Differences in the Photobleaching Process between 7-*cis*- and 11-*cis*-Rhodopsins: A Unique Interaction Change between the Chromophore and the Protein during the Lumi–Meta I Transition†

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ABSTRACT: The photochemical and subsequent thermal reactions of 7-*cis*-rhodopsin prepared from cattle opsin and 7-*cis*-retinal were investigated by low-temperature spectrophotometry and laser photolysis, and compared with those of 11-*cis*-rhodopsin prepared from cattle opsin and 11-*cis*-retinal. Low-temperature experiments revealed that the absorption maxima of batho and lumi intermediates from 7-*cis*-rhodopsin were at slightly shorter wavelengths than those of 11-*cis*-rhodopsin while the meta I intermediates of both rhodopsin isomers showed the same absorption maxima. Kinetic experiments of the photobleaching process of 7-*cis*-rhodopsin using picosecond and nanosecond laser pulses revealed the formation of intermediates corresponding to the batho, lumi, meta I, and meta II intermediates from 11-*cis*-rhodopsin. An intermediate of 7-*cis*-rhodopsin corresponding to photorhodopsin (a precursor of bathorhodopsin), however, was not detected. Batho and lumi intermediates from 7-*cis*-rhodopsin had shorter lifetimes ($\sim$40 ns and 300 μs) than those of 11-*cis*-rhodopsin (250 ns and 800 μs), but the lifetime of the meta I intermediate from 7-*cis*-rhodopsin was identical with that from 11-*cis*-rhodopsin (12 ms). These results indicate that the difference in configuration of the original chromophore between 7-*cis*- and 11-*cis*-rhodopsins is a cause of different chromophore–opsin interactions in the batho and lumi stages, while in the meta I stage the difference has disappeared by the relaxation of the protein near the chromophores. A possible interaction change between the 9-methyl group of the chromophore and its neighboring protein during the lumi–meta I transition will be discussed.

Rhodopsin is the visual pigment which is responsible for scotopic vision. It contains an 11-*cis*-retinal chromophore bound via a protonated Schiff base linkage to a specific lysine residue of an apoprotein, opsin. Upon absorption of a photon, rhodopsin bleaches to *all-trans*-retinal and opsin through several thermolabile intermediates [reviewed in Shichida (1986)]. The primary photochemical reaction of rhodopsin is an isomerization around the C_{11}=C_{12} bond of the chromophore to form photorhodopsin (Shichida et al., 1984), a highly twisted all-trans photoproduct (Kandori et al., 1989b). The subsequent thermal reactions result in the formation of several intermediates each of which has its own absorption spectrum and thermal stability (Wald et al., 1950; Hubbard & Kropf, 1958; Yoshizawa & Wald, 1963; Matthews et al., 1963). One of the intermediates called metarhodopsin II has the ability to bind transducin, a guanine nucleotide binding protein, and convert it to its active form, which can then activate cGMP phosphodiesterase [reviewed in Stryer (1986)].

The light-induced conformational change of the chromophore of rhodopsin has been investigated by various spectroscopic techniques together with synthetic retinal analogues (Mao et al., 1981; Eyring et al., 1982; Fukuda et al., 1984; Palings et al., 1989; Kandori et al., 1989b). Furthermore, the binding domain of metarhodopsin II to transducin has been characterized by spectroscopic and enzymatic techniques with the aids of synthetic peptides of the specific regions of opsin (Konig et al., 1989) and opsins modified by site-directed mutagenesis (Franke et al., 1988). However, little is known about changes in the chromophore–opsin interaction during and after the photoisomerization of chromophore which eventually causes the formation of metarhodopsin II. Recently our investigations using verious spectroscopies (Shichida et al., 1978; Maeda et al., 1978b, 1979; Yoshizawa et al., 1987; Okada et al., 1991) clearly showed that the strained chromophore of bathorhodopsin is relaxed through changes in the chromophore–opsin interaction near the cyclohexenyl ring binding site (Shichida, 1986). The aim of the present research is to get further information about specific changes in the chromophore–opsin interaction during the photobleaching process of rhodopsin using a synthetic rhodopsin having 7-*cis*-retinal as its chromophore (7-*cis*-rhodopsin).

Several years ago, we reported that cracks formed in a rhodopsin sample by cooling to liquid nitrogen temperature affected an absorption spectrum of the sample (Ono et al., 1986); the peak of the difference spectrum between rhodopsin and its photo-steady-state mixture containing mainly bathorhodopsin lay in the range from 559 to 568 nm, depending on the state of the cracks in the sample. This may be one of the

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reasons why contradictory results have been reported about the spectral shape of bathorhodopsin produced from rhodopsin and 9-*cis*-rhodopsin (Yoshizawa & Wald, 1963; Mao et al., 1980; Waddell et al., 1984). In order to overcome the spectral deviation due to the cracks, we applied a rapid-cooling technique to prepare a sample without cracks at low temperatures and showed that bathorhodopsin produced from 9-*cis*-rhodopsin is identical in spectral shape with that from rhodopsin (Ono et al., 1986).

Application of the rapid-cooling technique to the photochemical reaction of 7-*cis*-rhodopsin, however, revealed that contrary to an early report (Kawamura et al., 1980), the batho intermediate of 7-*cis*-rhodopsin had a different spectral shape from those of rhodopsin and isorhodopsin. These observations prompted us to reinvestigate the photochemical and subsequent thermal reactions of 7-*cis*-rhodopsin. The current findings clearly indicate that the difference in chromophore–opsin interaction between the original pigments forms intermediates with different spectroscopic and kinetic properties at the early stage of the bleaching process but the difference between the intermediates eventually disappears at the late stages of the bleaching process.

Materials and Methods

Preparation of 7-cis- and 11-cis-Retinals. 7-*cis*- and 11-*cis*-retinals were prepared according to the method described previously (Maeda et al., 1978a). Briefly, *all-trans*-retinal dissolved in acetonitrile was irradiated with a 2-kW xenon lamp (Usio) for about 1 h at 0 °C to convert it to a mixture of various retinal isomers, among which the 7-cis- or 11-cis isomer was isolated and purified by high-performance liquid chromatography (JASCO UVIDEC 100).

Preparation of Cattle Opsin. Cattle rod outer segments (ROS)[1] were isolaetd from fresh retinas by a conventional sucrose stepwise flotation method described previously (Shichida et al., 1987). The ROS thus obtained was suspended in 10 mM HEPES buffer containing 100 mM hydroxylamine (pH 7.0) and irradiated with an orange light (>540 nm) at 0 °C to bleach the rhodopsin to retinal oxime and opsin. After being washed 7 times with 10 mM HEPES buffer and once with distilled water by centrifugation, the ROS were lyophilized, followed by washing 7 times with light petroleum ether by centrifugation to remove the retinal oxime. The ROS thus obtained were suspended in 10 mM HEPES buffer (pH 7.0) and divided into two fractions.

Preparation of 7-cis- and 11-cis-Rhodopsins. 7-*cis*- and 11-*cis*-rhodopsins were prepared by incubating the ROS suspension containing opsin with a 2-fold molar excess of 7-*cis*- and 11-*cis*-retinals dissolved in a small amount of ethanol for about 20 and 2 h, respectively (the name "11-*cis*-rhodopsin" was used for the regenerated rhodopsin from 11-*cis*-retinal and opsin). In order to reduce any variation between individual preparations, both rhodopsin isomers were prepared from the same opsin preparation.

After formation of 7-*cis*- or 11-*cis*-rhodopsin, neutralized hydroxylamine (1 M) was added to each rhodopsin preparation at the final concentration of 10 mM to change the unreacted retinal into its oxime. Then the sample was washed 6 times with 10 mM HEPES buffer (pH 7.0), followed by lyophilization and washing 6 times with light petroleum ether by centrifugation. The pellet obtained was dried under a stream of nitrogen gas, and 7-*cis*- or 11-*cis*-rhodopsin contained in the pellet was extracted with 2% digitonin dissolved in 10 mM HEPES buffer (pH 7.0), followed by concentration using an ultrafiltration membrane (Amicon). Spectra of the extracts containing 7-*cis*- or 11-*cis*-rhodopsin were measured in a Shimadzu Model MPS-2000 spectrophotometer (Figure 1).

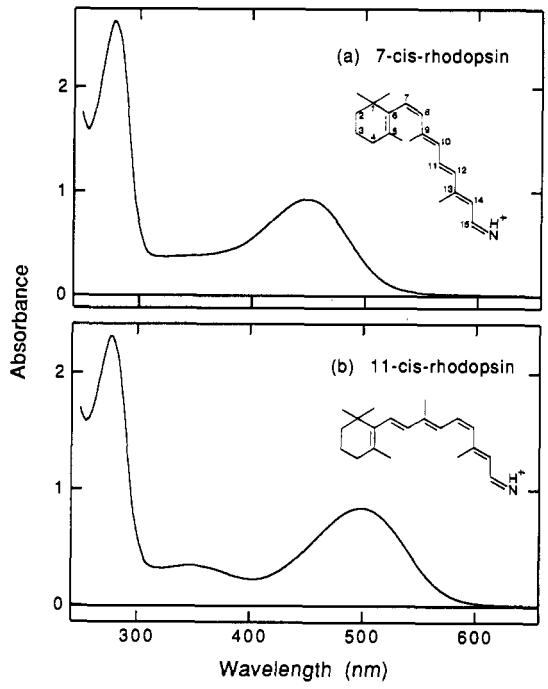

FIGURE 1: Absorption spectra of 7-*cis*- (a) and 11-*cis*-rhodopsins (b) prepared from the same opsin preparation. The measurements were performed by using an optical cell with 2-mm light path.

For low-temperature spectrophotometry, the neutralized hydroxylamine and glycerol were added to the extracts to final concentrations of 10 mM and 66%, respectively. Although 7-*cis*-rhodopsin is relatively unstable against the hydroxylamine at room temperature, it was added to the sample to diminish any spectral contribution of the random Schiff bases which may be produced on irradiation of 7-*cis*-rhodopsin. As a result, a part of 7-*cis*-rhodopsin in the sample was decomposed in the course of the preparation.

As a sample for picosecond or nanosecond laser photolysis, the concentrated extract was prepared. If necessary, the neutralized hydroxylamine was added to the sample immediately before the experiments in order to reduce the decomposition of 7-*cis*-rhodopsin by the hydroxylamine.

Spectrophotometry at Low Temperatures. To measure the absorption spectra at low temperatures (from 0 to −196 °C), a specially designed glass cryostat with quartz windows (Yoshizawa & Shichida, 1982) was used. The temperature of the sample was monitored with a copper–constantan thermocouple attached to the sample cell holder. The sample was irradiated with a light from a xenon lamp (2 kW, Ushio) which had passed through a glass cutoff filter (Toshiba) with or without an interference filter (Nihonshinku). A 5-cm water layer was placed in front of the lamp to remove heat radiation contained in the irradiation light. Absorption spectra were recorded on the Shimadzu spectrophotometer interfaced with an NEC PC-9801F computer. For correction of scattering due to the optical system, opal glass was placed in both sample and reference sides of the spectrophotometer.

An Optical System for Measurements of Picosecond Absorption Spectra. A double-beam spectrophotometer linked with a mode-locked Nd^{3+}:YAG laser (Yoshihara et al., 1979; Shichida et al., 1984) was used. From a train of picosecond pulses (1064 nm, 15 ps) generated by a laser oscillator, one

[1] Abbreviations: ROS, rod outer segment(s); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

of the most intense pulses was extracted by a single-pulse selector. The pulse was amplified and then divided into two pulses by a beam splitter. One of the pulses, having 99% of the total energy of the original pulse, was amplified again, while the other pulse having 1% of the total energy of the original pulse was amplified twice.

The former pulse was used for generating a blue pulse (449 nm), with which a sample was excited after passing through a movable prism for adjusting the arrival times of both excitation and probe pulses to the sample. The blue pulse was generated from the third harmonic pulse (355 nm) by passing through a set of phase-matched KDP crystals. After eliminating both the fundamental pulse (1064 nm) and the second harmonic pulse (532 nm) from the third harmonic pulse by a combination of filters (HA30, Hoya; UV33, Toshiba), it was focused on the center of a 10-cm optical cell containing cyclohexane to produce blue pulses owing to the Raman effect of the C–H elastic vibration (first Stokes, 400 nm; second Stokes, 449 nm). Then the second Stokes pulse was extracted from the pulses by passing through a combination of cutoff and interference filters (VY-42, -44, 448 nm; Toshiba) and used for excitation of the sample.

For generation of probe pulses to measure spectra of the sample, the fundamental pulse was focused onto ground glass (SPM) to convert it to a picosecond white pulse. The pulse was then divided into two pulses, one for monitoring an absorbance change of the sample (I^{sam}) and the other for checking the intensity and spatial distribution of the pulse (I^{ref}). Each pulse was focused onto the slit of each polychrometer, and its intensity was detected by a multichannel photodiode array (Unisoku).

An outline of each experiment is as follows: First, intensities of probe pulses, one of which was passed through the sample (10 μL) in a 2 × 2 mm optical cell with a 1.8-mm aperture and the other only through a 1.8-mm aperture, were monitored (I_n^{sam} and I_n^{ref}) without excitation of the sample. Then they were monitored (I_e^{sam} and I_e^{ref}) at an adequate time after excitation of the sample. Difference absorbance (ΔA) was calculated by the equation:

$$\Delta A = \log \frac{I_n^{sam} I_e^{ref}}{I_n^{ref} I_e^{sam}}$$

Difference spectra were calculated and analyzed by a computer (PC-9801, NEC).

During the course of each experiment, the intensity of the excitation pulse was monitored by a biplanar photodiode (Hamamatsu) connected with an oscilloscope, the signal of which was calibrated by a joule meter (ED100, Gentic) placed at the sample position at the end of the experiments. Since the absorbance increase at 530 nm (due to generating a bathochromic product; see Results) after excitation of 7-*cis*-rhodopsin was proportional to the excitation photon density up to 100 μJ/1.8 mmϕ, the sample was usually excited with a pulse of about 100 μJ/1.8 mmϕ. Under these conditions, any artificial absorbance change due to photoreaction of the photoproduct was not observed.

An Optical System for Measurements of Kinetics on the Nanosecond to Millisecond Time Scale. A sample was excited with a nanosecond blue pulse (17 ns, 449 nm) generated from an excimer-pumped dye laser (EMG 101 MSC and FL 3002, Lambda Physics), and the absorbance changes of the sample at selected wavelengths after the excitation were monitored by using a photographic flashlamp (622, Sanpak) or a xenon continuous lamp (L2274, Hamamatsu). When the xenon continuous lamp was used as the monitoring light, special care was taken to avoid any bleaching of the sample; the lamp was placed in a dark box with a special shutter system so that the light was passed through the sample only when necessary for monitoring the absorbance change of the sample. The monitoring light was focused onto a 1.5-mm aperture in front of the sample cell (2 × 2 mm) through an interference filter. The sample was excited perpendicularly. Magic-angle excitation was applied; the monitoring light was vertically polarized, and the excitation pulse was polarized at 54.7° from the vertical. By use of this technique, any effects of tumbling of the pigment or reorientation of the chromophore after photoexcitation of the pigment were nullified so that the absorbance changes that are not intrinsic to the photobleaching of the pigment were avoided (Nagle et al., 1982).

The monitoring light was focused onto a slit of a monochromator (H25, Jobin Yvon), and its intensity was measured by a photomultiplier (R666s, Hamamatsu) whose signals were monitored by a storage scope (TS8123, Iwatsu). The digitized light signals were then processed by a computer (NEC PC9801). The maximum time resolution of the storage scope was 0.39 ns per channel.

Photon density of the excitation pulse was about 280 μJ/1.5 mmϕ in the experiments of 7-*cis*-rhodopsin and 50 μJ/1.5 mmϕ in those of 11-*cis*-rhodopsin. Under these conditions, no photoreactions of intermediates were observed, because the absorbance changes were linearly proportional to the excitation photon densities.

Results

Low-Temperature Spectrophotometry of 7-cis-Rhodopsin. The experimental results which caused us to reinvestigate the photochemical reactions of 7-*cis*-rhodopsin are shown in Figure 2. A 7-*cis*-rhodopsin–66% glycerol sample was cooled to liquid nitrogen temperatures without any cracks in the sample (curve 1″ in Figure 2c) and irradiated with 440-nm light for 16 h, resulting in formation of a mixture containing mainly the batho intermediate of 7-*cis*-rhodopsin (curve 2″ in Figure 2c). The mixture was then irradiated with red light (>630 nm) to convert only the batho intermediate in the mixture to presumably 11-*cis*- and 9-*cis*-rhodopsins (curve 3″ in Figure 2c). The difference spectrum before and after irradiation with the red light was calculated (curve 5″ in Figure 2d) and compared with those calculated from similar experiments using 11-*cis*-rhodopsin– and 9-*cis*-rhodopsin–66% glycerol samples (curves 5 and 5′ in Figure 2d). Though the difference spectrum obtained from 9-*cis*-rhodopsin is identical in spectral shape with that from 11-*cis*-rhodopsin, that obtained from 7-*cis*-rhodopsin is clearly different from those of 11-*cis*- and 9-*cis*-rhodopsins. Although it is difficult to conclude from the difference spectra whether all the pigments contained in the mixture produced from 7-*cis*-rhodopsin were different in spectral shape from those from 11-*cis*- and 9-*cis*-rhodopsins, the fact that the long-wavelength tail of the spectrum of the photo-steady-state mixture produced from 7-*cis*-rhodopsin (curve 2″ in Figure 2c) had absorbances less than those from 11-*cis*- and 9-*cis*-rhodopsins (curves 2 and 2′ in Figure 2a,b) suggested that the spectrum of the batho intermediate produced from 7-*cis*-rhodopsin is blue-shifted from that of bathorhodopsin.

Irradiation of 7-*cis*-rhodopsin with 440-nm light for 16 h resulted in photoreversible reactions among the batho intermediate and 11-cis and 9-cis pigments produced from 7-*cis*-rhodopsin via the batho intermediate (Figure 2c). This was checked by the following experiments. The mixture of 11-cis and 9-cis pigments (curve 3″ in Figure 2c) was reirradiated with 440-nm light to form the mixture containing mainly the batho intermediate (curve 4″ in Figure 2c) whose spectrum

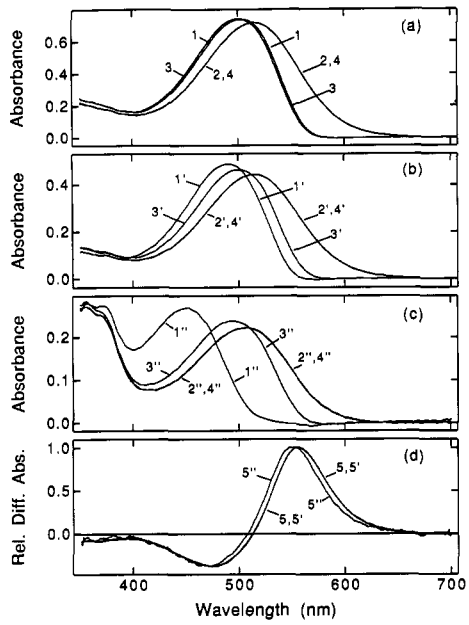

FIGURE 2: Formation of batho intermediates from 11-*cis*-, 9-*cis*-, and 7-*cis*-rhodopsins at −190 °C. 11-*cis*- (a), 9-*cis*- (b), and 7-*cis*-rhodopsin (c) samples were cooled to −190 °C without any cracks in the samples by the rapid-cooling technique (curves 1, 1′, and 1″) and irradiated with 440-nm light for 640, 640, and 61 200 s, respectively, to form their batho intermediates (curves 2, 2′, and 2″). Then they were irradiated with red light at wavelengths longer than 630 nm for 1280 s to convert the batho intermediates to a mixture of 11-cis and 9-cis pigments (curves 3, 3′, and 3″). The reirradiation with 440-nm light for 640 s produced a mixture whose spectra (curves 4, 4′, and 4″) were identical with those produced earlier under similar irradiation. Finally, difference spectra before and after irradiation with red light were calculated (curves 5, 5′, and 5″).

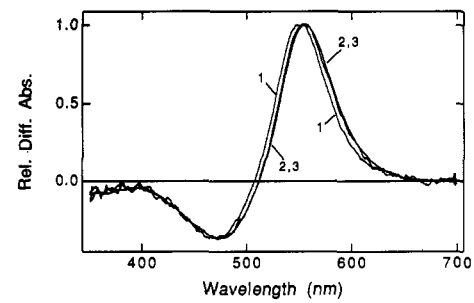

FIGURE 3: Difference spectra obtained from a series of irradiations of 11-cis and 9-cis pigments from 7-*cis*-rhodopsin before and after warming to −85 °C. A 7-*cis*-rhodopsin–66% glycerol mixture was cooled to −185 °C and irradiated with 436-nm light for 6 h to produce a mixture containing mainly the batho intermediate of 7-*cis*-rhodopsin. Then the sample was irradiated with red light at wavelengths longer than 630 nm for 1280 s. The difference spectrum before and after irradiation with red light was calculated (curve 1). After being warmed to −85 °C and then recooled to −185 °C, the sample was irradiated with 436-nm light for 1280 s, followed by irradiation with red light for 1280 s. The difference spectrum before and after irradiation with red light was calculated (curve 2). Curve 3 is the difference spectrum between bathorhodopsin produced from 11-*cis*-rhodopsin and 11-*cis*- and 9-*cis*-rhodopsins. That curve 2 was almost identical with curve 3 indicated that 11-cis and 9-cis pigments produced from 7-*cis*-rhodopsin change to 11-*cis*- and 9-*cis*-rhodopsins on warming to −85 °C, resulting in formation of the same bathorhodopsin produced from 11-*cis*- and 9-*cis*-rhodopsins.

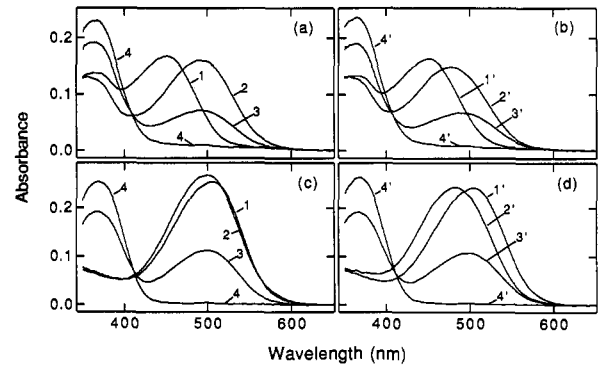

FIGURE 4: Low-temperature spectra used to calculate difference absorption spectra of lumi and meta I intermediates of 7-*cis*- and 11-*cis*-rhodopsins. (a) and (b) Low-temperature spectroscopy of 7-*cis*-rhodopsin. 7-*cis*-Rhodopsin was cooled to −80 °C (curve 1) or −30 °C (curve 1′) for measurements of the spectrum and then cooled to −190 °C, where the sample was irradiated with 436-nm light for 14 400 s to form the batho intermediate. Then the sample was warmed to −80 or −15 °C to convert the batho intermediate in the sample to the lumi or meta I intermediate whose spectrum was measured at −80 °C (curve 2) or −30 °C (curve 2′), respectively. The sample was further warmed to 10 °C to bleach the intermediate in the sample into retinal oxime and opsin. After measurement of the spectrum of the sample at −80 °C (curve 3) or −30 °C (curve 3′), the sample was irradiated with orange light (>500 nm) to completely bleach the sample. The spectrum was then measured at −80 °C (curve 4) or −30 °C (curve 4′). (c) and (d) Low-temperature spectroscopy of 11-*cis*-rhodopsin. The experimental procedures are identical with (a) and (b) except that the irradiation time of the 11-*cis*-rhodopsin sample with 436-nm light was 640 s.

was identical with that produced earlier under similar irradiation (curve 2″ in Figure 2c). Then the batho intermediate in the mixture was irradiated with red light to convert it to 11-cis and 9-cis pigments. The difference spectrum before and after red light irradiation displayed the same spectral shape as curve 5″ in Figure 2d. These facts also suggested that the 11-cis and 9-cis pigments produced from 7-*cis*-rhodopsin are different from those in the regenerated 11-*cis*- and 9-*cis*-rhodopsin samples. If 11-cis and 9-cis pigments produced from the batho intermediate of 7-*cis*-rhodopsin were identical with those contained in the 11-*cis*-rhodopsin sample, the same difference spectrum as that calculated from the 11-*cis*-rhodopsin sample (curve 4 in Figure 2d) would be obtained.

The difference might be because freezing of the protein moiety does not allow the 11-*cis*- and 9-*cis*-retinylidene chromophores to assume the same position relative to the protein moiety as in the regenerated samples. In fact, after the mixture is warmed to −85 °C and then recooled to −180 °C, almost the same difference spectrum as curve 5 in Figure 2d was obtained by the series of irradiations of the mixture (Figure 3). Therefore, warming the mixture causes some rearrangement of the protein moiety as well as the chromophores, resulting in forming the same 11-*cis*- and 9-*cis*-rhodopsins as those in the regenerated samples.

Second, we investigated whether or not the subsequent intermediates thermally produced from the batho intermediate of 7-*cis*-rhodopsin have the same spectral shapes as those of 11-*cis*-rhodopsin. Figure 4 shows the experiments used to calculate the spectra of lumi and meta I intermediates of 7-*cis*- and 11-*cis*-rhodopsins. For calculation of the lumi and meta I intermediates of 7-*cis*-rhodopsin, a 7-*cis*-rhodopsin sample was cooled to −185 °C after spectra were measured at −80 and −30 °C (curves 1 and 1′ in Figure 4a and Figure 4b, respectively) and irradiated with 436-nm light to convert it to a mixture of the batho intermediate and 11-cis and 9-cis pigments. Then it was warmed to −80 or −15 °C to convert the batho intermediate in the mixture to the lumi or meta I intermediate (curves 2 and 2′ in Figure 4a and Figure 4b, respectively). It should be noted that the 11-cis and 9-cis pigments produced from 7-*cis*-rhodopsin at −185 °C were also converted to 11-*cis*- and 9-*cis*-rhodopsins by the warming. The sample was warmed to 10 °C to decompose the lumi or meta I intermediate to retinal oxime and opsin (curves 3 and 3′ in Figure 4a and Figure 4b, respectively) and then irradiated with

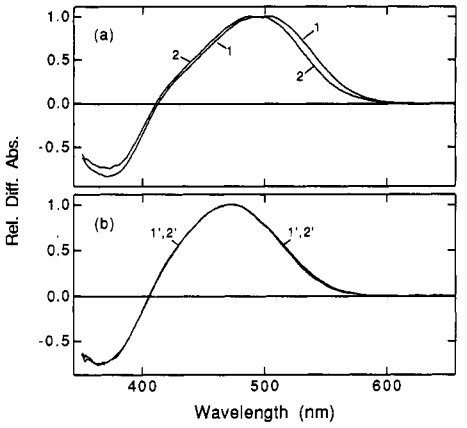

FIGURE 5: Difference absorption spectra of lumi (a) and meta I (b) intermediates of 11-*cis*- (curves 1 and 1') and 7-*cis*-rhodopsins (curves 2 and 2'). The spectrum represents the spectral difference between the lumi or meta I intermediate and retinal oxime.

orange light (>520 nm) to completely bleach the residual rhodopsin isomers in the mixture (curves 4 and 4' in Figure 4a, and Figure 4b, respectively). The spectra of the lumi and meta I intermediates (difference spectra between lumi and meta I intermediates and retinal oxime) were calculated by subtracting curves 3 and 3' from curves 2 and 2' in Figure 4a and Figure 4b, respectively (curves 2 and 2' in Figure 5a and Figure 5b, respectively). Similar experiments were done with the 11-*cis*-rhodopsin–66% glycerol sample (Figure 4c,d), and the spectra of lumirhodopsin and metarhodopsin I were also calculated (curves 1 and 1' in Figure 5a and Figure 5b, respectively). It is clearly indicated that the lumi intermediate of 7-*cis*-rhodopsin (λ_{max} 492 nm) is different in spectral shape from that of lumirhodopsin (λ_{max} 497 nm) while the meta I intermediate of 7-*cis*-rhodopsin (λ_{max} 475 nm) is identical with that of metarhodopsin I.

Picosecond Laser Photolysis of 7-cis-Rhodopsin. Since low-temperature spectrophotometry has clearly shown that the intermediates appearing at the early stages of the photobleaching process of 7-*cis*-rhodopsin have different spectra from those of rhodopsin, the primary photochemical reaction of 7-*cis*-rhodopsin was investigated by means of picosecond laser photolysis with attention to the formation of an intermediate corresponding to photorhodopsin. Figure 6 shows difference spectra between 7-*cis*-rhodopsin and the intermediate(s) measured at 0, 200, and 850 ps and 1.5 ns after excitation with a picosecond blue pulse (449 nm, 15 ps, 100 μJ/1.8 mmϕ), respectively. The spectra obtained were similar in shape, having peaks at 528 nm and intersection points at 495 nm, although the spectrum at 1.5 ns has less absorbance at wavelengths longer than 530 nm. This result showed that probably one bathochromic intermediate (the batho intermediate of 7-*cis*-rhodopsin) was produced immediately after the excitation and was stable on the picosecond time scale. Therefore, an intermediate corresponding to photorhodopsin in the 11-*cis*-rhodopsin system was not observed in the 7-*cis*-rhodopsin system within our experimental resolution.

Since it was very hard to estimate a precise percentage of bleaching of 7-*cis*-rhodopsin after excitation with a picosecond laser pulse, we could not determine the absorption maximum of the batho intermediate of 7-*cis*-rhodopsin at room temperature. However, it is clear that the peak (528 nm) of the difference spectrum between 7-*cis*-rhodopsin and its batho intermediate was located at a wavelength longer than the absorption maximum of the batho intermediate. In fact, the absorption maximum of the batho intermediate was estimated to be 523, 521, or 519 nm if the percentage of bleaching of

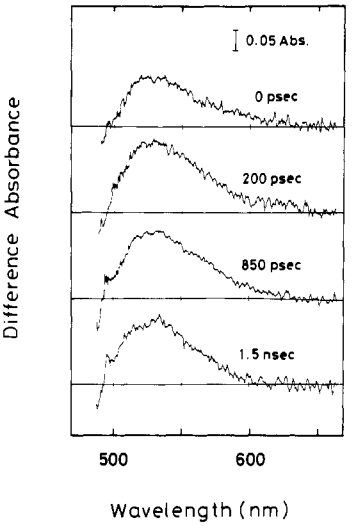

FIGURE 6: Transient absorption spectra measured at 0 ps, 200 ps, 850 ps, and 1.5 ns after excitation of 7-*cis*-rhodopsin with the 449-nm pulse (width, 15 ps). The excitation photon density was 100 μJ/1.8 mmϕ. Each spectrum represents the average of 50–80 measurements.

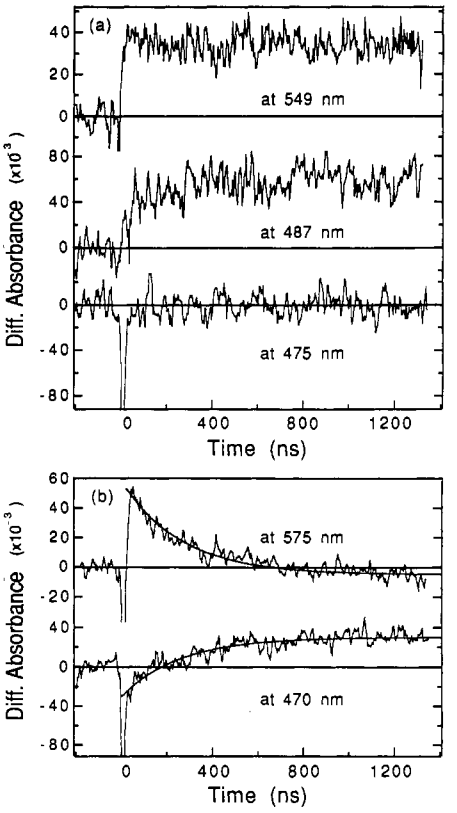

FIGURE 7: Kinetics of 7-*cis*- (a) and 11-*cis*-rhodopsins (b) on a nanosecond time scale measured after magic-angle excitation with a nanosecond blue pulse (449 nm, 17 ns). (a) Kinetic profiles of 7-*cis*-rhodopsin at 549 (top), 487 (middle), and 475 nm (bottom). Each curve is an average of 15–21 experiments. (b) Kinetic profiles of 11-*cis*-rhodopsin at 575 nm (top) and 470 nm (bottom). Each curve is the average of 27 or 28 measurements. The smooth curves are the fitting curves having a time constant of 250 ns.

7-*cis*-rhodopsin was assumed to be 10, 15, or 20%, respectively. Therefore, the batho intermediate of 7-*cis*-rhodopsin should have an absorption maximum at shorter wavelengths than that of 11-*cis*-rhodopsin (535 nm at room temperature; Kandori et al., 1989c).

Nanosecond Laser Photolyses of 7-cis- and 11-cis-Rhodopsins. The decay process of the batho intermediate of 7-*cis*-rhodopsin was also measured and compared with that

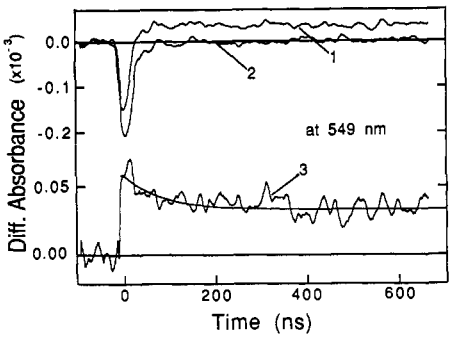

FIGURE 8: Estimation of the decay time constant of the batho intermediate of 7-*cis*-rhodopsin. The 7-*cis*-rhodopsin sample was excited with the nanosecond blue pulse for the kinetic measurements (curve 1). Then the sample was completely bleached with yellow light (>500 nm) without moving the cell in the optical setup for nanosecond photolysis, followed by measurement of the kinetics after excitation with a nanosecond blue pulse (curve 2). Curve 3 shows a kinetic profile calculated by subtraction of curve 2 from curve 1. Each curve is an average of nine measurements. The smooth curve is the fitted curve having a time constant of 40 ns.

of 11-*cis*-rhodopsin (Figure 7). Both 7-*cis*- and 11-*cis*-rhodopsin samples were excited by a nanosecond blue pulse (449 nm, 17 ns), and the absorbance changes were monitored by using the photographic flashlamp. Figure 7b shows a decay process of bathorhodopsin from 11-*cis*-rhodopsin monitored at 575 and 470 nm. Since the laser pulse we have used had a pulse width of 17 ns, the kinetic data shown in Figure 7b displayed some undershoot due to scattering of the excitation pulse. Therefore, the kinetic data monitored before 50 ns were neglected. Then the decay time constant of bathorhodopsin was estimated to be 250 ns by fitting the kinetic data monitored after 50 ns with a single-exponential curve.

On the other hand, excitation of 7-*cis*-rhodopsin with the nanosecond blue pulse caused an instantaneous increases of the absorbances at 549 and 487 nm, while the absorbance at 475 nm of the sample did not change after the excitation (Figure 7a). Since the isosbestic point between 7-*cis*-rhodopsin and its batho intermediate produced on the picosecond time scale is around 490 nm (Figure 6), these results indicated that the batho intermediate had already decayed and another intermediate, probably formed from the batho intermediate, had formed within 50 ns. In order to observe the decay of the batho intermediate, we tried to cancel the scattering effect of the excitation pulse on the kinetic data by subtracting the signals from a bleached sample from those of an unbleached sample (Figure 8). The data (curve 3 in Figure 8) demonstrated that the decay time constant of the batho intermediate of 7-*cis*-rhodopsin was ~40 ns.

The following decay processes were measured on submillisecond time scales using a xenon continuous lamp as the monitoring light. Figure 9a shows the decay processes of the lumi intermediate of 7-*cis*-rhodopsin monitored at 535 and 487 nm. Both processes were fit by a single-exponential curve whose time constant was 300 μs. The single-exponential fittings, however, were unable to be adopted to the decay process of lumirhodopsin (Figure 9b) in which the fitting needed at least two exponential curves. The reason is that the decay time constant of lumirhodopsin may be close to that of metarhodopsin I. In fact, the decay process of a sample composed of mainly metarhodopsin I measured on the millisecond time scale (Figure 10b) was also unable to be expressed by a single-exponential curve. Therefore, the decay of lumirhodopsin and then metarhodopsin I (Figures 9b and 10b) was approximated by two sequential single-exponential curves (lumirhodopsin → metarhodopsin I → metarhodopsin II), and their

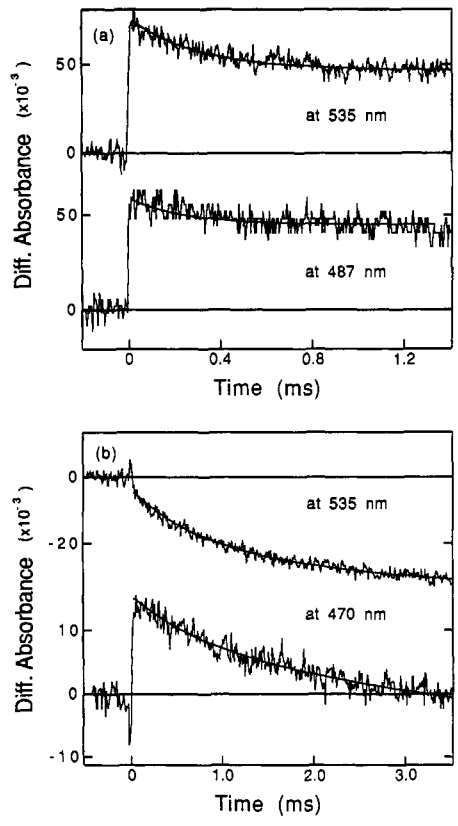

FIGURE 9: Kinetics of 7-*cis*- and 11-*cis*-rhodopsins on a submillisecond time scale measured after magic-angle excitation with a nanosecond blue pulse (449 nm, 17 ns). (a) Kinetics of 7-*cis*-rhodopsin at 535 nm (top) and 478 nm (bottom). They were expressed by the single-exponential curves (smooth lines), whose time constant was 300 μs. (b) Kinetics of 11-*cis*-rhodopsin at 535 nm (top) and 470 nm (bottom). The smooth curves represent the fitted curves which can be expressed by two sequential processes (L → MI → MII). Each time constant was estimated to be 800 μs and 15 ms, respectively.

time constants were estimated to be 800 μs and 12 ms, respectively.

Figure 10a shows the decay process of the meta I intermediate of 7-*cis*-rhodopsin, whose time constant was estimated to be 12 ms by a single-exponential fitting.

Discussion

The present experiments clearly show that the batho and lumi intermediates of 7-*cis*-rhodopsin have different spectroscopic and kinetic properties from those of 11-*cis*-rhodopsin. Furthermore, an intermediate corresponding to photorhodopsin was unable to be observed in 7-*cis*-rhodopsin. On the other hand, the meta I intermediate of 7-*cis*-rhodopsin was identical in spectrum and decay time constant with that of 11-*cis*-rhodopsin (Figures 5b and 11). Therefore, the different configuration of the chromophore between 7-*cis*- and 11-*cis*-rhodopsins led to different chromophore–opsin interactions in the early photobleaching processes, even when their chromophores are photoisomerized to the all-trans configuration.

It is important to know the kinds of differences in the chromophore–opsin interaction between the original 7-*cis*- and 11-*cis*-rhodopsins in order to elucidate the difference in photobleaching process between them. In rhodopsin, it has been supposed that both ends of the chromophore are fixed in a restricted chromophore binding site by a hydrophobic interaction between the trimethylcyclohexenyl ring (β-ionone ring) of the chromophore and surrounding protein and by the α-helix through the butyl tether of the lysine residue (Matsumoto & Yoshizawa, 1975; Liu & Asato, 1985). The fixation of the

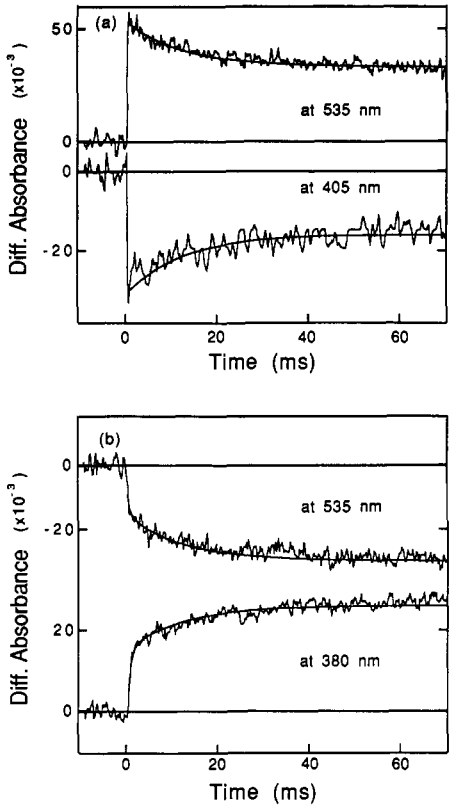

FIGURE 10: Kinetics of 7-*cis*- and 11-*cis*-rhodopsins on a millisecond time scale measured after excitation with the nanosecond blue pulse (449 nm, 17 ns). Kinetics of 7-*cis*-rhodopsin at 535 nm (a, top) and 405 nm (a, bottom) were expressed by a single-exponential curve, whose time constant was 12 ms. Kinetics of 11-*cis*-rhodopsin at 535 nm (b, top) and 380 nm (b, bottom) were composed of two sequential processes (L → MI → MII) whose time constants were estimated to be 800 μs and 12 ms, respectively.

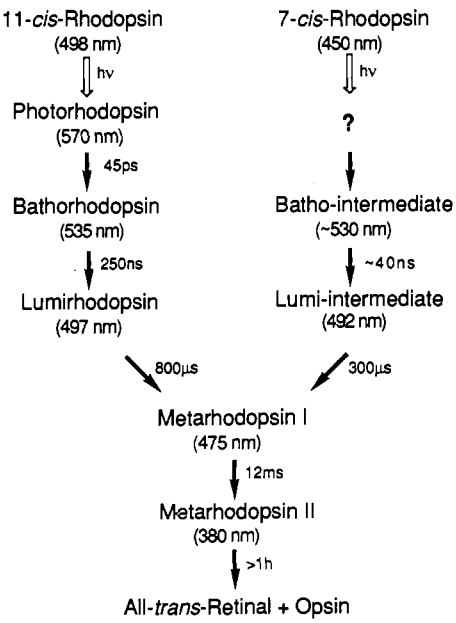

FIGURE 11: Photobleaching processes of 7-*cis*- and 11-*cis*-rhodopsins. The values in parentheses show absorption maxima. The decay time constants are shown on the right-hand side of the arrows.

cyclohexenyl ring may be important to explain the difference in the photobleaching between 7-*cis*- and 11-*cis*-rhodopsins. 11-*cis*-Retinal has a bent structure near the center of the side chain and has a slightly twisted structure between the cyclohexenyl ring and the side chain, while 7-*cis*-retinal has a planar side chain and a highly twisted structure between the cyclo-

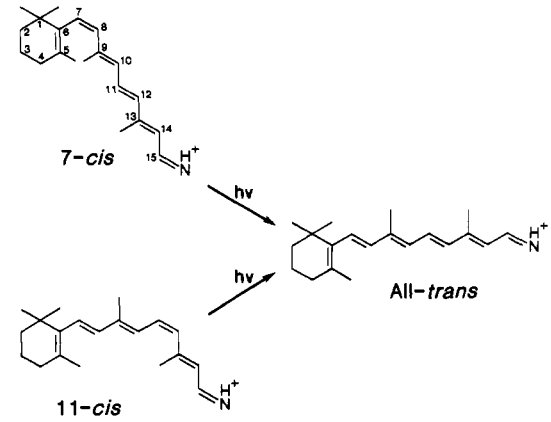

FIGURE 12: Schematic representation of the photoisomerization of 7-*cis*- and 11-*cis*-retinylidene chromophores to the all-trans form.

hexenyl ring and the side chain owing to the existence of high steric hindrance between the methyl groups at the 5- and 9-positions (Figure 12). Therefore, the interaction between the cyclohexenyl ring of the chromophore and the surrounding protein in 11-*cis*-rhodopsin may be different from that in 7-*cis*-rhodopsin (DeGrip et al., 1976). The difference in interaction may cause the difference in absorption maximum between them, because it depends on the distance between the protonated Schiff base and its counterion (Fukada et al., 1990). However, a recent investigation using 9-demethylretinal suggested that the absence of an interaction between the 9-methyl group of the chromophore and its surrounding protein causes a blue shift of the absorption maximum of rhodopsin (Blatz et al., 1969; Kropf et al., 1973; Ganter et al., 1989). This effect may be also applicable to the blue shift of the absorption maximum of 7-*cis*-rhodopsin, because in 7-*cis*-rhodopsin, the 9-methyl group of the 7-*cis*-chromophore is placed on the opposite side of that of the 11-cis chromophore in rhodopsin, resulting in a similar lack of such interaction.

On absorption of light, the chromophore of 11-*cis*-rhodopsin as well as those of 7-*cis*- and 9-*cis*-rhodopsins converts to the all-trans form. It was suggested that the photoisomerization of the chromophores in 11-*cis*- and 9-*cis*-rhodopsins proceeds by movement of the half of the polyene chain containing the Schiff base (Shichida et al., 1987). If a similar movement occurs in the chromophore isomerization of 7-*cis*-rhodopsin, a unique displacement of the 9-methyl group should take place (Figure 12), resulting in a new chromophore–opsin interaction between the 9-methyl group of the chromophore and the protein moiety. This interaction should be diferent from that in the bathoproducts of 11-*cis*- and 9-*cis*-rhodopsins because the bathoproduct of 7-*cis*-rhodopsin is different in absorption maximum from those of 11-*cis*- and 9-*cis*-rhodopsins. The difference is retained in the lumi stage as shown by the different absorption maxima between the lumi intermediates of 7-*cis*-rhodopsin and 11-*cis*- and 9-*cis*-rhodopsins. Furthermore, the batho and lumi intermediates of 7-*cis*-rhodopsin have shorter decay time constants than those of 11-*cis*- and 9-*cis*-rhodopsins. These facts may indicate that the 7-*cis*-rhodopsin system is less stable (or different) in interaction between the 9-methyl group of the chromophore and surrounding protein than the 11-cis and 9-cis systems. Therefore, the binding site of the 9-methyl group of the chromophore in the early intermediates of 7-*cis*-rhodopsin may have a more strained conformation than those of rhodopsin, because the 9-methyl group of 7-*cis*-rhodopsin would be unable to be fit into the right position of the binding site during the isomerization from the 7-cis to the all-trans form.

As already described, the meta I intermediate of 7-*cis*-rhodopsin is identical in spectrum and decay time constant with that of rhodopsin. Therefore, the unique interaction between the 9-methyl group of the chromophore and surrounding protein has disappeared by the meta I stage, probably owing to conformational changes in the protein.

There could be another explanation for the difference between 7-*cis*- and 11-*cis*-rhodopsins in the early photobleaching process. Since a twisted 6-s-trans conformer of 7-*cis*-retinal is expected to be very close in energy to the twisted 6-s-cis conformer, 7-*cis*-rhodopsin might have a 6-s-*trans*-7-*cis*-retinal as its chromophore. If this is true, the difference in spectroscopic character between the early intermediates of 7-*cis*- and 11-*cis*-rhodopsins could be explained by the difference in conformation of the cyclohexenyl ring of the chromophore. The weak point of this explanation, however, is one must assume the cyclohexenyl ring rotates during the lumi–meta I transition. Since the meta I intermediate as well as the lumi intermediate has induced circular dichroism due to the asymmetric binding of the chromophore to the protein (Waggoner & Stryer, 1971; Yoshizawa & Horiuchi, 1973; Ebrey & Yoshizawa, 1973), it is unlikely that during the transition the rather bulky cyclohexenyl ring could rotate in the protein. The fact that the lumi intermediate of 7-*cis*-rhodopsin is converted to the meta I intermediate faster than that of 11-*cis*-rhodopsin also does not support such a rotation. Furthermore, our experiments showed that 11-cis and/or 9-cis pigments produced by the irradiation of 7-*cis*-rhodopsin at liquid nitrogen temperature were identical with the 11-*cis*- and/or 9-*cis*-rhodopsins upon warming to −85 °C. It is also unrealistic that the cyclohexenyl ring could rotate at such a low temperature. Therefore, the difference in absorption spectrum between lumi intermediates as well as batho intermediates of 7-*cis*- and 11-*cis*-rhodopsins may not be explained only by the difference in the initial conformation of the cyclohexenyl ring.

From the present results together with those obtained previously, the change in chromophore–opsin interactions between bathorhodopsin and metarhodopsin I will be discussed. The chromophore is photoisomerized by movement of half of the polyene chain containing the Schiff base to form bathorhodopsin. The subsequent interaction change between the cyclohexenyl ring region of the chromophore and surrounding protein produces lumirhodopsin. A highly twisted conformation of the chromophore of bathorhodopsin may force inducement of a conformational change of the protein near the cyclohexenyl ring of the chromophore. Lumirhodopsin is then converted to metarhodopsin I by the disappearance of the interaction between the 9-methyl group of the chromophore and surrounding protein.

As shown in Figure 6, we failed to detect a putative "photorhodopsin" from 7-*cis*-rhodopsin, although photorhodopsin was detected as a precursor of bathorhodopsin in rhodopsin (Shichida et al., 1984; Kandori et al., 1989a). There are at least two possibilities to explain this. One is that the batho intermediate is directly produced from the excited state of 7-*cis*-rhodopsin, and the other is that the decay time constant of a putative photorhodopsin of 7-*cis*-rhodopsin was too short to be detected with our experimental conditions. The former suggests that the photochemical reaction pathway of 7-*cis*-rhodopsin is somewhat different from that of rhodopsin as described below, while the latter is consistent with the results that the following intermediates of 7-*cis*-rhodopsin have also smaller decay time constants than those of 11-*cis*-rhodopsin. Since the photosensitivity of 7-*cis*-rhodopsin is about 7 times less than that of rhodopsin (our unpublished results), the different interaction between the cyclohexenyl ring region of the chromophore and the surrounding protein and the absence of interaction between the 9-methyl group and the surrounding protein may cause the difference in the photochemical reaction pathway between 7-*cis*- and 11-*cis*-rhodopsins.

In the course of nanosecond laser photolytic experiments, we observed some interesting results concerning the rotational motion of the 7-*cis*-rhodopsin sample. Since we applied the magic-angle measurement to the photolytic experiments, no change of absorbance on the submicrosecond time scale was observed. This indicated that the lumi intermediate of 7-*cis*-rhodopsin is stable on this time scale. However, a decrease or increase of absorbance at 535 nm was observed when measured by the monitoring light polarized parallel or vertical to the excitation pulse, respectively. On the other hand, the plateau levels of the absorbance are the same as those measured by magic-angle excitation. The time constant of the absorbance change was estimated to be 630 ns at 22 °C. Since the changes of absorbance thus measured are due to the rotation of the 7-*cis*-rhodopsin–digitonin complex in the sample, the effective radius of the complex was calculated to be 60 Å on the assumption that the complex is spherical and the viscosity of the sample is the same as that of water (0.01 P).

It should be noted that in Figure 10a the positive absorbance at 535 nm was retained even at the meta II stage of 7-*cis*-rhodopsin. Since the absorbance changes at 535 nm were measured as the differences between 7-*cis*-rhodopsin and its intermediate, they should be zero or negative because 7-*cis*-rhodopsin has a small absorbance and the meta II intermediate has no absorbance at 535 nm. This may be attributed to the formation of a product different from the meta II intermediate. Possible formation of the meta III (or para) intermediate in the sample was denied by the observation that the product was stable even in the presence of the hydroxylamine. The formation of the product was linearly correlated with the photon density of the excitation pulse, suggesting that it is not produced by photon absorption of a bleaching intermediate of 7-*cis*-rhodopsin. A plausible interpretation is that it is 11-*cis*- or 9-*cis*-rhodopsin thermally produced from one of the bleaching intermediates of 7-*cis*-rhodopsin. Such a back-isomerization to 11-cis and/or 9-cis isomers was already reported in the case of iodopsin (Yoshizawa & Wald, 1967) and a rhodopsin analogue having 6-s-cis-fixed bicyclic retinal (Yoshizawa et al., 1987). If so, the lack of a stable interaction between the 9-methyl group of the chromophore and the surrounding protein may cause a thermal isomerization around the $C_{11}=C_{12}$ or $C_9=C_{10}$ bonds, although the percentage of conversion to the product was estimated to be less than 5% of the total conversion of the intermediate produced.

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Registry No. 7-*cis*-Retinal, 24315-14-8.

References

Blatz, P. E., Lin, M., Balasubramaniyan, P., Balasubramaniyan, V., & Dewhurst (1969) *J. Am. Chem. Soc. 91*, 5930–5931.

DeGrip, W. J., Liu, R. S. H., Ramamurthy, V., & Asato, A. (1976) *Nature 262*, 416–418.

Ebrey, T. G., & Yoshizawa, T. (1973) *Exp. Eye Res. 17*, 545–556.

Eyring, G., Curry, B., Broek, A., Lugtenburg, J., & Mathies, R. (1982) *Biochemistry 21*, 384–393.

Franke, R. R., Sakmar, T. P., Oprian, D. D., & Khorana, H. G. (1988) *J. Biol. Chem. 263*, 2119–2122.
Fukada, Y., Shichida, Y., Yoshizawa, T., Ito, M., Kodama, A., & Tsukida, K. (1984) *Biochemistry 23*, 5826–5832.
Fukuda, Y., Okano, T., Shichida, Y., Yoshizawa, T., Trehan, A., Mead, D., Denny, M., Asato, A. E., & Liu, R. S. H. (1990) *Biochemistry 29*, 3133–3139.
Ganter, U. M., Schmid, E. D., Perez-Sala, D., Rando, R. R., & Siebert, F. (1989) *Biochemistry 28*, 5954–5962.
Hubbard, R., & Kropf, A. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 130–139.
Kandori, H., Matuoka, S., Shichida, Y., & Yoshizawa, T. (1989a) *Photochem. Photobiol. 49*, 181–184.
Kandori, H., Matuoka, S., Shichida, Y., Yoshizawa, T., Ito, M., Tsukida, K., Balogh-Nair, V., & Nakanishi, K. (1989b) *Biochemistry 28*, 6460–6467.
Kandori, H., Shichida, Y., & Yoshizawa, T. (1989c) *Biophys. J. 56*, 453–457.
Kawamura, S., Miyatani, S., Matsumoto, H., Yoshizawa, T., & Liu, R. S. H. (1980) *Biochemistry 19*, 1549–1553.
Konig, B., Arendt, A., McDowell, J. H., Kahlert, M., Hargrave, P. A., & Hofmann, K. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6878–6882.
Kropf, A., Whittenberger, B. P., Goff, S. P., & Waggoner, A. S. (1973) *Exp. Eye Res. 17*, 591–606.
Liu, R. S. H., & Asato, A. E. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 259–263.
Maeda, A., Shichida, Y., & Yoshizawa, T. (1978a) *J. Biochem. 83*, 661–663.
Maeda, A., Ogurusu, T., Shichida, Y., Tokunaga, F., & Yoshizawa, T. (1978b) *FEBS Lett. 92*, 77–80.
Maeda, A., Shichida, Y., & Yoshizawa, T. (1979) *Biochemistry 18*, 1449–1453.
Mao, B., Ebrey, T. G., & Crouch, R. (1980) *Biophys. J. 29*, 247–256.
Mao, B., Tsuda, M., Ebrey, T. G., Akita, H., Balogh-Nair, V., & Nakanishi, K. (1981) *Biophys. J. 35*, 543–546.
Matthews, R. G., Hubbard, R., Brown, P. K., & Wald, G. (1963) *J. Gen. Physiol. 47*, 215–240.
Nagle, J. F., Parodi, L. A., & Lozier, R. H. (1982) *Biophys. J. 38*, 161–174.
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.
Ono, T., Shichida, Y., & Yoshizawa, T. (1985) *Photochem. Photobiol. 43*, 285–289.
Palings, I., van den Berg, E. M. M., Lugtenburg, J., & Mathies, R. A. (1989) *Biochemistry 28*, 1498–1507.
Shichida, Y. (1986) *Photobiochem. Photobiophys. 13*, 287–307.
Shichida, Y., Tokunaga, F., & Yoshizawa, T. (1978) *Biochim. Biophys. Acta 504*, 413–430.
Shichida, Y., Matuoka, S., & Yoshizawa, T. (1984) *Photobiochem. Photobiophys. 7*, 221–228.
Shichida, Y., Ono, T., Yoshizawa, T., Matsumoto, H., Asato, A. E., Zingoni, J. P., & Liu, R. S. H. (1987) *Biochemistry 26*, 4422–4428.
Stryer, L. A. (1986) *Rev. Neurosci. 9*, 87–119.
Suzuki, T., Ui, K., & Kito, Y. (1976) *Biochim. Biophys. Acta 428*, 321–328.
Waddell, W. H., Lecomte, J., West, J. L., & Younes, U. E. (1984) *Photochem. Photobiol. 39*, 213–219.
Waggoner, A. S., & Stryer, L. (1971) *Biochemistry 10*, 3250–3254.
Wald, G., Durrell, J., & St. George, R. C. C. (1950) *Science 111*, 179–181.
Yoshihara, K., Namiki, A., Sumitani, M., & Nakashima, N. (1979) *J. Chem. Phys. 71*, 2892–2895.
Yoshizawa, T., & Wald, G. (1963) *Nature 197*, 1279–1286.
Yoshizawa, T., & Horiuchi, S. (1973) in *Biochemistry and Physiology of Visual Pigments* (Langer, H., Ed.) pp 69–81, Springer, Berlin.
Yoshizawa, T., & Shichida, Y. (1982) *Methods Enzymol. 81*, 333–354.
Yoshizawa, T., Shinozawa, T., Shichida, Y., Matuoka, S., Ioshida, S., Kandori, H., & Sokabe, M. (1987) in *Retinal Proteins* (Ovchinnikov, Yu. A., Ed.) pp 75–84, VNU Science Press, The Netherlands.