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CIRCULAR DICHROISM OF SQUID RHODOPSIN AND ITS INTERMEDIATES

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Summary

Circular dichroism (CD) and absorption spectra of squid (*Todarodes pacificus*) rhodopsin, isorhodopsin and the intermediates were measured at low temperatures. Squid rhodopsin has positive CD bands at wavelengths corresponding the α- and β-absorption bands at liquid nitrogen temperature (CD maxima: 485 nm at α-band and 348 nm at β-band) as well as at room temperature (CD maxima: 474 nm at α-band and 347 nm at β-band). The rotational strength of the α-band has a molecular ellipticity about twice that of cattle rhodopsin. The CD spectrum of bathorhodopsin displays a negative peak at 532 nm, the rotational strength of which has an absolute value slightly larger than that of rhodopsin. The reversal in sign at α-band of the CD spectrum may indicate that the isomerization of retinal chromophore from twisted 11-*cis* form to twisted 11-*trans* form has occurred in the process of conversion from rhodopsin to bathorhodopsin. Lumirhodopsin has a small negative CD band at 490 nm, the maximum of which lies at 25 nm shorter wavelengths than the absorption maximum (515 nm), and a large positive CD band near 290 nm, which is not observed in rhodopsin and the other intermediates. This band may be derived from a conformational change of the opsin. In the process of changing from lumirhodopsin to LM-rhodopsin, the CD bands at visible and near ultraviolet regions disappear. Both alkaline and acid metarhodopsins have no CD bands at visible and near ultraviolet regions.

Introduction

Squid rhodopsin, like cattle rhodopsin, is a chromoprotein with 11-*cis* retinal as a prosthetic group. On absorption of light, it converts through several intermediates [1] to the final photoproduct, metarhodopsin. In this process, the prosthetic group is isomerized from 11-*cis* retinal to all-*trans* form [2]. There are several differences between squid and cattle rhodopsin systems. For example, squid metarhodopsins are stable at physiological temperature while

cattle metarhodopsin are not [3]. The squid photoreceptor cell is depolarized on irradiation, while the cattle photoreceptor cell is hyperpolarized [4]. It should be noted that squid rhodopsin lies in the plasma membrane of the photoreceptor cell, while cattle rhodopsin is mainly present in the disk membrane which has no contact with the plasma membrane of the photoreceptor cell. This fact indicates that the squid rhodopsin system may couple with the generation of the receptor potential more directly than the system in cattle, because the receptor potential is induced from the plasma membrane. Thus, we attempted to study a photobleaching process of squid rhodopsin.

Although the intermediates in the photobleaching process of rhodopsin have been measured by means of low temperature spectrophotometry [5,6], the conformation of the retinal chromophore and the non-covalent interaction between retinal and opsin (protein moiety of rhodopsin) in rhodopsin and its intermediates still remain unclear. In order to elucidate these problems, measurements of optical activities (especially circular dichroism (CD)) of rhodopsin and its intermediates are important because they reflect the small alterations and the conformational changes of the chromophore and its neighboring groups. In the present paper, the absorption and CD spectra of intermediates in photobleaching of squid rhodopsin are systematically measured and compared with those of cattle rhodopsin.

Materials and Methods

Squid (*Todarodes pacificus*) rhodopsin was prepared by a modification of a method described by Hubbard and St. George [3]. The bisected eyes were shaken in 0.1 M phosphate buffer (pH 6.8) for separating the outer limbs of the photoreceptor cells from the retinas. The suspension was filtered through double sheets of gauze to remove the retinal debris. The suspension was then mixed with an equal volume of 80% sucrose solution (w/v in distilled water) and centrifuged at 14 000 $\times g$ for 30 min. The supernatant fluid was diluted with more than 3 vols. of the phosphate buffer and centrifuged at 14 000 $\times g$ for 1 h. The precipitate was suspended in 38% sucrose solution (w/v in 0.1 M phosphate buffer, pH 6.8), and followed by centrifugation. The supernatant fluid was diluted again with more than 3 vols. of the phosphate buffer and centrifuged. The 38% sucrose floatation was repeated. The precipitate thus obtained contained mainly the rhabdomal parts of the photoreceptor cells. The precipitate was washed first with the phosphate buffer, then distilled water and finally 0.1 M sodium carbonate buffer (pH 10.0) by centrifugation at 14 000 $\times g$ for 40 min. These procedures were repeated five times. Finally, the precipitate was suspended in the phosphate buffer and centrifuged at 10 000 $\times g$ for 30 min. The precipitate thus obtained consisted of two layers; the upper layer, which was semi-transparent, was presumably the rhabdomal membranes (microvilli), and the lower layer was relatively dense, and was presumably the cores or the basal parts of the outer segments. After discarding about two-thirds of the supernatant fluid from the centrifuge tube by use of a syringe, the tube was gently shaken to collect the semi-transparent layer as a suspension. The collected layer was centrifuged at 24 000 $\times g$ for 1 h to obtain the microvilli as precipitate. The microvilli were washed at least three times with

the sodium carbonate buffer for removing a screening pigment (omochrome) and then washed three times with distilled water.

The rhodopsin was extracted from the microvilli with 2% digitonin dissolved in 0.1 M sodium carbonate buffer (pH 10.5) or 0.1 M phosphate buffer (pH 5.5). The extract has λ_{max} at 480 nm as Hara and Hara [7] reported. To test whether or not the extract contains retinochrome, a small amount of the extract was diluted with 5 vols. of 0.1 M phosphate buffer (pH 7.0) and then 1 M hydroxylamine (pH 6.5) was added to the extract at a final concentration of 0.1 M. No decrease in absorbance at 480 nm occurred except for the effect of dilution, indicating that no retinochrome was contained in the extract. In the dense lower layer, rhodopsin and retinochrome were present in roughly equal amounts. If necessary, the rhodopsin extract was concentrated by ultracentrifugation at 105 000 $\times g$ for more than 12 h.

Glycerol was added to the preparation at a final concentration of 75 or 66%. The mixture thus obtained was used as a sample for measuring the absorption and CD spectra at low temperatures.

For these measurements, a specially designed glass cryostat with quartz windows, as described by Yoshizawa [6], was used. The temperature of the sample was monitored with a copper-constantan thermocouple attached to the sample holder made of unoxygenated copper. The sample was irradiated with light from a Xenon lamp (2 kW) which has passed through a glass cut-off filter with or without an interference filter. Absorption spectra were measured by a Hitachi 323 recording spectrophotometer, and CD spectra by Jasco J-20 recording spectropolarimeter.

For measurement of CD at liquid nitrogen temperature, the preparation must not develop cracks. We used the following technique [8]. A quartz optical cell in the holder was immersed in liquid nitrogen. Immediately after taking the optical cell out of the liquid nitrogen, the sample was injected into it with a syringe. Thus, the sample was uniformly frozen without any cracks. The CD spectra, which were measured at liquid nitrogen temperature, were checked by re-scanning CD spectra to see whether or not they contained linear dichroism. If the sample is not uniformly frozen, a CD spectrum at the first scan will be different in shape and/or magnitude from that at the second scan, because some of linear dichroism will be induced by the measuring light of a spectropolarimeter like J-20. The change of true CD spectrum of the sample by the measuring light is negligible under our experimental conditions. Further discussion concerning the measurement of CD spectra at liquid nitrogen temperature will be reported elsewhere (Horiuchi et al., to be published).

For measurement of absorption spectrum, an opal glass was placed between the sample cell and the photomultiplier for correction of scattering of the measuring light.

Isorhodopsin was prepared by means of low temperature irradiation [1], i.e., the preparation (rhodopsin) was irradiated at liquid nitrogen temperature with orange light at wavelengths longer than 560 nm for more than 6 h. Rhodopsin virtually changed to isorhodopsin but contained a small amount of bathorhodopsin. In order to remove this bathorhodopsin, the preparation was irradiated at this temperature with red light at wavelengths longer than 610 nm. This preparation contained more than 95% of isorhodopsin, which was

estimated by a slight modification of the method described by Yoshizawa and Wald [1]. That is, the preparation which had been irradiated at liquid nitrogen temperature was warmed to 10°C and re-irradiated at this temperature with orange light at wavelengths longer than 560 nm for conversion of rhodopsin and isorhodopsin to alkaline metarhodopsin. Then the change of absorbance at 490 nm (an isosbestic point between rhodopsin and isorhodopsin) was plotted logarithmically against time. Because rhodopsin converts to alkaline metarhodopsin much faster than isorhodopsin does, the change in absorbance of the mixture displays a straight line in the later period of the irradiation after the rhodopsin has been converted. Extrapolation of the straight line portions (representing the conversion of isorhodopsin) to zero time yields a measure of the proportion of isorhodopsin present in the mixture.

Results

(1) Thermostabilities of intermediates in the photobleaching process

The experiment began with confirmation of intermediates in the photobleaching process. Fig. 1 shows the changes of λ_{max} on warming the photosteady-state mixture composing rhodopsin, isorhodopsin and bathorhodopsin which has been prepared by irradiation of rhodopsin at liquid nitrogen temperature. All the λ_{max} values were estimated from the spectra measured at —184°C after stepwise warming to required temperatures. Three plateau temperature regions were found, which indicate existence of intermediates, bathorhodopsin, lumirhodopsin and a new intermediate, which we shall call LM-rhodopsin, in the photobleaching process. We also confirmed that metarhodopsins are stable below 10°C.

Next, we examined in detail spectral changes of thermal conversion from one intermediate to another. When the photosteady-state mixture of rhodopsin, isorhodopsin and bathorhodopsin was warmed to any temperature below

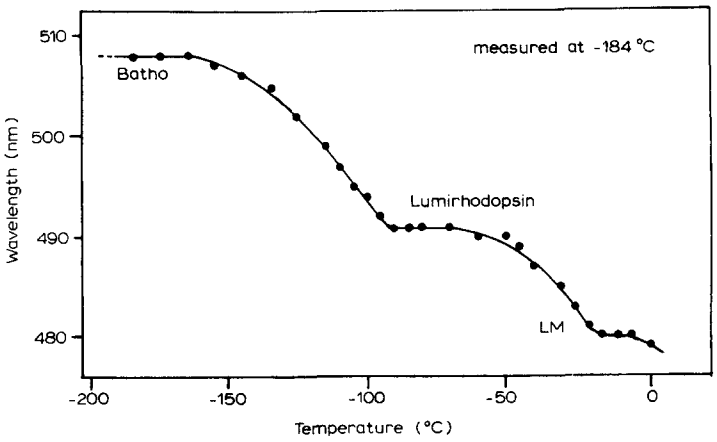


Fig. 1. Change in λ_{max} of photosteady-state mixture by stepwise warming. Rhodopsin/glycerol (pH 10.1) was irradiated with 437 nm light for 40 min at —184°C, resulting in a photosteady-state mixture composed of rhodopsin (8%), isorhodopsin (31%) and bathorhodopsin (61%). The mixture was then warmed to various temperatures (abscissa), and each time was re-cooled to —184°C at which the spectra were measured. The λ_{max} of the spectra are plotted on the ordinate.

−165°C and then re-cooled to −184°C, the spectra of the mixture did not change. Above −160°C, the spectra shifted to shorter wavelengths (λ_{max}: from 508 nm to 491 nm), forming an isosbestic point at 500 nm (Fig. 2a). Thus, bathorhodopsin was stable below −165°C and there was no intermediate between bathorhodopsin and lumirhodopsin.

Fig. 2b shows the conversion of lumirhodopsin to LM-rhodopsin. The experiment was started with the familiar photosteady-state mixture, formed by irradiating rhodopsin with 437 nm light at liquid nitrogen temperature in order to obtain a large amount of intermediate. Then the preparation was warmed to a required temperature in the range of −85°C to −35°C and then measured the spectrum at −85°C. As shown in Fig. 2b, lumirhodopsin was stable below −65°C and above this temperature it converted to LM-rhodopsin, forming an isosbestic point at 502 nm. This indicates that any intermediate did not exist between lumirhodopsin and LM-rhodopsin. The conversion of lumirhodopsin to LM-rhodopsin was so gradual that it could not complete even after warming to −25°C, while at this temperature a small amount of LM-rhodopsin began to convert to alkaline metarhodopsin. Thus, the thermostability region of LM-rhodopsin is very narrow, as shown in Fig. 1.

In Fig. 2c, the conversion of LM-rhodopsin to alkaline metarhodopsin is shown. The familiar photosteady-state mixture composed of rhodopsin, isorhodopsin and bathorhodopsin was warmed to −35°C (curve 1). The preparation was then warmed to the required temperature and re-cooled to −35°C for measurement of the spectrum. The spectra warmed to between −35°C and −20°C (curves 1—3) showed little irregular change in the visible region, owing to concurrent conversions of lumirhodopsin to LM-rhodopsin and of LM-rhodopsin to alkaline metarhodopsin. Above −20°C, the spectra (curves 3—8) made a sharp isosbestic point at 418 nm, indicating that lumirhodopsin completely converts to LM-rhodopsin below −20°C and LM-rhodopsin converted to alkaline metarhodopsin. Accordingly, there is no stable intermediate between LM-rhodopsin and alkaline metarhodopsin.

On warming the photosteady-state mixture produced by irradiating rhodopsin in an acidic preparation (pH 5.5—7.0) with 437 nm light at liquid nitrogen temperature, the same spectral changes as those in the alkaline preparation were observed below −35°C. The only difference in behaviour of the intermediates between acidic and alkaline conditions is that LM-rhodopsin converted to acid metarhodopsin under the acidic conditions, and under the alkaline condition it converted to alkaline metarhodopsin. Fig. 2d shows the conversion of LM-rhodopsin to acid metarhodopsin (pH 6.8). Below −25°C, the spectrum shifted to shorter wavelengths (curves 1—6) forming an isosbestic point at about 500 nm, which represents the conversion of lumirhodopsin to LM-rhodopsin. On warming above −10°C, the spectrum shifted to longer wavelengths (curves 6—9), presumably owing to the conversion of LM-rhodopsin to acid metarhodopsin.

According to a flash photolytic experiment by Ebina et al. [9], acid metarhodopsin can be formed before the formation of alkaline metarhodopsin even under alkaline conditions. However, we did not detect the formation of acid metarhodopsin under the alkaline condition [10], presumably owing to the rapid decay of acid metarhodopsin.

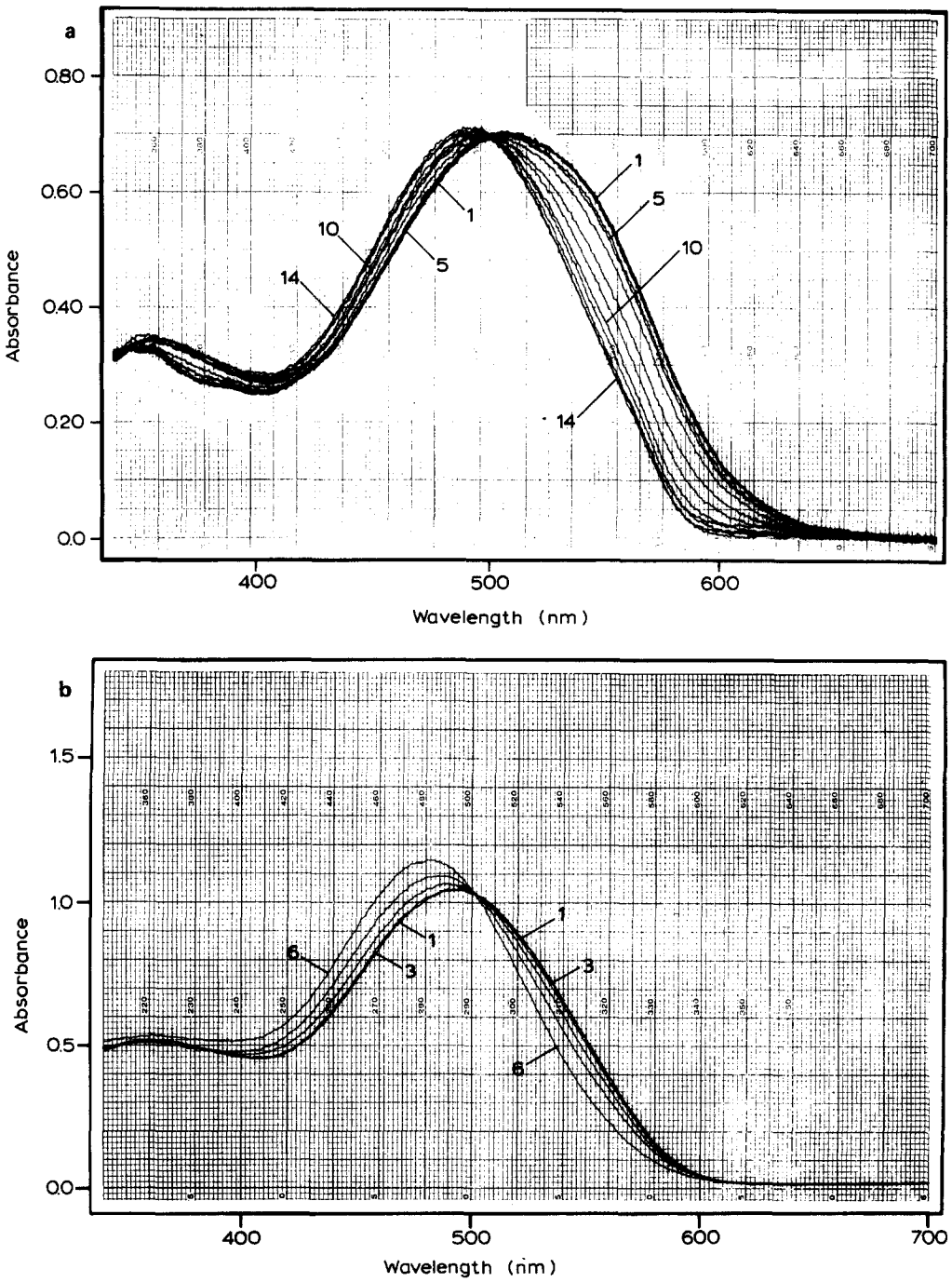
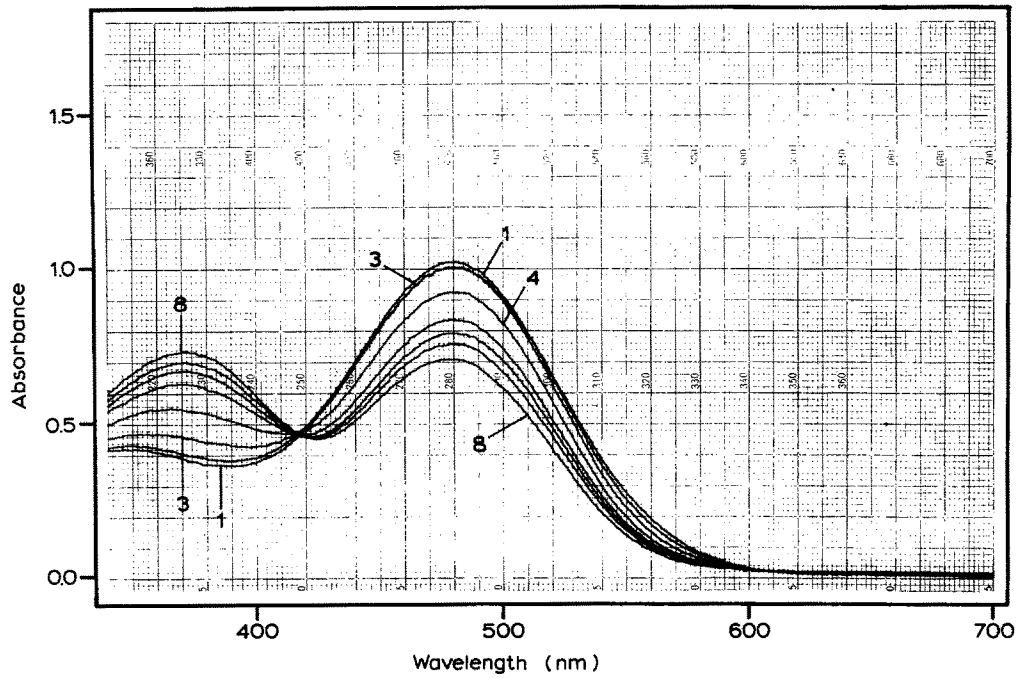
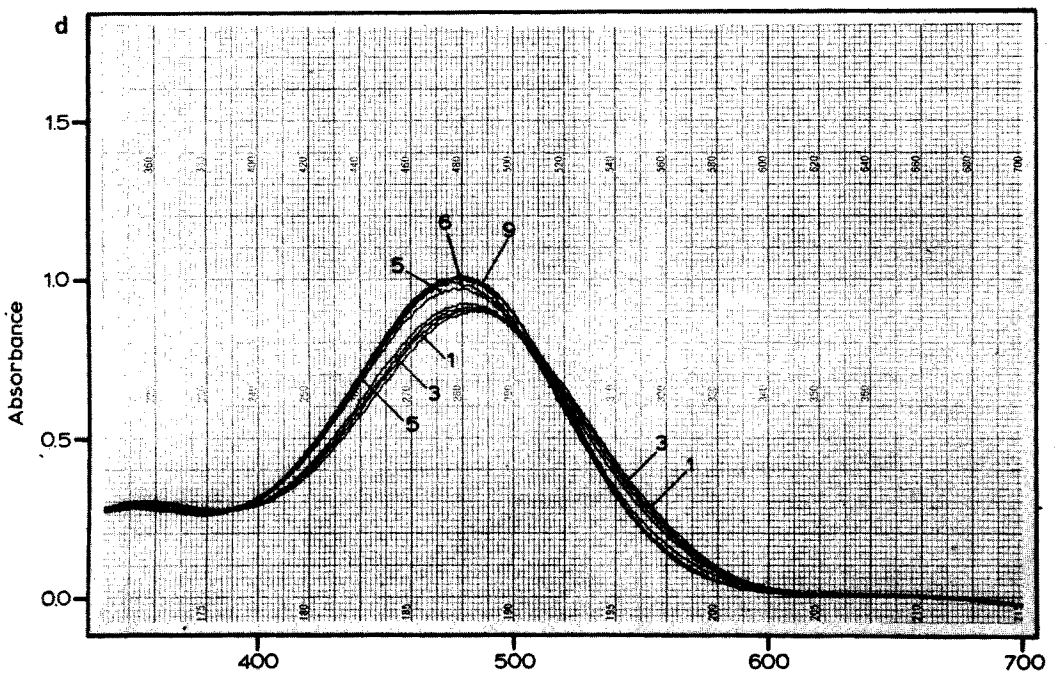


Fig. 2. a. Course of conversion of bathorhodopsin to lumirhodopsin by warming. A photosteady-state mixture composed of rhodopsin, isorhodopsin and bathorhodopsin, which had been prepared by irradiating rhodopsin/glycerol (pH 10.1) with 437 nm light at —185°C, was warmed to an appropriate temperature for conversion of bathorhodopsin to lumirhodopsin and then re-cooled to —185°C for measuring the spectrum. Curve 1, the photosteady-state mixture at —185°C; curves 2—14, products formed by warming in the dark to successive temperatures of —175, —165, —155, —145, —135, —125, —115, —110, —105, —100, —95, —90 and —85°C. b. Course of conversion of lumirhodopsin to LM-rhodopsin by warming. Rhodopsin/glycerol (pH 10.8) was irradiated with 437 nm at —188°C, then warmed to an appropriate temperature for conversion of lumirhodopsin to LM-rhodopsin. After re-cooling to —85°C, the spectrum was measured. Curves 1—6, products formed by warming in the dark to successive temperatures of —85,





—75, —65, —45, —35 and —25° C. c. Course of conversion from LM-rhodopsin to alkaline metarhodopsin. Rhodopsin/glycerol (pH 10.5) was irradiated with 437 nm light at —188° C, warmed to an appropriate temperature for conversion of LM-rhodopsin to alkaline metarhodopsin and then re-cooled to —35° C for measurement of the spectrum. Curves 1—8, products formed by warming in the dark to successive temperatures of —35, —25, —20, —15, —10, —5, 0 and 5° C. d. Course of conversion from LM-rhodopsin to acid metarhodopsin. Rhodopsin/glycerol (pH 6.8) was irradiated with 437 nm light at —190° C, warmed to an appropriate temperature for conversion of LM-rhodopsin to acid metarhodopsin and then re-cooled to —35° C for measurement of the spectrum. Curves 1—9, products formed by warming in the dark to successive temperatures of —35, —30, —25, —20, —15, —10, —5, 0 and 5° C.

(2) Absorption and CD spectra of rhodopsin and its intermediates

(a) Bathorhodopsin. CD measurement of bathorhodopsin may give some clues to whether or not the isomerization of 11-*cis* retinal to 11-*trans* may occur in the initial process of photobleaching of rhodopsin. Since the protein part of rhodopsin (opsin) may hardly change at liquid nitrogen temperature [11], a change of CD signal in the conversion of rhodopsin to bathorhodopsin may reflect some conformational changes of the retinal chromophore.

Conversion of rhodopsin to bathorhodopsin at liquid nitrogen temperature is shown in the top part of Fig. 3. When rhodopsin was cooled from room temperature (20°C) to liquid nitrogen temperature (—178°C in this case)

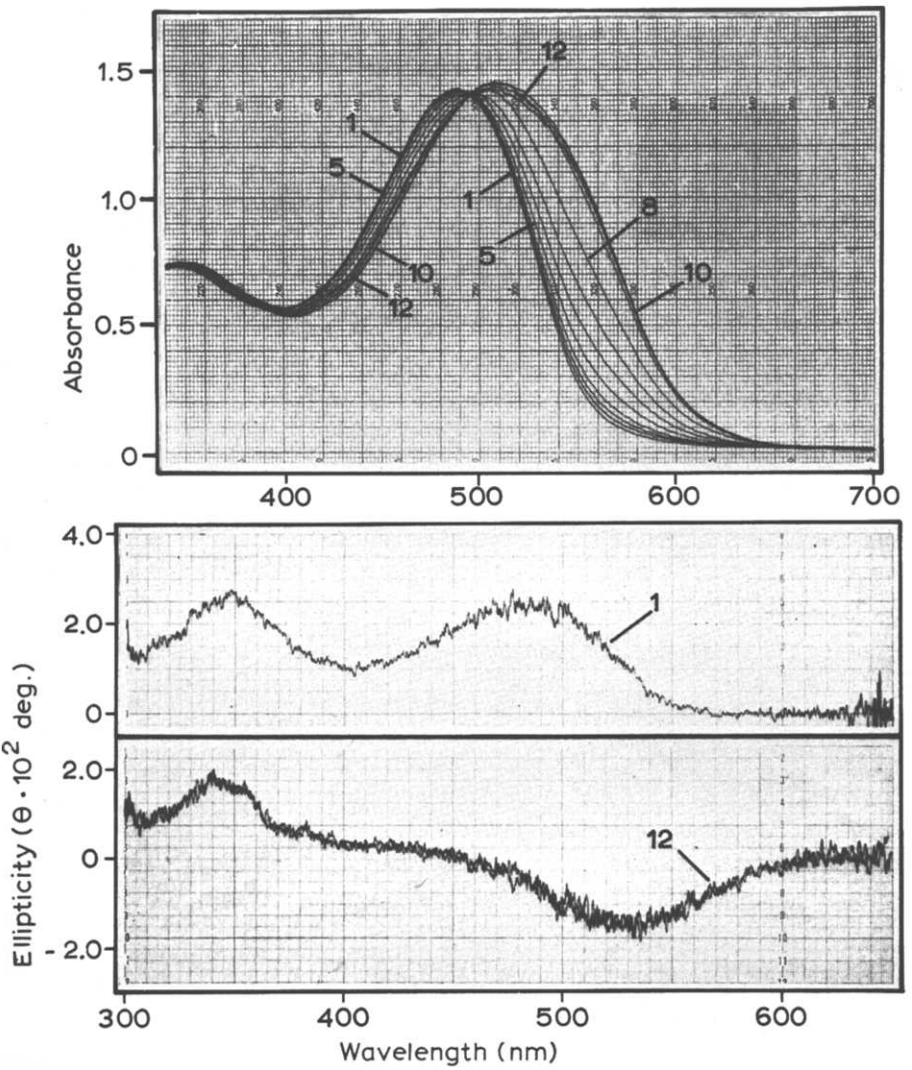


Fig. 3. Above: absorption spectral change in the course of conversion of rhodopsin to bathorhodopsin. Rhodopsin/glycerol (pH 10.5, curve 1) was successively irradiated with 437 nm light at —178°C for 5, 10, 20, 40, 80, 160, 320, 640, 1280, 2560, and 5120 s (curves 2—12). Below: CD spectra of rhodopsin (curve 1) and the photosteady-state mixture composed of rhodopsin, isorhodopsin and bathorhodopsin (curve 12). Curve 12 consists of two spectra.

without any traces of cracks, the λ_{max} of absorption spectrum shifted from 480 nm to 488 nm and the absorbance increased to 1.18-fold that at room temperature without any correction of volume contraction. Since the volume contraction of glycerol/2% digitonin (2 : 1) is about 7.7% at liquid nitrogen temperature [11], one can correct the ratio of extinction coefficient at liquid nitrogen temperature to that at room temperature as $1.18 \times (1-0.077) = 1.09$. This value is similar to those of all-*trans*, 9-*cis* and 13-*cis* retinals [12].

When rhodopsin was irradiated with 437 nm light at liquid nitrogen temperature (curve 1), the absorption spectrum shifted to longer wavelengths with a sharp isosbestic point at 495 nm at the early stage of irradiation (from curve 2 to curve 7). On further irradiation, the spectrum shifted to a longer wavelength and showed a slight decrease in absorbance near the maximum (curve 12), owing to some formation of isorhodopsin from bathorhodopsin. The composition of the photosteady-state mixture (curve 12) was estimated at 8% rhodopsin, 31% isorhodopsin and 61% bathorhodopsin, as described below. Using the same preparation, the CD spectrum was measured (Fig. 3, lower part). Curve 1 shows a CD spectrum of rhodopsin at liquid nitrogen temperature, the α-band of which shows almost the same molar ellipticity as the β-band, while the CD spectrum of the photosteady-state mixture (curve 12) displayed a negative α-band. In order to confirm that the CD spectrum contained no linear dichroism, the CD spectrum was recorded again. Curve 12 consists of two spectra which perfectly coincided with each other. This is an assurance that the CD spectrum does not contain any signal of photo-induced linear dichroism (ref. 8 and Horiuchi et al., to be published).

The absorption and CD spectra of bathorhodopsin were calculated as follows: after measurement of the absorption and CD spectra of the photosteady-state mixture (curve 12), the preparation was warmed to 10°C in the dark for conversion of bathorhodopsin to alkaline metarhodopsin and the absorption spectrum was measured. Then, the residual rhodopsin and isorhodopsin in the preparation were completely bleached to alkaline metarhodopsin by irradiation with light at wavelengths longer than 510 nm. The amount of the bathorhodopsin in the photosteady-state mixture was calculated from the ratio of the absorbance at 490 nm (an isosbestic point between rhodopsin and isorhodopsin at room temperature) after warming the photosteady-state mixture to 10°C to that of the original rhodopsin. The ratio between rhodopsin and isorhodopsin in the mixture was calculated by both the method of kinetic analysis of bleaching, as described in Materials and Methods, and the method of simultaneous equation reported by Hubbard [13]. Thus, the composition of the photosteady-state mixture was estimated at 8% rhodopsin, 31% isorhodopsin and 61% bathorhodopsin. From this ratio, one can calculate the spectrum of bathorhodopsin by subtracting the spectra of 8% rhodopsin and 31% isorhodopsin from the spectrum of the photosteady-state mixture. As shown in Fig. 6, bathorhodopsin displayed a large negative CD at the α-band. The conversion of sign in CD from positive to negative indicates that conformational changes of retinal may occur in the process of conversion from rhodopsin to bathorhodopsin.

(b) Lumirhodopsin. A typical experiment of absorption and CD measurements of lumirhodopsin is shown in Fig. 4. All the spectra were measured at

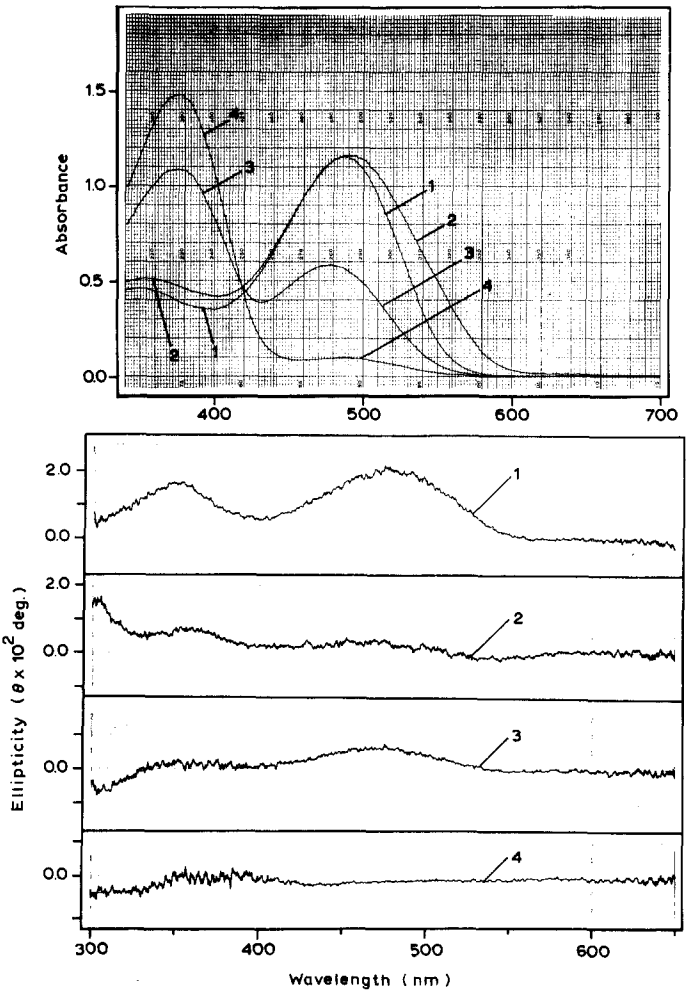


Fig. 4. An experiment for calculation of absorption (above) and CD (below) spectra of lumirhodopsin. Spectra of rhodopsin/glycerol (pH 10.5) were measured at −85°C (curves 1). After cooling to −188°C, the mixture was irradiated with 437 nm light for 40 min, and then warmed to −85°C for measurement of spectra (curves 2). After incubation at 2°C for 30 min, the spectra were measured at −85°C (curves 3). Finally, the preparation was irradiated with light at wavelengths longer than 510 nm at 2°C, and then re-cooled to −85°C to measure the spectra (curves 4).

−85°C. After the spectra of rhodopsin at −85°C were recorded (curves 1 in both parts of the figure), the preparation was cooled to liquid nitrogen temperature and then irradiated with 437 nm light until the photosteady-state mixture was formed. After warming to −85°C, spectra were measured (curve 2, above). In another experiment, we confirmed that the warming to −85°C completely converted bathorhodopsin in the photosteady-state mixture to lumirhodopsin; i.e., when the mixture which had been warmed to −85°C was re-cooled to liquid nitrogen temperature and re-irradiated with light at wavelengths longer than 630 nm for 15 min, the spectrum did not change at all. If bathorhodopsin were presented in the preparation, the spectrum should be changed by the irradiation, because spectrum of bathorhodopsin has some absorbance at 630 nm. Thus, it is confirmed that the mixture (curve 2) con-

tains no bathorhodopsin. After CD measurement (curve 2, below), the mixture was warmed to 2°C and incubated at this temperature for 30 min in order to complete the conversion of lumirhodopsin to alkaline metarhodopsin. The absorption and CD spectra were measured at −85°C (curves 3, above and below). Then the mixture was irradiated with longer wavelengths than 510 nm at 2°C, until the residual rhodopsin and isorhodopsin completely converted to alkaline metarhodopsin. After re-cooling to −85°C, absorption and CD spectra were measured (curves 4, above and below).

The absorption and CD spectra of lumirhodopsin were calculated as follows: difference spectra between lumirhodopsin and alkaline metarhodopsin were calculated by subtracting curves 3 in both parts of Fig. 4 from curves 2, and also the amounts of lumirhodopsin, rhodopsin and isorhodopsin were calculated in a similar manner to that of bathorhodopsin. Thus, the spectra of lumirhodopsin can be obtained by adding the spectrum completely bleached (curves 4, pure alkaline metarhodopsin) to the difference spectra which have been corrected to 100% conversion. As shown in Fig. 7, lumirhodopsin displays small negative CD at the α-band.

(c) LM-rhodopsin. An experiment for measurements of absorption and CD spectra of LM-rhodopsin was carried out at −40°C (Fig. 5). The procedure is almost the same in principle as that of lumirhodopsin except for the difference in temperature at which the spectra were measured. In order to determine the absorption spectrum of LM-rhodopsin more precisely, a sample containing LM-rhodopsin without any contamination of lumirhodopsin must be prepared. For preparing such a sample, one must pay attention to the thermostabilities of lumirhodopsin and LM-rhodopsin. After the spectra of rhodopsin were recorded at −40°C (curve 1), the preparation was irradiated with 437 nm light at liquid nitrogen temperature until the photosteady-state mixture was formed. Then, the preparation was warmed for incubation at −25°C. The absorption spectra were measured repeatedly at this temperature until the curve-intersection point moved to 418 nm, where an isosbestic point between LM-rhodopsin and alkaline metarhodopsin lies. The preparation was then re-cooled to −40°C for measurements of absorption and CD spectra (curves 2). This is the only way to obtain a preparation containing LM-rhodopsin without any trace of lumirhodopsin.

In another experiment, the preparation which had been irradiated at liquid nitrogen temperature was incubated at −40°C for 6 h until the spectrum hardly changed. This preparation still contained a small amount of lumirhodopsin because on warming to −35°C it showed some decrease of absorbance in the wavelength region longer than 530 nm. When another preparation which had been irradiated at liquid nitrogen temperature was directly warmed to −35°C, the absorbance in the region 420—510 nm increased. This indicates formation of LM-rhodopsin from bathorhodopsin via lumirhodopsin. When the preparation was kept for 3 h in the dark at the same temperature, the spectrum changed further. That is, the absorbance in the wavelength region shorter than 420 nm increased, that in the wavelength region longer than 510 nm decreased and that in the region 420—510 nm did not change. These spectral changes indicate that some alkaline metarhodopsin was formed from LM-rhodopsin concurrently with formation of LM-rhodopsin from lumirhodopsin.

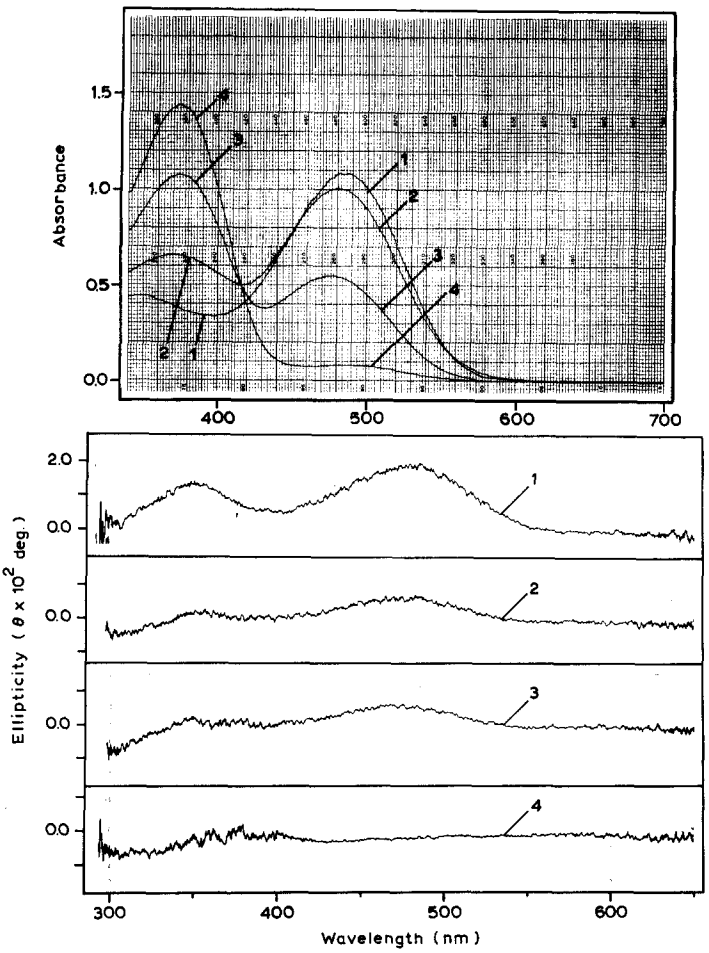


Fig. 5. An experiment for calculation of absorption (above) and CD (below) spectra of LM-rhodopsin. Rhodopsin/glycerol (pH 10.7) was cooled to −40°C for measurement of the spectra (curves 1), and then irradiated at −188°C with 437 nm light for 40 min. After incubation at −25°C for 10 min to convert lumirhodopsin to LM-rhodopsin, it was re-cooled to −40°C and measured the spectra (curves 2). Then, it was incubated at 2°C for 30 min for converting LM-rhodopsin into alkaline metarhodopsin and then re-cooled to −40°C for measurement of the spectra (curves 3). Finally, the preparation was irradiated at 2°C with light at wavelengths longer than 510 nm, and then re-cooled to −40°C to measure the spectra (curves 4).

Thus, we could not obtain a preparation containing only LM-rhodopsin without any contamination of lumirhodopsin and alkaline metarhodopsin. That is the reason why the preparation was warmed to −25°C and kept in the dark until the curve-intersection point moved to 418 nm.

Curves 2 in Fig. 5 above and below show a mixture of rhodopsin, isorhodopsin, LM-rhodopsin and alkaline metarhodopsin. After the mixture was kept at 2°C in the dark for 30 min to completely convert LM-rhodopsin to alkaline metarhodopsin, absorption and CD spectra were measured at −40°C (curves 3). Afterwards, the preparation was re-warmed to 2°C and irradiated with light at wavelengths longer than 510 nm for complete bleaching of the residual rhodopsin and isorhodopsin to alkaline metarhodopsin. Then the spectra were measured at −40°C (curves 4).

TABLE I

ABSORPTION AND CD SPECTROSCOPIC PROPERTIES OF RHODOPSIN AND ITS INTERMEDIATES

All values represent the averages of three to four experiments. Dipole strengths and rotational strengths were calculated under the assumption that the spectra fit Gaussian curves (see text).

	Absorption (λ_{max})	CD (λ_{max})	Dipole strength (10^{-35} cgs unit)	Rotational strength (Debye magneton)
Rhodopsin	488 nm	485 nm	5.93	0.80
Bathorhodopsin	532 nm	532 nm	7.79	−1.04
Lumirhodopsin	515 nm	490 nm	7.31	−0.34
LM-rhodopsin	486 nm	—	8.93	—
Isorhodopsin	472 nm	468 nm	7.34	0.72

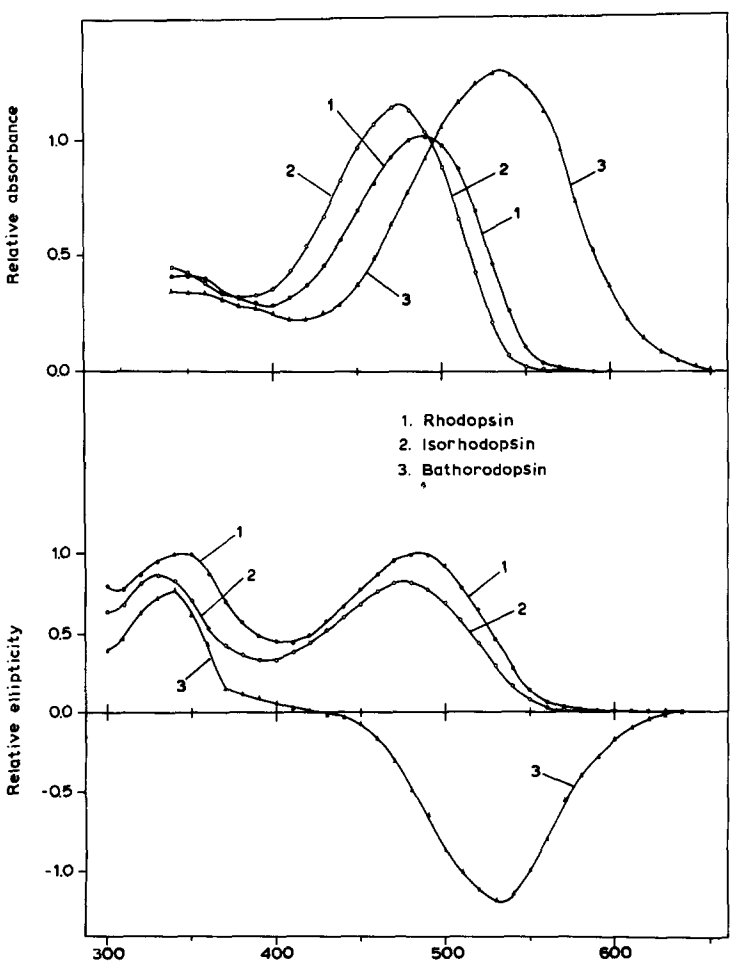


Fig. 6. Absorption (above) and CD (below) spectra of equivalent concentration of rhodopsin (curves 1), isorhodopsin (curves 2) and bathorhodopsin (curves 3) in glycerol/2% digitonin glass at liquid nitrogen temperature. All spectra represent averages of four to six experiments.

Since curve 2 coincides with curve 3 in the lower figure, it is clear that LM-rhodopsin does not display any CD at visible and near ultraviolet regions. Absorption and CD spectra of LM-rhodopsin (Fig. 8) were calculated as follows. The amount of rhodopsin converted to intermediate was calculated at 60% in a similar manner to that of bathorhodopsin. However, the mixture containing LM-rhodopsin (curves 2 in Fig. 5) has alkaline metarhodopsin as a contaminant, so that the amount of the alkaline metarhodopsin in the mixture must be estimated in order to determine the absorption and CD spectra of LM-rhodopsin. An isosbestic point of absorption spectra between lumirhodopsin and LM-rhodopsin at −40°C lies at 394 nm. Now, the absorbance at 394 nm of the spectrum which had been measured just after warming the photosteady-state mixture formed at liquid nitrogen temperature to −40°C (this mixture did not contain any amount of alkaline metarhodopsin) was denoted as A_1. The absorbances at 394 nm of curve 2 above and curve 3 above (Fig. 5) were denoted A_2 and A_3, respectively. The difference between A_1 and A_2

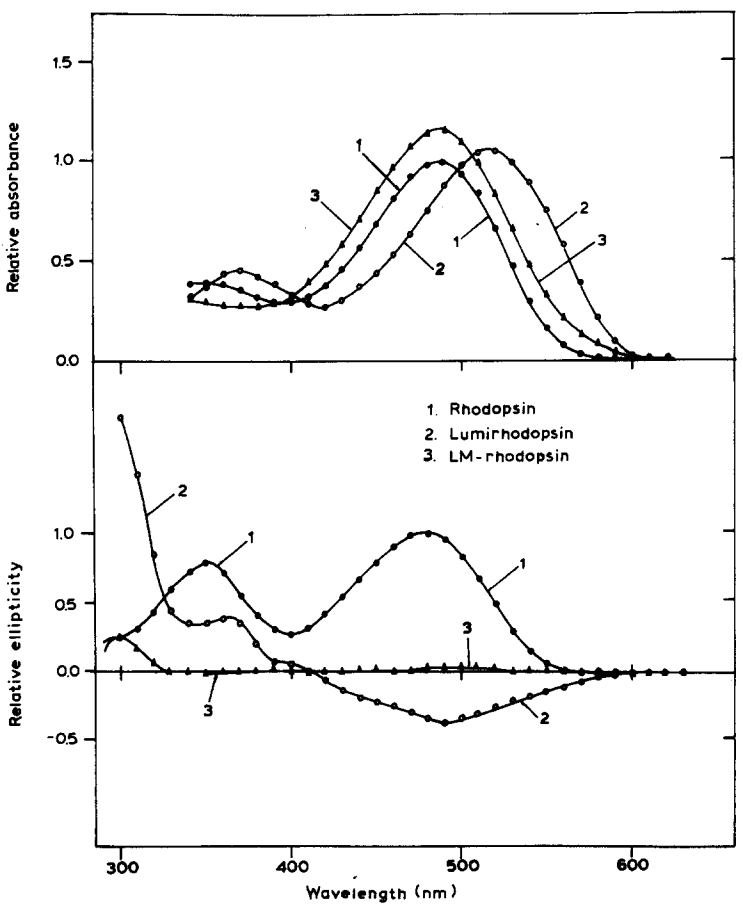


Fig. 7. Absorption (above) and CD (below) spectra of rhodopsin (curves 1), lumirhodopsin (curves 2) and LM-rhodopsin (curves 3). The maximum absorbance and ellipticity of rhodopsin at −85°C were normalized at 1.0 and spectra of equivalent concentration of lumirhodopsin measured at −85°C and those of LM-rhodopsin measured at −40°C were plotted. All spectra represent averages from four experiments.

($D_{A_1-A_2}$) corresponds to the absorbance change brought by conversion of LM-rhodopsin to alkaline metarhodopsin during the incubation of the photo-steady-state mixture at −25°C for 10 min. The difference between A_2 and A_3 ($D_{A_2-A_3}$) corresponds to the absorbance change brought about by conversion of the residual amount of LM-rhodopsin in the mixture (curve 2, upper part of Fig. 5) to alkaline metarhodopsin. Therefore, the ratio of the amount of alkaline metarhodopsin to LM-rhodopsin in the mixture (curves 2 in Fig. 5) was represented by $D_{A_1-A_2}/D_{A_2-A_3}$. From these calculations, the mixture (curves 2 in Fig. 5) consisted of 34% LM-rhodopsin, 26% alkaline metarhodopsin and 40% residual rhodopsin and isorhodopsin. Difference spectra between LM-rhodopsin and alkaline metarhodopsin were calculated by subtracting curves 3 from curves 2 (Fig. 5). Thus, the spectra of LM-rhodopsin (λ_{max}: 486 nm) can be obtained by adding the completely bleached spectrum (curve 4, pure alkaline metarhodopsin) to the difference spectrum which has been corrected to 100% conversion (Fig. 7).

(d) Acid and alkaline metarhodopsins. We confirmed that squid metarhodopsins display no CD at α-bands, as reported by Kito et al. [14].

Discussion

In the process of conversion of rhodopsin to bathorhodopsin, the CD signal at the α-band changes in sign from positive to negative, while that at the β-band does not change. Since the conformation of opsin seems hardly to change at liquid nitrogen temperature, the change of the CD signals seems to be due to a conformational change of retinal chromophore in the conversion of rhodopsin to bathorhodopsin. Recently it was reported that artificial rhodopsins with 5,6-dihydroretinal [15], in which no double bond in the ring structure is present, and with 5,6-epoxy-3-dehydroretinal [16], in which the conjugated double-bond system in the side chain is separated from the double bond in the ring structure, showed no CD at the β-band. These results suggest that the β-bands of rhodopsin and bathorhodopsin may be mainly due to a change in the electronic state of double bonds near the region of the ionone ring, at which the retinal chromophore binds with opsin through hydrophobic bond [17]. Accordingly, the fact that the positive CD at the β-band of rhodopsin is roughly equal in rotational strength to that of bathorhodopsin may indicate that the retinal-opsin interaction at the β-ionone-ring region does not change in the conversion of rhodopsin to bathorhodopsin. On the other hand, CD at the α-band seems to be due to a change in the electronic state at the whole conjugated double-bond system of retinal, and the fact that CD at the α-band inverses in sign in the conversion indicates that a large conformational change occurs in the side chain of the chromophore.

We confirmed that the CD spectrum in the near ultraviolet region (approx. 280 nm) did not change in the conversion of rhodopsin to bathorhodopsin. If CD of rhodopsin at the α- and β-bands should be induced by coupling between the retinal chromophore and an amino acid residue [15] (e.g. tryptophan), the conversion of rhodopsin to bathorhodopsin would show some change in CD in the near ultraviolet region as in the visible region. Therefore, the coupled oscillator model between the retinal and the amino acid residue seems unlikely to be an

explanation of the induced CD of rhodopsin. Furthermore, if one calculates the distance between the retinal chromophore and the amino acid residue (a tryptophan was assumed) of rhodopsin or bathorhodopsin by the coupled oscillator model, which is derived by approximating them to the point dipoles [18], one gets the value of 3.3 Å or 3.0 Å, respectively. These values are too small to apply the coupled oscillator model for the calculation. However, the possibility that their CD are induced by coupling between the retinal and the peptide bonds has still remained unsettled. Parker et al. [19] expressed their opinion that the coupled oscillator model was inadequate for the case of porphyropsins. However, our results on CD indicate that the isomerization from twisted 11-*cis* to twisted 11-*trans* already occurs in the conversion from rhodopsin to bathorhodopsin without dissolving the hydrophobic bond between the β-ionone ring and opsin. Recently, Kawamura et al. [20] reported that the direction of transition dipole moment of the retinal chromophore changes in the conversion of rhodopsin to bathorhodopsin. This fact is consistent with our results.

Lumirhodopsin has characteristic absorption and CD spectra. As shown in the upper part of Fig. 7, the β-band of absorption spectrum of lumirhodopsin is larger than that of rhodopsin. This fact is contrary to expectation from the general belief that lumirhodopsin has all-*trans* retinal as the prosthetic group, because all-*trans* retinal in solution has a β-band much smaller than that of 11-*cis* retinal [21]. Also the λ_{max} of the α-band of the absorption spectrum lies at wavelengths 25 nm longer than that of the CD spectrum. Furthermore, lumirhodopsin has a large positive CD band at the region of near ultraviolet (λ_{max}: approx. 290 nm). In this region rhodopsin and the other intermediates have a small shoulder of CD spectra (Shichida et al., unpublished results). One possible explanation of the absorption and CD spectra of lumirhodopsin is that coupling between the retinal chromophore and the amino acid residues of opsin may occur. If so, the β-absorption band, which is larger in magnitude than that of rhodopsin, may be induced by coupling between the electronic transition from the ground state of retinal chromophore to the second excited state (β-band) and that from the grand state of amino acid residue(s) (such as tryptophan) to the excited state(s). Therefore, CD spectra of lumirhodopsin may be expressed as the sum of three parts; intrinsic CD of retinal chromophore and amino acid residues, CD induced by coupling between the transitions of the α-band of retinal chromophore and amino acid residues, and CD induced by coupling between the transitions of the β-band of retinal chromophore and amino acid residues.

LM-rhodopsin has no CD in the visible region and the extinction coefficient is 1.23 times larger than that of rhodopsin. This indicates that the retinal chromophore of LM-rhodopsin may be free in the cavity of opsin except for the Schiff base linkage. In our previous report [10], we showed the absorption and CD spectra of lumirhodopsin and intermediate LM formed by irradiation of rhodopsin at −85°C and −40°C, respectively. The intermediate LM showed small positive CD peaks at the α- and β-bands. LM-rhodopsin described in this paper, which is thermally formed from bathorhodopsin through lumirhodopsin, does not display any CD in the visible and near ultraviolet regions, as shown in Fig. 7. These discrepancies may be due to the formation of photoproducts which have isomeric chromophores other than all-*trans* retinal, as Hubbard and Kropf [22] and Hubbard et al. [23] have suggested. If one irradiates squid rho-

dopsin with orange light (wavelengths longer than 530 nm) at −40°C, the photosteady-state mixture consists of five components, which have all-*trans*, 7-*cis*, 9-*cis*, 11-*cis* and 13-*cis* retinals as chromophores according to an analysis by high performance liquid chromatography (Maeda et al., to be published). Therefore, intermediate LM, which we reported previously, is a mixture of LM-rhodopsin and photoproducts with 7-*cis* and 13-*cis* retinals as chromophores. One of the photoproducts, which has the absorption maximum at about 475 nm and small positive CD at the α- and β-bands, has 7-*cis* retinal as a chromophore. When rhodopsin was irradiated at −85°C, the photosteady-state mixture also consisted of five components, each of which was different in absorption maxima from those produced by irradiaton at −40°C, except for rhodopsin (11-*cis*) and isorhodopsin (9-*cis*). On irradiating rhodopsin at liquid nitrogen temperature, however, only rhodopsin (11-*cis*), isorhodopsin (9-*cis*) and bathorhodopsin (all-*trans*) were found in the photosteady-state mixture. Thus, bathorhodopsin, lumirhodopsin and LM-rhodopsin which we have described in this paper should be all-*trans* intermediates. It is not yet clear whether or not one of the photoproducts formed at −40°C is the same as *P*-465 reported by Suzuki et al. [24].

Finally, let us compare the rhodopsin systems of squid and cattle. Cattle bathorhodopsin (ref. 8 and Horiuchi et al., to be published), as well as squid bathorhodopsin, has negative and positive CD at the α- and β-bands, respectively. Therefore, in both systems, the isomerization of retinal chromophore from twisted 11-*cis* to twisted 11-*trans* form may occur in the process of formation of bathorhodopsin.

Cattle lumirhodopsin has a positive CD peak at the α-band [25], while lumirhodopsin from squid, and also octopus, [26] has a negative CD peak. However, both lumirhodopsins have large positive CD peaks at the near ultraviolet region (approx. 290 nm). This fact indicates that in both systems, the β-ionon ring-opsin interactions change in this stage, and new interactions between the ring and amino acid residue such as tryptophan may be formed.

It is interesting that with squid rhodopsin there is no intermediate having positive CD at the α-band and all-*trans* retinal as a chromophore, though cattle metarhodopsin I and II have large positive CD at the α-bands [8,18]. Therefore, the first remarkable difference in the retinal-opsin interaction of both systems can be seen in the process of decay of lumirhodopsin. Since the decay of lumirhodopsin may couple with the triggering mechanism of the generation of receptor potential, the physiological meaning of the difference in circular dichroism between the both systems must be the subject of further research.

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