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SPECIFIC PHOTOISOMERIZATION OF RETINAL IN SQUID RHODOPSIN AND METARHODOPSIN

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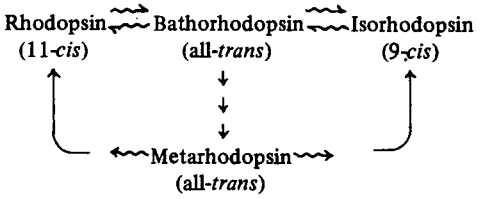
The composition of retinal isomers in the photosteady-state mixtures formed from squid rhodopsin and metarhodopsin was determined by high-pressure liquid chromatography. A large amount of 9-*cis*-retinal was obtained at liquid N_2 temperature when rhodopsin was irradiated with orange light, but only small quantities of 9-*cis*-retinal were obtained at 15°C. Scarcely any 9-*cis*-retinal was produced from metarhodopsin by irradiation at liquid N_2 temperature. A large quantity of 7-*cis*-retinal was found in the photoproduct of rhodopsin irradiated at solid carbon dioxide temperature, but not at 15°C and liquid N_2 temperature. 7-*cis*-Retinal was not produced from metarhodopsin at any temperatures. These results indicate that the photoisomerization of retinal is regulated by the structure of the retinal-binding site of this protein. The formation of 9-*cis*- and 7-*cis*-retinals is forbidden in the metarhodopsin protein.

Introduction

Squid rhodopsin has 11-*cis*-retinal as its chromophore and light induces isomerization of 11-*cis*-retinal into all-*trans*-retinal as in cattle rhodopsin [1]. Cumulative evidence indicates that the isomerization of retinal is followed by conformational changes in the protein moiety of squid rhodopsin [2,3]. The reaction cycle of squid rhodopsin is simplified as follows (the wavy line indicates photoreaction):



Squid metarhodopsin is stable below 20°C and is efficiently converted to rhodopsin by reabsorbing light. One of us studied spectroscopically the formation of isorhodopsin (9-*cis* pigment) at various temperatures [4]. A small amount of isorhodopsin was produced by irradiating rhodopsin at 10°C with orange light while a great amount of isorhodopsin was formed at liquid N_2 temperature. This suggests that the photoisomerization of retinal is dependent on the protein structure of the pigment.

In the present study, we investigate the isomeric composition of retinal in photosteady-state mixtures produced from squid rhodopsin and metarhodopsin with high-pressure liquid chromatography (HPLC). We will demonstrate that the photoisomerization of retinal is dependent on the protein conformation but not on temperature.

Material and Methods

Squid (*Todarodes pacificus*) rhodopsin was extracted and purified by using the method of Suzuki et al. [4]. No change in the absorption spectrum of the purified rhodopsin solution was observed on addition of NH_2OH, indicating no contamination of retinochrome, another photosensitive retinoprotein in the squid retina. Lipid binding is an important factor not only in the photoisomerization, but also in the

extraction of retinal. The rhodopsin preparation used here contained 50 mol phospholipid/mol rhodopsin.

The absorption spectrum was determined at 15°C with a Shimadzu UV-200 spectrophotometer. Alkaline metarhodopsin was made by irradiating rhodopsin with orange light (λ>560 nm, Toshiba V-O 56) for 10 min at pH 11.0 adjusted with saturated Na_2CO_3. Acid metarhodopsin was made from the alkaline metarhodopsin by changing the pH from 11.0 to 6.5 with saturated KH_2PO_4. Photosteady-state mixtures were made by irradiating rhodopsin and acid metarhodopsin with orange light (λ>560 nm) for 30 min in a solvent of 0.2% digitonin, 100 mM NaCl and 10 mM Tris-HCl buffer (pH 6.5). A 1 kW projection lamp was used for irradiation as a light source with neutral density filters.

Retinal was extracted according to the method of Groenendijk et al. (retinaloxime method) [5]; About 95% retinal was extracted by this method from squid rhodopsin. HPLC analysis was performed with a JASCO HPLC system equipped with a Sorbax SIL column (2.1 × 250 mm, Shimadzu-Du Pont). Hexane/diethyl ether (100 : 10, v/v) was used as the eluant and the flow rate was 0.4 ml/min. The absorbance at 350 nm was recorded with a JASCO UVIDEC-100-III apparatus and peak area was determined by integrating with a Shimadzu chromatopac E 1A. The percentage of retinal isomer in each sample was calculated from the peak area and absorption coefficients previously reported [6,7]. The absorption coefficient of 7-*cis*-retinaloxime was determined to be 53 000 by comparing with free 7-*cis*-retinal (44 100 at 356 nm [8]) in the peak area of HPLC.

Results and Discussion

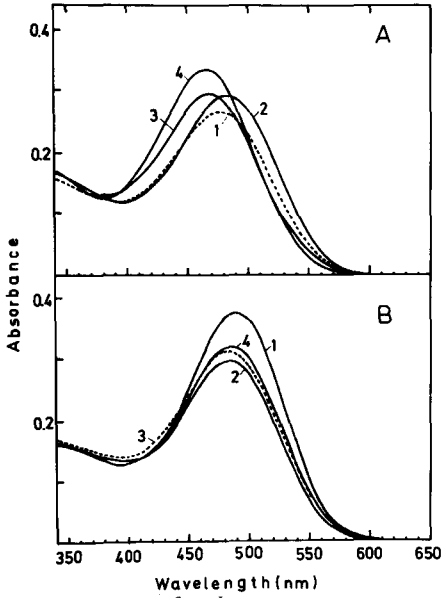
In order to check for artifactual isomerization of retinal during the extraction from the protein, a photosteady-state mixture was made by irradiating rhodopsin solution at 15°C with blue light (λ = 440 nm) for 30 min. Retinal was extracted from the mixture four times, independently, and subjected to HPLC analysis. The composition of retinal isomers was 38.4 ± 0.7% 11-*cis*, 60.2 ± 0.7% all-*trans*, 1.2 ± 0.2% 13-*cis* (mean ± S.E.) and a trace amount of 9-*cis*-retinal. The composition of retinal isomers extracted from unirradiated rhodopsin was 99.0 ± 0.3% 11-*cis* and 1.0 ± 0.3% all-*trans* (n = 4). From these results we judged that artifactual isomerization of retinal is negligible under these experimental conditions.

Subsequently, we made the photosteady-state mixtures by irradiating rhodopsin and metarhodopsin at three characteristic temperatures, 15°C (metarhodopsin is stable at this temperature), solid CO_2 temperature (lumirhodopsin stable) and liquid N_2 temperature (bathorhodopsin stable).

Fig. 1 shows absorption spectra of rhodopsin, acid metarhodopsin and their photoproducts. All spectral curves were determined at 15°C. The photoproduct of rhodopsin irradiated at liquid N_2 temperature showed an absorption spectrum different from that of the photoproduct at 15°C (Fig. 1A). This is due to the formation of isorhodopsin (λ_{max} = 468 nm) at liquid N_2 temperature and to the formation of acid metarhodopsin (λ_{max} = 490 nm) at 15°C. The photoproduct from acid metarhodopsin at liquid N_2 temperature had almost the same λ_{max} value as that of the product at 15°C (Fig. 1B), suggesting a similar composition of retinal isomers. The absorption maximum of the photoproduct at solid CO_2 temperature was at slightly shorter wavelengths than those of two other preparations.

Fig. 2 shows chromatograms of the four samples. The *syn* isomer of 9-*cis*-retinaloxime was not separated from that of 13-*cis*-retinaloxime. The *anti* isomers of all retinaloximes were well separated from each other. According to Groenendijk et al. [5], the *syn*/*anti* ratio varies from 3 to 4 when retinal isomers are reacted with NH_2OH in methanol/water, and the ratio is a constant 1.9 in membrane preparations. We confirmed that the detergent-solubilized preparation of rhodopsin had almost the same *syn*/*anti* ratio (about 2.0) as that in the membrane preparation. Therefore, we can determine the total peak area in each retinaloxime isomer. The absorption coefficients used here for calculation were determined by Hubbard [6,7] using mixtures of *syn* and *anti* isomers in unknown ratios. Since the absorption coefficient of the *anti* isomer is slightly different from that of the *syn* isomer [9], our results include some errors although they are not critical. The percentage of each retinal isomer in the sample was calculated from the total peak area and the absorption coefficient.

Table I shows the composition of stereoisomers of retinal in rhodopsin, acid metarhodopsin and their



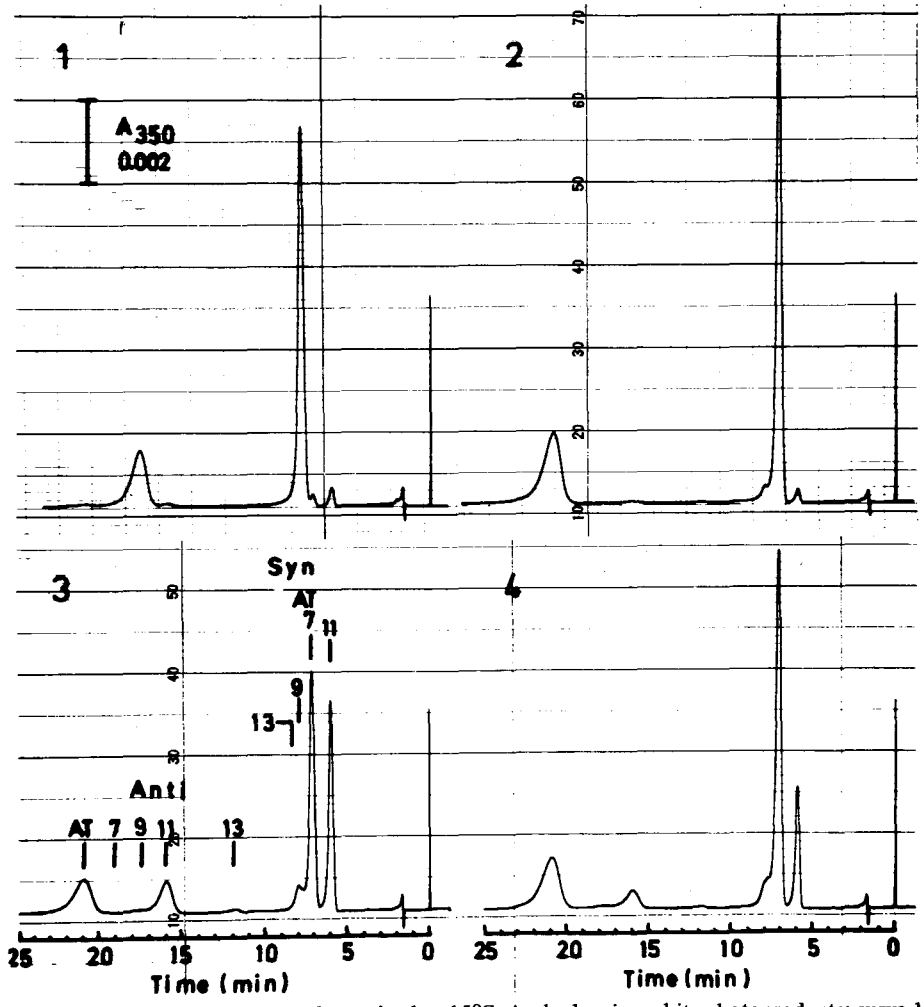


Fig. 1. Absorption spectra of rhodopsin, metarhodopsin and their photoproducts. All curves were determined at 15°C. A, rhodopsin and its photoproducts; curve 1, unirradiated rhodopsin; curve 2, irradiated at 15°C; curve 3, irradiated at solid CO_2 temperature; curve 4, irradiated at liquid N_2 temperature; B, acid metarhodopsin and its photoproducts, curve 1, unirradiated acid metarhodopsin; curves 2–4, acid metarhodopsin irradiated at the same temperature as in A, respectively. Samples were irradiated with orange light (λ>560 nm) for 30 min in the solvent of 0.2% digitonin, 100 mM NaCl, 10 mM Tris-HCl (pH 6.5).

Fig. 2. HPLC analyses of four samples. 1, rhodopsin irradiated at liquid N_2 temperature; 2, acid metarhodopsin, unirradiated; 3, acid metarhodopsin irradiated at 15°C; 4, acid metarhodopsin irradiated at liquid N_2 temperature. The peak positions of retinaloxime isomers were determined using authentic isomers and shown in the figure. Samples used here are the same as those in Fig. 1.

TABLE I

COMPOSITION OF RETINAL ISOMERS IN THE PHOTOSTEADY-STATE MIXTURES FORMED FROM RHODOPSIN AND METARHODOPSIN

Samples were irradiated with orange light ($\lambda > 560$ nm) for 30 min. All values are expressed in %.

	11-*cis*	all-*trans*	9-*cis*	13-*cis*	7-*cis*
Rhodopsin					
Unirradiated	99.1	0.9	0	0	0
Irradiated at 15°C	50.2	45.9	1.5	1.5	0
Irradiated at solid CO_2 temperature	14.1	14.1	18.1	11.4	42.3
Irradiated at liquid N_2 temperature	5.3	2.2	92.5	0	0
Metarhodopsin					
Unirradiated	3.3	95.8	0	0.9	0
Irradiated at 15°C	52.4	43.9	1.4	2.3	0
Irradiated at solid CO_2 temperature	27.2	56.8	1.9	12.2	1.9
Irradiated at liquid N_2 temperature	29.7	65.4	3.2	1.8	0

photoproducts at three temperatures. The irradiation of rhodopsin at liquid N_2 temperature with orange light produced more than 90% 9-*cis*-retinal. The amount of 9-*cis*-retinal produced was only 1.5% when rhodopsin was irradiated at 15°C. The all-*trans* pigment formed at liquid N_2 temperature is bathorhodopsin, the protein conformation of which is considered to be the same as that of rhodopsin. The all-*trans* pigment at 15°C is metarhodopsin which has a protein conformation different from that of rhodopsin. It is suggested that 9-*cis* formation is prohibited in the metarhodopsin protein.

When acid metarhodopsin was irradiated at liquid N_2 temperature, 3% 9-*cis*-retinal was produced. The original metarhodopsin preparation contained 3% rhodopsin. Since most of rhodopsin is converted to isorhodopsin at liquid N_2 temperature, the 9-*cis*-retinal in the photoproduct formed from metarhodopsin by irradiation at liquid N_2 temperature must come from isorhodopsin formed from rhodopsin of the orifinal metarhodopsin preparation. Therefore, 9-*cis*-retinal is not formed in the metarhodopsin protein even at liquid N_2 temperature. The irradiation of acid metarhodopsin at solid CO_2 temperature yielded a mixture containing an amount of 9-*cis*-retinal of the same order as that at 15°C. These results clearly show that the all-*trans* → 9-*cis* reaction is forbidden in the metarhodopsin protein irrespective of the irradiation temperature.

42% 7-*cis*-retinal was produced by irradiating rhodopsin at solid CO_2 temperature where lumirhodopsin (all-*trans* pigment) is stable. No 7-*cis*-retinal was produced from metarhodopsin at liquid N_2 temperature and only a little at solid CO_2 temperature. The 7-*cis*-retinal in the photoproduct formed from metarhodopsin at solid CO_2 temperature must come from 7-*cis* pigment formed from the rhodopsin contaminating the original metarhodopsin preparation. The results indicate that 7-*cis*-retinal is formed only in the lumirhodopsin protein and not in rhodopsin and metarhodopsin proteins.

All the results described above lead to the following conclusions: (1) the conformation of metarhodopsin protein is frozen (does not follow the stereoisomerization of retinal) at liquid N_2 temperature; (2) the formation of 9-*cis*-retinal is not allowed in the metarhodopsin protein; (3) no 7-*cis*-retinal is formed in the rhodopsin and metarhodopsin proteins. Thus, the photoisomerization of retinal is dependent upon the conformation of the retinoprotein. Some steric factors of the retinal-binding site of rhodopsin may play an important role in the specific photoisomerization of retinal.

Recently, Maeda et al. [10] reported that 7-*cis*-retinal was produced by irradiating squid rhodopsin at any temperature where bathorhodopsin is unstable. They used microvillar membranes as a starting material and a different extraction method from ours. These differences are not important. We detected a considerable quantity of 7-*cis*-retinal (7.5%) when

rhodopsin was irradiated at 10°C with 20-times stronger light than the light used in our experiment. The yellow light (λ>530 nm) used by Maeda et al. [10] produced more 7-*cis*-retinal than the orange light (λ>560 nm). It is considered that the 7-*cis* pigment is formed from lumirhodopsin and mesorhodopsin (P465, the photoproduct formed at solid CO_2 temperature, reported by Suzuki et al. [4], must be the 7-*cis* pigment). The mesorhodopsin has a relatively long life-time, about 10 ms at 10°C (Ref. 11 and Ebina, Y., personal communication). Therefore, the probability of mesorhodopsin absorbing light must increase considerably when the stronger light is used for the irradiation. The discrepancies between the results of Maeda et al. [10] and ours may be due to the different conditions of irradiation.

A considerable quantity of 13-*cis*-retinal was formed in all preparations except for rhodopsin irradiated at liquid N_2 temperature. The photosteady-state mixture formed from rhodopsin at solid CO_2 temperature contained 11% 13-*cis*-retinal besides 42% 7-*cis*-retinal. The specificity of the stereoisomerization of retinal may be lost in the lumirhodopsin protein. However, the photosteady-state mixture produced by irradiating metarhodopsin at solid CO_2 temperature contained an amount of 13-*cis*-retinal of the same order as that of the rhodopsin irradiated at this temperature. The mechanism of the formation of 13-*cis*-retinal is not clear and further study concerning this problem is now in progress. 13-*cis* pigment was not detected by the previous analysis with spectroscopy [4]. 13-*cis* pigment may have a bleaching behaviour similar to that of 9-*cis* pigment in solution.

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