Photochemical Reactions of 13-Demethyl Visual Pigment Analogues at Low Temperatures†

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ABSTRACT: The photobleaching reaction of 13-demethylisorhodopsin (hereafter designated as 9-*cis*-13-dm-rhodopsin), which was synthesized from 9-*cis*-13-demethylretinal and cattle opsin, was investigated by low-temperature spectrophotometry in order to elucidate the role of the 13-methyl group of retinal in photobleaching. When 9-*cis*-13-dm-rhodopsin was irradiated at −190 °C, batho-13-dm-rhodopsin was produced. Its absorption maximum lay at 532 nm, 11 nm shorter than that of cattle bathorhodopsin (λ_{max} 543 nm), and batho-13-dm-rhodopsin had an extinction coefficient about 0.6 times that of bathorhodopsin. Batho-13-dm-rhodopsin was thermally unstable. Above −180 °C, it converted to a new intermediate, BL-13-dm-rhodopsin, which in turn changed to lumi-13-dm-rhodopsin above −140 °C. BL-13-dm-rhodopsin was "photoinsensitive" at temperatures around −188 °C, though batho-13-dm-rhodopsin and lumi-13-dm-rhodopsin were "photosensitive" at the same temperature. In the photobleaching process, lumi-13-dm-rhodopsin and meta-I-13-dm-rhodopsin were observed. Their thermostabilities were very similar to those of lumirhodopsin and metarhodopsin I, but each dm intermediate differed from its methylated counterpart in its value of λ_{max} and extinction coefficient.

It is well-known that the 11-*cis*-retinylidene chromophore in the rhodopsin molecule is fixed in an opsin cavity by a covalent Schiff-base linkage to the ϵ-amino group of a lysine residue of the opsin moiety (Bownds, 1967; Akhtar et al., 1967) and a hydrophobic interaction between the β-ionone ring region of retinal and a hydrophobic region of opsin (Matsumoto & Yoshizawa, 1975). The interaction of the polyene chain of the chromophore with opsin has been investigated with particular emphasis on the methyl groups of the polyene chain. Studies with demethylretinal analogues have shown that the 9-methyl group of the chromophore exhibits a specific interaction with opsin, and this interaction affects the absorption spectra of rhodopsin analogues (Blatz et al., 1969; Kropf et al., 1973), while the 13-methyl group has only a small effect on λ_{max} of pigment analogues (Nelson et al., 1970). However, the reactivity of rhodopsin toward hydroxylamine is raised and the stability constant of the 11-cis pigment is noticeably lowered when the 13-methyl group is absent (Nelson et al., 1970). These latter differences in properties of the 13-demethyl pigments prompted us to study the low-temperature photochemical behavior of these pigments in order to obtain information about bleaching intermediates of this demethyl analogue of the visual pigment rhodopsin.

When rhodopsin absorbs light, it bleaches through several intermediates. In the primary process, the retinylidene chromophore is isomerized from its 11-cis to an all-trans form (Hubbard & Kropf, 1958). We investigated the photobleaching reaction of 9-*cis*-13-demethylrhodopsin (9-*cis*-13-dm-rhodopsin) by means of low-temperature spectrophotometry. The present paper reports a number of spectroscopic properties of the intermediates which were observed in the photobleaching process, including a new intermediate.

Materials and Methods

Synthesis and Purification of 13-Demethylretinal. 13-Demethylretinal was prepared by methods described previously (Van den Tempel & Huisman, 1966). The isomers of 13-dm-retinal were isolated and purified by means of high-performance liquid chromatography with a silica gel column and an eluting solvent consisting of petroleum ether and diethyl ether (Rotmans & Kropf, 1975).

Extraction of Cattle Opsin. Opsin was prepared from cattle retinas by standard methods (Hubbard et al., 1971). The rod outer segments (ROS) floated at a phosphate buffer/40% sucrose solution interface were collected and then washed at least 3 times with 0.1 M phosphate buffer (pH 6.8), followed by irradiation with orange light (>520 nm) during which time the ROS remained in the phosphate buffer containing 0.1 M hydroxylamine (pH 6.5). Such irradiation conditions convert rhodopsin in ROS to retinal oxime and opsin. For removal of the excess hydroxylamine and some retinal oxime the ROS were washed with phosphate buffer. After lyophilization, the dried ROS were treated with petroleum ether to remove lipid and remaining retinal oxime. Digitonin solution (2%) in 0.1 M phosphate buffer (pH 6.5) was added to the lyophilized ROS in order to extract opsin, the ROS were then centrifuged (24000*g* for 1 h), and the supernatant solution, containing opsin, was drawn off.

Synthesis of 9-cis-13-dm-rhodopsin. 9-*cis*-13-dm-retinal in hexane was placed in a reaction vessel, and the solvent was evaporated by a stream of nitrogen gas. The opsin extract was then added and the mixture incubated overnight at room temperature. For removal of the unreacted 9-*cis*-13-dm-retinal from the solution, 1 M hydroxylamine solution (pH 6.5) was added to give a final concentration of 0.1 M. When necessary, the solution was concentrated by ultracentrifugation at 105000*g* for more than 12 h in order to form a 9-*cis*-13-dm-rhodopsin pellet. For low-temperature experiments, glycerol was added to the solution to yield a final glycerol concentration of 66%.

Spectroscopy at Low Temperatures. For low-temperature spectroscopy, a specially designed glass cryostat with quartz windows (Yoshizawa, 1972) was used. The temperature of the sample was monitored with a copper-constantan thermocouple attached to the sample cell which had a light path of 2 mm. The sample was irradiated with a 2-kW xenon lamp whose light passed through a glass cutoff filter with or without an interference filter. Absorption spectra were measured with a Hitachi 323 recording spectrophotometer. For correction

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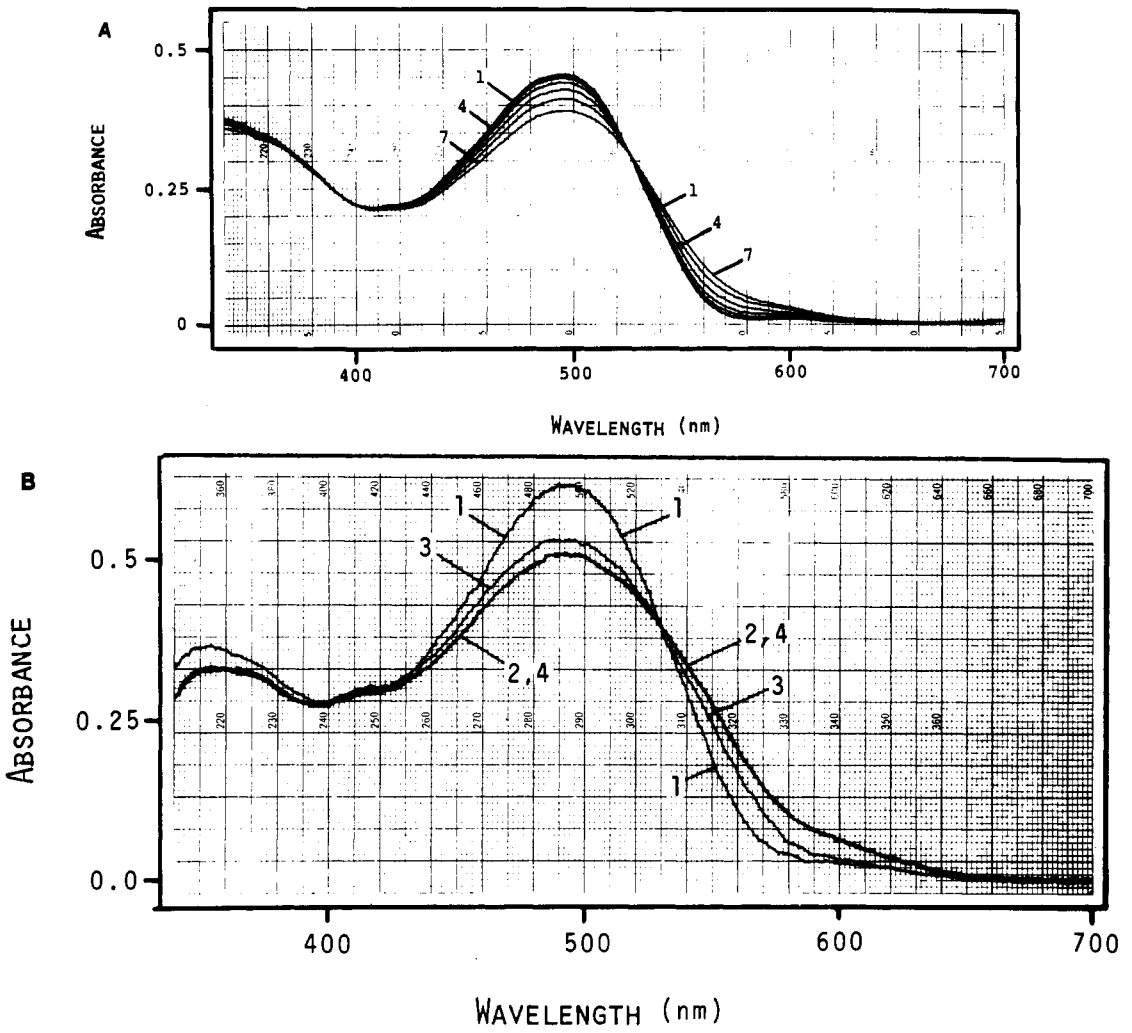

FIGURE 1: (A) Spectral change in the course of the conversion of 9-*cis*-13-dm-rhodopsin to batho-13-dm-rhodopsin. A 9-*cis*-13-dm-rhodopsin–glycerol mixture (pH 6.5, curve 1) was successively irradiated with 437-nm light at −188 °C for a total of 5, 10, 20, 40, 80, and 160 s (curves 2–7). All spectra were measured at −188 °C. (B) Photoreversibility among 11-*cis*-13-dm-rhodopsin, 9-*cis*-13-dm-rhodopsin, and batho-13-dm-rhodopsin at liquid nitrogen temperatures. A 9-*cis*-13-dm-rhodopsin–glycerol mixture (pH 6.5, curve 1) was irradiated with 437-nm light at −188 °C for 640 s (curve 2). The sample was then irradiated with light containing wavelengths longer than 560 nm for 20 s. After measurement of the spectrum (curve 3), the sample was irradiated with 437-nm light again for 640 s, and the spectrum was recorded (curve 4).

of scattering of the sample, opal glasses were placed between the sample and reference beams.

Results

Absorption Spectrum of 9-cis-13-dm-rhodopsin. When the solution was cooled from room temperature to liquid nitrogen temperature, −190 °C, the λ_{max} of 9-*cis*-13-dm-rhodopsin shifted from 486 to 494 nm, and the extinction coefficient increased. When a rapid cooling technique was used (Yoshizawa & Horiuchi, 1973), the sample was frozen at liquid nitrogen temperatures without any cracks. The intensification of absorbance with cooling was about 1.1 times (average of three experiments), taking into account the volume contraction of the sample.

Interconversion of 9-cis-13-dm-rhodopsin, 11-cis-13-dm-rhodopsin, and Batho-13-dm-rhodopsin. When 9-*cis*-13-dm-rhodopsin was irradiated with 437-nm light at −188 °C, the spectrum shifted to longer wavelengths, owing to the formation of a batho product (Figure 1A). We call this product batho-13-dm-rhodopsin. During the course of the spectral change, a sharp isosbestic point at 525 nm was evident, indicating that 9-*cis*-13-dm-rhodopsin converts directly to batho-13-dm-rhodopsin. The ratio of the increase in absorbance at 580 nm (mainly due to batho-13-dm rhodopsin) to the decrease in absorbance at 460 nm is much smaller than that observed when 9-*cis*-rhodopsin is irradiated under similar conditions (T. Yoshizawa and G. Wald, unpublished observation), suggesting that batho-13-dm-rhodopsin has a smaller extinction coefficient than bathorhodopsin. This conclusion is predicated on the knowledge that the values of λ_{max} and the extinction coefficient of 9-*cis*-13-dm-rhodopsin are the same as those of 9-*cis*-rhodopsin (Nelson et al., 1970).

Prolonged irradiation formed a photo-steady-state mixture composed of 9-*cis*-13-dm-rhodopsin, 11-*cis*-13-dm-rhodopsin, and batho-13-dm-rhodopsin (Figure 1B, curve 2). When the photo-steady-state mixture was irradiated with orange light containing wavelengths greater than 560 nm, absorbance at wavelengths longer than 530 nm decreased while there was some increase of absorbance at wavelengths shorter than 530 nm, presumably owing to the conversion of batho-13-dm-rhodopsin to a mixture of 9-*cis*-13-dm-rhodopsin and 11-*cis*-13-dm-rhodopsin (Figure 1B, curve 3). When the photo-steady-state mixture was reirradiated with 437-nm light, the same photo-steady-state mixture was produced as was formed by the first irradiation of 9-*cis*-13-dm-rhodopsin with this wavelength light (Figure 1B, curve 4). These spectral changes clearly indicate that the components in the mixture are perfectly interconvertible by light at around −188 °C.

Absorption Spectrum of Batho-13-dm-rhodopsin. 13-dm rhodopsin, which has an 11-cis chromophore, is decomposed by hydroxylamine at room temperature (Nelson et al., 1970).

Special care is thus needed when calculating the absorption spectrum of batho-13-dm-rhodopsin. As already mentioned, the mixture composed of only 9-*cis*-13-dm-rhodopsin and batho-13-dm-rhodopsin can be formed by a brief irradiation of 9-*cis*-13-dm-rhodopsin at −188 °C. In fact, for a brief irradiation, we were unable to convert more than 35% of the original 9-*cis*-13-dm-rhodopsin to batho-13-dm-rhodopsin. In order to calculate the absorption spectrum of batho-13-dm rhodopsin precisely, we used the following procedure:

A 9-*cis*-13-dm-rhodopsin preparation was first rapidly cooled to about −188 °C, and the spectrum was recorded (A_1). Rapid cooling produces a glycerol–water glass with no cracks, and, hence, an undistorted absorption spectrum. The preparation was then irradiated with 437-nm light for 1 min, and the spectrum of the resulting mixture of 9-*cis*-13-dm-rhodopsin and batho-13-dm-rhodopsin was recorded (A_2). (In another experiment, we confirmed that we could irradiate 9-*cis*-13-dm-rhodopsin under the above conditions for 2 min with no shift in the isosbestic point, indicating that only the two species, 9-*cis*-13-dm-rhodopsin and batho-13-dm-rhodopsin, were present.) The preparation was then warmed to 20 °C. All of the batho-13-dm-rhodopsin decomposed to *all-trans*-13-dm-retinal oxime and opsin. The preparation was rapidly recooled to −188 °C, and the spectrum was recorded (A_3). After the measurement of A_3, the sample was warmed to 20 °C and irradiated with orange light (>510 nm) until the remaining 9-*cis*-13-dm-rhodopsin was completely bleached, and the spectrum was again recorded (A_4).

The difference spectrum between batho-13-dm-rhodopsin and 13-dm-retinal oxime was calculated by subtracting curve A_3 from curve A_2. The difference absorbance at wavelengths longer than 440 nm is almost identical with that of the absolute absorption spectrum of batho-13-dm-rhodopsin, because 13-dm-retinal oxime has no significant absorbance at wavelengths greater than 440 nm. The amount of batho-13-dm-rhodopsin in the mixture was estimated from the difference in absorbance at 486 nm (λ_{max} of 9-*cis*-13-dm-rhodopsin) between the 20 °C spectra measured before and after irradiation at −188 °C. Under our conditions, 30% of 9-*cis*-13-dm-rhodopsin converted to batho-13-dm-rhodopsin. It is interesting to note that the extinction coefficient of batho-13-dm-rhodopsin is 0.66 times that of 9-*cis*-13-dm-rhodopsin, as shown in Figure 4. On the other hand, bathorhodopsins in all the rhodopsin systems which have been studied [cattle, Yoshizawa & Wald (1963); chicken, Yoshizawa & Wald (1967); frog, Kawamura et al. (1977); squid, Yoshizawa & Wald (1964), and Shichida et al. (1978)] show larger extinction coefficients than the corresponding isorhodopsins.

Intermediates in the Photobleaching. For estimatation of the number of intermediates which exist in the photobleaching sequence of 9-*cis*-13-dm-rhodopsin, the photo-steady-state mixture formed as a result of irradiating 9-*cis*-13-dm-rhodopsin with 437-nm light at −188 °C was warmed in a stepwise manner, and the spectra were measured. In Figure 2, λ_{max} of each spectrum was plotted as a function of the temperature to which the photo-steady-state mixture was warmed. Four plateaus were observed in the process of warming. When a similar experiment was carried out with 9-*cis*-rhodopsin, three plateaus were observed (Figure 2B), corresponding to the sequence bathorhodopsin, lumirhodopsin, and metarhodopsin I. In Figure 2A, the first, third, and fourth plateaus correspond to batho, lumi, and meta I intermediates, respectively. We base these designations on the temperature ranges of the intermediate states. Thus, the second plateau represents a new state between batho and lumi intermediates. We call the

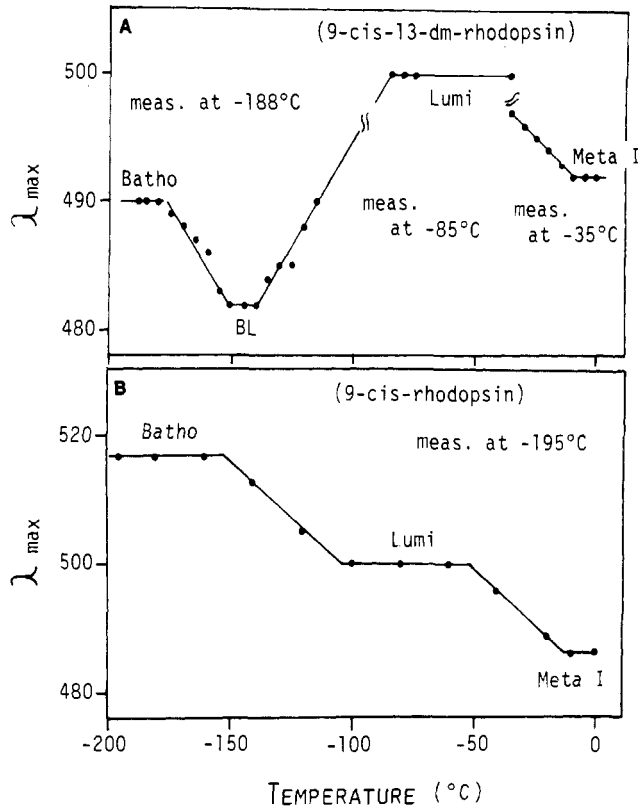

FIGURE 2: Changes in λ_{max} of a photo-steady-state mixture by stepwise warming. (A) A 9-*cis*-13-dm-rhodopsin–glycerol mixture (pH 6.5) was irradiated with 437-nm light for 20 min at −188 °C, resulting in a photo-steady-state mixture composed of 9-*cis*-13-dm-rhodopsin, 11-*cis*-13-dm-rhodopsin, and batho-13-dm-rhodopsin. After irradiation, the mixture was warmed to various temperatures (abscissa) and then recooled to −188 °C (in those cases where warming was to <−115 °C), −85 °C (in those cases where warming was between −85 and −35 °C), and −35 °C (in those cases where warming was between −35 and 0 °C) for measurement of the spectra. (B) 9-*cis*-Rhodopsin was iradiated with 437-nm light, and the photo-steady-state mixture at −195 °C was transformed by stepwise warming [Yoshizawa & Wald (1963); T. Yoshizawa and G. Wald, unpublished results].

compound(s) which exists in this range "BL-13-dm-rhodopsin".

BL-13-dm-rhodopsin. When the mixture containing 9-*cis*-13-dm-rhodopsin and batho-13-dm-rhodopsin is warmed above −180 °C in the dark, its spectrum shifts to shorter wavelengths and its maximum absorbance increases (Figure 3). This spectral change indicates the formation of BL-13-dm-rhodopsin from batho-13-dm-rhodopsin. A difference spectrum between BL-13-dm-rhodopsin and batho-13-dm-rhodopsin was calculated by subtracting curve 1 from curve 7 in Figure 3. From this difference spectrum, a difference spectrum between BL-13-dm-rhodopsin and 13-dm-retinal oxime was estimated as follows: BL-13-dm-rhodopsin is stable in a very narrow range of temperature (Figure 2). For this reason, a certain amount of BL-13-dm-rhodopsin will convert to lumi-13-dm-rhodopsin before all of the batho-13-dm-rhodopsin in the mixture has changed into BL-13-dm-rhodopsin. In order to overcome this complication, we have utilized the fact that λ_{max} of BL-13-dm-rhodopsin is at a shorter wavelength than that of either 9-*cis*-13-dm-rhodopsin or batho-13-dm-rhodopsin. We can thus safely assume that BL-13-dm-rhodopsin has no absorbance at wavelengths longer than 590 nm. The fraction of batho-13-dm-rhodopsin which converted at BL-13-dm-rhodopsin can be estimated as the ratio of the absorbance difference at 600 nm between curves 1 and 7 in Figure 3 to that between curve 1 in Figure 3 and the 600-nm absorbance of 9-*cis*-13-dm-rhodopsin sample at −188

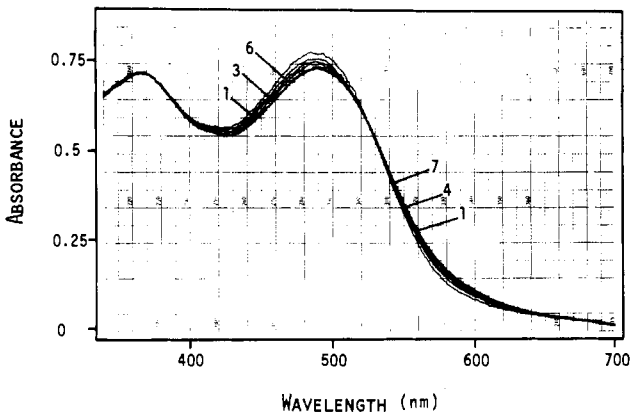

FIGURE 3: Thermal conversion of batho-13-dm-rhodopsin to BL-13-dm-rhodopsin. A photo-steady-state mixture composed of 11-*cis*-13-dm-rhodopsin, 9-*cis*-13-dm-rhodopsin, and batho-13-dm-rhodopsin, which had been prepared by irradiating a 9-*cis*-13-dm-rhodopsin–glycerol mixture (pH 6.5) with 437-nm light at −188 °C, was warmed to an appropriate temperature for conversion of batho-13-dm-rhodopsin to BL-13-dm-rhodopsin and then recooled to −188 °C for measurement of the spectra. Curve 1: Photo-steady-state mixture at −188 °C. Curves 2–7: Products formed by successively warming in the dark to −185, −180, −175, −170, −165, and −160 °C.

°C. Such a calculation shows that the mixture represented by curve 7 of Figure 3 still contained 11% batho-13-dm-rhodopsin. By use of this result, the difference spectrum between BL-13-dm-rhodopsin and 13-dm-retinal oxime can be estimated by adding the difference spectrum between BL-13-dm-rhodopsin and batho-13-dm-rhodopsin to an equal amount of the difference spectrum between batho-13-dm-rhodopsin and 13-dm-retinal oxime. This rather complicated procedure leads to spectrum 3 in Figure 4.

Lumi-13-dm-rhodopsin. BL-13-dm-rhodopsin converts to lumi-13-dm-rhodopsin above −140 °C (Figure 2). In this process, λ_{max} of the mixture shifts to longer wavelengths, and its maximum absorbance increases. A difference spectrum between lumi-13-dm-rhodopsin and 13-dm-retinal oxime, as shown in Figure 4, was calculated by a procedure analogous to that used earlier to calculate the difference spectrum of batho-13-dm-rhodopsin.

After 9-*cis*-13-dm-rhodopsin was cooled to −188 °C and then irradiated with 437-nm light to form a mixture containing only 9-*cis*-13-dm-rhodopsin and batho-13-dm-rhodopsin, the mixture was warmed to −85 °C to convert its batho-13-dm-rhodopsin component to lumi-13-dm-rhodopsin. The amount of lumi-13-dm-rhodopsin in the mixture was estimated by comparing the original amount of 9-*cis*-13-dm-rhodopsin with the amount of 9-*cis*-13-dm-rhodopsin remaining after irradiation at −188 °C.

Lumi-13-dm-rhodopsin has its absorption maximum at 517 nm and has an extinction coefficient 1.36 times larger than that of 9-*cis*-13-dm-rhodopsin. It is also larger than those of the other intermediates (Figure 4).

Meta-I-13-dm-rhodopsin. In the process of conversion of lumi-13-dm-rhodopsin to meta-I-13-dm-rhodopsin above −35 °C, (Figure 2), λ_{max} of the mixture shifted to a shorter wavelength and its maximum absorbance decreased. A difference spectrum between meta-I-13-dm-rhodopsin and 13-dm-retinal oxime was also calculated by the method described earlier.

Meta-I-13-dm-rhodopsin has its absorption maximum at 512 nm and has an extinction coefficient 0.95 times that of 9-*cis*-13-dm-rhodopsin (Figure 4).

Photoreversibilities of BL-, Lumi-, and Meta-I-13-dm-rhodopsins at Liquid Nitrogen Temperature. Yoshizawa &

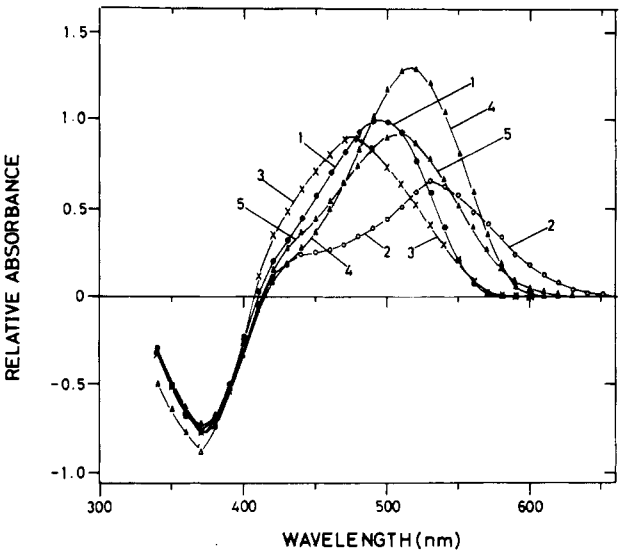

FIGURE 4: Difference spectra of 9-*cis*-13-dm-rhodopsin and its intermediates. The difference spectrum between each intermediate and 13-dm-retinal oxime is calculated relative to the difference spectrum between 9-*cis*-13-dm-rhodopsin and 13-dm-retinal oxime. All the difference spectra are the averages of four experiments. The spectra are (1) 9-*cis*-13-dm-rhodopsin, (2) batho-13-dm-rhodopsin, (3) BL-13-dm-rhodopsin, (4) lumi-13-dm-rhodopsin, and (5) meta I-13-dm-rhodopsin.

Wald (1963) reported that upon irradiation at −188 °C cattle lumirhodopsin reverted to rhodopsin or 9-*cis*-rhodopsin and then to bathorhodopsin. They also confirmed an earlier result of Hubbard et al. (1959) that metarhodopsin I did not photoisomerize to rhodopsin or 9-*cis*-rhodopsin when cooled below about −70 °C. We examined the photoreversibilities of BL-, lumi-, and meta-I-13-dm-rhodopsins at liquid nitrogen temperature in order to ascertain the effect of the altered chromophore structure on the photoconversion of intermediates at −190 °C.

Figure 5 shows that lumi-13-dm-rhodopsin is photoconvertible at liquid nitrogen temperature. When 9-*cis*-13-dm-rhodopsin was irradiated at −190 °C with 437-nm light, a photo-steady-state mixture containing 11-*cis*-13-dm-rhodopsin, 9-*cis*-13-dm-rhodopsin and batho-13-dm rhodopsin resulted (curve 2). When the photo-steady-state mixture was warmed to −110 °C and incubated at this temperature for 30 min, batho-13-dm-rhodopsin converted to lumi-13-dm-rhodopsin as is seen in curve 3 of Figure 5. When the mixture was irradiated again with 437-nm light, its maximum absorbance decreased with a slight shift to shorter wavelengths. Finally, a photo-steady-state mixture was produced (curve 4) which exactly coincided with curve 2. We interpret these results to mean that lumi-13-dm-rhodopsin photochemically converts to batho-13-dm-rhodopsin, presumably via 11-*cis*-13-dm-rhodopsin and/or 9-*cis*-13-dm-rhodopsin.

On the other hand, our experiments showed that BL-13-dm-rhodopsin at −190 °C cannot be converted by light to either 11-*cis*-13-dm-rhodopsin or 9-*cis*-13-dm-rhodopsin and thus not to batho-13-dm-rhodopsin either (Figure 6). The experimental procedure was similar to the one shown in Figure 5, except that the photo-steady-state mixture obtained by irradiating 9-*cis*-13-dm-rhodopsin at −190 °C was warmed to −160 °C instead of −110 °C. Curves 1 and 2 of Figure 6 represent, respectively, the absorption spectra at −190 °C of 9-*cis*-13-dm-rhodopsin and the photo-steady-state mixture obtained by irradiating 9-*cis*-13-dm-rhodopsin with 437-nm light. When the photo-steady-state mixture was warmed to −160 °C and incubated at this temperature for 20 min, most of the batho-13-dm-rhodopsin in the mixture converted to BL-13-dm-

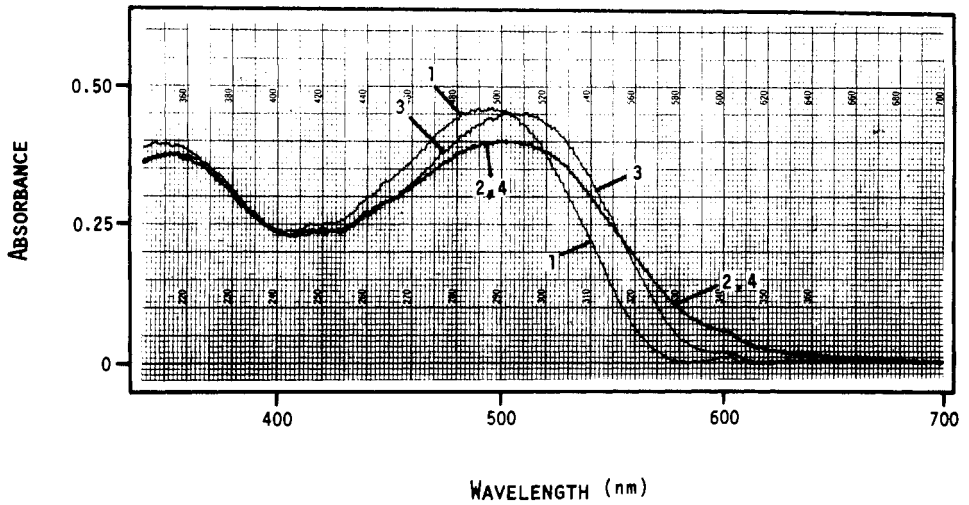

FIGURE 5: Photoreversal of lumi-13-dm-rhodopsin at −190 °C. 9-*cis*-13-dm-rhodopsin was cooled with liquid nitrogen (curve 1) and irradiated with 437-nm light for 10 min until a photo-steady-state mixture was formed (curve 2). After being warmed to −110 °C and incubated at this temperature for 30 min, the sample was recooled to −190 °C for measurement of the spectrum (curve 3). The sample was then irradiated with 437-nm light for 10 min until a photo-steady-state mixture was formed again (curve 4). All spectra were measured at −190 °C.

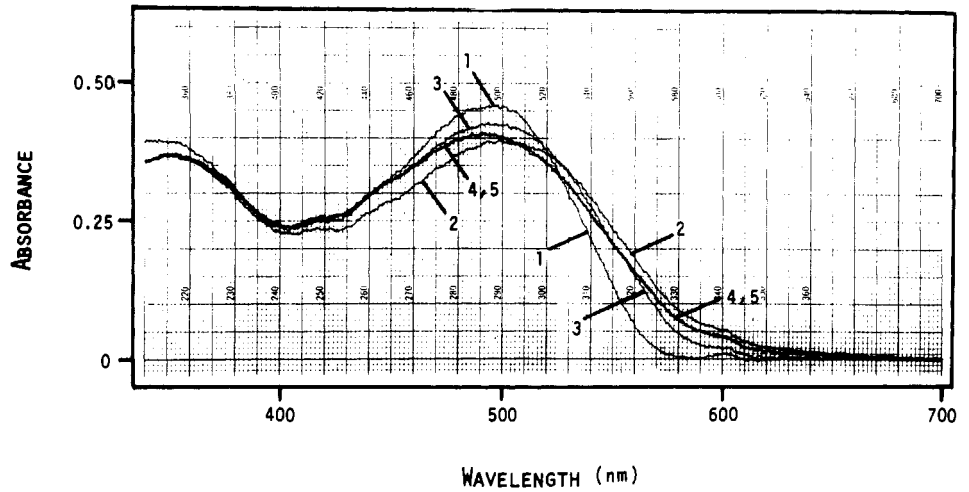

FIGURE 6: Failure of BL-13-dm-rhodopsin to revert to batho-13-dm-rhodopsin upon irradiation at −190 °C. A 9-*cis*-13-dm-rhodopsin–glycerol mixture was cooled to −190 °C (curve 1) and then irradiated with 437-nm light for 10 min until a photo-steady-state mixture was formed (curve 2). After being warmed to −160 °C and incubated at this temperature for 20 min, the sample was recooled to −190 °C for measurement of the spectrum (curve 3). Then it was irradiated with 437-nm light for 10 min until a photo-steady-state mixture was formed (curve 4). The preparation was further irradiated with 437-nm light for 20 min in order to confirm formation of the photo steady state (curve 5).

rhodopsin. Thus the mixture represented by curve 3 consisted of 11-*cis*-13-dm-rhodopsin, 9-*cis*-13-dm-rhodopsin, and BL-13-dm-rhodopsin, with a small amount of batho-13-dm-rhodopsin. After being recooled to −190 °C, the mixture was again irradiated with the same (437 nm) light. A photo-steady-state mixture was produced (curve 4) which is clearly different from that produced by the first irradiation (curve 2). Further irradiation of the preparation with 437-nm light did not produce any measurable spectral changes (curve 5). The fact that curves 4 and 5 do not coincide with curve 2 shows BL-13-dm-rhodopsin to be "photoinsensitive".

Discussion

The photobleaching processes of 9-*cis*-13-dm-rhodopsin and 9-*cis*-rhodopsin (i.e., isorhodopsin) are summarized in Figure 7. On absorption of light, 9-*cis*-13-dm-rhodopsin converts to batho-13-dm-rhodopsin which then bleaches to *trans*-13-dm-retinal and opsin through BL-, lumi-, and meta-I-13-dm-rhodopsins.

Like 9-*cis*-rhodopsin, 9-*cis*-13-dm-rhodopsin is converted by light into batho-13-dm-rhodopsin at liquid nitrogen temperatures. However, batho-13-dm-rhodopsin differs in its spectroscopic properties from bathorhodopsin. Batho-13-dm-rhodopsin has a λ_{max} at 532 nm, which is 11 nm smaller than the λ_{max} of bathorhodopsin, and its extinction coefficient is only 0.6 times that of bathorhodopsin. The lack of a 13-methyl group on the side chain of the chromophore has a significant effect on the absorption spectral properties of the batho intermediate. Since the presence of the 13-methyl group has only a small effect on the spectral properties of 9-*cis*-rhodopsin and rhodopsin compared with their 13-dm analogues (Nelson et al., 1970), it seems clear that the 13-methyl group shows a particular interaction with opsin when 9-*cis*-rhodopsin or rhodopsin converts bathorhodopsin. The appearance of this interaction suggests that a conformational change of the retinylidene chromophore, such as a cis to trans isomerization of the 11-cis double bond, may well occur during the formation of the batho intermediate. If the batho intermediate had a chromophore with a retro-type structure (Oroshnik et al., 1952), such as would be produced by a hydrogen transfer and without a conformational change of the retinylidene chromophore (Van der Meer et al., 1976), batho-13-dm-rhodopsin would be similar in λ_{max} and extinction coefficient to batho-rhodopsin, just as 9-*cis*-13-dm-rhodopsin and 11-*cis*-13-dm-rhodopsin are, respectively, similar to 9-*cis*-rhodopsin and rhodopsin.

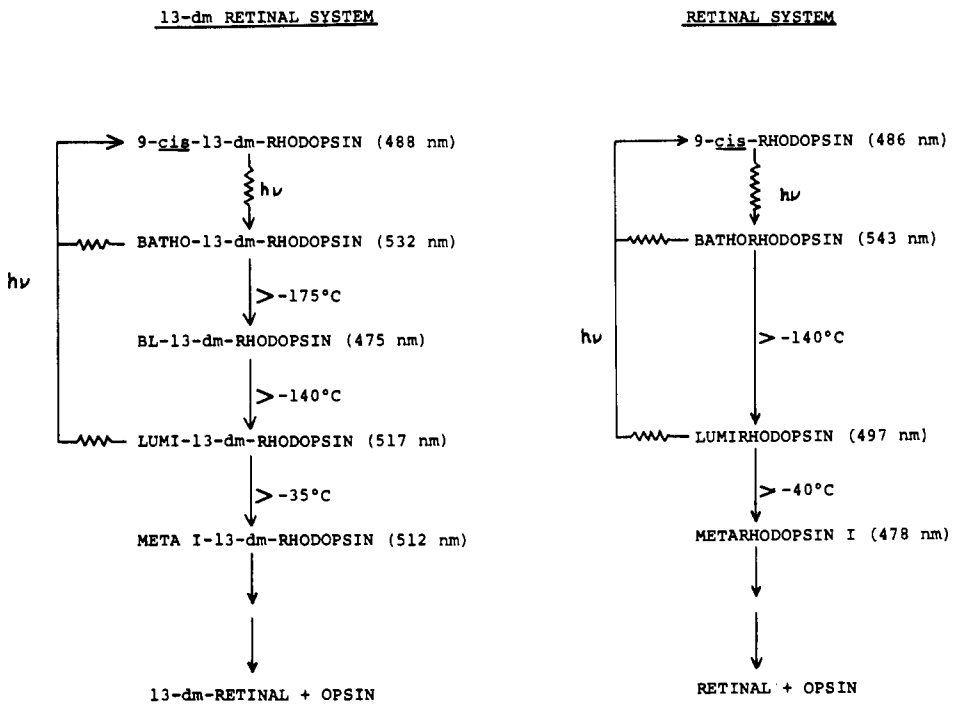

FIGURE 7: Photobleaching processes of 9-*cis*-13-dm-rhodopsin and 9-*cis*-rhodopsin. Photochemical reactions at liquid nitrogen temperatures are denoted by wavy lines. Absorption maxima are shown in parentheses.

Yoshizawa & Wald (1963) suggested that the formation of lumirhodopsin may be due to a relaxation of the strained chromophore of bathorhodopsin accompanied by small conformational changes of opsin around the chromophore. That the absence of a 13-methyl group does not alter the transition temperature for the formation of the lumi intermediate is consistent with their suggestion. But batho-13-dm-rhodopsin is more unstable than bathorhodopsin. Perhaps only a small conformational change of opsin, near the 13 position, accounts for the change of batho-13-dm-rhodopsin to BL-13-dm-rhodopsin.

BL-13-dm-rhodopsin is apparently unchanged by light at −190 °C in spite of the fact that batho-13-dm-rhodopsin and lumi-13-dm-rhodopsin are both photosensitive at this temperature. We infer that the chromophore–opsin interaction in BL-13-dm-rhodopsin results in a rigidity which blocks the photoisomerization of the chromophore from a trans to a cis geometry, although other hypotheses are possible.

As shown in Figure 7, BL-13-dm-rhodopsin has a λ_{max} at 475 nm, which is 57 and 42 nm shorter than those of batho-13-dm-rhodopsin (λ_{max} 532 nm) and lumi-13-dm-rhodopsin (λ_{max} 517 nm), respectively. There are at least two explanations for the blue shift. One is that the distance(s) between the 13-dm chromophore and the charged group(s) of opsin changes as a result of the conversion of batho-13-dm-rhodopsin to BL-13-dm-rhodopsin, resulting in a less favorable protein–chromophore interaction (Kropf & Hubbard, 1958). The other is that one single bond or more of the 13-dm chromophore twist due to the specific interaction between the 13-dm chromophore and opsin in BL-13-dm-rhodopsin (Honig & Ebrey, 1974).

As seen in Figure 2, the transition temperature for the conversion of lumi-13-dm-rhodopsin to meta-I-13-dm-rhodopsin is almost the same as that of lumirodopsin to metarhodopsin I, indicating that this conversion is not affected by the presence or absence of a 13-methyl group on the chromophore. It is presumably determined by structural factors peculiar to opsin.

As already mentioned, BL-13-dm-rhodopsin is apparently "photoinsensitive" at around −190 °C. Now let us assume that BL-13-dm-rhodopsin is also photoinsensitive at −160 °C, a temperature at which batho-13-dm-rhodopsin can change in the dark to BL-13-dm-rhodopsin. Irradiation of 11-*cis*-13-dm-rhodopsin or 9-*cis*-13-dm-rhodopsin at −160 °C should eventually yield a preparation containing only BL-13-dm-rhodopsin. When we irradiated 9-*cis*-13-dm-rhodopsin at −160 °C for several hours with light containing wavelengths longer than 430 nm only, we were unable to produce a preparation containing only BL-13-dm-rhodopsin. We can propose two possible explanations: (1) BL-13-dm-rhodopsin may not be totally photoinsensitive at −160 °C. (2) Since the thermostable region for BL-13-dm-rhodopsin is very narrow, as shown in Figure 2, some BL-13-dm-rhodopsin might convert to lumi-13-dm-rhodopsin due to local heating generated by the radiationless transition of electronically excited BL-13-dm-rhodopsin decaying to its ground state (melting effect; Yoshizawa & Wald, 1963). Since lumi-13-dm-rhodopsin is photosensitive, any "leakage" of BL-13-dm-rhodopsin to lumi-13-dm-rhodopsin would defeat the funneling of all pigment into the BL-13-dm state. We have observed other instances of the local melting effect produced by visible radiation. Metarhodopsin I can be produced by irradiating rhodopsin even at −60 °C where lumirhodopsin is very stable (Kawamura et al., 1977). In addition, cattle bathorhodopsin as well as squid bathorhodopsin can be produced by irradiating rhodopsin at liquid helium temperatures (4 °K) where hypsorhodopsin is the stable intermediate (Yoshizawa, 1972; Shichida et al., 1978).

In the course of these experiments, a photoproduct which has its λ_{max} at a wavelength shorter than that of BL-13-dm-rhodopsin was produced by irradiating 9-*cis*-13-dm-rhodopsin at −160 °C with light containing wavelengths longer than 430 nm only. The spectrum of this product did not change when it was warmed from −160 to −110 °C. Recently, Maeda et al. (1978, 1979) observed that a photo-steady-state mixture, which was produced by irradiating either cattle rhodopsin or squid rhodopsin at −85 °C with light containing wavelengths

longer than 530 nm, consisted of at least five pigments. An analysis by high-performance liquid chromatography showed that the isomeric composition of the pigment chromophore was all-trans, 7-cis, 9-cis, 11-cis, and 13-cis. On irradiation of rhodopsin at −190 °C, however, only rhodopsin (11-cis), 9-*cis*-rhodopsin, and bathorhodopsin (all-trans) were found in the photo-steady-state mixture. Therefore, one or another of the photoproducts which were observed in the 9-*cis*-13-dm-rhodopsin system may have been either 7-*cis*- or 13-*cis*-13-dm-rhodopsin.

Though future studies will undoubtedly answer this last question, the present study has shown that visual pigment analogues containing the 13-demethyl chromophore bleach through a series of distinct intermediates which closely resemble those of the rhodopsin system. But the one striking difference, the appearance of a new intermediate, called BL-13-dm-rhodopsin, may provide us with new insight into the chromophore–protein interaction in visual pigments.

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