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PHOTOCHEMICAL REACTION OF 7,8-DIHYDRORHODOPSIN AT LOW TEMPERATURES

OSAMU MUTO [a,*], FUMIO TOKUNAGA [a,**], TÔRU YOSHIZAWA [a,***], VINAYAK KAMAT [b], HARD A. BLATCHLY [b], VALERIA BALOGH-NAIR [b] and KOJI NAKANISHI [b]

[a] *Department of Biophysics, Faculty of Science, Kyoto University Sakyo-ku, Kyoto 606 (Japan) and* [b] *Department of Chemistry, Columbia University, New York, NY (U.S.A.)*

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The photoreaction of 9-*cis*-7,8-dihydrorhodopsin was examined at liquid nitrogen temperatures (−180°C) in order to elucidate the photochemical events in visual pigments. This rhodopsin analog was prepared by incubating 9-*cis*-7,8-dihydroretinal with bovine opsin in the dark. 9-*cis*-7,8-Dihydrorhodopsin (λ_{max} = 427 nm) was cooled to −180°C, and then irradiated at −180°C with a 390 nm light, resulting in formation of its bathochromic product (λ_{max} = roughly 465 nm). This result indicates that the presence of four double-bonds adjacent to the Schiff base nitrogen is sufficient to allow formation of a bathochromic product. Thus, the mechanism of formation of bathorhodopsin (in bovine rhodopsin system) may be considered as some change of the interaction between the conjugated double-bond system from C-9 to the Schiff base nitrogen and its surrounding charges in opsin, caused by rotation of 11-12 double-bond.

Introduction

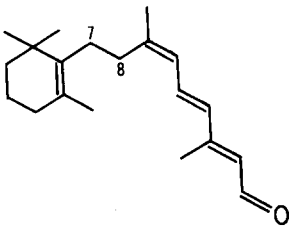
Many retinal analogs have been used for studying the interaction between retinylidene chromophore and opsin in visual pigments [1,2]. Among these, some dihydroretinals [3], in which the conjugated double-bond system is shortened or dissected into the ene and enal moieties by reduction of various double-bonds, represent a unique series of retinal analogs. Binding studies carried out with the dihydroretinal series have led us to the two contrasting external point charge models to account for the absorption maxima of bovine rhodopsin at 498 nm [4] and bacteriorhodopsin at 560 nm [5].

The initial reaction of visual photoreception has been considered to be the photoconversion of rhodopsin to bathorhodopsin [6,7] and several models for the structure of the retinylidene chromophore in bathorhodopsin have been proposed [6–10]. Some of the artificial visual pigments have played significant functions in clarifying various aspects of these models [11–16]. Here, we report low temperature absorption spectroscopic study aiming to elucidate the requirements of the opsin moiety leading to formation of the bathochromic product, or more specifically, what role the unsaturation of the retinal moiety plays in the formation of bathorhodopsin. The paper deals with 9-*cis*-7,8-dihydrorhodopsin derived from 7,8-dihydroretinals. We have found that 7,8-dihydrorhodopsin was converted into bathochromic product by irradiation at liquid nitrogen temperatures. This fact indicates that the entire double-bond

* Present address: Pharmacological Division, Institute of Biological Science, Mitsui Pharmaceuticals Inc. 1900-1 Togo, Mobara-shi, Chiba-ken Japan.
** Present address: Department of Physics, Faculty of Science, Tohoku University, Aobayama, Sendai 980, Japan.
*** To whom all correspondence should be addressed.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

system is not required for bathochromic shift, i.e., four double-bonds conjugated to the Schiff base nitrogen suffice for this spectral shift.



Materials and Methods

Bovine rod outer segments were prepared according to the method of Papermaster and Dreyer [17]. The purified outer segments were suspended in 10 mM Hepes buffer (pH 7.0). After bleaching of rhodopsin in the outer segments with a sodium lamp (Toshiba, NA 60) in the presence of 100 mM hydroxylamine, the suspension was centrifuged and then the pellet was washed with 10 mM Hepes buffer to remove the hydroxylamine.

For preparation of 9-*cis*-7,8-dihydrorhodopsin, the bleached outer segment suspension was mixed with seven-times excess in absorbance of 9-*cis*-7,8-dihydroretinal dissolved in a small amount of ethanol (less than 3% of total volume). The mixture was incubated for 8 h at 25 °C in the dark in order that almost all the opsin in the outer segment except the denatured portion during the preparation might be converted to the pigment. After centrifugation at 10000 × *g* for 30 min, the pellet was washed seven times with hexane (−20 °C) to remove the free unbound retinal analog. 9-*cis*-7,8-Dihydrorhodopsin thus prepared was stable at room temperatures. It was suspended in 10 mM Hepes buffer, mixed with 2 vol. glycerol and then served as the sample for low temperature spectrophotometry.

The sample was placed in a specially designed glass-cryostat for measurements of spectra at the low temperature [7]. The spectra were recorded with a Hitachi 323 spectrophotometer. The light source for irradiation of the sample was a xenon lamp (2 kW, Ushio). Wavelengths for irradiation were selected either by a monochromator (MPF Flash Photolysis, JASCO) or a glass cutoff filter (Toshiba) inserted in front of the light source.

Results

9-*cis*-7,8-Dihydrorhodopsin showed a broad spectrum with a single peak at 427 nm in the range between 340 and 600 nm. On cooling to −180 °C, it displayed vibrational fine structure (415, 433 and 450 nm) in its absorption spectrum at liquid nitrogen temperature (−180 °C) (curve 1 in Fig. 1); it should be noted that 7,8-dihydroretinal dissolved in hexane exhibited vibrational fine structure in its absorption spectrum at 305, 322 and 335 nm at room temperature.

Irradiation of 9-*cis*-7,8-dihydrorhodopsin (curve 1) with light at 390 nm increased absorbances at wavelengths longer than 460 nm and shorter than 376 nm, and decreased between these wavelengths, owing to formation of a bathochromic product. The final spectrum (curve 7) should be a photo-steady-state mixture, presumably composed of the original pigment, the bathochromic product and other isomers.

In order to examine the photoreversibility among these components, the sample containing the bathochromic product (curve 2 in Fig. 2, identical with curve 7 in Fig. 1) was irradiated with yellow light longer than 470 nm which could be

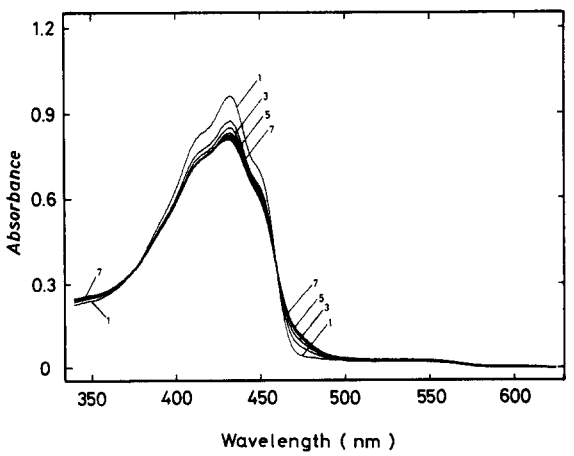


Fig. 1. Photoconversion of 9-*cis*-7,8-dihydrorhodopsin at −180 °C. 9-*cis*-7,8-Dihydrorhodopsin at −180 °C (curve 1) was successively irradiated with light at 390 nm for a total of 1, 2, 4, 8, 16 and 32 min, respectively (curves 2–7).

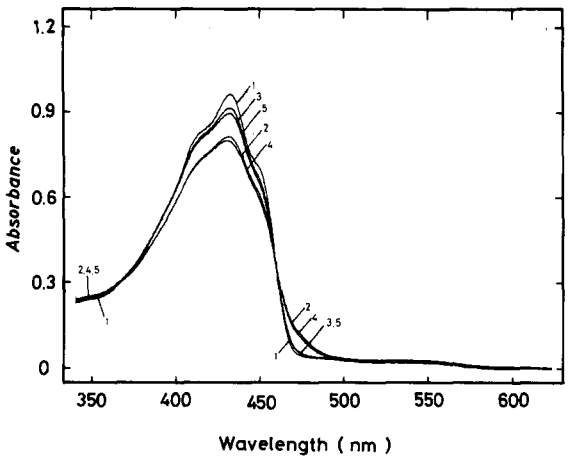


Fig. 2. Interconversion among 7,8-dihydrorhodopsin, bathochromic product and other isomer(s) by light at −180 °C. 9-*cis*-7,8-Dihydrorhodopsin was cooled to −180 °C (curve 1, identical with curve 1 in Fig. 2) and then irradiated with light at 390 nm for 32 min (curve 2, identical with curve 7 in Fig. 1). Then, the sample was irradiated with light longer than 470 nm for 160 min (curve 3). The sample was reirradiated with light at 390 nm for 32 min (curve 4) and then again with light longer than 470 nm for 180 min (curve 5).

absorbed only by the bathochromic product. The absorbance between 460 and 495 nm due to the bathochromic product decreased, while that near 430 nm mainly due to the original pigment (9-*cis*-7,8-dihydrorhodopsin) and its isomer(s) increased (curve 3). Reirradiation with light at 390 nm caused a bathochromic shift due to formation of the bathochromic product. The spectrum measured (curve 4) was almost identical with curve 2. Further irradiation with the yellow light caused the reversion of the bathochromic product to the mixture of the original pigment and its isomers (curve 5). This curve was almost coincidental with curve 3. These reversible spectral changes could be repeated many times.

When a photosteady-state mixture containing the bathochromic product (corresponding to curve 7 in Fig. 1) was warmed to −170 °C, the absorbance between 422 and 486 nm increased, whereas that between 354 and 422 nm decreased as shown in Fig. 3. Such spectral changes upon warming have not been observed in the natural visual pigment systems [7].

On warming above −140 °C, another spectral change took place, i.e., the absorbance between 421 and 496 nm decreased as shown in Fig. 4. In analogy to formation of lumirhodopsin above −140 °C in the bovine rhodopsin system, this spectral change in the present 7,8-dihydrorhodopsin system can also be regarded as the formation

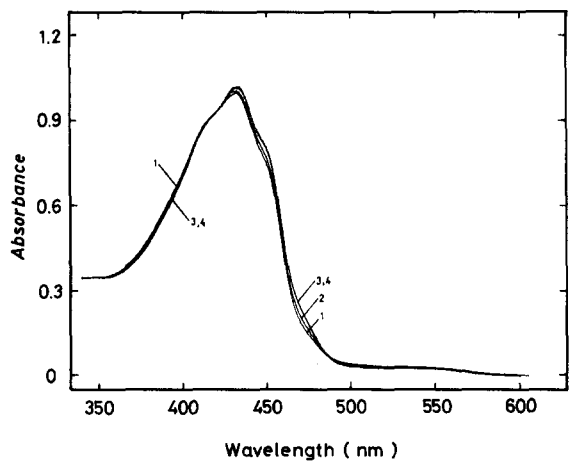


Fig. 3. Spectral change on warming a sample containing batho-7,8-dihydrorhodopsin from −180 to −150 °C. 9-*cis*-7,8-Dihydrorhodopsin was irradiated at −180 °C with light at 390 nm for 32 min, resulting ina photosteady-state mixture containing the bathochromic product (curve 1). This sample warmed to −170 °C (curve 2), −160 °C (curve 3) and then −150 °C (curve 4). All measurements of spectra were done at −180 °C.

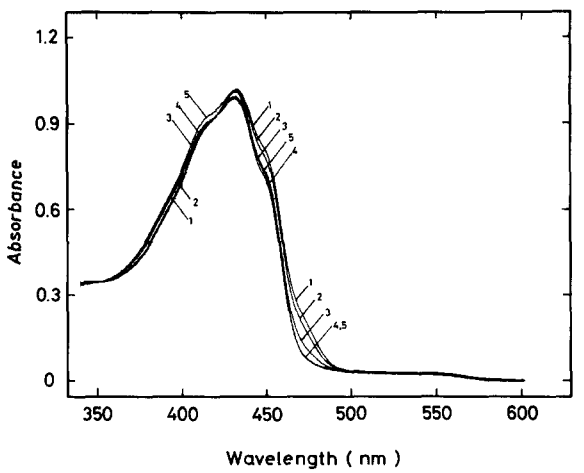


Fig. 4. Spectral change on warming a sample containing batho-7,8-dihydrorhodopsin from −150 to −110 °C. The sample (curve 4 in Fig. 3) was redrawn as curve 1 and then warmed to −140 °C (curve 2), −130 °C (curve 3), −120 °C (curve 4) and −110 °C (curve 5). All measurements of spectra were done at −180 °C.

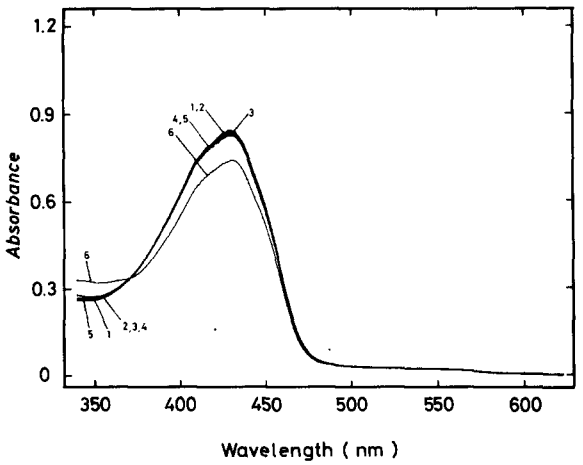


Fig. 5. Spectral change on warming a sample containing batho-7,8-dihydrorhodopsin from −90 to +10 °C. The sample (curve 5 in Fig. 4) was warmed to −90 °C (curve 1), −70 °C (curve 2), −50 °C (curve 3), −30 °C (curve 4), −10 °C (curve 5) and +10 °C (curve 6). All measurements of spectra were done at −90 °C.

of an intermediate corresponding to lumirhodopsin. Curve 5, which does not pass through an isosbestic point at 421 nm, is somewhat distorted in shape, probably due to some change in the state of the medium, e.g., change in degree of cracks on the recooling process for measuring the spectrum.

From −110 to −10 °C, no spectral change was observed (curves 1–5 in Fig. 5). Above 0 °C, the absorbance above 370 nm decreased, owing to decomposition of the intermediate, resulting in a mixture (λ_{max} = 427 nm) of the original pigment (λ_{max} = 427 nm) and its final photoproducts (curve 6). The pigment in the preparation was completely bleached at 10 °C with yellow light (greater than 470 nm). The absorbance at 470 nm of the residual pigment at 10 °C after the irradiation at −180 °C was estimated to be 78% of that of the original pigment. On the assumption that the residual pigment is composed of only 9-*cis*-dihydrorhodopsin, the amount of batho-9-*cis*-dihydrorhodopsin in the photosteady-state mixture (curve 4 in Fig. 2) was calculated to be 22% and the absorption maximum of batho-9-*cis*-dihydrorhodopsin was located around 465 nm. The relatively little formation of the batho-intermediate may be due to strong overlap in the α-band between the original pigment and its batho-intermediate and to a high relative quantum efficiency in conversion of the batho-intermediate to the original pigment.

Discussion

The conjugated double-bond system of 9-*cis*-7,8-dihydrorhodopsin which is responsible for absorbance in the visible region, is composed of four double-bonds adjacent to the Schiff base nitrogen. The finding that irradiations of dihydrorhodopsin at liquid nitrogen temperatures led to an increase of absorbance in the long wavelength range indicates that the formation of a bathochromic product in rhodopsin and its analogs does not require the whole conjugated double-bond system present in retinal. A similar conclusion had also been reached in the cases of 9-*cis*-retro-γ-rhodopsin [11] and 9-*cis*-5,6-dihydrorhodopsin [18] which also possess shorter conjugated chains. On the other hand, 7-membered rhodopsin which has an 11-*cis*-locked chromophore cannot be isomerized by light, so that it does not produce the batho-product by irradiation at liquid nitrogen temperatures [15,16]. Therefore, the formation of the batho-product requires some rotation of the conjugated double-bond system of the chromophore. This rotation causes some changes in interaction between the chromophore and charge(s) within the retinal binding site of opsin moiety which is (are) localized near the retinal side-chain terminal; these charges could be the external point charge located close to C-12/C-14 and the counteranion [9], resulting in bathochromic spectral change.

It should be noted that the chromophore of 9-*cis*-7,8-dihydrorhodopsin has a rectilinear conjugated double-bond system as shown in the structure in Introduction. Now a question arises as to which double-bond(s) in the chromophore can be isomerized for the formation of its bathochromic product. Since the α-band of 9-*cis*-7,8-dihydrorhodopsin is due to absorption by the conjugated double-bond system adjacent to the Schiff base nitrogen, some of the double-bonds in the system may possibly be isomerized. The most probable photoisomerization may occur at the 9-10 double-bond from the *cis* to *trans* form, and at other double-bonds, it seems unlikely for several reasons described below.

According to a series of binding experiments of

retinal isomers with bovine opsin, 13-*cis*- and all-*trans*-retinals cannot bind with opsin to form pigments stable at room temperature. All the other retinal isomers that had been examined have been confirmed to form their own stable pigments [19,20]. Since the bathochromic product derived from 9-*cis*-7,8-dihydrorhodopsin is unstable at room temperatures, the chromophore of the bathochromic product should not be in any form of the isomers which can form their own stable pigments at room temperature.

Some of the isomers (11,13-*dicis*-, 7,9,11-*tricis*-, 7,11,13-*tricis*-, 9,11,13-*tricis*- and 7,9,11,13-*tetracis*-retinals), however, have not yet been examined [19]. These isomers can probably form their own stable pigments in consideration of the longitudinal restriction to the retinal binding site of opsin [21,22]. In addition, it should be noted that all the unexamined isomers contain 11-*cis* bond. When 11-*cis*-locked-7-membered rhodopsin was irradiated at room or liquid nitrogen temperature, no spectral change was observed [16]. This fact may support the view that 11-*cis* chromophore would not convert into any of the multi-*cis* forms.

Furthermore, a high performance liquid chromatographic examination of samples produced by irradiation of rhodopsin at liquid nitrogen temperatures revealed only three isomers, 9-*cis*, 11-*cis* and all-*trans* forms, though that at dry ice temperatures displayed two additional isomers, 7-*cis* and 13-*cis* forms [23,24]. These facts clearly indicate that the retinal binding site of opsin at liquid nitrogen temperatures can accomodate only the three forms as its chromophore. Thus, the possibility that the bathochromic product may have the 13-*cis* form as its chromophore, may be eliminated. Therefore, the formation of the bathochromic product by irradiation of 9-*cis*-7,8-dihydrorhodopsin at liquid nitrogen temperature should be attributed to photoisomerization of the chromophore from 9-*cis* to all-*trans* forms.

Change occurring upon warming the bathochromic product from 9-*cis*-7,8-dihydrorhodopsin is apparently different from that from natural rhodopsin, but is similar to that of the primary intermediate in the 13-demethyl(dm)-rhodopsin system: Batho-13-dm-rhodopsin converted into BL-13-dm-rhodopsin above $-180\,^{\circ}C$, and on warming above $-140\,^{\circ}C$, it converted into lumi-13-dm-rhodopsin [25]. Thus, the bathochromic intermediate formed from batho-7,8-dihydrorhodopsin by warming to $-170\,^{\circ}C$ may correspond to BL-13-dm-rhodopsin.

One of the possible mechanisms of formation of BL-7,8-dihydrorhodopsin is that the thermal conversion of batho-7,8-dihydrorhodopsin above $-170\,^{\circ}C$ might be attributed to some rotation in the single-bond system, i.e., the irradiation with light at 390 nm may produce a twisted form of the single-bond system, which relaxes upon warming. But it is unlikely that the twisted form is the complete 7-s-*cis* conformation, because 7-*cis*-rhodopsin has λ_{max} at the shorter wavelength region and has not been produced with the irradiation at liquid nitrogen temperature [12,19]. We may consider the spectral change above $-140\,^{\circ}C$ to correspond to a conversion to the lumi-intermediate. At higher temperatures, no meta-intermediate was observed. However, since the spectral change in the conversion from lumi-intermediate to meta-intermediate is very small in retro-γ-rhodopsin (Muto, O., unpublished observation), we cannot exclude the intermediacy of a meta-intermediate in 7,8-dihydrorhodopsin system. On warming above $0\,^{\circ}C$, the lumi-intermediate decomposed to opsin and 7,8-dihydroretinal.

Acknowledgements

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