final concentration, 71% (v/v)].

Spectrophotometry. The absorption spectra were recorded with Shimadzu MPS-2000 recording spectrophotometer interfaced to a personal computer (NEC PC9801VM). A glass optical cryostat (Yoshizawa & Shichida, 1982) was used to study the photobleaching processes of rhodopsin and its analogs at low temperatures. The temperature of the sample was regulated to within 1 °C by dropping liquid nitrogen into the coolant sink of the cryostat and monitored by a copper−constantan thermocouple. Opal glasses were set into both sample and reference beams of the spectrophotometer to compensate for light scattering by cracks of the sample formed by freezing. A 1-kW tungsten−halogen lamp (Rikagaku-Seiki) was used as a light source for irradiation of the sample. Wavelength of the irradiation light was selected with a glass cut-off filter (R-65 or R-61, Toshiba) or an interference filter (501 nm, Nihonshinku). A 5-cm water layer was placed in front of the light source to remove heat from the irradiation light.

RESULTS

Absorption Spectra of Rhodopsin, 6,5-Rhodopsin, and 6,7-Rhodopsin. 11-*cis*-Retinal or its analog (Figure 1) dissolved in ethanol was added to the opsin preparation and incubated at 23 °C in the dark. After absorbance increase in the visible region was saturated, the sample was applied to a DEAE-Sepharose column to remove excess retinal or analog. Absorption spectra of unmodified rhodopsin, 6,5-rhodopsin, and 6,7-rhodopsin are compared in Figure 2. The maxima are located at 498, 535, and 503 nm, respectively.

Low-Temperature Spectrophotometry of Rhodopsin. Low-temperature spectrophotometry has been extensively applied to the studies on photochemistry of bovine rhodopsin, but

the sample previously used was rod outer segment membrane (Sasaki et al., 1980) or digitonin solution (Yoshizawa & Wald, 1963). Thus, we first tested whether or not the photochemical and subsequent thermal reactions of rhodopsin in a CHAPS/PC system are similar to those in other conditions.

Figure 3 shows a typical experiment using a rhodopsin/71% glycerol mixture in CHAPS/PC system. Irradiation with 501-nm light at −190 °C caused the formation of a photo-steady-state mixture mainly containing bathorhodopsin (Figure 3a, curve 2). A photoreversibility among rhodopsin, bathorhodopsin, and isorhodopsin at −190 °C was demonstrated by changing the irradiation lights (Figure 3a). These results indicated that rhodopsin in CHAPS/PC system displays photoreactions similar to those in 2% digitonin and in rod outer segment membrane.

The thermal reaction of bathorhodopsin as well as those of the subsequent intermediates was investigated by warming the photo-steady-state mixture in a stepwise manner (Figures 3b,c). To visualize the thermal conversions, we plotted the absorption maxima of the sample against the temperatures to which the sample was warmed (Figure 3d). In Figure 3d, three phases of the shift of maxima were observed. They correspond to bathrhodopsin to lumirhodopsin (Figure 3b), lumirhodopsin to metarhodopsin I (Figure 3c, curves 20−22), and metarhodopsin I to metarhodopsin II (Figure 3c, curves 22−24) transitions.

The transition temperature of bathorhodopsin was estimated to be −120 °C, which was 20 °C higher than that observed in 2% digitonin (−140 °C; Yoshizawa & Wald, 1963). Those of lumirhodopsin and metarhodopsin I were also estimated to be −30 and −10 °C, respectively, both of which were about 10 °C higher than those observed in 2% digitonin (Yoshizawa & Wald, 1963). Formation of the metarhodopsin III was not observed below 0 °C under our experimental conditions (Figure 3d, curve 24).

Low-Temperature Spectrophotometry of 6,5-Rhodopsin. Similar experiments were performed using 6,5-rhodopsin sample. Irradiation with 501-nm light resulted in formation of batho-intermediate (Figure 4a, curve 2). The photoreversibility among original pigment, batho-intermediate, and iso-pigment at −190 °C was demonstrated by changing the irradiation lights although their absorption maxima were ∼40 nm red-shifted from those of rhodopsin counterparts (Figure 4a). Therefore, the photochemical reactions of 6,5-rhodopsin at −190 °C are qualitatively similar to those of rhodopsin.

The transition temperatures of the intermediates appearing in the bleaching process of 6,5-rhodopsin were also investigated by warming the photo-steady-state mixture in a stepwise manner (Figures 4b−d). The results showed that the profile of the changes in absorption maxima (Figure 4d) was qualitatively similar to that of rhodopsin (Figure 3d), indicating that 6,5-rhodopsin exhibits a bleaching process similar to that of rhodopsin. However, batho-intermediate of 6,5-rhodopsin shows transition temperature (−90 °C) much higher than that of bathorhodopsin, while the other intermediates show transition temperatures similar to those of the corresponding intermediates of rhodopsin.

Low-Temperature Spectrophotometry of 6,7-Rhodopsin. In spite of almost identical absorption and CD spectra between rhodopsin and 6,7-rhodopsin (Wada et al., 1993), the photobleaching process of 6,7-rhodopsin is significantly different from that of rhodopsin.

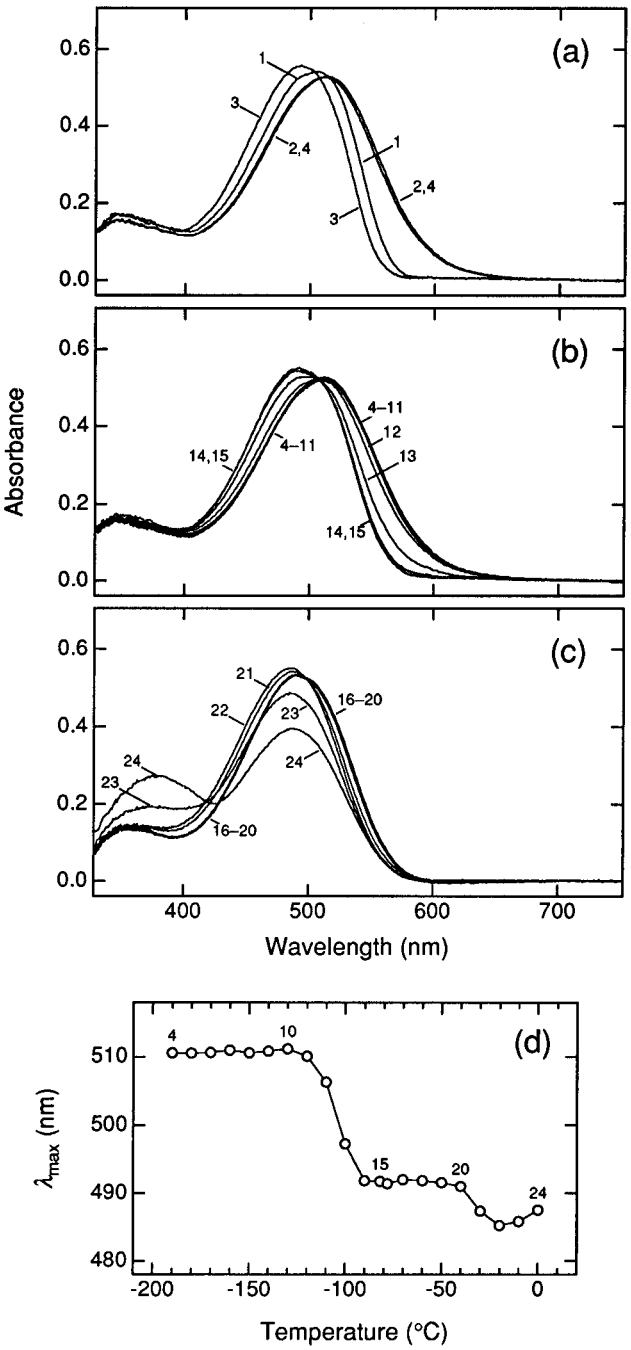

FIGURE 3: Low-temperature spectrophotometry of rhodopsin. (a) Rhodopsin/71% glycerol mixture was cooled to −190 °C (curve 1) and irradiated with 501-nm light for 160 s to produce a photo-steady-state mixture containing mainly bathorhodopsin (curve 2). Then it was irradiated with >550-nm light for 640 s to produce isorhodopsin (curve 3). Finally, it was irradiated with 501-nm light for 160 s to convert isorhodopsin to the photo-steady-state mixture previously formed (curve 4). (b) The sample mainly containing bathorhodopsin (curve 4) was warmed in a stepwise manner to −180, −170, −160, −150, −140, −130, −120, −110, −100, −90, and −80 °C, and the spectra were recorded at −190 °C (curves 5−15, respectively). (c) Absorption spectrum of the sample was recorded at −80 °C (curve 16). 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 17−24, respectively). (d) The maxima at α-band of the spectra shown in panels b and c were plotted against the temperatures to which the sample was warmed. The numbers in the panel are the curve numbers in panels b and c.

Irradiation of 6,7-rhodopsin at −190 °C converted it to batho-intermediate (Figure 5a, curve 2), and prolonged

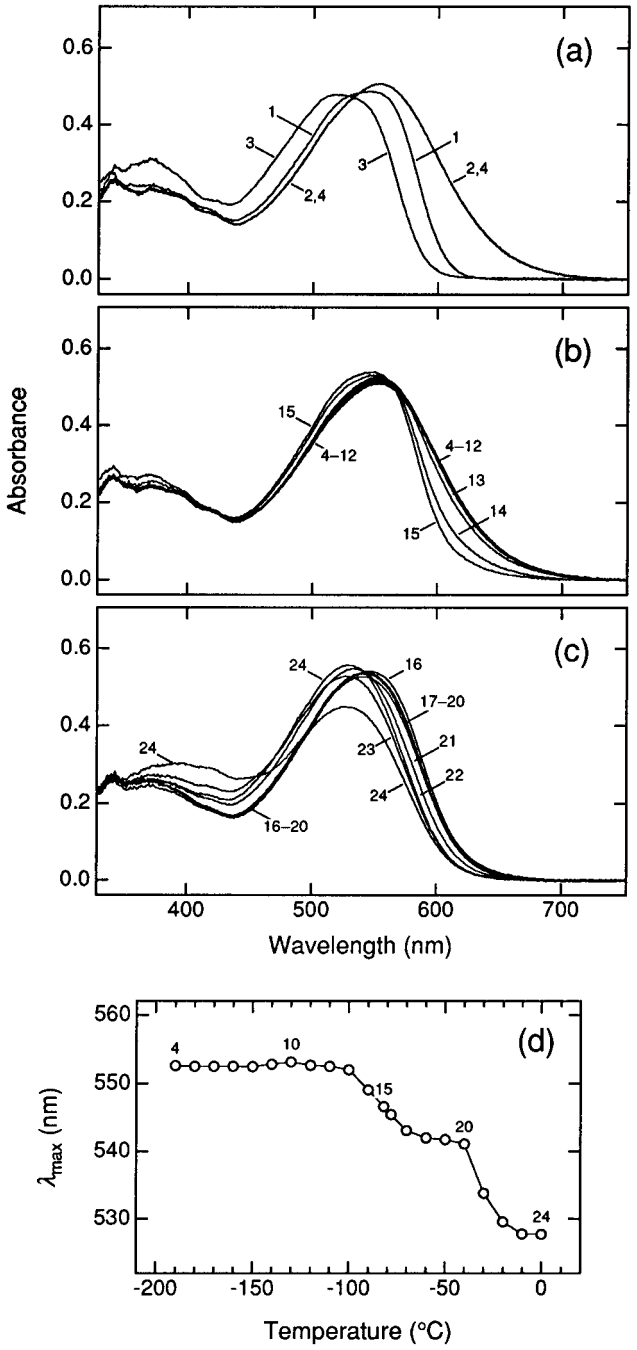

FIGURE 4: Low-temperature spectrophotometry of 6,5-rhodopsin. (a) 6,5-Rhodopsin/71% glycerol mixture was cooled to −190 °C (curve 1) and irradiated with 501-nm light for 320 s to produce a photo-steady-state mixture containing mainly batho-intermediate (curve 2). It was then irradiated with >550-nm light for 640 s to produce iso-pigment (curve 3) and with 501-nm light for 320 s to reconvert the photo-steady-state mixture previously formed (curve 4). (b) The sample mainly containing batho-intermediate (curve 4) was warmed in a stepwise manner to −180, −170, −160, −150, −140, −130, −120, −110, −100, −90, and −80 °C, and the spectra were recorded at −190 °C (curves 5−15, respectively). (c) Absorption spectrum of the sample was recorded at −80 °C (curve 16). 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 17−24, respectively). (d) The maxima at α-band of the spectra shown in panels b and c were plotted against the temperatures to which the sample was warmed. The numbers in the panel are the curve numbers in panels b and c.

irradiation caused the formation of a photo-steady-state mixture composed of original pigment, batho-intermediate, and iso-pigment, which were interconvertible among each

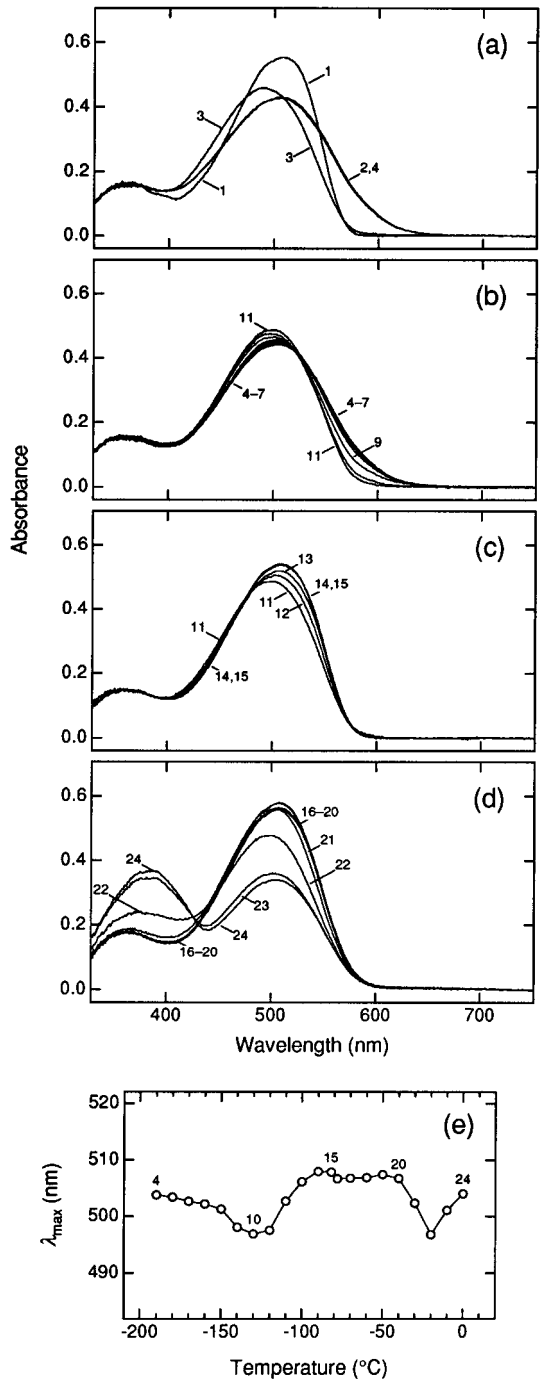

FIGURE 5: Low-temperature spectrophotometry of 6,7-rhodopsin. (a) 6,7-Rhodopsin/71% glycerol mixture was cooled to −190 °C (curve 1) and irradiated with 501-nm light for 320 s to produce a photo-steady-state mixture containing mainly batho-intermediate (curve 2). It was then irradiated with >550-nm light for 640 s (curve 3) and with 501-nm light for 320 s (curve 4) to demonstrate photoreversibility among original-, batho-, and iso-pigments. (b) The sample mainly containing batho-intermediate (curve 4) was warmed in a stepwise manner to −180, −170, −160, −150, −140, −130, and −120 °C, and the spectra were recorded at −190 °C (curves 5−11, respectively). (c) The sample was further warmed to −110, −100, −90, and −80 °C, and the spectra were recorded at −190 °C (curves 12−15, respectively). (d) Absorption spectrum of the sample was recorded at −80 °C (curve 16). 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 17−24, respectively). (e) The maxima at α-band of the spectra shown in panels b, c, and d were plotted against the temperatures to which the sample was warmed. The numbers in the panel are the curve numbers in panels b, c, and d.

other (Figure 5a). However, the extinction coefficient of the batho-intermediate was considerably smaller than that of original pigment, unlike rhodopsin and 6,5-rhodopsin.

Thermal behavior of batho-intermediate of 6,7-rhodopsin was also different from that of bathorhodopsin (Figure 5b−e). Namely, the absorption spectrum of the photo-steady-state mixture was first blue-shifted (−150 to −130 °C) and then red-shifted (−120 to −90 °C), whereas that from rhodopsin was only blue-shifted (−120 to −90 °C). These results indicate the presence of an additional intermediate between batho- and lumi-intermediates of 6,7-rhodopsin, the latter of which has a transition temperature (−30 °C) similar to that of lumirhodopsin. Since the spectrum of the additional intermediate is similar to that of BL-intermediate found in other rhodopsin analogs (Shichida et al., 1981; Okada et al., 1991), we denote it as BL-intermediate of 6,7-rhodopsin.

The transition temperature of meta I-intermediate of 6,7-rhodopsin seems to be slightly lower than that of metarhodopsin I, because a significant amount of meta II-intermediate was formed even at −20 °C (curve 22 in Figure 5d).

DISCUSSION

We previously reported that the absorption and CD spectra of 6,7-rhodopsin are almost identical in shapes with those of rhodopsin (Wada et al., 1993), while those of 6,5-rhodopsin are significantly different (Ito et al., 1992). These results strongly suggest that the C_6-C_7 bond of the chromophore in rhodopsin is twisted to the extent that the seven-membered ring allows and is in a form different from the five-membered ring (Figure 1). On the other hand, the present study clearly demonstrates that the spectral shift and the thermal behavior of bathorhodopsin are considerably different from those of 6,7-rhodopsin but qualitatively similar to those of 6,5-rhodopsin, although batho-intermediate of 6,5-rhodopsin shows thermal stability much higher than that of rhodopsin (Figures 4 and 5). Thus, 6,7-retinal and 6,5-retinal might be regarded as the models of the chromophores in rhodopsin and bathorhodopsin, respectively.

We first discuss the structural features of 6,5- and 6,7-retinals to implicate the structure of bathorhodopsin chromophore. Because of the fixed five-membered ring, 6,5-retinal has a completely planer structure at $C_5=C_6-C_7=C_8$ bonds and in-plane bent structure at C_6-C_7 bond (Figure 1). On the other hand, 6,7-retinal never has such structures but has a twisted C_6-C_7 bond structure due to the bulky seven-membered ring. Retinal can twist and/or bend at the C_6-C_7 bond but hardly forms a planar structure at $C_5=C_6-C_7=C_8$ bond because of the steric hindrance between the methyl group at C_5 and the hydrogen at C_8.

Among the above structural features possible for retinal, the possibility that the $C_5=C_6-C_7=C_8$ bonds in the bathorhodopsin chromophore become planar due to the interaction with opsin is also unlikely in the following reason. If it is the case, a part of the red-shift in absorption maximum from rhodopsin to bathorhodopsin could be accounted for the planarization, because the planarization induces a red-shift of absorption maximum. However, the experimental results showed that red-shift observed in 6,5-rhodopsin system (about 30 nm) is very close to that in rhodopsin system. These results also suggest that the planarization of the bonds is not the main reason, if any, for the red-shift of absorption maximum from rhodopsin to bathorhodopsin (batho-opsin shift).

Because of different spectral characteristics between batho-intermediates of rhodopsin and 6,7-rhodopsin, bathorhodopsin has a conformation at C_6-C_7 bond different from that of 6,7-retinal. This is in contrast with the fact that rhodopsin has a twisted conformation at C_6-C_7 bond similar to that of 6,7-retinal (Wada et al., 1993). Thus it is of interest to discuss the mechanism leading to the different conformation around C_6-C_7 bond of the chromophore between batho-intermediates of rhodopsin and 6,7-rhodopsin. Since 6,7-retinal has an extra ethylene bridge between C_{18} and C_8, it is possible that the ethylene bridge might interfere with the chromophore conformation in bathorhodopsin due to interaction with nearby amino acid residue(s), while, in rhodopsin, it has little interaction with the nearby amino acid residue(s). Thus a chromophore/opsin interaction additionally induced by the ethylene bridge in batho state might reflect the different absorption maxima as well as the different bleaching pathways between these bathorhodopsins. However, accumulated evidence has now suggested that the photoisomerization of the chromophore occurs mainly through rotation of the imine portion of the chromophore (Shichida et al., 1987) and only minor rearrangement of amino acid residues constituting the chromophore-binding site near the β-ionone ring region could occur, although the polyene part of the chromophore including $C_5=C_6$ double bond is highly distorted due to the isomerization in a restricted chromophore binding site (Yoshizawa & Wald, 1963; Eyring et al., 1982). Furthermore, binding experiments of various retinal isomers and analogs to opsin suggested that the chromophore binding site of opsin has enough space in the region in which the ethylene bridge would be located (Liu & Mirzadegan, 1988). Therefore, there could be another possibility that the difference in chromophore conformation of batho-intermediate between rhodopsin and 6,7-rhodopsin is due to intrinsic nature of the chromophores, retinal and 6,7-retinal. Namely, the chromophore of bathorhodopsin could be in an in-plane bent structure at the C_6-C_7 bond (Figure 6) due to the extension of the longitudinal distance of the chromophore upon photoisomerization in the restricted chromophore binding site, and the bent structure is hardly accomplished in the case of seven-membered ring, thereby resulting in the different absorption spectra between batho-intermediates of rhodopsin and 6,7-rhodopsin. The different conformation around C_6-C_7 bond between batho-intermediates of rhodopsin and 6,7-rhodopsin might induce the different interaction between β-ionone ring of the chromophore and surrounding protein. Thus the bleaching pathway of batho-intermediate of 6,7-rhodopsin is different from that of bathorhodopsin.

Since the chromophore conformations around C_6-C_7 bond between native retinal and 6,5-retinal are so different, it is hard to speculate on the difference or similarity in chromophore conformation and/or chromophore/opsin interaction between batho-intermediates of rhodopsin and 6,5-rhodopsin. Furthermore, similarities in batho-opsin shift and transition to lumi-intermediate between these systems might suggest that structural changes in this part of the chromophore play no role in the formation and decay of batho-intermediate. However, the highly stable property of batho-intermediate of 6,5-rhodopsin relative to that of rhodopsin gives us some hint to elucidate the conformational changes of the chro-

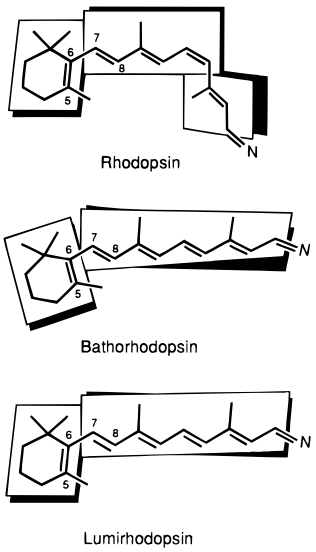

FIGURE 6: Chromophore structure of rhodopsin, bathorhodopsin, and lumirhodopsin. The chromophore of rhodopsin is represented to be in a twisted conformation at the C_6-C_7 and $C_{12}-C_{13}$ bonds. The chromophore of bathorhodopsin is in a distorted conformation in the conjugated double bond system as well as an in-plane bent structure at the C_6-C_7 bond. The in-plane bent structure at the C_6-C_7 bond might be relaxed upon conversion to lumirhodopsin.

mophore, because fixation of this bond by five-membered ring blocks a part of the relaxation of the energy stored in the native bathorhodopsin.

As already described, retinal can bend at the C_6-C_7 bond. If the chromophore of bathorhodopsin is in the bent structure around the C_6-C_7 bond as speculated from the results of 6,7-rhodopsin, the photon energy stored at this conformation could accelerate the transition from bathorhodopsin to lumirhodopsin. The same bleaching pathways between batho-intermediates of rhodopsin and 6,5-rhodopsin also suggest that the batho to lumi transition would result in the relaxation of the whole region of the chromophore including the β-ionone ring (Figure 6). On the other hand, the bent structure in 6,5-retinal is fixed by the five-membered ring, and therefore the transition from batho to lumi-intermediates in 6,5-rhodopsin needs more thermal energy from the environment. Thus the highly stable property of the batho-intermediate of 6,5-rhodopsin might be due to the fixed conformation of the bent structure at the C_6-C_7 bond by the five-membered ring. This is consistent with the notion from the NMR experiments (Smith et al., 1991) that the in-plain distortion of the retinal chromophore could be one of the essential factors for the storage of photon energy in bathorhodopsin. It should be noted that our speculation stands on the assumption that both the retinal and 6,5-retinal bind the same binding site of opsin and only minor changes near the β-ionone ring region of the chromophore occur during the conversion from rhodopsin to bathorhodopsin. These assumption should be examined by future research such as vibrational spectroscopy.

The thermal behaviors of lumi- and meta I-intermediates are qualitatively similar among three pigments. These results suggest that modification of the β-ionone ring has little effect on the later bleaching process. Thus the chromophore of lumirhodopsin would be in a more relaxed conformation near the β-ionone ring region, which is consistent with those reported earlier (Okada et al., 1991). It would result from a loose structure of the chromophore binding site near the β-ionone ring region of the chromophore.

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