# Spectroscopic Study of the Batho-to-Lumi Transition during the Photobleaching of Rhodopsin Using Ring-Modified Retinal Analogues†

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ABSTRACT: Photochemical and subsequent thermal reactions of rhodopsin containing 9-*cis*-retinal [Rh(9)] or one of four analogues with 9-cis geometries formed from ring-modified retinals, α-retinal [αRh(9)], acyclic retinal [AcRh(9)], acyclic α-retinal [AcαRh(9)], and 5-isopropyl-α-retinal [PαRh(9)] were investigated by low-temperature spectrophotometry and nanosecond laser photolysis. Irradiation of each pigment at −180 °C produced a photosteady-state mixture containing the original 9-cis pigment, its 11-cis pigment, and a photoproduct, indicating that the primary process of each pigment is a photoisomerization of its chromophore. The photoproduct produced by the irradiation of AcRh(9) had an absorption spectrum red shifted from the original AcRh(9) and was identified as the batho intermediate of AcRh(9). It was converted to the lumi intermediate through a metastable species, the BL intermediate, which has never been detected in Rh(9) at low temperature and whose absorption maximum was at shorter wavelengths than that of the batho intermediate. In contrast, the absorption maxima of the photoproducts produced from the other analogue pigments were at shorter wavelengths than those of the original pigments. They were identified as BL intermediates on the basis of their absorption maxima and thermal stabilities. The formation time constant of the lumi intermediate at room temperature was found to be dependent on the extent of modification of the ring portion of the chromophore, decreasing with the complete truncation of the cyclohexenyl ring [AcαRh(9)] and increasing with the attachment of the isopropyl group to the ring [PαRh(9)]. However, partial truncation of cyclohexenyl ring [AcRh(9)] or saturation of the $C_5{=}C_6$ bond [αRh(9)] resulted in little change. The results are consistent with the notion that changes in chromophore–opsin interaction in the batho–lumi transition are primarily limited to the ring portion of the chromophore.

The visual transduction process in the rod photoreceptor cells is initiated by photon absorption by the visual pigment, rhodopsin. The initial step of the photoreaction of rhodopsin is isomerization of the 11-cis double bond of the retinylidene chromophore to its trans form (Kropf & Hubbard, 1958; Yoshizawa & Wald, 1963; Shichida et al., 1988). For many years bathorhodopsin was considered to be the primary photoproduct. However, recently an earlier intermediate photorhodopsin has been detected (Shichida et al., 1984). Subsequent thermal reactions eventually led to the enzymatically active metarhodopsin II, which initiates the enzymatic cascade system in the photoreceptor cell (Fukada & Yoshizawa, 1981; Bennett et al., 1982; Falk & Applebury, 1986). In the course of conversion from photorhodopsin to metarhodopsin II, a series of intermediates is formed whose absorption maxima are distinct from each other. In order to clarify the role of these intermediates in leading to the enzymatic transformation processes of rhodopsin, a variety of spectroscopic techniques have been used to elucidate the chromophore configuration and/or conformation and the specific nature of chromophore–opsin interaction in each intermediate (Shichida, 1986; Derguini & Nakanishi, 1986; Mathies et al., 1987; Ottolenghi & Sheves, 1989).

Because of the short period of time elapsed (45 ps) between light absorption and formation of bathorhodopsin, it is generally believed that only minor rearrangement of amino acid residues constituting the chromophore-binding site could occur. On the other hand, a relatively larger change in chromophore–opsin interactions could take place during the batho-to-lumi transition. In recent years, the Kyoto group has devoted a considerable amount of its time to acquiring information that would lead to a better understanding of specific structural changes involved during the batho-to-lumi transition. Thus, low-temperature CD mesurements revealed that the strong α-band of bathorhodopsin decreases in intensity in lumirhodopsin (Ebrey & Yoshizawa, 1973; Shichida et al., 1978; Horiuchi et al., 1980). This result supported the original idea by Yoshizawa and Wald (1963) that bathorhodopsin has a distorted all-trans chromophore that changes to a relaxed all-trans form during the batho–lumi transition. Recent FTIR measurements of these intermediates also supported this idea (DeGrip et al., 1988; Ganter et al., 1988). Furthermore, similar measurements revealed that, unlike rhodopsin and bathorhodopsin (Yoshizawa & Horiuchi, 1973; Shichida et al., 1978), lumirhodopsin has a uniquely strong CD band in the near-ultraviolet region (~310 nm) (Ebry & Yoshizawa, 1973; Tokunaga et al., 1975; Suzuki et al., 1977; Shichida et al., 1978). The appearance of this large CD band was at-

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tributed to a newly generated interaction between the ring region of the chromophore and nearby aromatic amino acid residue(s) during the batho–lumi transition (Shichida et al., 1978).

The different photochemical behavior of the retinyl chromophore encapsulated by opsin at the batho and lumi stages, as elucidated in chromophore-extraction experiments (Maeda et al., 1978), also revealed alterations of the protein–substrate interaction in these intermediates. Thus, irradiation of the all-trans chromophore of lumirhodopsin produced a 7-cis chromophore as well as 11-cis (rhodopsin) and 9-cis (isorhodopsin) chromophores, while similar irradiation of the all-trans chromophore of bathorhodopsin produced only 11-cis and 9-cis chromophores. Since 7-*cis*-retinal is known to have a highly twisted ring–chain conformation (Liu et al., 1983), these results also suggest that during the batho–lumi transition, the chromophore-binding pocket must have expanded in the region near the cyclohexenyl ring.

Furthermore, synthetic retinal analogues and isomers have been used to elucidate the changes in specific chromophore–opsin interactions during the photobleaching process of rhodopsin. Using a 6-cis-locked retinal of which the $C_6$–$C_7$ single bond is fixed by a planar cyclopentadiene ring, Yoshizawa et al. (1987) showed that loss of flexibility of this bond is detrimental to the batho–lumi transition. All the results described above suggested that the effect on the batho–lumi transition of changing the interaction between the chromophore and opsin is likely to involve the region adjacent to the cyclohexenyl ring of the chromophore.

To further clarify the nature of the changes of the chromophore–opsin interaction around the binding site of the cyclohexenyl, we compared the properties of pigments derived from four 9-cis ring-modified retinal analogues with 9-*cis*-rhodopsin (rhodopsin analogue containing 9-*cis*-retinal). The photobleaching behavior of these pigment analogues was examined by means of low-temperature spectrophotometry and nanosecond laser photolysis at room temperature. The result of such a study is summarized in this paper. Furthermore, it reports the appearance of the new BL intermediates in the bleaching process of all the analogue pigments investigated and the effect of modification of the cyclohexenyl ring on relative ease of formation of the lumi intermediates at room temperature.

The choice of the analogues used in this study was assisted by the reported finding of stable pigment analogue formation from a series of acyclic retinal analogues (Crouch & Or, 1983; Zhang et al., 1989). And also, there has been undisputable evidence that 9-*cis*-retinal occupies the same binding site as the 11-*cis*-retinal (Ono et al., 1986). This allows us to take advantage of the synthetic experience in working with the more readily available 9-cis isomers of the analogues instead.

## Materials and Methods

*Synthesis and Purification of 9-cis-Retinal Analogues.* The synthesis and spectroscopic properties of 9-*cis*-α-retinal, αRet(9),[1] 5-isopropyl-9-*cis*-α-retinal, PαRet(9) (Asato et al., 1989), and the open-chain retinal analogue, AcRet(9), (Zhang et al., 1989) have been described elsewhere. The acyclic α-analogue, AcαRet(9), was prepared in a manner similar to that reported by Rao et al. (1985).

NMR (300 MHz, $CDCl_3$, TMS): AcαRet(9) 0.94 ppm, d, 6.6 Hz (6 H); 1.96 ppm, s (3 H); 2.11 ppm, t, 6.9 Hz (2 H); 2.33 ppm, s (3 H); 5.91 ppm, dt, 7.4 and 15.4 Hz ($C_7$–H); 5.97 ppm, d, 8.2 Hz ($C_{14}$–H); 6.03 ppm, d, 11.6 Hz ($C_{10}$–H); 6.29 ppm, d, 15.1 Hz ($C_{12}$–H); 6.66 ppm, d, 15.3 Hz ($C_8$–H); 7.24 ppm, dd, 11.5 and 14.9 Hz ($C_{11}$–H); and 10.11 ppm, d, 8.2 Hz ($C_{15}$–H).

*Preparation of Cattle Rod Outer Segments.* Cattle rod outer segments (ROS) were isolated under dim red light conditions from fresh retinas by a sucrose flotation method described previously (Shichida et al., 1987) and suspended in 10 mM HEPES buffer containing 67 mM NaCl (pH 7.0). After complete bleaching of rhodopsin in the ROS suspension by irradiation with orange light (>500 nm) in the presence of 100 mM $NH_2OH$, the ROS in the suspension was washed six times with 10 mM HEPES buffer (pH 7.0) by centrifugation to remove extrinsic membrane proteins from the ROS and the excess $NH_2OH$ in the suspension. Then the ROS suspension was lyophilized, followed by washing 10 times with petroleum ether to remove retinal oxime. The final pellet was resuspended in the HEPES buffer containing 67 mM NaCl (purified ROS suspension). All the procedures were performed under ice-chilled conditions.

*Preparation of Analogue Pigments.* A 2-fold molar excess of 9-*cis*-retinal or one of its analogues (0.5–1.0 mL of ethanol solution) was added to the purified ROS suspension (40 mL), and the mixture was incubated for 20–22 h in the dark. After formation of each pigment, 4 mL of 1 M neutralized $NH_2OH$ was added to the suspension for conversion of unreacted retinal analogues to their retinal oximes, and then the ROS containing the pigment were washed five times with 10 mM HEPES buffer containing 67 mM NaCl followed by lyophilization and washing five times with petroleum ether. Finally they were dried under a stream of nitrogen gas. Each pigment in the pellet was extracted with 2% digitonin in 10 mM HEPES buffer (pH 7.0). Absorption spectra of the samples used for nanosecond laser photolysis experiments are shown in Figure 1. It should be noted that each pigment was stable in the presence of 100 mM $NH_2OH$ at 4 °C.

*Spectrophotometry at Low Temperatures.* Measurements of the absorption spectra at low temperature were carried out by using a system identical with that reported recently (Imamoto et al., 1989). The samples were prepared by adding 66.6% glycerol in the final concentration to another fraction of the same sample used for the photolysis experiments.

*Nanosecond Laser Photolysis at Room Temperature.* Each pigment in a 2% digitonin solution was irradiated by a 17-ns pulse (FWHM) from an excimer-pumped dye laser (Lambda Physik EMG101 MSC, FL3002). The excitation energy was 1.0–1.2 mJ (/1.8 mm $\phi$), and the wavelength was 460 nm. A photographic flash lamp (622, Sanpak) or a xenon continuous lamp (L2274, Hamamatsu) coupled with a shutter system was used as a light source of a probe light for kinetic measurements, while only the flash lamp was used for spectral measurements.

The probe light was focused onto an aperture (1.8 mm $\phi$) that was attached in front of the sample cell (2 × 2 mm), and the sample (10 μL) containing 100 mM $NH_2OH$ was excited laterally. In order to eliminate the effects of rotation and diffusion of the pigment molecules in the sample on the kinetic and spectral measurements, the method of the magic angle excitation was applied; the angle between the plane of polarization of the excitation and probe lights was 54.7°.

For the kinetic experiments, the intensities of the probe light passing through the sample and a monochromator (Jobin

[1] Abbreviations: Rh, rhodopsin; Rh(9), rhodopsin "analogue" with 9-*cis*-retinal; AcRh(9), rhodopsin "analogue" with acyclic 9-*cis*-retinal; αRh(9), rhodopsin "analogue" with 4,5-dehydro-5,6-dihydro-9-*cis*-retinal; PαRh(9), rhodopsin "analogue" with 5-isopropyl-9-*cis*-α-retinal; AcαRh(9), rhodopsin "analogue" with acyclic 9-*cis*-α-retinal; Ret, retinal; ROS, rod outer segments; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

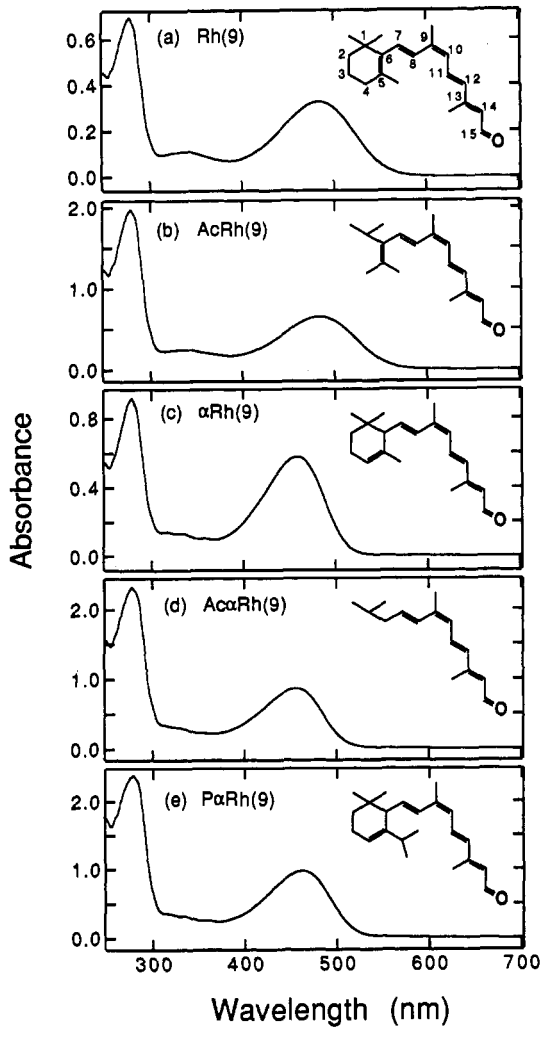


FIGURE 1: Absorption spectra (2-mm light path length) of the pigments prepared from the 9-cis isomers of retinal analogues and cattle opsin: (a) Rh(9), (b) AcRh(9), (c) αRh(9), (d) AcαRh(9), (e) PαRh(9), and structure of the retinal analogues used to form the pigments. Absorption maximum of each pigment is 483, 481, 459, 457, or 462 nm, respectively.

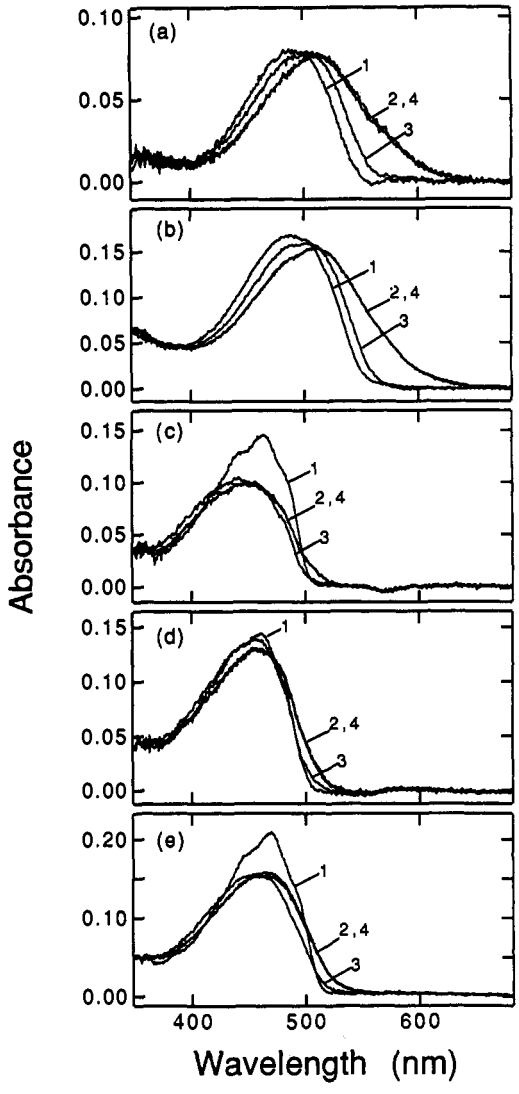


FIGURE 2: Spectral changes during the photoconversion of each pigment measured at −180 °C: (a) Rh(9), (b) AcRh(9), (c) αRh(9), (d) AcαRh(9), and (e) PαRh(9). Each sample (curve 1) was subjected to a series of irradiations with 436-nm light (curve 2), then with >630-nm light for Rh(9) and AcRh(9) or with >500-nm light for αRh(9), AcαRh(9), and PαRh(9) (curve 3), and with 436-nm light again (curve 4). In all cases, irradiation was continued until a photosteady-state was reached. The reirradiation with the 436-nm light produced a photosteady-state mixture whose absorption spectrum is identical in shape with that produced earlier with 436-nm irradiation, thus interconversion of the photoproducts by light was perfectly reversible. All measurements were done at −180 °C.

Yvon) were monitored by a photomultiplier (R666S, Hamamatsu), the output of which was recorded in a Stragescope (TS-8123, Iwatsu). Then they were transferred to a personal computer (PC-9801, NEC), which calculated the absorbance changes of the sample after excitation.

Transient spectra of the sample at selected times after excitation were recorded by a Spectrometric Multichannel Analyzer (SMA) system (Princeton Instruments; PI) in which two sets of cooled (−20 °C) multichannel photodiode arrays were used as detectors. A light pulse from a photographic flash lamp was divided into two pulses: one was focused on a 1.8 mm $\phi$ aperture in front of the sample cell and the other on another aperture of the same diameter. Then each pulse was focused onto the slit of a polychrometer (82-499, Jarrell Ash) to which the SMA detector (IRY-700G, PI) was connected. Signals from the detectors were digitized by the SMA controllers (ST-100, PI) and transferred to a personal computer (PC-286, EPSON), followed by calculation of difference absorbances. The gating width of the SMA detectors was controlled by a high-voltage pulse generator (FG-100, PI); the gate width was set at 18 ns, which was comparable to the laser pulse width. The gating jitter was negligible.

Before and after each experiment, the energy of the excitation laser pulse was monitored by a joule meter (ED100A, Gentec), which was set behind the sample cell. The triggering time of each device was controlled by a four-channel digital delay/pulse generator (DG535, Stanford). Data taken by 5–15 and 30–36 laser shots in kinetics and spectral measurements, respectively, were averaged.

## RESULTS

*Photoreactions at Liquid-Nitrogen Temperature.* The spectra shown in Figure 2 are typical of those of photochemical reactions of the five pigments at −180 °C. On irradiation with a 436-nm light, Rh(9) (curve 1 in Figure 3a) was converted to a bathochromic photoproduct. Prolonged irradiation caused the formation of a photosteady-state mixture containing the original 9-cis pigment, its 11-cis pigment (Rh), and batho-Rh (curve 2). The latter was converted to the 11-cis and 9-cis pigments by irradiation with a red light at wavelengths longer than 630 nm, resulting in formation of a mixture of 11-cis and 9-cis pigments (curve 3). We subsequently showed that this

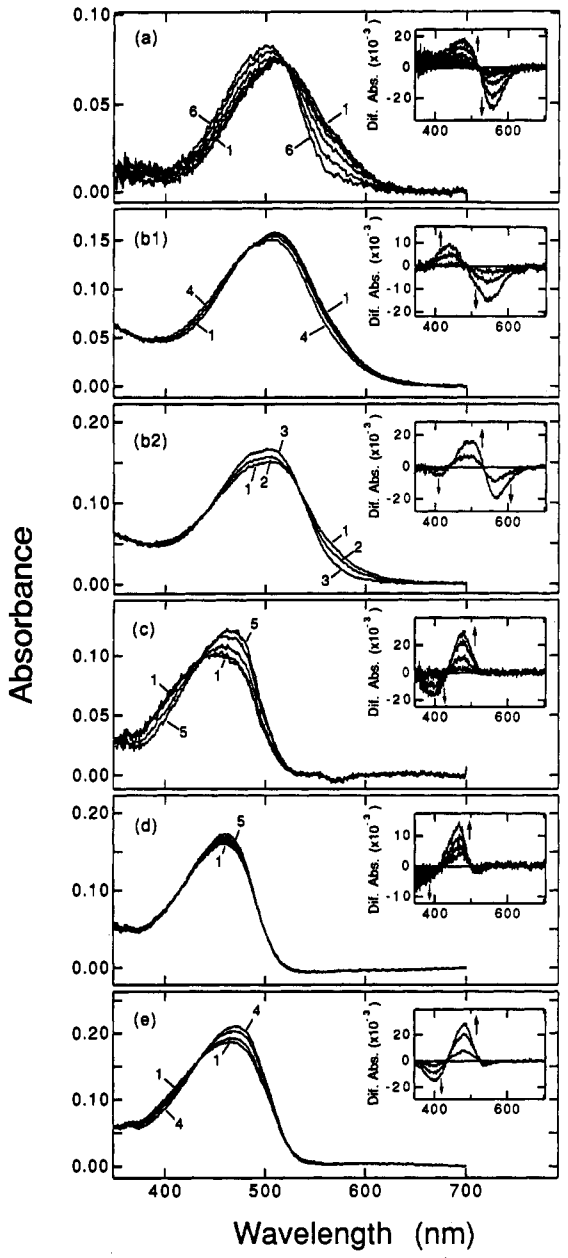


FIGURE 3: Changes in the absorption spectrum induced by warming the photosteady-state mixture obtained as in Figure 2, and their difference spectrum: (a) Rh(9), (b) AcRh(9), (c) αRh(9), (d) AcαRh(9), and (e) PαRh(9). Each sample was warmed in a stepwise manner (10 °C interval), and the spectra were measured after recooling to −180 °C. (a) Curves 1–6: −150 to −100 °C. (b1) Curves 1–4: −160 to −130 °C. (b2) Curves 1–3: −120 to −100 °C. (c) Curves 1–5: −150 to −110 °C. (d) Curves 1–5: −160 to −120 °C. (e) Curves 1–4: −110 to −80 °C. Inserts: Difference spectra between the products formed by successively warming and products of the photosteady-state mixture (curve 1). Arrows indicate the direction of the spectral changes during warming.

cycle of changes could be reproducibly repeated.

AcRh(9) exhibited similar spectral changes (curves 1–4 in Figure 2b). Therefore, partial truncation of the cyclohexenyl ring does not affect the photochemical reaction of the pigment at liquid-nitrogen temperature. On the other hand, spectral changes of αRh(9) and PαRh(9) (Figure 2c,e) were remarkably different. Thus, irradiation with the 436-nm light caused a substantial decrease of absorbance around the peak near 460 nm but only a slight concurrent increase of absorbance around 400 nm and discernible tailing into the red (curves numbered in Figure 2c,e). Subsequent irradiation with yellow light (>500 nm) caused a minor increase of the absorbance at the blue side of the spectrum and a similar decrease at longer wavelength side (curve 3 in Figure 2c,e). This cycle was also found to be reversibly reproducible. Spectral changes of AcαRh(9) were similar to those of αRh(9) and PαRh(9) except that the extent of decrease of absorbance around the peak by irradiation with the 436-nm light was not so prominent. In each of these three pigments, the initial slight red shift produced by the 436-nm light is likely to be due to the formation of the 11-cis pigment because the extent of the red shift is comparable to the spectral difference between the 9-cis (around 460 nm) and 11-cis (around 470 nm) pigments. A slight increase of absorbance at the shorter wavelength side upon irradiation of the original pigment with the 436-nm light as well as yellow light indicated the formation of a hypsochromic intermediate at this temperature. We shall call the latter the "BL intermediate" (see below).

*Formation Processes of Lumi Intermediates.* Figure 3 shows the spectral changes corresponding to the formation of the lumi intermediate resulted from warming of the photosteady-state mixture formed from irradiation with 436-nm light at −180 °C (curve 4 in Figure 2a–e). A comparison of the curves in Figure 3b1,b2 to those in Figure 3a revealed that AcRh(9) has a different bleaching process from Rh(9) in the temperature range from −180 to −80 °C. Instead of a single isosbestic point for curves in Figure 3a, the presence of two isosbestic points are discernible for AcRh(9). We, therefore, present the data for the latter in two parts: those from −160 to −130 °C (Figure 3b1) and from −120 to −100 °C (Figure 3b2). Thus, the conversion of batho-Rh to lumi-Rh is a single-step process while that of batho-AcRh(9) is by way of a new intermediate. The assignment of the final intermediate to lumi-AcRh(9) was based on its similar absorption characteristics and thermal stability [stable below −40 °C and converted to meta I AcRh(9) above this temperature] to those of lumi-Rh. The new intermediate has its absorption spectrum ($\lambda_{max}$ = 517 nm) blue shifted from the batho intermediate ($\lambda_{max}$ = 532 nm) and its extinction coefficient slightly lower than that of the lumi intermediate ($\lambda_{max}$ = 507 nm).[2] Furthermore, the absorption spectrum is broader than that of the lumi intermediate.[2]

It should be noted that this intermediate has spectral and thermal characteristics similar to those reported for new intermediates formed in the bleaching process of other rhodopsin analogues, first detected by us in low-temperature spectroscopic studies of the 13-demethyl and dihydro analogues (Shichida et al., 1981; Yoshizawa et al., 1984) and subsequently verified in room-temperature fast kinetics studies (Albeck et al., 1989; Einterz et al., 1990). Therefore, by analogy, again we wish to label the new intermediate "BL".

The spectral changes taking place during the warming process for the other three analogue pigments were quite similar to each other (Figure 3c–e): an increase of absorbance near the maximum with a simultaneous decrease at shorter wavelengths. These changes seemed to correspond to the transition from BL to lumi intermediates as detected in AcRh(9) and the 13-demethyl systems although the decrease of absorbance on the longer wavelength side of AcRh(9) was more prominent than in these pigments (Figure 3b2).

[2] Absorption spectra of batho, BL, and lumi intermediates of AcRh(9) were calculated by the usual method (Yoshizawa & Shichida, 1982), i.e., by subtracting the spectra of the 9-cis pigment and the 11-cis pigment from the spectrum of the photosteady-state mixture produced by irradiation with 436-nm light at −180 °C (for batho) or of the mixture produced by warming the photosteady-state mixture to −130 °C (for BL) or −100 °C (for lumi).

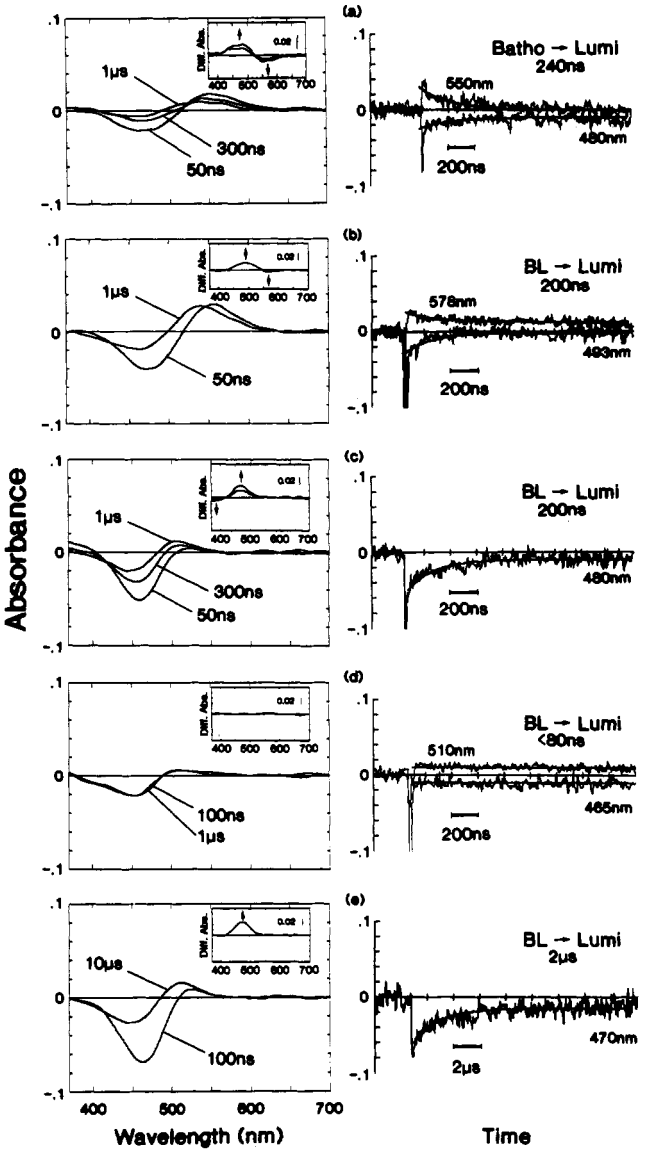


FIGURE 4: Transient absorption spectra at selected times after the excitation (left panel) and kinetics at selected wavelengths (right panel) measured by nanosecond laser photolysis of (a) Rh(9), (b) AcRh(9), (c) αRh(9), (d) Acα Rh(9), and (e) Pα Rh(9) at room temperature (22 °C). Left panel: Each sample was excited with a nanosecond blue pulse (460 nm), and the difference spectra before and after the laser excitation were measured. The times shown in the figures indicate when each spectrum was taken after excitation. (Inserts: The difference spectrum corresponds to the difference between the two recorded spectra or between the spectrum recorded at the earliest time and the spectra at subsequent times. Arrows indicate the direction of the spectral changes.) Right panel: Each sample was excited with the nanosecond blue pulse, and the changes of absorbance at selected wavelengths were measured as a function of time. Solid curves represent the fitting curves estimated by single exponential fitting. The transition time constants ($\tau = 1/e$) calculated are shown in the figures.

The transition temperature for the BL to lumi intermediate in each of the analogues was different: −140 °C for αRh(9) [same as for Rh(9)], −150 °C for Acα Rh(9), and −110 °C for the bulkier Pα Rh(9).

*Nanosecond Photolysis at Room Temperature.* Figure 4 shows the transient absorption spectra measured at selected times and the absorbance changes at selected wavelengths after exciting each pigment with a single nanosecond pulse of blue light at room temperature. In AcRh(9), the transient absorption spectrum [the difference between spectra of AcRh(9) and its intermediate] measured at 50 ns after excitation displayed positive absorbances at 578 nm and a negative absorbance at 493 nm (Figure 4b). This indicated the formation of a bathochromic product within 50 ns of excitation. Subsequent spectral changes reflect the decay process of this intermediate. It should be noted that the difference spectrum between the transient spectra measured at 50 ns and 1 μs (Figure 4b, inset) is, within experimental error, identical in its difference maximum and isosbestic point with those in the difference spectrum from BL and lumi intermediates of AcRh(9) (Figure 3b2), suggesting that the process measured on the nanosecond time scale is the BL-to-lumi transition. The decay process was also measured by monitoring the absorbance changes at 578 and 493 nm. The time constant was estimated to be 200 ± 30 ns by fitting the kinetic data with a single exponential curve. This value is comparable with that determined for the transition from batho to lumi in Rh(9) (240 ± 46 ns) (Figure 4a). Our attempts to measure the conversion process from batho to BL intermediates in AcRh(9) at room temperature were unsuccessful, probably because the transition is too rapid for our experimental setup as suggested by its lower transition temperature (−150 °C).

As shown in Figure 4c,e, similarities between αRh(9) and Pα Rh(9) in spectral changes induced by the transition from BL to lumi were also detected by means of room-temperature photolysis. The estimated time constants for the transitions in these two pigments were 200 ± 18 ns for αRh(9) and 2 ± 0.6 μs for Pα Rh(9). On the other hand, excitation of Acα Rh(9) with a nanosecond blue pulse caused an instantaneous increase in absorbance at 510 nm and a decrease at 465 nm, whose absorbance did not change on the nanosecond time scale (Figure 4d). This fact indicated that the time constant of conversion from the BL to lumi intermediates in the Acα Rh(9) system is too short to be detected by our experimental setup. The next transition from the lumi to meta I intermediate of Acα Rh(9) was, however, detectable with the time constant of 90 μs (data not shown).

## Discussion

Of the four visual pigment analogues for rhodopsin containing 9-*cis*-retinal used in the study, one retains the 5,6-double bond [AcRh(9)], resembling the β-retinylidene chromophore. The other three fall into the different category of α-like pigments; αRh(9) derived from α-retinal, Acα Rh(9) from a much truncated analogue containing the same tetraene chromophore as in α-retinal and Pα Rh(9) from 5-isopropyl-9-*cis*-α-retinal (see Figure 1 for structures). The difference of the two groups is evident from the different absorption characteristics of the pigment analogues: 481 nm for AcRh(9), similar to that of Rh(9) (483 nm), while the others are blue shifted to 457–462 nm (Figure 1). The low-temperature spectrum of Acα Rh(9) is also characteristic of α-pigments exhibiting fine structure similar to the corresponding retinals in hexane (Asato et al., 1989; Zhang et al., 1989), most likely as a consequence of the removal of the $C_6$–$C_7$ bond twist. The structural differences are also reflected in their photochemical and bleaching characteristics, summarized in Figure 5 and to be discussed below.

*The Primary Photoproducts.* It should be mentioned that the present experimental conditions are not conducive for detecting the shorter lived photorhodopsin intermediate as reported for rhodopsin (Shichida et al., 1984). Hence, only batho and later intermediates will be considered in the current discussion of formation and reaction of the primary photoproducts.

Of these four analogues, only the β-like AcRh(9) analogue gave a batho intermediate (Figure 2b) with its absorption

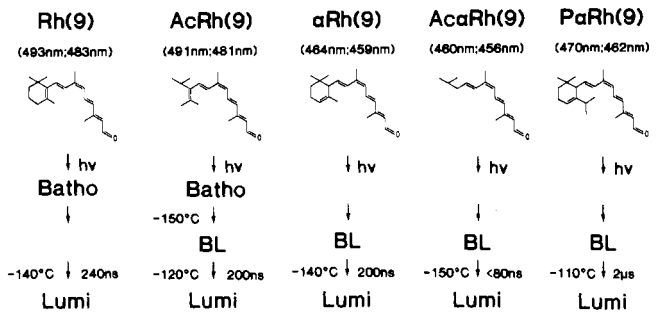


FIGURE 5: Transitions between photoproducts (batho) and subsequent intermediates of Rh(9) and four analogue pigments measured at room and low temperatures. The absorbance maxima of the pigments are shown above the structural formula of the retinals (left, at −180 °C; right, at 20 °C). The values shown at the left of arrows are the transition temperatures and those at the right are the time constants at room temperature.

maximum (532 nm) substantially red shifted (51 nm) from that of the pigment, a phenomenon similar to that of Rh(9). All others led to the formation of intermediates with absorption maxima only slightly blue shifted from the corresponding pigments. Thus, in the latter cases, instead of formation of a batho-Rh-like intermediate, a different intermediate designated as the BL intermediate is formed (see the next section for additional discussion).

The 5-methyl group in the β-like analogue AcRh(9), should cause a 6,7-twist in the chromophore as in the parent retinal. On the other hand, the α-like chromophore of the other three analogues should lead to a different conformation near the $C_6$–$C_7$ bond. The tetrahedral center at $C_6$ should make the polyene chain adopt a different conformation (Seki et al., 1985). This altered protein conformation apparently facilitates the batho-to-BL conversion, rendering direct conversion to the latter intermediates at liquid-nitrogen temperature for the α-like analogues. In fact, irradiation of α-like analogue pigments at liquid-helium temperature produced batho intermediates that were then converted to BL intermediates upon warming (manuscript in preparation).

Regardless of the spectral properties associated with the primary photoproduct of these analogues, the observed photoreversible reactions among 9-cis and 11-cis pigments and the photoproduct at liquid-nitrogen temperature in all cases indicate that the primary processes are the photoisomerization of the chromophores. The present analogue results show that the isomerization of the chromophore is neither affected by modification of the cyclohexenyl ring nor by the attachment of a bulky group to the ring portion of the chromophore. This is reasonable when one considers that the isomerization of the chromophore is believed to proceed by the movement of the half of the polyene chain containing the Schiff base, which may be accommodated by the movement of the butyl tether of the lysine residue (Shichida et al., 1987).

*The BL Intermediate.* The BL intermediates from the three α-Rh analogues are slightly blue shifted from the stable 9-cis and 11-cis pigments as shown from the difference spectra obtained during warming of the initial product mixtures (Figure 3c–e). Even for the β-like analogue, AcRh(9), the initial loss of the batho pigment during the warming process (Figure 3b1) was replaced by a buildup of a slightly blue-shifted pigment (Figure 3b2) upon continued warming at more elevated temperatures. Hence, the blue-shifted intermediate is common to all four analogues formed during the photobleaching process. In fact, they were also observed in two other pigment analogues, formed from 13-demethylretinal (Shichida et al., 1981) and 5,6-dihydroretinal (Yoshizawa et al., 1984) as reported earlier. It is interesting to note that at liquid-nitrogen temperatures the β-like 13-demethyl analogue is also converted to the batho intermediate, which is then converted to the BL intermediate on warming, while the α-like 5,6-dihydro analogue is directly converted to the BL intermediate because the batho intermediate of 5,6-dihydro analogue is unstable at this temperature. Thus again, the implication is that the protein interacting with the nonplanar β-like pentaene chromophore serves not only as a source of induced dichroic activity of the pigment's chromophore but also as a restraining force to prohibit ready conversion of the batho intermediate to the BL intermediate.

*BL-to-Lumi Transition.* Lumi intermediates are stable at higher temperatures (−80 °C) than BL intermediates. Thus, continued warming of the BL intermediate led to a shift of the absorption spectrum. The process was followed spectroscopically in Figure 3 at low temperatures and in Figure 4 at room temperature; the data are summarized in Figure 5. It is clear that the formation constant of lumirhodopsin at room temperature and the transition temperature to lumirhodopsin at low temperatures are affected by the modification of the cyclohexenyl ring of the chromophore. We suggested earlier that some changes of the chromophore–opsin interaction around the cyclohexenyl ring binding site occur during formation of lumirhodopsin (see the introduction). The delay of the time constant for the transition from BL to lumi intermediate in PαRh(9) seems to be reasonable when one considers that the bulkier isopropyl group attached to the ring is expected to hinder the changes in chromophore–opsin interaction that accompanies the conversion from the BL to lumi intermediate, thus slowing down the formation of the lumi intermediate. In a similar vein, the acceleration of the transition time from the BL to lumi intermediate in the AcαRh(9) system is consistent with the much truncated thus smaller chromophore of AcαRh(9), resulting in the rapid transition to its lumi intermediate.

Our experiments on αRh(9) and AcRh(9) showed that the formation time constants of the lumi intermediates were not affected by partial truncation of the cyclohexenyl ring [AcRh(9)] or saturation of the $C_5$=$C_6$ bond [αRh(9)]. These facts suggest that, in the BL-to-lumi transition, the changes in the chromophore–opsin interaction take place near the entire hydrophobic binding site around the cyclohexenyl ring, while, in the batho-to-BL transition, the changes are restricted to a region around the $C_5$=$C_6$ bond of the chromophore.

*Other General Comments.* We believe specific information on changes of chromophore–substrate interaction during photoexcitation and subsequent dark processes as defined in this and related studies will be useful toward helping the design of other retinal analogues with strategically located reporting groups (e.g., NMR or photoaffinity labels). Also, it will be of interest to determine whether or not each of the four analogue pigments has the capacity to bind and activate transducin. Our preliminary experiments showed that all of these analogue pigments are converted to meta II-like intermediates through meta I following the decay of the lumi intermediates. Therefore, it is reasonable to assume that these analogue pigments can activate transducin.

*Summary.* Through a photochemical and spectroscopic study of four ring-modified visual pigment analogues, we have successfully extended our continued investigation on structural changes of the chromophore of visual pigments during the photoexcitation and the subsequent dark processes. The accumulated evidence now points to the following sequential changes of the chromophore. The primary photochemical

process leads to the formation of a strained all-trans chromophore in photo- and bathorhodopsins through rotation of the imine portion of the chromophore. Subsequent relaxation of the strained chromophore and the surrounding protein residues to the BL and lumi intermediates primarily involves the region near the ring, i.e., the hydrophobic pocket. Therefore, knowledge of the structure of the hydrophobic pocket will be important in better understanding the dark relaxation processes of the visual chromophore.

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